Structure of Nucleophosmin DNA-binding Domain and Analysis of Its Complex with a G-quadruplex Sequence from the c-*MYC* Promoter*[S]

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Background: Nucleophosmin leukemia-associated domain binds G-quadruplex DNA.
Results: NMR structural analysis of the 70-residue nucleophosmin C-terminal domain and its interaction with G-quadruplex DNA from the c-*MYC* promoter was carried out.
Conclusion: The interaction involves helices H1 and H2 of the nucleophosmin terminal three-helix bundle mainly through electrostatic contacts with G-quadruplex phosphates.
Significance: Learning how nucleophosmin interacts with nucleic acids may be crucial in rescuing its function in leukemia.

Nucleophosmin (NPM1) is a nucleocytoplasmic shuttling protein, mainly localized at nucleoli, that plays a key role in several cellular functions, including ribosome maturation and export, centrosome duplication, and response to stress stimuli. More than 50 mutations at the terminal exon of the *NPM1* gene have been identified so far in acute myeloid leukemia; the mutated proteins are aberrantly and stably localized in the cytoplasm due to high destabilization of the NPM1 C-terminal domain and the appearance of a new nuclear export signal. We have shown previously that the 70-residue NPM1 C-terminal domain (NPM1-C70) is able to bind with high affinity a specific region at the c-*MYC* gene promoter characterized by parallel G-quadruplex structure. Here we present the solution structure of the NPM1-C70 domain and NMR analysis of its interaction with a c-*MYC*-derived G-quadruplex. These data were used to calculate an experimentally restrained molecular docking model for the complex. The NPM1-C70 terminal three-helix bundle binds the G-quadruplex DNA at the interface between helices H1 and H2 through electrostatic interactions with the G-quadruplex phosphate backbone. Furthermore, we show that
the 17-residue lysine-rich sequence at the N terminus of the three-helix bundle is disordered and, although necessary, does not participate directly in the contact surface in the complex.

Nucleophosmin (also called B23, numatrin, and herein NPM1[4]) is a protein involved in a variety of crucial cellular functions, including ribosome maturation and export, centrosome duplication, and response to stress stimuli (1, 2). NPM1 is a highly mobile protein capable of shuttling between nucleus, nucleoplasm, and cytoplasm, although the bulk of the protein is mainly localized in the nucleoli (3).

The *NPM1* gene, overexpressed in a number of solid tumors, has been proposed as a marker for colon, gastric, ovarian, and prostate carcinomas (1). *NPM1* is also frequently modified in hematopoietic tumors. For instance, in both lymphoid and myeloid disorders, *NPM1* chromosomal translocations lead to the production of several oncogenic fusion proteins (1). Furthermore, *NPM1* is the most frequently mutated gene in acute myeloid leukemia, accounting for 35% of all cases (2, 4); over 50 different mutations, always heterozygous and largely associated to a normal karyotype, were discovered (3, 4). Mutations involve duplication or insertion of small base sequences at the last exon of the gene and lead to a C-terminal domain that has acquired four additional residues compared with wild type and a completely different sequence in the last seven. The nucleolar localization signal is compromised with the loss of one or both of the two critical tryptophan residues, and a third nuclear export signal appears. Moreover, the mutated domain is largely destabilized or totally unfolded (5–7). Both the destabilization of NPM1 C-terminal domain and the presence of an additional nuclear export signal contribute to the aberrant and stable

[S] This article contains a supplemental file showing the coordinates for the lowest energy NPM1-C70·Pu24I structural model.
The atomic coordinates and structure factors (code 2llh) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).
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[4] The abbreviations used are: NPM1, nucleophosmin; NPM1-C70, 70-residue NPM1 C-terminal domain; *SOD2*, superoxide dismutase 2; AIRs, ambiguous interaction restraints; NHE III, nuclease hypersensitive element III.

localization of the mutated protein in the cytoplasm, which is the salient feature of this type of leukemia (2).

