Nucleic Acid Binding Properties of the Simian Immunodeficiency Virus Nucleocapsid Protein NCp8*

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The nucleocapsid protein of simian immunodeficiency virus (SIV) NCp8 has two copies of conserved sequences (termed zinc fingers, ZF) of 14 amino acids with 4 invariant residues (CCHC) that coordinate Zn(II). Each of its two ZFs has a Trp residue. A significant quenching of NCp8 Trp fluorescence was seen in nucleic acid complexes, suggesting stacking of the indole ring with nucleobases and the simultaneous involvement of both ZFs in the binding process. Both ZFs contribute to the nucleic acid binding free energy of NCp8, albeit in a not additive manner. NCp8 exhibited a base preference analogous to that of NCp7: G > I > T > U > C > A. Alternating base sequences that bind HIV-1 NCp7 in a sequence-specific manner were also bound selectively by NCp8. Specific sequence recognition required at least five bases and the presence of bound Zn(II). The two ZFs account for the net displacement of 3 out of 4 sodium ions upon binding (2 by the first and one by the second finger), and for most (88%) of the hydrophobic stabilization in complex formation. Based on the sequence and functional similarity of SIV NCp8 and HIV-1 NCp7, and using available structural information for free and oligonucleotide bound NCp7, we propose a structural model for NCp8-oligonucleotide complexes.

NCP8, the NC† protein of SIV1 is encoded within a polyprotein Gag precursor that binds and packages genomic RNA during viral assembly (1). Retroviral NC proteins (2, 3), except those of spumaviridae, have conserved sequences (zinc fingers) of 14 amino acids with 4 invariant residues (CCHC) that coordinate Zn(II). ODMR studies have shown intercalation of Trp side chains with nucleobases in several single-stranded nucleic acid-binding proteins (4–8). ODMR spectroscopic investigation of the NC proteins from HIV-1 (8, 9) and MuLV (10, 11) and their nucleic acid complexes has confirmed that Trp stacking occurs in their association with nucleic acids. SIV NCp8 presents distinct spectroscopic advantages regarding its use in fluorescence and ODMR spectroscopy studies, as it contains one Trp residue in each of its two zinc fingers. This feature allows us to monitor the response of both zinc fingers domains upon complex formation. Preliminary ODMR results suggest that Trp stacking occurs as well in SIV NCp8 bound to oligonucleotide sequences.

From a functional standpoint, the close phylogenetic relation between HIV-1 NCp7 and SIV NCp8 renders a comparative analysis of their nucleic acid interactive properties relevant. In particular, we have recently described the preferential binding of HIV-1 NCp7 to certain alternating base oligonucleotide sequences (e.g., d(TT)₄ and d(TG)₄) (12, 13). Such sequence-specific interaction was not seen with NC proteins from more distantly related retroviruses (e.g., human T-cell leukemia virus type 1 p15₀ or MMTV p14 (15)). Thus, we set out to determine whether the sequence recognition capacity was species-specific by characterizing the interaction of SIV NCp8 with alternating base sequences. Functional homologies between SIV NCp8 and HIV-1 NCp7 may originate from the high percentage of identical and similar amino acid residues in their primary amino acid sequences. Then, the identification of the number and nature (electrostatic, hydrophobic) of interactions between the NC protein and its cognate nucleic acid ligand may result in the assignment of specific residues in complex formation. Computer modeling of the NCp8-oligonucleotide complex can be applied to check that the hypothetical structure satisfies the functional constraints derived from thermodynamic analysis. Such models may be relevant in visualizing the surface of nucleocapsid protein that is exposed to chemical attack by electrophilic agents known to react with the zinc finger thioreuddates (16–19) in several retroviruses, and result in their loss of infectivity. Wild-type SIV NCp8, as well as mutants carrying replacements of either aromatic or Zn(II)-coordinating amino acids within the zinc finger domain, are being actively pursued as agents in whole virus-inactivated vaccine strategies in primary models (20, 21).

MATERIALS AND METHODS

Protein Purification of SIV NCp8—The nucleotide sequence encoding the NC protein NCp8 of SIV-1 Mne has been cloned into an inducible Escherichia coli expression vector (p-Mal-c, New England Biolabs, Beverly, MA) and used to express recombinant NC protein. The protein was produced as a fusion construct with the NC sequence tethered to the C-terminal end of a maltose-binding protein through a tetrapeptide linker that confers a cleavage site for Factor Xa. A method for purification of the maltose-binding-NC fusion protein, cleavage by Factor Xa, and subsequent purification of the recombinant NC protein has been described (22). The final purification step was accomplished by high pressure liquid chromatography as described previously for HIV-1 NCp7 (23). The protein was subject to extensive analytical characterization, including polyacrylamide gel electrophoresis migration, amino acid composition, sequence analysis, and molecular weight determination by matrix-assisted laser desorption ionization-time of flight mass

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† The abbreviations used are: SIV, simian immunodeficiency virus; DTT, dithiothreitol; HIV-1, human immunodeficiency virus type 1; MMTV, mouse mammary tumor virus; MuLV, Moloney murine leukemia virus; NEM, N-ethylmaleimide; NC, nucleocapsid; ODMR, optically detected magnetic resonance; ZF, zinc finger.

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§ D. R. Morcock, B. P. Kane, and J. R. Casas-Finet, submitted for publication.

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Nucleic Acid Sequences—The deoxyoligonucleotides d(A), d(C), d(G), d(U), d(A), d(U), and d(T) (with n = 4, 6, 8, 10, 16, and 64); d(U), d(TG), d(GT), 5′-pd(TGT), 5′-pd(GTG), 5′pd(TGT)p3, 5′pd(GTG)p3, d(TGTG)p3, d(GTGT)p3, d(TGTGp5), d(GTGT)p5, d(TGTGp7), d(GTGT)p7, d(TGTGp9), d(GTGT)p9, where - denotes an abasic site; d(TGG), d(GTG), d(GTG), and d(TGG) were synthesized by the Recombinant DNA Laboratory, SAIC-Frederick, NCI-FCRDC. PolcDTYD and poly(U) were purchased from Amersham Pharmacia Biotech. The oligoribonucleotides r(G)8 and r(U)8 were obtained from Macromolecular Resources (Fort Collins, CO).

