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STRUCTURE/FUNCTION MAPPING OF AMINO ACIDS IN THE N-TERMINAL ZINC FINGER OF THE HUMAN IMMUNODEFICIENCY VIRUS TYPE 1 NUCLEOCAPSID PROTEIN: RESIDUES RESPONSIBLE FOR NUCLEIC ACID HELIX DESTABILIZING ACTIVITY*

Nirupama Narayanan¹, Robert J. Gorelick², and Jeffrey J. DeStefano^{1,+}

¹Department of Cell Biology and Molecular Genetics, University of Maryland, College Park, MD

²AIDS Vaccine Program, SAIC-Frederick, Inc., NCI at Frederick, Frederick, MD

Abstract

The nucleocapsid protein (NC) of HIV-1 is 55 amino acids in length and possesses two CCHC-type zinc fingers. Finger one (N-terminal) contributes significantly more to helix destabilizing activity than finger two (C-terminal). Five amino acids differ between the two zinc fingers. To determine at the amino acid level the reason for the apparent distinction between the fingers, each different residue in finger one was incrementally replaced by the one at the corresponding location in finger two. Mutants were analyzed in annealing assays with unstructured and structured substrates. Three groupings emerged: (1) those similar to wild type levels (N17K, A25M), (2) those with diminished activity (I24Q, N27D), and (3) mutant F16W which had substantially greater helix destabilizing activity than wild type. Unlike I24Q and the other mutants, N27D was defective in DNA binding. Only I24Q and N27D showed reduced strand transfer in *in vitro* assays. Double and triple mutants F16W/I24Q, F16W/N27D, and F16W/I24Q/N27D all showed defects in DNA binding, strand transfer, and helix destabilization, suggesting that the I24Q and N27D mutations have a “dominant negative” effect and abolish the positive influence of F16W. Results show that amino acid differences at positions 24 and 27 contribute significantly to finger one’s helix destabilizing activity.

Keywords

Nucleocapsid protein; NC; zinc finger; helix-destabilization; HIV replication

INTRODUCTION

Human immunodeficiency virus belongs to the lentivirus genus of retroviruses. The mature virion has two copies of the RNA genome, which are coated by a 55 amino acid, highly

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+Corresponding author: Address: Department of Cell Biology and Molecular Genetics, University of Maryland, Building 231, College Park, MD 20742 (p) 301-405-5449; (f) 301-314-9489; (e) jdestefa@umd.edu.

basic nucleocapsid protein (NC)¹. NC is produced as a result of proteolytic cleavage of the Gag polyprotein upon maturation of the virion. Because of its high lysine and arginine content (15 total), NC has a net positive charge of 13 and a pI between 10.0 and 11.0. All known orthoretroviruses contain one or two -Cys-X₂-Cys-X₄-His-X₄-Cys- (CCHC) motifs. HIV NC contains two CCHC motifs, each of which is bound to a zinc ion with high affinity (Fig. 1). The two zinc fingers of NC are connected by a highly basic linker (RAPRKKG) (1–3).

NC acts as a nucleic acid chaperone (3, 4). Upon binding to nucleic acids it can transiently break base pairs and catalyze their reformation, thereby enabling them to assume optimal conformations with the maximum number of base pairs (5). Results indicate that NC is involved at multiple steps in the viral life cycle and has several functions including: coating and protecting the viral genome, recognition and packaging of the genome (6–11) and enhancing binding of the host tRNA primer to the viral primer binding site (as part of the Gag precursor protein) (12–15), promoting dimerization between the two genomes within the viral capsid (16–18), increasing the processivity (average number of nucleotides added to the primer in a single binding event between primer-template and enzyme) of RT (19–21), stimulating and modulating RNase H activity of RT (22, 23), enhancing strong-stop minus and plus strand transfers as well as viral recombination in general (24–30), and integration and protection of the newly synthesized viral DNA (31–34).

Both the rigid fingers and flexible backbone regions of NC play important roles in its function. The zinc finger architecture of NC does not seem to play a significant role in tRNA annealing. In vitro studies conducted using NC mutants revealed that a mutant in which the CCHC zinc binding motifs of the zinc fingers are replaced by SSHS motifs (SSHS NC), is comparable in performance to wt NC in enhancing tRNA annealing to the PBS (35). However, recent studies suggest that this may result from the mutual cancellation of two factors: SSHS NC is less effective than wild-type NC as a duplex destabilizer, but more effective as a duplex nucleating agent due to increased flexibility (36). Many zinc finger mutants also retain most of the non-specific nucleic acid binding ability of wild type NC (37). Specific finger mutants with little apparent helix destabilizing activity can also promote the annealing of non-structured nucleic acid complementary strands, presumably because they retain wild type NC's ability to aggregate nucleic acids (38). In contrast, in vitro analysis indicates that the fingers are involved in specific recognition of the viral genomic RNA ψ signal element (39, 40), and other functions including minus strand strong stop transfer and recombination in general (41, 42). Cellular infections with NC mutants have also demonstrated the pivotal role of both fingers in packaging, dimer formation, and general viability (43). Despite this, NC can apparently tolerate significant changes at the amino acid level without drastic effects on function. A recent study revealed that six of seven zinc fingers, found in a family of human cellular nucleic acid binding proteins can replace the first zinc finger of HIV-1 NC with very little loss in replication (44).

The ability of NC to destabilize strong helix structures also requires zinc fingers, in particular finger one (38). In vitro assays showed that mutant NC proteins with two copies of finger one where finger two is replaced by a second copy of finger one (termed 1.1), or with an inactivated finger two where the CCHC motif is replaced by SSHS, retain helix

destabilizing activity. In contrast, mutants with two copies of finger two (2.2) or replacement of finger one CCHC with SSHS have little unwinding activity (38). A finger switch mutant with the sequences of the fingers reversed (termed 2.1), also retained partial helix destabilizing activity suggesting that both context and the finger amino acid composition play important roles (38). Interestingly, virus clones with the 1.1 mutation, though clearly defective, packaged RNA at near wild type levels and reverted over time to a virus with wild type phenotype. Viral clones with 2.2 or 1.2 mutations were highly defective in packaging and showed no reversion (45). Overall the results show that finger one but not two is pivotal to NC's helix destabilizing activity.