The NPM1 C-terminal domain is known to bind both duplex and single-stranded DNA as well as RNA with no sequence specificity and with a preference for single-stranded nucleic acids (8). This picture was suggestive of a protein playing mainly a chaperone and transport role for preribosomal RNA particles (1, 9). Recently, however, it was shown that NPM1 binds a specific G-rich sequence at the superoxide dismutase 2 (*SOD2*) gene promoter and participates in the transcriptional activation of this gene (10).

Starting from these premises, we recently showed (11) that (i) NPM1 binds with high affinity DNA sequences that form G-quadruplexes, including the one found at the c-*MYC* oncogene promoter (see later); (ii) NPM1 is able to induce G-quadruplex formation in G-rich unstructured oligos; and (iii) the region of the *SOD2* promoter recognized *in vivo* by NPM1 is indeed folded as a G-quadruplex *in vitro* (11). We also investigated the domain boundaries necessary for DNA binding and demonstrated that a 17-residue segment preceding the C-terminal three-helix bundle (5) is necessary for high affinity recognition (11).

G-quadruplex DNA is gaining increasing attention because it is highly represented (especially in selected regions of the genome, including telomeres and gene promoters) and is involved in a number of regulatory processes (12, 13). Interestingly, G-quadruplex regions are frequently found at oncogene promoters, whereas a reduced frequency at tumor suppressor genes is observed (14). For instance, a well characterized G-quadruplex-forming sequence present at the NHE$_{III}$ region of the c-*MYC* oncogene promoter (15) is recognized by NPM1 C-terminal domain *in vitro* (11) and folds as a G-quadruplex both *in vitro* and *in vivo*, regulating up to 90% of total c-MYC transcription (15–17). The interaction with proteins that stabilize the G-quadruplex fold, such as nucleolin, causes a marked down-regulation of the gene (18, 19), whereas the opposite happens through the interaction with G-quadruplex-unwinding proteins, such as NMR-H2 (20).

Given its selective localization at oncogene promoters and telomeres, G-quadruplex DNA is an attractive target for tumor treatment, and the structures of several G-quadruplex regions alone and in complex with drugs have been reported (21). Conversely, although the list of proteins that bind G-quadruplex DNA is rapidly increasing and the importance of such interactions for a variety of physiological processes is now clear, very little structural information is available concerning the molecular recognition mechanism of complex formation (22). To the best of our knowledge, only two structures are available: (i) thrombin in complex with a synthetic aptamer that folds as a G-quadruplex (23) and (ii) the *Oxytricha nova* telomere-binding protein heterodimer bound to its telomeric sequence (24).

Here we present the high resolution NMR structure of the NPM1 DNA-binding domain and analyze its interaction with the G-quadruplex DNA from the NHE$_{III}$ region of the c-*MYC* promoter. We show (i) that the contact surface involves largely amino acids belonging to helices H1 and H2 of the terminal three-helix bundle and (ii) that a well defined G-quadruplex region is recognized through several electrostatic interactions

with the phosphate backbone. The N-terminal lysine-rich region of the NPM1 C-terminal domain, which we show to be unstructured, does not participate directly in the interacting surface although it proved necessary to increase affinity (11). The data presented below unveil the interaction surface between G-quadruplex DNA and NPM1 and may inspire the search for small molecules or aptamers aimed at restoring a native-like fold in NPM1 leukemic mutants.

EXPERIMENTAL PROCEDURES

Oligonucleotides—The oligonucleotides used in this study are Pu27 of sequence 5′-T**GGGG**A**GGG**T**GGGG**A**GGG**T**GG-GG**AAGG-3′ and Pu24I of sequence 5′-**TG**A**GGG**T**GGIG**AG-**GG**T**GGGG**AAGG-3′. Pu27 and Pu24I were purchased from Primm (Milan, Italy) and Integrated DNA Technologies, Inc. (Coralville, IA), respectively, and were both HPLC-purified. Lyophilized oligos were dissolved in 20 mM phosphate buffer pH 7.0, 100 mM KCl and annealed. For annealing, oligos were heated to 95 °C for 15 min and then allowed to gently cool down to room temperature overnight. After annealing, the parallel G-quadruplex assembly of both oligos was assessed by inspecting CD spectra collected with a Jasco J-710 spectropolarimeter.