Chemicals—Sodium monophosphate and biphosphate, sodium chloro- dithiothreitol, and EDTA (tetrasodium salt) were purchased from Sigma-Aldrich Chemical Inc. (Milwaukee, WI). NEM was from Pierce (Rockford, IL).

Fluorescence Spectroscopy—Equilibrium binding isotherms for SIV NCp8 were obtained by monitoring its tryptophan fluorescence quenching upon binding to nucleic acid. Fluorescence measurements were carried out with a SPEX Fluoromax-2 spectrofluorometer, exciting at 295 nm (1 nm bandwidth) and monitoring emission at 356 nm (5 nm bandwidth). Data were acquired at 25 °C followed by a titration of a concentrated oligo- or polynucleotide solution to a 1.0-mL protein sample (1 μM) in 10 mM sodium phosphate buffer, pH 7.0, placed in a Teflon-capped, 0.5 × 1.0-cm path length Suprasil quartz cell (Hellma Cells, Inc., Jamaica, NY). Readings were taken every 5 s for 2 min, averaged, and corrected for dilution and inner filter effects. NCp8-nucleic acid complex formation was monitored by the reduction in the initial fluorescence of the protein.

Thermodynamic Calculations—The fractional saturation of NCp8 was inferred from the ratio of observed quenching to maximal quenching (at saturation) for each of the nucleic acid stepwise additions. The occluded binding site size (n) was derived from the extrapolation of the initial slope with the limiting fluorescence plateau in equilibrium binding isotherms carried out in near stoichiometric conditions (see, for instance, Fig. 2A). Salt-back titrations were carried out upon addition of a concentrated NaCl solution to a preformed NC-nucleic acid complex. Double-logarithmic plots of log K versus log [Na+] (see Fig. 2B) were used to derive binding constants at various ionic strength conditions (see Ref. 24). The fitting of our experimental data to a regression line made it possible to resolve the binding free energy in two components, the electrostatic contribution from the slope and the hydrophobic or non-electrostatic contribution from the y intercept (24). In the absence of net anion uptake or release during binding, dividing the slope value by 0.65 (number of ions displaced from the polyphosphate lattice after salt-back titrations) yields the number of ions displaced from the polyphosphate lattice. The inhomogeneity in microenvironment resulting from side chain motion and dipolar relaxation by water appears to be the main cause for the emission linewidth. Each of the constituent zinc finger peptides peaked at the same wavelength as NCp8, and had a linewidth similar to that of the full-length sequence (Fig. 1). Furthermore, ZF1 and ZF2 had comparable fluorescence emission intensities that were close to half of that seen for SIV NCp8 (Fig. 1).

RESULTS

Fluorescence Spectroscopy—As a preliminary step to using the intrinsic fluorescence emission from SIV NCp8 as the observable physical parameter in monitoring the protein’s binding to nucleic acids, we acquired the excitation and emission spectra from the full-length protein, as well as several synthetic zinc finger peptides. Steady state fluorescence measurements on NCp8 showed a relatively broad band (full-width at half-maximum = 4800 cm−1 or 64 nm) peaking at about 357 nm (Fig. 1). Such red-shifted wavelength of maximal emission points to a Trp indole side chain with nearly complete exposure to the solvent. The inhomogeneity in microenvironment resulting from side chain motion and dipolar relaxation by water appears to be the main cause for the emission linewidth. Each of the constituent zinc finger peptides peaked at the same wavelength as NCp8, and had a linewidth similar to that of the full-length sequence (Fig. 1).

The fluorescence emission intensity of SIV NCp8 or its synthetic zinc finger peptides decreased drastically (by about 60%) upon addition of 10 mM EDTA or 0.1 mM NEM. EDTA is able to compete with the ZF sequences for Zn(II) uptake when present in large excess, while NEM is a sulfhydryl alkylating agent that reacts with the Cys thiolate sulfurs of NCp8 preventing zinc chelation by the NC protein. These results indicated that...
DNA Binding by SIV Nucleocapsid Protein NCp8

**TABLE I**

| Oligo   | $Q_{max}$ | $K_{eq} (M^{-1})$ | $m^a$ | log $K^c$ |
|---------|-----------|-------------------|-------|-----------|
| Poly(dT) | 0.950     | $1.44 \times 10^8$ | -2.65 | 3.16      |
| d(A)$_8$ | 0.679     | $1.35 \times 10^5$ | -2.53 | 0.38      |
| d(C)$_8$ | 0.867     | $7.96 \times 10^5$ | -2.45 | 1.28      |
| d(U)$_4$dT | 0.956   | $3.74 \times 10^6$ | -2.61 | 1.65      |
| d(T)$_8$ | 0.935     | $9.51 \times 10^5$ | -2.56 | 2.15      |
| d(I)$_8$ | 0.978     | $1.83 \times 10^8$ | -2.70 | 3.17      |
| d(G)$_8$ | 0.967     | $2.49 \times 10^8$ | -2.75 | 3.21      |

$a^a$ $m$ is the slope in double-logarithmic plots of log $K$ versus log[Na$^+$]. The average value for all lattices is given in bold.

**TABLE II**

| Oligo   | $Q_{max}$ | $m$ | log $K$ |
|---------|-----------|-----|---------|
| Poly(dT) | 0.950     | -2.65 | 3.16       |
| Poly(U)  | 0.945     | -3.12 | 2.91       |
| d(G)$_8$  | 0.967     | -2.75 | 3.21       |
| r(G)$_8$  | 0.930     | -2.64 | 3.65       |
| d(U)$_8$dT | 0.956   | -2.61 | 1.65       |
| r(U)$_8$  | 0.935     | -2.74 | 1.55       |

**Fig. 2.** A, fluorimetric titration of SIV NCp8 with poly(dT). The normalized fluorescence was plotted versus the molar ratio of nucleobase to protein. B, double logarithmic plot of the apparent binding affinity versus monovalent ion concentration for the interaction of SIV NCp8 with poly(dT). C, Scatchard plot according to the McGhee and von Hippel model for the interaction of SIV NCp8 with poly(dT). The solid line represents the best fit, and yields the values for the occluded binding site size ($n$), intrinsic affinity ($K$), and cooperativity parameter ($\omega$).