Of the 14 amino acids that compose each finger, five differ between the two zinc fingers (in the pNL4-3 clone of HIV-1): (finger one to finger two): phenylalanine to tryptophan (F to W), asparagine to lysine (N to K), isoleucine to glutamine (I to Q), alanine to methionine (A to M), and asparagine to aspartic acid (N to D) at positions 16, 17, 24, 25, and 27 of finger one, respectively. Presumably these differences underlie the much stronger helix destabilizing activity of finger one. To understand this at the amino acid level, we replaced residues in finger one with those at the corresponding locations in finger two. Each mutant was analyzed in annealing assays with unstructured and structured substrates and in strand transfer and nucleic acid binding assays. To our surprise replacing the phenylalanine in finger one with tryptophan from finger two (F16W) actually enhanced helix-destabilizing activity. Two changes were essentially neutral (N17K and A25M) while two others (N27D and I24Q) showed strongly negative effects. Combining the F16W mutation with one or both of the negative mutations also produced a protein with little unwinding activity. Results show that the isoleucine at position 24 and asparagine at 27 contribute most significantly to the difference between the two fingers with respect to helix destabilizing activity.

MATERIALS AND METHODS

Materials

DNA oligonucleotides for the fluorescence resonance energy-transfer (FRET) assay were purchased from Integrated DNA Technologies (Coralville, IA). T4 polynucleotide kinase and RNase-free DNase I were from New England Biolabs (Ipswich, MA). Taq polymerase was from Eppendorf North America (Westbury, NY). SP6 polymerase, dNTPs, and DNase-free RNase were from Roche Applied Science (Indianapolis, IN). RNase inhibitor was from Promega Corp. (Madison WI). Radiolabeled [γ - 32 P] ATP was obtained from Amersham Biosciences (Piscataway, NJ) and Sephadex G-25 spin columns were from Harvard Apparatus (Holliston, MA). PCR primers for the strand transfer assay were purchased from Integrated DNA Technologies. DE-81 filters and cellulose nitrate membrane filters were from Whatman Inc. (Florham Park, NJ). Recombinant HIV-RT was purchased from Worthington Biochemical Corp. (Lakewood, NJ). Aliquots of HIV-RT were stored at 80°C, and a fresh aliquot was used for each experiment. All other chemicals were from Sigma Aldrich (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA).

Preparation of wt and mutant HIV-1 nucleocapsid protein—Wild-type HIV-1 NC from either the MN strain (GenBank accession number: M17449), the ARV strain or

pNL4-3 was used in this study. Wild-type MN NC was expressed and purified as described (46). The construct that expresses wild-type ARV NC (GenBank accession number: K02007) was graciously provided by Dr. Charles McHenry (University of Colorado) and this protein was prepared as described previously (47). Wild type and mutant NC proteins from the pNL4-3 sequence (GenBank accession number AF324493) were prepared essentially as described (32). For the mutant NC proteins, the following sequence changes were made in the gene coding for NC (at the nucleotide level of the pNL4-3 sequence): F16W (t1967g/c1968g), N17K (t1971a), I24Q (a1990c/t1991a), I24E (a1990g/t1991a), A25M (g1993a/c1994t/c1995g), N27D (a1999g/t2001c), and mutants with multiple changes were made with combinations of the above listed changes. The three wild type NC proteins differ by no more than five amino acids, which are all functionally conserved. NC aliquots were stored at -80°C in 50 mM Tris-HCl (pH 7.5), 10% glycerol, and 5 mM 2-mercaptoethanol.

FRET assay to detect DNA/DNA annealing—The 5' end of DNA oligonucleotides were tagged with a fluorescein-6-carboxamidoethyl (FAM) label. The complementary DNAs were obtained with a 4-[(4-dimethylamino) phenyl]-azo]benzenesulfonamido (DABCYL) moiety at the 3' ends. The annealing experiments were carried out using a Cary Eclipse fluorescence spectrophotometer (Varian, Inc., Palo Alto, CA). The final concentrations of the FAM and DABCYL DNAs were 5 and 10 nM respectively. FAM and DABCYL complements in the presence or absence of 2 μ M (unless otherwise indicated) wt/ mutant NC protein, were separately preincubated in 35 μ l of buffer containing 50 mM Tris-HCl (pH 8.0), 1 mM DTT, 1 or 6 mM $MgCl_2$ (as indicated), 80 mM KCl, and 25 μ M $ZnCl_2$ for 5 min at 30°C. The solutions were then mixed in a quartz cuvette to start the time course reaction. The time course was monitored over 4 min for the unstructured (0.0dna) and the 5.8dna substrates (see Fig. 2). With the 9.0dna significant annealing could be observed only by 16min. The FAM molecule was excited at 494 nm and fluorescence emissions were observed at 520 nm. Recordings were made every 10s for the 4 min and every minute for the 16 min time course. The intensity ratio (I_t) was obtained by dividing the peak intensity at every time point (I_t) by the peak intensity observed at time zero (I_0). Plots of I_t vs. time were then constructed. Annealing assays with each wt NC and NC mutants were performed at least three times and an average of the results was used for constructing the plots.

0.0dna end labeling for filter binding assay—Fifty pmoles of the 0.0dna was labeled at the 5'-end with [γ - ^{32}P] ATP using T4 polynucleotide kinase according to the manufacturer's protocol. The labeled DNA was then passed over a hydrated Sephadex G-25 spin column to remove any random dNTPs, and processed according to the manufacturer's protocol, then stored at -20°C.