Protein Sample Preparation—A DNA construct for residues 225–294 of human NPM1 was cloned into pET28+(a) vector and transformed into *Escherichia coli* BL21(DE3) cells. For isotope enrichment, cells were grown in a minimal medium with (^{15}NH$_4$)$_2$SO$_4$ and [^{13}C]glucose. Protein expression was induced with 1.0 mM isopropyl β-D-thiogalactopyranoside at 20 °C, and cells were harvested after 16 h. NPM1-C70 was purified as reported previously (11). The hexahistidine tag at the N terminus of the protein was thrombin-cleaved and removed by nickel-nitrilotriacetic acid affinity column.

Structure Calculations of the Free Protein—The ^{1}H, ^{13}C, and ^{15}N resonance frequencies of NPM1-C70 were assigned using all classical NMR experiments. NMR experiments used for resonance assignment and structure calculations were performed on ^{13}C,^{15}N-labeled NPM1-C70 sample containing 10% D$_2$O. NMR spectra were collected at 298 K, processed using standard Bruker software (TOPSPIN 2.1), and analyzed with CARA (25).

Structure calculations were performed with the software package UNIO using as input the amino acid sequence; the chemical shift lists; and three ^{1}H-^{1}H NOE experiments, two-dimensional NOESY, three-dimensional ^{13}C-edited NOESY, and three-dimensional ^{15}N-edited NOESY, recorded at 900 MHz with a mixing time of 100 ms. The standard protocol included in UNIO with seven cycles of peak picking using ATNOS (26), NOE assignment with CANDID (27), and structure calculation with CYANA-2.1 (28) was used. φ and ψ dihedral angles were obtained from the chemical shift analysis using TALOS+ software (29–31). In each ATNOS/CANDID cycle, the angle constraints were combined with the updated NOE upper distance constraints in the input for subsequent CYANA-2.1 structure calculation cycle.

The 20 conformers with the lowest target function values were subjected to restrained energy minimization in explicit water with AMBER 11.0 (32, 33). NOE and torsion angle constraints were used. The quality of the structures was evaluated using the programs PROCHECK, PROCHECK-NMR (34), and

WHAT IF (35). Statistics about the energy-minimized family of conformers are reported in Table 1. The atomic coordinates and structural restraints for NPM1-C70 have been deposited in the Protein Data Bank with accession code 2llh. Resonance assignments are also available at BioMagResBank (accession number 18048).

Structure Calculations of the Complex—To identify intermolecular NOEs in the NPM1-C70·Pu24I complex, a ω_1-^{13}C-edited, ω_2-^{13}C-filtered NOESY experiment was recorded in a two-dimensional plane (^{1}H-^{1}H plane) on ^{13}C,^{15}N-labeled NPM1-C70·unlabeled Pu24I (36). The selected temperature was 290 K, and the mixing time used was 120 ms. ^{1}H, ^{13}C, and ^{15}N backbone resonances of NPM1-C70 in the complex were assigned by performing all the typical experiments for backbone assignment.

To calculate a structural model for the interaction between NPM1-C70 and Pu24I, a data-driven molecular docking was performed using the HADDOCK protocol. HADDOCK comprises a series of Python scripts that run on top of the structure determination programs ARIA and CNS (37–41). The method relies on the definition of ambiguous interaction restraints (AIRs) derived from experimental data.

For our docking calculations, we defined as unambiguous restraints residues resulting from intermolecular NOE cross-peaks on both protein and DNA. Furthermore, we defined as AIRs (i) residues experiencing chemical shift variations above the average variations plus one standard deviation ($\delta_{HN} = 0.043 \pm 0.025$ ppm; Fig. 3*B*, *black line*); (ii) residues whose signals broadened their line width (Glu^{245}, Lys^{248}, and Phe^{268}) and/or disappeared during the titration (Phe^{276}); and (iii) DNA atoms interacting with the protein, also identified by chemical shift perturbation, as detected in homonuclear experiments (one-dimensional and two-dimensional). Residues used as AIRs are listed in Table 2.