**Discussion:**

All systems were capable of binding Zn(II), as previously reported for HIV-1 ZF sequences (28), and that coordination of Zn(II) ions by the zinc finger sequences greatly increased their Trp lifetime, as reported for HIV-1 NCp7 and other retroviral NC proteins (29, 30).

Circular dichroism spectroscopy confirmed the presence of a small amount of $\alpha$-helical and $\beta$-turn structure that was lost in the presence of NEM (not shown). This observation demonstrates the structural unraveling that ZF peptides undergo in the absence of zinc coordination.

Addition of saturating amounts of nucleic acid ligands quenched the NCp8 Trp fluorescence dramatically, without a displacement in the maximum emission wavelength. For homopolymeric sequences the limiting quenching varied from 68% for A to 97% for I or G (Tables I and II), suggesting: (i) stacking of the indole ring with nucleobases; and (ii) the simultaneous involvement of both zinc fingers in complex formation. The occurrence of Trp stacking was supported by a 3-fold increase in the fluorescence emission intensity (see above and Fig. 1), our synthetic ZF1 and ZF2 zinc finger peptides had comparable Trp lifetime, as reported for HIV-1 NCp7 and other retroviral NC proteins (29, 30).

Alternating base oligonucleotides (d(IT)$_4$ and d(TG)$_4$) that bind HIV-1 NCp7 in a sequence-specific manner (13) induced a 3-fold increase in emission of a fluorescent RNA, poly(eA), induced by NCp8 (data not shown); this effect ensues from an increase in base-base distance and ring overlap of ethenoadenine. It is also in agreement with ODMR spectroscopic analysis on NCp8-oligonucleotide complexes.

**Polymerization Binding—**The single-stranded nucleic acid binding properties of SIV NCp8 were characterized using homopolymeric oligo- and polynucleotides of various base composition. Addition of poly(deoxythymidylic acid) in saturating amounts extinguished 95% of the intrinsic tryptophan fluorescence emission of NCp8 (Fig. 2A; Table I). Poly(dT) titrations performed under near stoichiometric conditions yielded the NCp8 occluded binding site size ($n = 5.2$). This value was somewhat smaller than that ($n = 6.2$) observed for NCp7 from HIV-1 (31). The extent of quenching was almost identical to that seen with the octanucleotide d(T)$_8$ (Table I), capable of accommodating only one protein molecule per oligo strand (see below), suggesting that the decrease in fluorescence emission was not caused by vicinal protein-protein interactions. As the synthetic ZF1 and ZF2 zinc finger peptides had comparable Trp fluorescence emission intensity (see above and Fig. 1), our measurements strongly suggest that both ZF domains had to be involved simultaneously in the interaction of full-length NCp8 with nucleic acids to result in the nearly complete quenching observed with several lattices.

SIV NCp8 bound poly(dT) with affinity 1 order of magnitude higher than to the single-site ligand d(T)$_8$. The difference arose from higher hydrophobic contribution to the binding process in the case of the polynucleotide lattice (compare log $K^c$ for the two ligands in Table I), while the electrostatic component of the association was comparable (Table I). Higher binding affinity could result from protein-protein interactions arising from contiguously occupied sites in the polynucleotide. However, a Scat-
chard analysis (Fig. 2C) according to the model of McGhee and von Hippel (25, 26) showed marginal positive cooperativity ($\omega = 3$) in the interaction of NCp8 with poly(dT). This cooperativity value was roughly comparable to the measured occluded site size ($n = 5.2; \omega/n \approx 0.6$), partially compensating for the gap creation observed in the binding of extended ligands to overlapping binding sites as those present in nucleic acids. As a result, a Scatchard plot of the interaction of NCp8 with poly(dT) was almost rectilinear (Fig. 2C), showing only a slight concavity. Yet, such small cooperativity was only able to account for half of the difference between the $\Delta G^0$ values of $d(T)_8$ and poly(dT). Non-nearest neighbor positive cooperativity factors (e.g. a propagated distortion of the DNA lattice that renders up- or downstream aromatic stacking with nucleobases energetically more favorable), “covert” electrostatic contributions in polynucleotide complex formation (e.g. salt-bridges between contiguously bound NCp8 molecules that result in no net ion uptake or release) or some structural difference between the bound NCp8 structure in its oligo- and polynucleotide complexes must account for the remaining 3-fold difference in $K^\alpha$. The thermodynamic parameters for the association of SIV NCp8 with poly(U) were almost identical to those observed with poly(dT) (Table I). Thus, NCp8 bound to DNA or RNA polynucleotides with comparable affinity, similar ionic dependence, and marginal cooperativity. The limiting Trp fluorescence quenching, however, was somewhat reduced in the RNA complex (Table II).

**Octanucleotide Binding**—The relative affinity of SIV NCp8 for DNA oligonucleotides of different base composition was determined using homopolymeric octanucleotides in fluorimetric titrations to obtain equilibrium binding isotherms. Octanucleotides were chosen to allow binding of only one NCp8 molecule per oligo monomer (on the basis of its occluded binding site size determined from polynucleotide titrations), while permitting a comparison with NC proteins from other retroviral sources that exhibit $n$ values as large as 8 bases per NC molecule (15, 32). As expected, NCp8 bound all octanucleotides used with a 1:1 stoichiometry (not shown). The effect of oligonucleotide size on the binding properties of SIV NCp8 is addressed below. In binding homopolymeric deoxyoctanucleotides, NCp8 exhibited a base preference analogous to that of NCp7 (13): $G > I > T > U > C > A$, with a range spanning 3 orders of magnitude in binding constant (Table I). The extent of fluorescence quenching induced by the various DNA octanucleotides at saturation varied from 68% for $d(A)_8$ to 97–98% for $d(T)_8$ and $d(G)_8$. There was a direct correlation between the extent of fluorescence quenching and the magnitude of the hydrophobic stabilization for the association of a given sequence (see below). Most of the hydrophobic contribution to the binding free energy is thought to originate from the occurrence of stacking interactions between the aromatic amino acid side chains (including Trp) in NCp8 and the nucleic acid bases. Intercalated Trp structures are known to present near null quantum yield (33–35); thus, the correlation between quenching and hydrophobic stabilization found in our study may point to a more intimate or more frequent Trp stacking (or both) with certain oligo sequences, leading to a more complete extinction of NCp8 Trp fluorescence.