Nitrocellulose filter binding assay to monitor binding of wild type and mutant NC proteins to nucleic acids—Whatman nitrocellulose membrane filters with a pore size of 0.2 μ m were presoaked for 15 min in nitrocellulose binding buffer (NB buffer: 50 mM Tris-HCl (pH 8.0), 1 mM DTT, 6 mM $MgCl_2$, 80 mM KCl, and 25 μ M $ZnCl_2$). NC (0.047–2 μ M) was mixed with 0.0dna (1 nM) in 10 μ l of NB buffer plus 0.1 μ g/ μ l BSA and incubated for 5 min at room temperature. The entire reaction was spotted onto the center of a

freshly-pres soaked nitrocellulose membrane filter. The filter was then subjected to vacuum and washed 3× with 1 ml of wash buffer consisting of 10 mM Tris-HCl (pH 8.0) and 10 mM KCl. The filters were then air dried. The dried filters were counted using a LKB Wallac 1209 Rackbeta liquid scintillation counter. The fraction of the total substrate that bound to the nitrocellulose filter was then calculated as follows: The counts obtained for each concentration of NC used was initially subtracted from background (determined using a reaction without NC). This value was then divided by the total counts added to the reaction (calculated by counting a reaction applied to a DE81 filter that was not presoaked or washed) to obtain the fraction of the total substrate bound. A plot of fraction substrate bound vs. NC concentration in the reaction was then constructed for each NC protein.

PCR amplification of DNA substrates for strand transfer assays—Two sets of primers (5'-**gatttaggtgacactatag***atata*gaatgtggaaagga-3' and 5'-ttgtgtctctaccccagac-3' for the donor and 5'-**gatttaggtgacactatag***tata*cccctaggaaaaagggtgt-3' and 5'-ctgaagctctctctgtgg-3' for the acceptor) were designed to generate the donor and acceptor RNAs derived from the pNL4-3 plasmid (obtained from NIH AIDS Research and Reference Reagent Program). An SP6 promoter sequence (in bold above) was added to the forward primer along with 5 additional non homologous nucleotides (in italics) to prevent transfer of DNA products from the end of the donor to the acceptor. One hundred pmoles of each primer was used in the PCR reactions in a volume of 100 µl including 0.1 µg plasmid DNA, 5 units Taq polymerase, 10 mM Tris-HCl (pH 8.3 @ 25°C), 50 mM KCl, 1.5 mM MgOAc. The cycling parameters used were as follows: 35 cycles of successive denaturation, annealing and extension reactions were carried out for 1 min each at 94, 50 and 72°C, respectively. This was followed by one 5 min extension cycle at 72°C. The PCR products were then resolved on an 8% native polyacrylamide gel (48). Excised gel slices containing DNA were eluted overnight in 550 µl of 10 mM Tris-HCl (pH 7.5), 1 mM EDTA (pH 8.0). The eluate was filtered through a 0.45 µm cellulose acetate syringe filter and DNA was precipitated with two volumes of ethanol and 1/10th volume 3 M NaOAc (pH 7.0) and resuspended in water

Preparation of RNA substrates—DNA products obtained by the PCR described above were used to generate donor and acceptor RNA transcripts with SP6 RNA polymerase (Roche Applied Science) using the manufacturer's protocol. Approximately two µg of the purified PCR DNAs were used. The transcription products were digested with 20 units of RNase free DNase I for 15 min to remove any remaining template DNA. Reactions were then extracted with phenol:chloroform:isoamyl alcohol (25:24:1) and precipitated with ethanol. The pellet was resuspended in 70 µl of water and centrifuged through two successive hydrated G-25 spin columns according to the manufacturer's protocol. The RNA was then quantified by measuring absorbance at 260 nm in a Pharmacia Biotech Gene Quant II RNA/DNA spectrophotometer (Piscataway, NJ). The integrity of the RNAs was analyzed to ensure it was comprised of predominately fully extended RNAs by running approximately 40 pmoles on an 8% denaturing polyacrylamide gel and staining with ethidium bromide.

Preparation of RNA/DNA hybrids—DNA primers were labeled at the 5' end with γ-P³²ATP as described above. The donor RNA and the labeled primer DNA were mixed in 10

μl of 50 mM Tris-HCl (pH 8.0), 1 mM DTT and 80 mM KCl. The donor:primer ratio used was 1:5. The donor/primer reaction was heated to 65–70°C for 5 min and then slow cooled to room temperature.

Strand transfer assay and NC time course reaction—Hybrids from above were preincubated with acceptor RNA in the presence/absence of wild-type or mutant NC proteins for 1 min in 42 μl of reaction buffer (described below). HIV-1 RT (8 μl at 0.5 pm/μl) was added to start the time course. Final concentrations of reagents in the reactions were: 80 nM RT, 2 nM donor -10 nM primer, 10 nM acceptor, 2 μM NC, 50 mM Tris-HCl (pH 8.0), 80 mM KCl, 6 mM MgCl₂, 5 mM AMP (pH 7.0), 1 mM DTT, 25 μM ZnCl₂, 100 μM dNTPs, and 0.4 units/μl RNasin. Six μl aliquots were taken out at 2, 4, 8, 16, 32 and 64 min and stopped by the addition of 4 μl of 25 mM EDTA (pH 8.0) and 5 ng of DNase-free RNase and incubated for additional 15 min at 37°C. Two μl of proteinase-K solution (2mg/ml proteinase-K, 10 mM Tris-HCl (pH 8), 15 mM EDTA (pH 8), 1.25% SDS) was then added to all of the samples, which were incubated at 65°C for 45 min to digest protein. Twelve μl of 2X formamide dye (90% formamide, 10 mM EDTA (pH 8.0), 0.1% xylene cyanol and 0.1% bromophenol blue) was then added to each sample. The samples were electrophoresed on 8% denaturing polyacrylamide gels (48). Dried gels were imaged and quantified using a Bio Rad FX Pro Plus molecular imager with Quantity One software (Hercules, CA).

RESULTS

Structure of DNA substrates for annealing assay

All of the DNA substrates were 42 nucleotides in length but differed in sequence composition. Structures were designed using mfold. RNA versions of the substrates had been used in earlier experiments (38). The predicted folded structures are shown in Fig. 2A. Only one of the two complementary strands is shown for each substrate. The Gibbs free energy of unfolding (ΔG) is indicated next to the corresponding structure and each structure is referred to throughout the text by the energy value followed by “dna” (for example, 9.0dna). ΔG values for the complementary DNA strands were also determined using mfold and are indicated in the legend. All of the substrates formed a stem-loop except 0.0dna which was predicted by m-fold analysis to have no structure. The binding strength of the stem in the substrates was increased from one substrate to the next. Successive GT repeats were avoided, since NC has been shown to have a preference for GT repeats (49). Because of the stem-loop structure of the substrate, the complementary strands must be unwound before they can completely hybridize. The assay essentially tests the ability of NC protein to facilitate annealing by aiding in the unwinding process. Substrate 0.0dna tests the ability of NC to accelerate annealing in the absence of structure. In this case the aggregation/condensation activity of NC is presumably responsible for the observed rate increase (38).