The HADDOCK docking protocol consists of (i) randomization of orientation and rigid body minimization, (ii) semirigid simulated annealing in torsion angle space, and (iii) final refinement in Cartesian space with explicit solvent. The rigid body docking step was performed five times with 1000 structures generated at each stage, the best 200 of which were refined in the semiflexible stage and subsequently in explicit water. Electrostatic and van der Waals terms were calculated with an 8.5-Å distance cutoff (37).

For the docking procedure, the structures of NPM1-C70 (here determined) and Pu24I (Protein Data Bank code 2A5P) were used as starting points. The coordinates for the lowest energy NPM1-C70·Pu24I structural model are included as supplemental material.

Relaxation Data—Heteronuclear relaxation experiments were performed on ^{15}N-labeled samples of NPM1-C70 at 700 MHz. The ^{15}N backbone longitudinal (R_1) and transverse (R_2) relaxation rates as well as heteronuclear ^{15}N{^{1}H} NOEs were measured using a standard protocol (42, 43).

RESULTS

The Structure of the NPM1-C70 Domain—The short C-terminal domain of NPM1 (residues 242–294; hereafter NPM1-C53) was shown by Grummitt *et al.* (5) to fold as a three-helix

TABLE 1

Statistical analysis of the energy-minimized family of conformers of NPM1-C70

r.m.s., root mean square; r.m.s.d., root mean square deviation; rad, radian; BB, backbone; HA, heavy atoms.

	AMBER 10.0[a] (20 structures)
r.m.s. deviations per meaningful distance constraint (Å)[b]	
Intraresidue (189)	0.0065 ± 0.0063
Sequential (279)	0.0208 ± 0.0062
Medium range (220)[c]	0.0198 ± 0.0031
Long range (40)	0.0106 ± 0.0122
Total (728)	0.0178 ± 0.0015
r.m.s. violations per meaningful dihedral angle constraints (°)[b]	
ϕ (42)	1.20 ± 1.01
ψ (42)	6.17 ± 4.19
Average no. of NOE violations larger than 0.3 Å	0.05 ± 0.22
Average NOE deviation (Å^2)[d]	0.01 ± 0.03
Average angle deviation (rad^2)[d]	0.66 ± 0.10
r.m.s.d. to the mean structure (Å) (BB)[e]	0.80 ± 0.21
r.m.s.d. to the mean structure (Å) (HA)[e]	1.40 ± 0.15
Structural analysis	
Residues in most favorable regions (%)[f,g]	96.5
Residues in allowed regions (%)[f,g]	3.3
Residues in generously allowed regions (%)[f,g]	0.1
Residues in disallowed regions (%)[f,g]	0.0
G-factor[f,g]	0.03
Structure Z-scores[g,h]	
First generation packing quality	1.27
Second generation packing quality	4.51
Ramachandran plot appearance	−1.50
χ_1/χ_2 rotamer normality	−3.04
Backbone conformation	−0.50

[a] AMBER indicates the energy-minimized family of 20 structures.
[b] The number of meaningful constraints for each class is reported in parentheses.
[c] Medium range distance constraints are those between residues (i, $i + 2$), (i, $i + 3$), (i, $i + 4$), and (i, $i + 5$).
[d] NOE and torsion angle constraints were applied with force constants of 20 kcal mol^{-1} Å^{-2} and 20 kcal mol^{-1} rad^{-2}, respectively.
[e] The r.m.s.d. to the mean structure is reported considering the segment 293–291.
[f] As it results from the Ramachandran plot analysis. For the PROCHECK statistics, an overall G-factor larger than −0.5 is expected for a good quality structure.
[g] The statistical analysis is reported considering the segment 293–291.
[h] Values based on WHAT IF output. A Z-score is defined as the deviation from the average value for this indicator observed in a database of high resolution crystal structures expressed in units of the standard deviation of this database-derived average. Typically, Z-scores below a value of −3 are considered poor, and those below −4 are considered bad.

bundle. However, NPM1-C53 is poorly competent for DNA binding, and the 17-residue lysine-rich region at its N terminus (residues 225–241) is necessary for high affinity (11). Therefore, we decided to determine by NMR the structure of this longer construct encompassing the last 70 residues of the NPM1 sequence (residues 225–294; hereafter NPM1-C70). Statistics about structure determination are shown in Table 1.