Salt-back titrations demonstrated that SIV NCp8 exhibits identical dependence of its nucleic acid binding affinity on salt concentration for all homopolymeric DNA or RNA lattices longer than $\approx 10$ bases (Tables I and II), independent of base composition. All lattices showed a rectilinear trace of binding affinity versus monovalent ion concentration in double-logarithmic plots. The magnitude of the slope ($-2.6 \pm 0.1$) was consistent with the net release of 4 Na$^+$ ions per bound NCp8.

| Oligo | $Q_{\max}$ | $m$ | $\log K^\alpha$ |
|-------|------------|-----|-----------------|
| $d(T)_8$ | 0.809 | -1.97 | 2.04 |
| $d(T)_9$ | 0.883 | -2.23 | 1.67 |
| $d(T)_9'$ | 0.935 | -2.56 | 2.15 |
| $d(T)_{10}$ | 0.979 | -2.71 | 2.36 |
| $d(T)_{15}$ | 0.995 | -3.21 | 3.32 |
| $d(T)_{64}$ | 0.952 | -2.85 | 3.45 |
| $d(T)_{100}$ | 0.956 | -2.65 | 3.16 |

We observed identical counterion release in the nucleic acid association of HIV-1 NCp7 in a previous study (31). The standard binding free energies, $\Delta G^\alpha$, were derived from the ordinate intercept of such plots. At 1 m Na$^+$ (the thermodynamic reference point), $\log [\text{Na}^+] = 0$, and the extrapolated binding affinity ($K^\alpha$, Table I) reflects solely the contribution of hydrophobic interactions, since by definition all ionic contributions to $\Delta G$ vanish in the reference state. $\Delta G^\alpha$ ranged from 2.1 kJ/mol for A to 18.3 kJ/mol for G; this variation was solely responsible for the dissimilar binding affinity of SIV NCp8 for various DNA oligonucleotides, which at a given ionic strength may differ by approximately 1000-fold. We have observed earlier a similar result for the association of HIV-1 NCp7 which was, on average, only 5-fold weaker than that of SIV NCp8 for a given oligonucleotide. Since the energetic contribution of electrostatic interactions between NCp8 and nucleic acids is identical to that shown by HIV-1 NCp7 (see above), the somewhat tighter NCp8 association may originate in the replacement of Phe$^{16}$ in HIV-1 NCp7 by a Trp at the analogous NCp8 position. Theoretical considerations (36) and experimental results (37) strongly suggest that nucleobase stacking with amino acid side chains is energetically more favorable in the order Ile $>$ Phe $>$ Tyr $>$ Trp. In physiological salt concentrations (about 150 mM monovalent ion), the fractional contribution of hydrophobic interactions to the total $\Delta G$ varied between 14% for $d(A)_8$ and 60% for $d(G)_8$. Note that the thermodynamic properties of the association of NCp8 with $d(G)_8$ or $d(I)_8$ were nearly identical (Table I), suggesting that the additional exocyclic amino group of guanine does not participate in a direct interaction (e.g. hydrogen bond) with NCp8. On the other hand, there is a measurable difference in the $\log K^\alpha$ of $d(T)_8$ and $d(U)_8$dT, resulting in a 3-fold difference in overall binding affinity. This result suggests that the methyl group in the 5 position of thymine, may contact NCp8 directly.

The Trp quenching induced by RNA oligonucleotides was slightly smaller (Table II) than that seen with analogous DNA lattices. In the association with RNA octanucleotides, however, NCp8 showed electrostatic and hydrophobic values very similar to those measured with the corresponding DNA sequences (Table II). The RNA phosphodiester backbone, while more flexible than that of DNA, exhibits similar base-base distance (the major determinant of the number of counterions released in the binding of an extended ligand to nucleic acids).

**Effect of Oligonucleotide Size on Binding Parameters**—We have characterized the thermodynamic parameters describing the association of SIV NCp8 with deoxyoligonucleotides of various length. NCp8 showed similar $\Delta G^\alpha$ values in binding tetra-, hexa-, or octanucleotides (Table III), but displayed increasing fluorescence quenching (Table III; Fig. 3A) and salt dependence (Table III; Fig. 3B) with lattice length, reaching an asymptotic limit at $10 \pm 1$ bases (Fig. 3, A and B). The apparent increase in salt dependence may originate from the incomplete formation of a counterion atmosphere around a short DNA lattice.
While a decanucleotide (the asymptotic limit length found) may still be insufficient for a fully developed equipotential surface along the DNA axis, it engulfs fully the bound NC protein (with a footprint of 5–6 nucleotide bases).

Similarly, the increase in NCp8 limiting Trp quenching with oligonucleotide length is likely to reflect a more stable intercalation of the indole side chain between the nucleic acid bases. A maximal quenching is reached below a lattice length sufficient for binding of two NC protein molecules to contiguous sites, suggesting that protein-protein interactions are not responsible for the decrease in Trp emission intensity. A double-logarithmic plot (Fig. 4B) showed a linear dependence between the fractional quenching in NCp8-oligonucleotide complexes and their hydrophobic stabilization (log K° values calculated from salt-back titrations). In this representation, Qₘ is meant to represent the “static” component of the limiting quenching of NCp8 Trp emission observed upon oligonucleotide binding. In other words, Qₘ (obtained by iterative fitting) is defined as the fraction of the maximal quenching that would be predicted to occur in the absence of any stabilizing non-electrostatic interaction (presumed to be dominantly aromatic intercalation); that is, at log K° = 0. It is termed static by analogy with the pre-collisional deactivation of the excited state observed in a Stern-Volmer model, although it may in fact involve dynamic quenching of the indole ring with groups other than the nucleic acid bases. It is worth noting that 37% of NCp8 Trp fluorescence in the absence of aromatic stacking.

Binding to Specific Sequences—We and others have recently reported (9, 13) that certain G/T-rich alternating base sequences are recognized and bound preferentially by HIV-1 NCp7. Among these, complexes of NCp7 with d(IT)₄ and d(TG)₄ have been characterized thermodynamically (19) and spectroscopically (9). NCp8 showed a binding affinity in the standard state (K°) that exceeded the mean value for the constituent homopolymeric octanucleotides in d(IT)₄ and d(TG)₄, by 20- and 130-fold, respectively. These K° factors were similar to those that we reported earlier (13) for HIV-1 NCp7 (10- and 145-fold).