Annealing assays performed with wt and NC finger mutants—Work previously done in this and other laboratories has shown that the first zinc finger of NC is primarily responsible for unwinding nucleic acid secondary structures (helix-destabilizing activity), while the second finger plays an accessory role (see Introduction). We wanted to determine what residues in finger one were responsible for its unwinding advantage over finger two.

Figure 1 shows a schematic diagram of HIV-1 pNL4-3 NC protein. There are five amino acids which differ between the two finger sequences. These include (finger one to finger two): F to W, N to K, I to Q, A to M, and N to D at positions 16, 17, 24, 25, and 27 of finger one, respectively. NC point mutants were constructed where the amino acid residues in finger one were incrementally replaced by those at the corresponding locations in finger two. Double and triple NC mutants were also constructed, which contained two or three mutations in finger one (See “Experimental procedures”). Finger mutants in which the CCHC zinc coordinating amino acids in the fingers were replaced by SSHS were also tested for their unwinding activities. Three SSHS mutants were used; SSHS1 had the three cysteines in finger one replaced by three serine residues, SSHS2 had the three cysteines in its second zinc finger replaced with serine residues, and SSHSd had the cysteines in both fingers replaced with serines. These SSHS finger mutants allowed us to confirm previous findings about the roles of the fingers with respect to annealing and helix destabilization. In addition NC finger mutants 1.1 and 2.2 were also tested (see Introduction). Annealing was detected by FRET as described under “Experimental Procedures”. Assays were performed with mutant and wild-type NC proteins using the various substrates.

The role of the two zinc fingers of NC was previously investigated in our laboratory on an rna/dna hybrid gel shift annealing assay (38). However we decided to test them using the dna/dna FRET-based system. The effect of the NC finger mutants with the 0.0dna, 5.8dna, and 9.0dna substrates is shown Table 1. For each NC protein (2 μ M final concentration) and DNA substrate, a rate constant for complement annealing was calculated by fitting the intensity profile data to a semi-logarithmic plot (30). This is illustrated for one of the point mutants and wild type NC in Fig. 2F. With the unstructured substrate (0.0dna) the complementary nucleic acids annealed very rapidly even in the absence of NC, and wild type NC clearly enhanced annealing even further resulting in about a 2-fold increase in the rate constant (Table 1, see Fig. 2B also). This demonstrates NC’s ability to enhance annealing even in the absence of secondary structure, presumably by aggregation/condensation. All of the finger mutants enhanced annealing with this substrate in comparison to reactions without NC. There were some small differences with SSHSd showing the least stimulation, however, all of the mutants appeared to retain aggregation/condensation activity based on this assay. This is consistent with the idea that this activity results mostly from the highly positively charged NC backbone amino acids (4) that act to neutralize negative charges on the phosphate backbone of the nucleic acids.

The structured DNA substrates (5.8 and 9.0dna) showed much slower annealing rates than 0.0dna, presumably because they require helix destabilization in order to anneal. Clear groupings emerged with these substrates. Very little annealing was observed in reactions without NC and no rate values were obtained. All the finger mutants showed some stimulation. In general the annealing rates were about 4–7 fold slower on 9.0dna than 5.8dna reflecting the greater stability of the former. Consistent with previous results, NC mutants without an active finger one (2.2, SSHS1, and SSHSd) were clearly more defective in helix destabilization. These mutants showed an annealing rate about 20–30% of the wild type level. Results support earlier findings indicating that the first zinc finger of NC is required for unwinding of strong secondary structures. Further, the various SSHS mutants behave

very similarly to the corresponding finger mutants that contain only one of the two zinc finger sequences (1.1 and 2.2).

Effect of NC point mutants on annealing of structured and unstructured DNAs

—Figure 2B and Table 1 show the effects of various NC point mutants on annealing of the unstructured 0.0dna detected by FRET. As with the finger mutants a clear distinction was observed between reactions with and without NC; NC clearly enhanced annealing. There was no obvious difference between the point mutants. This was expected given that the more highly mutated finger mutants showed no strong differences with this substrate.

Figure 2C and Table 1 show the effect observed with the point mutants on 5.8dna. On this substrate clear groupings emerged. Mutants N17K and F16W were able to stimulate annealing as well as wt NC. Mutant A25M showed some reduction in annealing in comparison to N17K and F16W, but was clearly better than N27D and I24Q. The latter two showed about a 60–70% rate reduction in comparison to wild type NC.

The same general pattern was observed with the strongest structure, 9.0dna (Fig. 2D and Table 1). This assay was performed over 16 min rather than 4 min as with 0.0dna and 5.8dna because of the much slower annealing rate. Once again N17K was similar to wild type and A25M showed a small reduction in activity. Mutants N27D and I24Q showed reduced stimulation with about 50% the rate of wild type. Interestingly, the F16W mutant annealed much better than wt NC (Fig. 2E) showing about a 4-fold increase in annealing rate. This was also the case for an even stronger 42 nucleotide DNA substrate that folded with a ΔG value of -15.8 kcal/mole (15.8dna). On this substrate wt NC stimulated annealing less than half as well as F16W (data not shown).

Optimal magnesium concentrations (6 mM) for reverse transcriptase assays were used in the above described experiments. However, the concentration of non-complexed magnesium in cells may be much lower (50) and results have shown that NC is more active with lower ionic strength (14). Wild type, N17K, I24Q, and N27D NC proteins were also tested using 1 mM magnesium on 5.8dna (Table 1). As expected the annealing rates increased about 3–4 fold with the lower magnesium concentration. Differences between the wild type and mutant proteins remained consistent at both concentrations, indicating that the magnesium concentration was not a factor in this observation.