The structure depicted in Fig. 1*A* comprises a well defined three-helix bundle, similar to the NPM1-C53 construct (5), in terms of length, relative orientation, and hydrophobic interactions between all the paired helices (Fig. 1*A*). On the other hand, the lysine-rich region (residues 225–241) that enhances DNA binding is unstructured as indicated by high values for ^{15}N-^{1}H R_1 (Fig. 1*B*) and low values for ^{15}N-^{1}H R_2 (Fig. 1*C*) and heteronuclear NOEs (Fig. 1*D*) with no secondary structure elements nor any propensity to fractionally take up secondary structures as clearly shown from the chemical shift index analysis (Fig. 1, *B* and *C*). Conversely, the folded part of the NPM1-C70 construct shows ^{15}N-^{1}H R_1 and ^{15}N-^{1}H R_2 values typical of an 8-kDa protein (Fig. 1, *B* and *C*) and positive values for het-
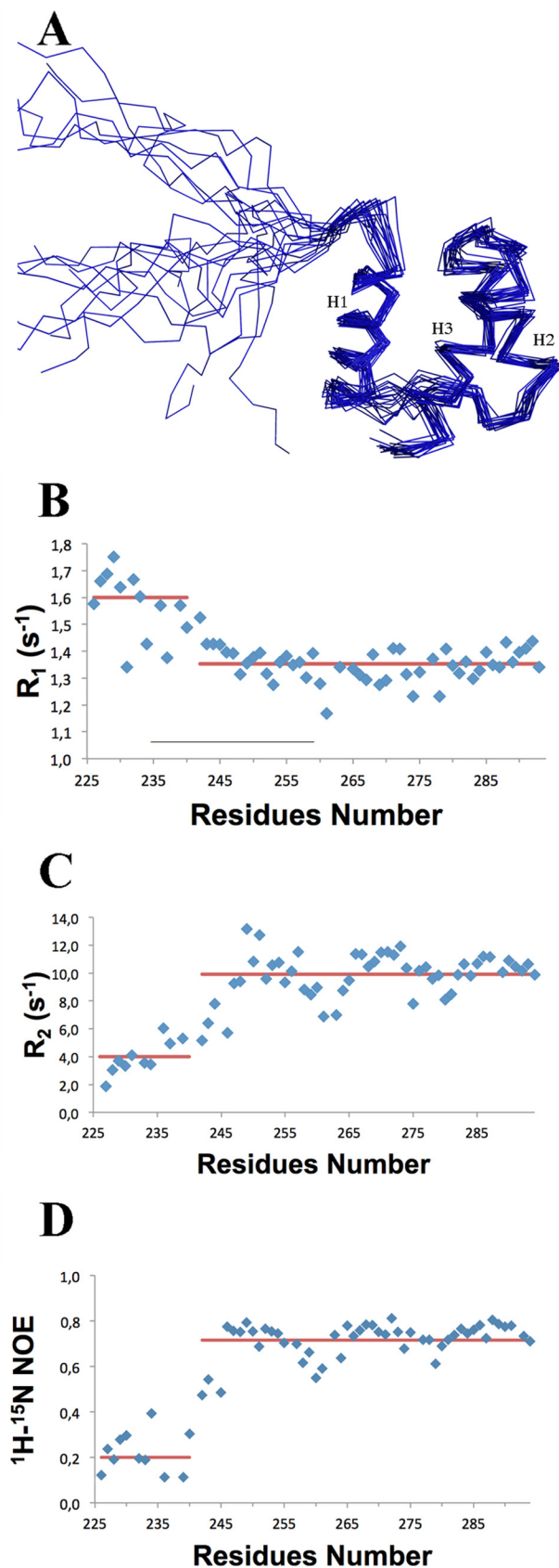
FIGURE 1. NPM1-C70 quadruplex-binding C-terminal domain encompassing residues 225–294. *A*, NMR solution structure of NPM1-C70 showing the 20 lowest energy structures. A lysine-rich natively unstructured segment (amino acids 225–242) precedes the terminal three-helix bundle. *B*, ^{15}N-^{1}H R_1

eronuclear ^{15}N{^{1}H} NOEs values (Fig. 1*D*), although the latter are lower than those of a rigid protein, thus indicating the occurrence of fast internal motions.