The salt dependence exhibited by such sequence-specific lattices (m = –2.45 ± 0.03; Table IV) was comparable to that (m = –2.60 ± 0.10; Table I) observed for homopolymeric octanucleotides. In conclusion, SIV NCp8 was able to recognize and bind preferentially to the same sequences as HIV-1 NCp7, and with similar selectivity. The sequence recognition capacity depended entirely on a hydrophobic interaction of the NC protein with the nucleobase, as expected on the basis of the similar charge density and electrostatic character of nucleic acid lattices of different sequence. This “supernumerary” energetic contribution of the hydrophobic interactions that take place in the association of NCp8 with specific octanucleotide sequences is reflected in the practically complete (over 99%) quenching of the protein Trp fluorescence (Table IV).

Alternating base tetranucleotides that are still capable of inducing (less than maximal) specific sequence recognition in SIV NCp8 (Table IV), exhibit limiting fluorescence quenching that exceeds that seen with homopolymeric tetranucleotides. This finding suggests that the preferential binding of the former sequences stems from an enhanced hydrophobic stabilization involving the Trp side chains, in agreement with the similar number of ion pairs but increased non-electrostatic standard free energy of the alternating base versus homopolymeric tetranucleotides (see below). Moreover, their limiting quenching was somewhat lower than that seen with the alternating base octanucleotides, an effect concomitant with the reduction in specific sequence recognition seen with these shorter lattices (see below).

We have proposed earlier that full-sequence recognition by HIV-1 NCp7 requires at least 5 bases, a value for the interactive binding site size that is in agreement with the upper limit imposed by the occluded binding site size (6.2 for HIV-1 NCp7) and the observed 2 NCp7: d(TG)₄ stoichiometry (13). We measured somewhat smaller K° factors (7- and 30-fold, respectively) in the binding of SIV NCp8 to d(IT)₄ and d(TG)₄, relative to those seen with the longer lattices d(IT)₆ and d(TG)₆, suggesting that full recognition of specific sequences by NCp8 requires also at least five bases (in agreement with BIAcore results). The salt dependence for the binding of NCp8 to d(IT)₂ and d(TG)₂ (m = −1.74 ± 0.03) was comparable to that (m = −1.87 ± 0.09) measured for homopolymeric tetranucleotides (see Table IV). It is worth noting that NCp8 exhibited an approximately 6-fold decrease in the standard binding affinity (K°) for the alternating base tetra- or octanucleotides, upon substitution of the G ring by I (Table IV). We observed this effect in the binding of HIV-1 NCp7, as well, but neither protein showed a significant difference in binding affinity upon replacement of G for I in homopolymeric octanucleotides or tetranucleotides (Table IV), strongly suggesting that the nature of the hydrophobic interactions that take place in the formation of specific complexes are quite different from those involved in the association with nonspecific lattices. This hypothesis is further supported by the observation that, on average, the K° values for homopolymeric tetranucleotides were only 33 ± 3% lower than for octanucleotides (compare the log variability...
**Fig. 4.** A, monotonically correlation between the limiting Trp fluorescence quenching ($Q_{\text{max}}$) and the hydrophobic stabilization energy (represented here as $\log K^\circ$ = −ΔG°/2.3 R T) for various deoxyoligonucleotides. B, linearized dependence (according to a Boltzman model) of $Q_{\text{max}}$ induced by oligonucleotides of various length and base composition.

### Table IV

| Oligo  | $Q_{\text{max}}$ | $m$ | $\log K^\circ$ |
|--------|-----------------|-----|----------------|
| d(T)₈  | 0.935           | −2.56 | 2.15        |
| d(G)₈  | 0.967           | −2.75 | 3.21        |
| d(IT)₄ | 0.978           | −2.69 | 3.17        |
| d(TG)₄ | 1.000           | −2.21 | Observed = 3.97 |
| d(T)₄  | 0.809           | −1.97 | 2.04        |
| d(G)₄  | 0.905           | −1.77 | 3.07        |
| d(I)₄  | 0.907           | −1.78 | 3.05        |
| d(IT)₂ | 0.933           | −1.71 | Observed = 3.42 |
| d(TG)₂ | 0.942           | −1.77 | Observed = 4.01 |

3B reflects a discontinuity in slope for the trinucleotide sequences, whereas alternating T/G oligos ranging in size from 5 to 8 exhibited the modest increase in salt dependence expected from the denser ionic atmosphere present in the larger lattices. These findings strongly suggest that sequence-specific lattices involve (at least) 3 contiguous phosphate groups in electrostatic interactions with the basic residues of NCp8. Modeling and functional homology considerations suggest that the basic residues implicated are contained within the two zinc finger domains of NCp8 (see below).

Finally, the effect of terminal phosphorylation on NCp8-oligonucleotide association was investigated. Addition of terminal phosphate groups to alternating base T/G oligos increased their salt dependence (Table VI), in agreement with our model of three contiguous interactive phosphates on the nucleic acid lattice. The effect was similar at either the 3’ or 5’ termini, or for TGT or GTG deoxyribonucleotides (Table VI). No noticeable increase in $m$ values was observed upon terminal phosphorylation of alternating T/G tetranucleotides (Table VI). In general, the standard binding free energy and limiting Trp quenching (which we have shown earlier are positively correlated) increased measurably for the 3’-phosphorylated tri- and tetranucleotides, suggesting that additional electrostatic stabilization of complex formation imparts a non-electrostatic effect on Trp that is maximal at that position.