An NC titration was also performed using 5.8dna and each of the above mutants (Table 2). For wild type NC, the rate of annealing was proportional to the [NC] up to 4 μ M NC. Increasing to 8 μ M had little effect. This was also observed with N17K. The more defective mutants (I24Q and N27D) also showed proportional increases but did not saturate at 4 μ M NC. For both mutants rate values at a given NC concentration were always significantly lower than wild type NC.

Effect of double and triple mutants on annealing of structured and unstructured DNA substrates

—Results from the single point mutants in annealing assays showed that two of the mutants, N17K and A25M, were comparable to wild type with the latter being slightly less stimulatory. In contrast, I24Q and N27D mutants were

inhibitory while F16W showed enhanced activity on highly structured substrates. Therefore, of the five different amino acids between fingers one and two, only two (I24 and N27) seemed to be important to finger one's advantage in helix-destabilization. One change (F16W) actually indicated that finger two has a more effective amino acid for helix-destabilization at that position. It is important to note that single point mutations ignore the possibility of "context effects" in contributing to the activities of the fingers. A particular amino acid could function differently in the context of one group of amino acids vs. another. Testing all combinations of mutations in which amino acids in finger one are replaced by corresponding ones from finger two, would require 30 separate mutants, many of which would yield little information. We took a directed approach based on the results from the single mutants. Our working hypothesis was that the changes at positions 24 and 27 cause finger one to lose helix-destabilizing activity and these would have "dominant negative" effects with respect to the apparent gain in activity from the F16W change. This would explain why finger two (as judged from mutants 2.2 and SSHS1) shows little destabilizing activity. To test this, two double mutants (F16W/I24Q and F16W/N27D) containing the positive change along with each negative change, and a triple mutant (F16W/I24Q/N27D) which combined the positive mutant with both negatives were constructed.

On the non-structured 0.0dna substrate (Table 1) the double and the triple mutants displayed a similar annealing pattern with a slightly slower rate than wild type but a clear increase over reactions without NC. Again, the results suggest that the mutants retain most of the aggregation/condensation activity of NC. In contrast, these mutants were all highly defective on the structured substrates and were comparable to 2.2 and SSHSd (Table 1). Overall the results support the hypothesis and suggest that I24 and N27 are pivotal to the helix-destabilizing function of finger one. Changes in either position to the corresponding amino acid in finger two essentially mimic replacing finger one with finger two (2.2) or deactivating finger one (SSHS1). These changes also completely mask the strong destabilizing activity of the phenylalanine to tryptophan mutation at position 16 of finger one (F16W).

Binding of NC mutants to nucleic acid substrates detected by nitrocellulose filter binding assay

—One possible reason for the observed defects in some of the mutants could be reduced binding affinity for the substrate nucleic acid. To determine if any of the mutations affected the affinity of NC for DNA, filter binding assays were performed. Figures 3A and 3B show graphs obtained by plotting the fraction of total amount of substrate in the assay that bound to nitrocellulose filters at various NC concentrations. The FAM-derivatized complement of 0.0dna made without the FAM group, but 5' end labeled with P-32 was used for the assays. The three mutants that are comparable to wild type in the annealing assays (F16W, N17K and A25M) also show similar levels of DNA binding. In contrast, of the mutants that showed reduced activity in the annealing assays (I24Q, N27D, F16W/I24Q, F16W/N27D, and F16W/I24Q/N27D), only I24Q (Fig. 3A) was comparable to wt NC in binding. The other four showed significantly lower affinities than wild type (Fig. 3B). Consistent with these results, finger mutant 2.2 but not 1.1 showed reduced binding in the assay (data not shown). It was interesting that the double mutant F16W/I24Q showed reduced binding despite neither of the single mutants showing reductions. Mutants I24Q,

and N27D were also compared to the wild type for binding to 9.0dna in filter binding assays. The apparent binding affinity to this structured substrate was lower for all the NC proteins as more NC was required to retain a comparable amount of substrate on the filters. Consistent with binding to 0.0dna, only N27D showed reduced binding in comparison to wild type. Overall the results indicate that mutations that reduce NC binding lead to an apparent reduction in helix-destabilizing activity as expected. However, this activity is not solely determined by binding as I24Q binds similar to wild type but is defective in helix-destabilization.

Effect of NC mutants on strand transfer—An *in vitro* strand transfer assay has previously been used in our laboratory to investigate recombination in different regions of the genome (51). Figure 4A shows a schematic model of the system. The donor and acceptor RNA templates represent the two RNA strands of the viral genome. For these experiments, the templates were derived from a highly structured region of the HIV genome that included the *gag-pol* frameshift sequence (41). DNA synthesis is primed at the 5' end of the donor by a 5' end labeled DNA primer. The donor and acceptor RNA strands are designed to have a 150 base pair region of homology where strand transfer can potentially occur. The transfer zone is indicated by a dotted box. Full length DNA products from DNA synthesis directed by the donor would be 175 nucleotides in length. Products made after transfer and extension on the acceptor would be 197 nucleotides in length. This assay tests for internal strand transfers since transfers from the end of the donor were prevented by adding a 5 nucleotide non-homologous region to the 5' end of the donor template. Transfer could also occur between two donor templates. However, this would be negligible since the amount of acceptor RNA used was five times in excess over the donor RNA. The percent of transfer efficiency was calculated by: $\{\text{transfer DNA products}/(\text{transfer DNA products} + \text{full length donor-directed DNA})\} \times 100$.

Figure 4B shows an autoradiogram obtained from a strand transfer assay performed with the *gag-pol* substrate using F16W/I24Q/N27D and F16W/N27D mutants. The positions of transfer (T) and donor-directed products (D) are indicated as is the primer position (P). The four sets of assays show reactions (from left to right) without NC, those with wild type NC, and those with F16W/I24Q/N27D and F16W/N27D. The six lanes for each set were reactions stopped after 2, 4, 8, 16, 32, and 64 min from left to right. Strand transfer was greatly enhanced upon the addition of wt NC. A graph of % transfer efficiency vs. time for the double and triple mutants, and wild type is shown in Fig. 4C. All the mutants enhanced transfer considerably compared to reactions without NC. However there was clearly less enhancement, especially at early time points, in comparison to wild type NC. Graphs for the other single point mutants are shown in Figs. 4D and 4E. Mutants N17K, A25M and F16W were comparable to wt NC with N17K showing slightly more stimulation. Mutants I24Q and N27D were less stimulatory than wild type with N27D showing the lowest stimulation. Again, the differences were more evident at the early time points. The results indicated that even the mutants which show low helix-destabilizing activity enhance strand transfer considerably on this highly structured substrate. However, those with near wild type levels of unwinding activity showed more stimulation.