**Effect of Metal Ion Removal on Nucleic Acid Binding Affinity—** Displacement of bound Zn(II) from SIV NCp8 or its constituent ZF peptides results in a loss of secondary structure reflected in their peptidic band circular dichroism spectra (data not shown), a drastic decrease of the fluorescence emission intensity, and a large change in fluorescence decay lifetimes and dynamic anisotropy. These changes are indicative of the unfolding of the ZF domain upon sequestering of Zn(II) by excess EDTA or chemical reaction of the cysteine sulfur thiolates in p8 with a suitable electrophilic reagent. Apo-p8 exhibited also a dramatic decrease in the extent of fluorescence quenching observed upon addition of a saturating amount of nucleic acid (Table VII), suggesting reduced Trp stacking and
TABLE VI
Effect of terminal phosphorylation on the binding properties of SIV NCp8 for sequence-specific oligonucleotides of various length

| Oligo          | Q_{max} | m  | log K° |
|----------------|---------|----|--------|
| d(TG)          | 0.890   | -1.61 | 4.24  |
| d(TG)lg        | 0.976   | -1.84 | 4.24  |
| p[d(TG)lg]l    | 0.782   | -1.69 | 4.23  |
| d(TG)G         | 0.943   | -1.77 | 4.01  |
| d(TG)Glg       | 0.952   | -1.72 | 5.15  |
| p[d(TG)Glg]l   | 0.966   | -1.80 | 4.31  |
| d(TG)G         | 0.893   | -1.14 | 3.82  |
| d(TG)Glg       | 0.958   | -1.45 | 4.93  |
| p[d(TG)Glg]l   | 0.858   | -1.39 | 3.94  |
| d(TG)G         | 0.904   | -1.15 | 3.83  |
| d(TG)Glg       | 0.947   | -1.57 | 4.09  |
| p[d(TG)Glg]l   | 0.933   | -1.39 | 3.75  |

TABLE VII
Comparison of the octanucleotide binding properties of Zn(II)-NCp8 versus apo-NCp8

| Oligo          | Q_{max} | m  | log K° |
|----------------|---------|----|--------|
| Zn-p8 + d(T)_8 | 0.94    | -2.56 | 2.15  |
| Apo-p8 + d(T)_8 | 0.36   | -2.52 | 0.86  |
| Zn-p8 + d(G)_8 | 0.97    | -2.75 | 3.21  |
| Apo-p8 + d(G)_8 | 0.10   | -2.73 | 1.61  |
| Zn-p8 + d(TG)_8 | 1.00   | -2.21 | 4.80  |
| Apo-p8 + d(TG)_8 | 0.49   | -2.43 | 1.81  |

hydrophobic stabilization in the complex, relative to the Zn(II)-bound form. Zinc displacement from NCp8 by the alkylating agent NEM (added to a final 5:1 molar ratio to cysteine) did not alter the salt dependence of p8 association. While both Zn(II)-p8 and apo-p8 induce the net release of 4 Na⁺ ions upon binding (Table VII), it is unclear whether the same basic residues interact with the nucleic acid phosphates in both cases. The hydrophobic component of the interaction of apo-p8 with homopolymeric oligonucleotides was drastically reduced, showing a 30-fold reduction in K°. The impairment in hydrophobic stabilization was even more dramatic for the binding of apo-p8 with d(TG)_8, which lead to a 3 order of magnitude decrease in K°, relative to the zinc-coordinated protein. Moreover, most (73%) of the specific sequence recognition capacity was lost in the absence of Zn(II) binding by SIV p8; the measured K° for apo-p8 binding to d(TG)_8 was only 4 times that expected on the basis of the standard affinity of apo-p8 for d(T)_8 and d(G)_8, instead of the 130-fold factor observed with Zn(II)-bound p8 (see above and Table IV).

Binding Properties of p8 Zinc Finger Peptides—ZF1 and ZF2 are 18-amino acid synthetic peptides spanning the sequence of the first and second zinc fingers of SIV NCp8. Both peptides have a single aromatic residue (Trp) in homologous positions, but differ in net charge (+3 for ZF1, +1 for ZF2). Their nucleic acid binding properties were investigated in fluorimetric titrations with d(T)_8 or poly(U).

ZF1 exhibited 94% of the Trp quenching observed for NCp8 with either nucleic acid lattice, and displayed 62% of the salt dependence and 28% of the hydrophobic contribution to ΔG of full-length NCp8. ZF2 bound weaker than ZF1 to either lattice, and showed only 36% of the Trp quenching seen for SIV NCp8. ZF2 displayed 33% of the salt dependence and 86% of the hydrophobic contribution of full-length NCp8. Thus, taken together the NCp8 zinc finger peptides would account for 98% of the salt dependence and 114% of the hydrophobic stabilization of the full-length protein. However, as reported for HIV-1 NCp7 (31), the contribution of the constituent zinc finger domains to the binding free energy of NCp8 is less than that inferred from the isolated ZF peptides. For instance, the binding affinity of NCp8 for d(IT)_8 is 300 times that of either zinc finger peptide, but 400 times less than their product (data not shown). On average, only 74% of the total binding free energy that is available from the zinc finger peptides is found in the full-length protein. This non-additivity may stem from differences in the number and nature of the interactions with the nucleic acid that may take place when the two ZF domains are covalently linked. It has been proposed that, for proteins that have a single aromatic residue (Trp) in homologous positions, but differ in net charge (+3 for ZF1, +1 for ZF2). Their nucleic acid binding properties were investigated in fluorimetric titrations with d(T)_8 or poly(U).

ZF1 exhibited 94% of the Trp quenching observed for NCp8 with either nucleic acid lattice, and displayed 62% of the salt dependence and 28% of the hydrophobic contribution to ΔG of full-length NCp8. ZF2 bound weaker than ZF1 to either lattice, and showed only 36% of the Trp quenching seen for SIV NCp8. ZF2 displayed 33% of the salt dependence and 86% of the hydrophobic contribution of full-length NCp8. Thus, taken together the NCp8 zinc finger peptides would account for 98% of the salt dependence and 114% of the hydrophobic stabilization of the full-length protein. However, as reported for HIV-1 NCp7 (31), the contribution of the constituent zinc finger domains to the binding free energy of NCp8 is less than that inferred from the isolated ZF peptides. For instance, the binding affinity of NCp8 for d(IT)_8 is 300 times that of either zinc finger peptide, but 400 times less than their product (data not shown). On average, only 74% of the total binding free energy that is available from the zinc finger peptides is found in the full-length protein. This non-additivity may stem from differences in the number and nature of the interactions with the nucleic acid that may take place when the two ZF domains are covalently linked. It has been proposed that, for proteins that
contain repeats of nucleic acid interactive domains, the total affinity is directly proportional to (but not a straight multiple of) the number of domains and the length of the connecting intervening sequences (39).

Using the experimentally derived scaling factor of 74%, we attribute 73% (0.74 \times 98\%) of the electrostatic interactions in NCp8-nucleic acid complexes to the zinc finger sequences. This translates into the net displacement of 3 Na\(^+\) ions upon direct binding of the nucleic acid to the zinc finger domains (73% of 4 counterions ejected by full-length NCp8). Since the electrostatic contribution of ZF1 was twice that of ZF2 (see above), our calculations suggest that the first zinc finger domain is involved in two electrostatic interactions, whereas the second zinc finger domain would participate in one. One more electrostatic interaction would be established with basic groups located elsewhere in the protein sequence. We proposed an identical assignment of electrostatic interactions in the highly homologous NC protein NCp7 from HIV-1 based on a completely different strategy (31). In that study, differences in ionic dependence of the nucleic acid binding were observed following truncation of the NCp7 amino acid sequence, or in duplication or interchange of its constituent ZF domains (31). One electrostatic contact was ascribed to Arg\(^{27}\) in the flanking N-terminal region, and two and one, respectively, to within the first and second ZF domains (31). A comparison of the primary amino acid sequences of SIV NCp8 and HIV-1 NCp7 (Fig. 6) shows that basic residues are found at positions analogous to those proposed earlier (31). These results suggest that the scaling factor used in our calculations may reflect the occurrence in NCp8 of a subset of the interactions found in the nucleic acid association of the isolated ZF peptides. In this case, 85% (0.74 \times 118\%) of the hydrophobic stabilization observed in NCp8-nucleic acid complexes would be predicted to reside in the zinc finger domains. This is also the experimental observation with a double-zinc finger peptide that spans residues 13–51 of HIV-1 NCp7 (31). It suggests that the vast majority of the base-discriminating power, as well as the specific-sequence recognition capacity (both of which depend on dissimilar hydrophobic stabilization with different ligands), resides in the zinc finger motifs of SIV NCp8 and HIV-1 NCp7. This prediction is in good agreement with the drastic loss of binding affinity and sequence discrimination seen experimentally in apo-p8 and apo-p7 (13). It also suggests that there is probably no more than one hydrophobic contact with the nucleic acid outside the zinc finger regions of p7 or p8, and that the residue implicated is non-aromatic.

**Computer-assisted Structural Modeling of SIV NCp8 and NCp8-Nucleic Acid Complexes—**A three-dimensional structure of the complex between NCp8 and a short oligonucleotide was constructed based on the known solution structure of HIV-1 NC protein (40) and its complex with a linear pentadeoxynucleotide (27). The highly homologous NCp8 sequence was constructed in the computer by replacement of the appropriate side chains, was template-forced at the zinc finger thiolate sulfur and imidazole nitrogen atoms atop that of HIV-1 NCp7, and was subsequently energy-minimized. As shown in Fig. 6, the two NC proteins can adopt a similar conformation in which the two zinc fingers are in close spatial proximity to each other (30, 40, 41). This structural homology is likely reflected in their similar thermodynamic parameters and functional properties. Thus, we attempted to build a computer model of HIV-1 NCp7/SIV NCp8 bound to the minimal specific RNA sequence GU-GUG (Fig. 7), based on the structure of the p7-d(ACGGCC) complex (27), in two steps. Replacement of p7 by p8 in the d(ACGGCC) complex structure required minimal adjustments, as expected from our thermodynamic results and earlier func-
in the Consistent Valence Force Field. The nucleic acid bases are colored red for G and green for U, and the phosphate groups in the polynucleotide chain are labeled magenta. The 5’ end of the RNA is to the left of the figure and the first zinc finger of the p8 monomer on the left is at the bottom.

In the present study, we have characterized the affinity and modeled the structure of the SIV nucleocapsid protein. NCp8 has one Trp residue in each of its two zinc fingers, allowing us to monitor simultaneously their interaction with nucleic acids using the intrinsic fluorescence emission of the indole side chain. Steady state and dynamic fluorescence measurements show similar contribution of each ZF to the overall emission of NCp8, and comparable and drastic extinction of fluorescence intensity upon association with nucleic acid lattices. The quenching of NCp8 Trp fluorescence varied from 68% for A to 97% for I or G, suggesting stacking of the indole ring with nucleobases in both zinc fingers simultaneously. Alternating base sequences that bind HIV-1 NCp7 in a sequence-specific manner induced total quenching of NCp8 fluorescence. Analytical findings. Most of the unfavorable contacts were located in the vicinity of the intercalating Phe16 in p7, whose benzyl side chain was replaced by the bulkier imidazole ring of Trp in the p8 sequence. Local side chain energy minimization of the first ZF-oligonucleotide binding interface readily gave a complex structure without unfavorable contacts. During this process, the structure of the d(ACGCC) oligonucleotide was kept invariant. Replacement of d(ACGCC) for the specific RNA sequence GUGUG in the complex was easily accommodated as well, given their identical size and similar purine-pyrimidine alternation. A comparable structure for the p8-GUGUG complex was reached independently of the order of the structural replacements (HIV-1 p7 for SIV p8, or DNA for RNA pentanucleotide; not shown). The similarity between the experimental p7-pentanucleotide complex and the computer-derived p8-pentanucleotide structure provides additional support for the functional homology seen between these two proteins.

In addition, the structure of the p8 complex was used to model binding to a long polynucleotide lattice. For this purpose, p8 was “polymerized” in silico by generating multiple copies of the pentanucleotide complex structure and “ligating” the oligonucleotide sequences together by overlap of the phosphodiester backbone and elimination of the terminal base. Structures were rigid body-fitted to each other; no structural adjustment was allowed on the neighboring p8 monomers. The resulting structure (Fig. 8) shows several noteworthy features. Vicinal NC binding can be achieved without steric hindrance of the ZF-flanking sequences between contiguous bound proteins. In fact, the protein-protein interface shows neat docking of the adjacent NC proteins. Binding is polar, with all p8 units in identical disposition of both their N- and C-terminal domains, relative to the nucleic acid backbone vector. While a direct interaction of the protein’s hydrophobic core occurs with the three central bases covered by the binding site, the number of bases (5–6) between successive p8 monomers is in excellent agreement with experimental determinations of its occluded binding site. Finally, a curvature of the polynucleotide lattice was imposed by NC binding, a feature with profound implications both in the involvement of NC proteins in the formation of a compact core in mature retrovirions, as well as in the requirement for an active NC domain within the Gag polyprotein precursor and the presence of nucleic acid of moderate length for efficient particle assembly in vitro. Note that the walls of nucleic acid binding groove in p7 or p8 (facing forward in Fig. 8) are lined with basic residues that can interact with the negatively charged phosphate groups in the nucleic acid backbone; the opposite surface, in contrast, has an excess of acidic residues. Such spatial segregation of charge may contribute to an electrostatic preorientation prior to complex formation that favors presentation of the binding groove and increases the association rate constant of the binding process. In addition, apposition of successive “turns” of the coiled nucleoprotein complex may be stabilized by vertical electrostatic interactions, resulting in a compact structure resembling that of histone-packed cellular DNA that is consistent with the core dimensions observed in ultramicrographs of virion particles.