DISCUSSION

In this report we attempt to understand at an amino acid level why zinc finger one is more important than finger two to the helix destabilizing activity of HIV-1 NC (see Introduction). The approach used was to make mutations in finger one using the corresponding amino acids in finger two (see Fig. 1). Five of the 14 total amino acids in each finger are different. Of the 5 changes, three of them represent a significant chemical alteration including two neutral to charge changes: (finger one to two) N17 to K38 and N27 to D48, and a hydrophobic to polar change: I24 to Q45. The other two are amino acids of similar hydrophobicity with those in finger 2 being more bulky: F16 to W37 and A24 to M46. Overall the five changes result in finger one being considerably more hydrophobic than two. The results showed that only I24Q and N27D mutations significantly decreased NC's helix destabilizing activity while A25M caused a small reduction. The N17K mutation had no measurable effect while F16W imparted greater helix destabilizing activity on NC. Double and triple mutant combinations with F16W combined with I24Q and N27D showed that the latter two were "dominant negatives" in that all the combination mutants were highly defective in both binding DNA and helix destabilization.

The simple approach of drawing conclusions from altering just these 5 amino acids partly ignores potentially important context effects. For example, NMR studies have shown that several interactions occur between finger one amino acids and other parts of the NC protein during binding to specific HIV stem-loop structures (6–8, 11). These include for example, F16 and N17 interacting with W37, I24 with valine and phenylalanine residues at positions 13 and 6, respectively, and N17 interacting with proline and lysine at positions 31 and 33. Also, recent results implicate finger two in helix destabilizing as a W37 to leucine mutation in finger two greatly decreased NC binding to nucleic acids and helix destabilizing activity on the HIV TAR region (52). Clearly, several interactions between finger one and two, and finger one and non-finger amino acids in NC are important to its biological functions, however, results suggest they may be less important to chaperone and helix destabilizing activity. Experiments show that finger mutants without an active finger two (1.1 or SSHS2) are still potent chaperone proteins *in vitro* and retain nearly wild type helix-destabilizing activity (see Introduction). In addition, truncated NC proteins missing the first 12 N-terminal non-finger amino acids (NC 12–55) showed strong helix-destabilizing activity. A double switch mutant in which F16 is replaced by tryptophan and W37 by phenylalanine retained most of its binding and helix destabilizing activity on TAR implying that the aromatic nature of the amino acids rather than a specific residue at a specific position are important to activity (52). This is further supported by the high activity of NC 1.1 which has a phenylalanine at positions 16 and 37.

The genetic flexibility of NC is further illustrated by cell culture experiments using mutated viruses. In one report, replacement of finger one with 7 different zinc fingers from a family of human cellular nucleic acid binding proteins (CNBPs) resulted in infectious viruses in 6 of the 7 cases (44). In another report, alanine scanning mutagenesis was performed on finger one (53); excluding F16 and the four amino acids that are part of the zinc binding motif (C15, C18, H23, and C28), alanine mutagenesis of the other 9 amino acids (in one case alanine 25 was changed to glycine) produced viruses with essentially wild type infectivity

that in some cases showed mild to moderate RNA packaging defects. Although F16A was defective, an F16W mutant was comparable to wild type virus, a result that correlated with our finding that F16W is as good or better than wild type in helix destabilization and strand transfer. Also interesting with respect to our results was the finding that N27 could be changed to alanine with little effect on infectivity. Although this position is fairly highly conserved, HIV isolated with serine, histidine, isoleucine, and tyrosine at this position have been reported (HIV genome sequence information was obtained from the HIV sequence database provided by Los Alamos National Laboratory (<http://hiv-web.lanl.gov/content/hiv-db/mainpage.html>)). The N27D mutant in our studies was quite defective in DNA binding and helix destabilization, but this is a much more drastic change than those changes noted above. The NC protein of the virus used in the above study had a threonine at position 24 rather than isoleucine as is present in the strain we used, but the fact that it could be changed to alanine without loss of infectivity suggests some flexibility at this position. This position is not as strongly conserved as the other 4 examined in our assays. Many HIV isolates have leucine at this position and some have threonine and valine. The I24Q and an I24E (glutamic acid) mutant that we also tested (data not shown) were quite defective in helix destabilization and the latter bound DNA poorly. Perhaps there is a limit to how polar the amino acid at this position can be. Isoleucine 24 along with V13, F16, and A25 have been implicated as part of an important hydrophobic cleft in finger one that is required for binding and destabilizing nucleic acids (47). The fact that some virus strains have threonine at position 24 and that alanine can be substituted without loss of viability suggests that the amino acid at this position need not be highly hydrophobic. It is also possible that NC proteins with reduced helix destabilizing activity may be functional during infection. The NC protein of Moloney murine leukemia virus (M-MuLV) has very little helix destabilizing activity in *in vitro* assays (54) and it seems unlikely that the 6 functional fingers from the human CNBP proteins (see above) would all have high destabilizing activity. Therefore it is possible that HIV-1 NC could get by with less, as long as other important NC functions were not compromised. We are in the process of testing the mutants made in these experiments in the context of cellular infections. Preliminary experiments suggest that there is no defect in replication with N27D mutant viruses in H9 cells (data not shown); supporting the hypothesis that reduced helix destabilizing activity is not detrimental to virus fitness. It would also be interesting to test the helix destabilizing activity of viable mutations at position 24 as well.

The N17K mutation tested here has also been examined in cell culture infections (55). Interestingly, viruses with this mutation show no replication defects and actually have a 7–9 fold increased transduction frequency compared to wild type. In addition to packaging viral RNA better than wild type, there was also an increased packaging of non-viral RNA. Several other mutations in the finger amino acids that increased the net positive charge of the fingers also lead to viable viruses with transduction levels as good as or better than wild type. The N17K mutation was essentially equivalent to the wild type in all the assays we tested it in so it is not surprising that it would function well. Despite this, BLAST searches recovered no isolated viruses with N17K mutations, suggesting that this change is selected against *in vivo* (55).