**Fig. 8.** Model structure of two contiguous SIV p8 molecules bound to an alternating-base (GU)₄G polynucleotide. p8 is rendered as a Connolly solvent-accessible surface using a 1.4-Å probe (i.e. water molecule radius); basic residues are indicated in blue, acidic residues in red, and uncharged residues are shown in white except for the indole side chains of Trp, depicted in magenta. Atom charges were assigned as defined in the Consistent Valence Force Field. The nucleic acid bases are colored red for G and green for U, and the phosphate groups in the polynucleotide chain are labeled magenta. The 5′ end of the RNA is to the left of the figure and the first zinc finger of the p8 monomer on the left is at the bottom.
sis of our data supports an empirical relationship between standard (non-electrostatic) binding free energy and fluorescence quenching observed in oligonucleotide complexes. The base preference and ability to recognize specific sequences reside in the different extent of non-electrostatic stabilization that NCp8 exhibits with different lattices. No significant discrimination of RNA versus DNA was found, at least among homopolymeric sequences, a feature that has been reported earlier for HIV-1 NCp7 and other retroviral NC proteins, and that may be related to their involvement with both ligands in RNA chaperone (42) and reverse transcriptase facilitation reactions (43).

Although NCp8 Trp quenching increased with lattice length, it levels off at or just below the size needed for two NCp8 molecules, suggesting that vicinal NCp8 binding does not induce additional quenching. This is in agreement with the similar standard free energy values in binding tetra-, hexa-, or octanucleotides, and the location of the two Trp residues in the nucleic acid binding groove. Computer modeling did not provide evidence for the occurrence of vicinal salt-bridge interactions between nucleic acid-bound NCp8 molecules. Regardless of base sequence, NCp8 induced the release of 4 sodium ions upon binding homopolymeric lattices. Zinc fingers account for the net displacement of 3 of 4 sodium ions upon binding (2 by the first and one by the second finger). 85% of the hydrophobic stabilization in bound NCp8 resides in the zinc fingers. The energetic contribution of the two zinc fingers to the full-length NCp8 protein is synergistic but not additive.

In alternating T/G oligonucleotides, electrostatic interactions with at least three contiguous phosphate groups were established. Most of the non-electrostatic stabilization was seen when a GT or TG motif was present. Modified dinucleotides with TG or GT sequences have been used in nucleomimetic strategies for the inhibition of NCp7 from HIV-1, due to their high affinity binding to NCp7 (44). Modeling suggest that their base-base distance increases considerably in the complex due to flanking of the two Trp side chains in NCp8. This structure requires that, in the complex, the protein adopts a closed conformation in which the two zinc fingers are in close proximity. Such closed structures have been reported in solution for the highly homologous NC protein of HIV-1 (40, 45, 46). In these studies, a transient close approach of the side chains of Phe16 and Trp17 has been invoked to be functionally important for NC binding to its packaging signal. It is assumed that, in the absence of coordinated Zn(II), the adjacent disposition of the indole side chains in the unfolded zinc fingers would be much less likely. Indeed, apo-p8 binds nucleic acids with the same number of ionic interactions as the Zn(II)-chelated protein, but with a dramatic loss of hydrophobic stabilization, Trp fluorescence quenching, and specific sequence recognition ability. It is worth noting that, given that the two zinc fingers in NCp7 account for the vast majority of the electrostatic and non-electrostatic binding free energy in the association of SIV NCp8 (or HIV-1 NCp8) with nucleic acids, we would predict that a double zinc finger peptide would also be capable of recognizing specific sequences with specificity approaching that of the full-length protein. For HIV-1, sequence-specific RNA binding occurs with affinity only slightly higher than that seen with homologous DNA sequences (13). SIV NCp8 binds to stem-loop sequences that are contained within the HIV-1 Psi packaging site with affinity comparable to NCp7 from HIV-1. This observation, the similar thermodynamic properties of SIV NCp8 and HIV-1 NCp7 and their sequence homology strongly suggest that the structure of its protein-nucleic acid complexes resem-

5 M. A. Urbaneja and J. R. Casas-Finet, unpublished results.

In summary, SIV NCp8 and HIV-1 NCp7 are nearly identical in the nucleic acid binding properties characterized so far. Both NC proteins bound with similar size comparable affinity to a given lattice, and showed identical specific sequence recognition. It has been noted (48) that both HIV-1 and SIV encapsidate genomic RNA by efficient binding to very short regions at the 5′ end of the viral RNA containing similar secondary structures. The similarity in functional properties for the two phylogenetically closely related proteins is in agreement with their similar complex structure, as suggested by computer modeling, and may account for the viability of chimeric constructs in which the open reading frame of the HIV-1 nucleocapsid domain of Gag has been substituted by that of SIV.

Apo-NCp7 and apo-NCp8 exhibited a large decrease in affinity and recognized specific sequences poorly. Recent studies (49) of zinc finger mutants in SIV NC protein showed loss of infectivity in in vitro replication assays, albeit the level of RNA packaging was less affected than observed earlier for similar mutations in the MuLV and HIV-1 virions. This may be accounted for, in part, by the higher affinity of SIV NCp8 for nucleic acids (either in its zinc-bound or apo forms), compared with MuLV NCp10 and HIV-1 NCp7. The dramatic loss of affinity observed upon Zn(II) ejection from SIV NCp8 accounts for the good antiviral activity of agents known to react with the zinc finger thiolate sulfurs and to result in SIV inactivation (14, 20, 21). Virions inactivated by NC-targeted reaction with anti-zinc finger reagents are currently investigated as candidate antigens in whole killed vaccine strategies.

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Nucleic Acid Binding Properties of the Simian Immunodeficiency Virus Nucleocapsid Protein NCp8

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