The strand transfer assays were reasonably consistent with helix-destabilization assays in that those mutants defective in the latter generally showed some decrease in strand transfer. Mutant N27D and the double and triple mutants (F16W/N27D and F16W/I24Q/N27D) containing this mutation appeared to be the most defective while I24Q and F16W/I24Q were moderately less stimulatory than wild type (Fig. 4). The other point mutants were essentially the same as wild type with N17K showing a small enhancement. The *gag-pol* genome region used in the assays is highly structured (51) and it seems surprising that all the mutants, even the double and triple mutants and N27D which were highly defective in helix-destabilization and DNA binding, showed a clear enhancement. However, previous assays with finger mutant 2.2, which is highly defective in helix destabilization assays (Table 1 and (38)), showed stimulation of strand transfer on the *gag-pol* substrate, albeit at a reduced level compared to wild type. In addition, 2.2 was essentially identical to wild type for stimulation on a low-structured substrate (51). These results suggest that the aggregation/condensation activity of NC, which all the mutants appeared to retain (see Results), is a major driving force for strand transfer while helix destabilization may be less important, especially in low structured genome regions.

From our results, the most important amino acid differences between finger one and two with respect to chaperone activity are I24Q and N27D. Both are extreme chemical changes that strongly decrease the hydrophobic nature of finger one. The overall more hydrophobic nature of finger one could be important to its apparent advantage for helix destabilization. On a mechanistic level, mutant N27D caused a large defect in nucleic acid binding and this alone could have been responsible for its low activity. Low binding may have occurred from repulsion of the nucleic acid by the additional negative charge of the aspartic acid side chain or a dramatic change in protein folding. The latter is unlikely since viral clones with this mutation showed no defect in replication in H9 cells (see above). Mutant I24Q did not significantly alter nucleic acid binding, suggesting that no deleterious change in overall structure had occurred. Perhaps by decreasing the hydrophobicity of finger one, substitution of glutamine for isoleucine reduces NC's ability to interfere with hydrophobic base stacking. This would be consistent with the previously proposed role of the hydrophobic pocket of finger one (see above, (52)).

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ABBREVIATIONS

NC	nucleocapsid protein
HIV	human immunodeficiency virus

RNase H	ribonuclease H
RT	reverse transcriptase
nt(s)	nucleotide(s)
BSA	bovine serum albumin
PNK	T4 polynucleotide kinase
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic
PBS	primer binding site
TAR	trans-activation response element
FRET	fluorescence resonance energy transfer
FAM	fluorescein-6-carboxamidoethyl
DABCYL	4-[[[(4-dimethylamino) phenyl]-azo]benzenesulfonicamino
AMP	adenosine monophosphate
PCR	polymerase chain reaction

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Figure 1. Schematic diagram of HIV-1 NL4-3 nucleocapsid protein

Shown here is the amino acid sequence of HIV-1 NL4-3 NC protein. The five amino acids that differ between the two fingers are italicized and numbered. NC point mutants were constructed by incrementally replacing the amino acid residues in finger one with those at the corresponding locations in finger two. NC double and triple point mutants were also constructed which contained two and/or three mutations in finger one.

Fig. 2B

Fig. 2C

Fig. 2D

Fig. 2E

Fig. 2F

Figure 2. (A–F). Assays to detect helix destabilizing activity of NC

(A) DNA substrates used in annealing assays. The DNA substrates used were 42 nucleotides in length, but differed in their sequence composition. Structures were predicted using mfold with 80 mM Na⁺, 6 mM Mg²⁺, and 30°C. The Gibbs free energy of unfolding in kcal/mol is indicated next to the corresponding structure. The structures were also named in accordance to their ΔG of unfolding. The binding strength of the stem was increased from one structure to the next: 0.0dna did not possess any predicted base pairs; 5.8dna has 14 A-T base pairs and no G-C base pairs; and 9.0dna has 11 A-T base pairs and 4 G-C base pairs. The ΔG values for the complementary sequences were -0.3, -1.2, and -6.6 for 0.0dna, 5.8dna, and 9.0dna, respectively. In cases where more than one structure was predicted, the ΔG for the

most stable structure is reported. (B–E) FRET assays conducted with 0.0dna (B), 5.8dna (C), or 9.0dna (D and E) were carried out at 30°C in the absence or presence of wt or mutant NC proteins as described in Experimental Procedures. The 5'-FAM derivatized DNA (5 nM final) substrates and 3'-DABCYL derivatized (10 nM final) complements were separately preincubated in the presence of wt or finger mutant NC proteins (2 μ M final concentration). These were mixed to start the annealing reaction. Fluorescence was monitored over time using a fluorescence spectrophotometer. The Intensity ratio (I_t) (fluorescence intensity at each time point (I_t) divided by fluorescence intensity at time zero (I_0)) is plotted versus time (see “Experimental Procedures”). The duration of the time course was varied depending upon the substrate used. A control reaction in which no complementary DABCYL DNA was present was also performed. The lines show a representative experiment. At least three independent experiments were used to determine the rates shown in Tables 1 and 2. (F) Semi-log plot of the reaction with wild type and N27D NC constructed from the trace shown in Fig. 2D. The I_t value from the graph was plotted on a log scale vs. time and the data points were fit to a straight line using linear regression analysis (Sigma Plot). The slope of the line was used to determine the $t_{1/2}$ value of the reaction and the equation $k=0.693/t_{1/2}$ was used to calculate the rate constant k (30). At least three k values were obtained for each mutant on each substrate. Rate constant values for this particular experiment are shown next to each line. The average values $\pm$ standard deviations are shown in Tables 1 and 2.

Figure 3. (A and B). Binding of NC mutants to nucleic acid substrates detected by nitrocellulose filter binding assay

Nitrocellulose filter binding assays were carried out to monitor binding affinities of each of the NC mutants to DNA. The unstructured 0.0dna without a FAM group was 5' P-32 end-labeled and used as substrate (final concentration 1 nM). The final concentration of wt NC and mutant NC proteins ranged between 0.047 to 2 μ M. The 0.0dna was separately preincubated with each of the NC dilutions in reaction buffer and applied to nitrocellulose filters that were subsequently washed. The amount of substrate that remained bound to the filter was determined using a liquid scintillation counter. A plot of the fraction total substrate bound to nitrocellulose filters versus various NC concentrations was generated. A representative experiment is shown and each assay was repeated at least once. (A), binding of F16W, N17K, I24Q, and A25M NC mutants; (B), binding of N27D, F16W/I24Q, F16W/N27D, and F16W/I24Q/N27D NC mutants.

Figure 4. (A–E). Strand transfer assay

(A), Schematic model of the strand transfer system. The donor and acceptor RNAs were derived by PCR amplification of the *gag-pol* frameshift region. The 5' end of the 20 nucleotide primer (complementary to the donor) was radio-labeled with P-32 and is indicated with an *asterisk* symbol. The donor and acceptor RNAs were 175 and 177 nucleotides (*nt*) in length, respectively. They are homologous only in the *boxed region* which spans 150 nucleotides. Full length DNA synthesized on the donor RNA template is indicated by *dotted lines*. Transfer DNA synthesized on the acceptor RNA template is indicated by *broken lines*. Note that the donor-directed DNA product is shorter in length in comparison to the transfer DNA product. (B), An autoradiogram obtained from a strand transfer assay carried out on the *gag-pol* substrate using the F16W/I24Q/N27D and F16W/N27D mutants is shown. The assay was performed as described under “Experimental Procedures”. The four sets of assays shown from left to right were reactions carried out without NC, with wt NC, and those with F16W/I24Q/N27D and F16W/N27D mutants. Aliquots from each reaction were stopped at 2, 4, 8, 16, 32, and 64 min and analyzed on a 8% denaturing polyacrylamide gel. The positions of the donor-directed and transfer products and the primer are indicated by D, T, and P respectively. (C–E), Graphs of efficiency of strand transfer *versus* time for different NC mutants are shown. The graphs were made from quantification of experiments as shown in 4B. The % efficiency of strand transfer was obtained using the following formula: Transfer DNA products (T)/ {Transfer DNA products (T) + Full length donor-directed DNA products (D)} * 100, or $(T / (T+D)) * 100$. It was then plotted against time in min. (C), Graph obtained for the double and triple NC mutants; (D), Graph obtained for the N17K, A25M and F16W mutants; (E), Graph obtained for the N27D and I24Q mutants. Each graph also shows results with wt NC and no NC. A representative experiment is shown and each experiment was repeated 1–2 times.

Table 1Rate constant (k) calculation for NC proteins for DNA substrates

<i>I</i> Name	0.0dna	5.8dna	5.8dna (1 mM Mg ²⁺)	9.0dna
-NC	2.0549 ± 0.046	ND	ND	ND
wt NC	1.16 ± 0.05	0.161 ± 0.005	0.733 ± 0.019	0.024 ± 0.001
SSHS2	1.18 ± 0.11	0.149 ± 0.023	ND	0.017 ± 0.001
SSHS1	1.06 ± 0.05	0.048 ± 0.001	ND	0.012 ± 0.001
SSHSd	0.862 ± 0.111	0.026 ± 0.002	ND	0.005 ± 0.001
1.1	1.11 ± 0.05	0.097 ± 0.020	ND	0.018 ± 0.002
2.2	1.11 ± 0.13	0.038 ± 0.002	ND	0.006 ± 0.001
F16W	1.02 ± 0.03	0.191 ± 0.029	ND	0.102 ± 0.009
N17K	1.18 ± 0.02	0.162 ± 0.021	0.566 ± 0.049	0.025 ± 0.002
I24Q	1.15 ± 0.03	0.069 ± 0.008	0.281 ± 0.010	0.012 ± 0.001
A25M	0.957 ± 0.149	0.109 ± 0.035	ND	0.019 ± 0.001
N27D	0.928 ± 0.135	0.049 ± 0.002	0.170 ± 0.022	0.012 ± 0.001
F16WI24Q	0.792 ± 0.010	0.057 ± 0.012	ND	0.006 ± 0.001
F16WN27D	0.838 ± 0.171	0.058 ± 0.022	ND	0.006 ± 0.001
F16WI24QN27D	0.949 ± 0.102	0.058 ± 0.036	ND	0.005 ± 0.001

¹HIV strain pNL4-3 NC was used as backbone for making NC mutants. The SSHS mutants replace the CCHC zinc binding motif with SSHS in finger 2 (SSHS2), finger 1 (SSHS1), or both (SSHSd). 1.1 and 2.2 are mutants with two copies of finger 1 or finger 2, respectively.

² k (rate constant) values were calculated from the $t_{1/2}$ values by dividing 0.693 by $t_{1/2}$ as described in (30). Results are an average of 3–4 experiments $\pm$ standard deviations. Experiments were performed with 2 μ M NC, 6 mM MgCl₂ and 80 mM KCl in standard buffer except for the 5.8dna (1 mM Mg²⁺) column. For the 0.0dna substrate, although experiments were performed over 4 min, only values from the first min were used in calculations due to the rapid kinetics resulting in saturation. –NC values were only determined for 0.0dna since no significant hybridization was observed for the other substrates in the absence of NC. ND-Not determined.

Table 2Rate constant (k) calculation using varied [NC] on 5.8dna substrate^{*l*}

NC (μ M)	Wild type	N17K	I24Q	N27D
8	0.383 ± 0.206	0.268 ± 0.012	0.178 ± 0.028	0.184 ± 0.097
4	0.335 ± 0.055	0.356 ± 0.025	0.091 ± 0.003	0.078 ± 0.041
2	0.161 ± 0.005	0.162 ± 0.021	0.069 ± 0.008	0.049 ± 0.002
1	0.082 ± 0.027	0.069 ± 0.016	0.032 ± 0.008	0.034 ± 0.007
0.5	0.043 ± 0.001	0.053 ± 0.005	0.034 ± 0.001	0.019 ± 0.011

^{*l*}Refer to Table 1 for details.