The Complex of NPM1-C70 with c-MYC G-quadruplex DNA—NPM1-C70 binds a DNA oligonucleotide resembling a specific sequence found at the NHE$_{III}$ region of the c-*MYC* promoter (11). This 27-mer region (called Pu27) of sequence 5′-T**GGGG**A**GGG**T**GGGG**A**GGG**T**GGGG**AAGG-3′ is known to form a parallel G-quadruplex structure in the presence of K$^+$ at physiological concentration (100–150 mM) (11, 18). Pu27 contains runs of three or more consecutive guanines and therefore can, in principle, populate several G-quadruplex structures with different topologies (13). These multiple conformations are indeed observed in the free state as monitored by multiple sets of NMR signals in slow exchange in the one-dimensional ^{1}H NMR spectra (Fig. 2*A*). Moreover, Pu27 maintains different quadruplex topologies also when bound to NPM1-C70 (Fig. 2*A*). The structural heterogeneity of the Pu27 sample complicates the analysis of its interaction with NPM1-C70. However, recently, Patel and coworkers (44) showed that a c-*MYC*-derived shorter oligonucleotide of 24 residues containing a guanine to inosine substitution in one of the guanine runs populates only one of the possible G-quadruplex conformations (called Pu24I; 5′-T**GAGGG**T**GGIG**A**GGG**T**GGGG**AA-GG-3′). Therefore, we decided to use this oligonucleotide for further studies. The assignment of the ^{1}H nuclei of Pu24I was provided by courtesy of Anh Tuan Phan and Vitaly Kuryavyi (44). First, by comparing the one-dimensional ^{1}H NMR spectra of Pu24I in the free state and bound to NPM1-C70, we confirmed that Pu24I displays only a single G-quadruplex topology that is retained after NPM1-C70 binding (Fig. 2*B*). Then, to assess whether Pu24I undergoes major conformational changes upon NPM1-C70 binding, we performed intramolecular NOE experiments for the bound state of Pu24I and compared it with its free state. To characterize the bound state, a ω_1-^{13}C-filtered, ω_2-^{13}C-filtered NOESY experiment was recorded in a two-dimensional plane (^{1}H-^{1}H plane), whereas, in the case of the Pu24I free state, a standard two-dimensional NOESY was performed. As shown in Fig. 2*C*, no major variations are visible in the superimposition of the two spectra, indicating that the G-quadruplex structure of Pu24I is maintaining its conformation when bound to NPM1-C70.

To further investigate the interaction of NPM1-C70 with Pu24I, we titrated ^{15}N-labeled NPM1-C70 with increasing amounts of unlabeled Pu24I. We observed both the appearance of new peaks and the disappearance of others, indicating formation of a new species that exchanges slowly with the free protein on the NMR time scale, *i.e.* $< 10^{-2}$ s^{-1} (Fig. 3*A*). The ratio of the intensity of the signals for a given resonance in the two species changes linearly with the amount of Pu24I and reaches a plateau upon addition of stoichiometric amounts of Pu24I, consistent with a 1:1 protein·Pu24I complex. NPM1-

NOE values. *C*, ^{15}N-^{1}H R_2 NOE values. Low R_1 and high R_2 values for segment 225–242 are consistent with the N-terminal tail being unstructured. *D*, heteronuclear ^{15}N{^{1}H} NOEs are positive but smaller than expected for an 8-kDa protein, indicating fast internal motion in the three-helix bundle. *Red lines* indicate average values for the 225–242 and the 243–294 segments, respectively.
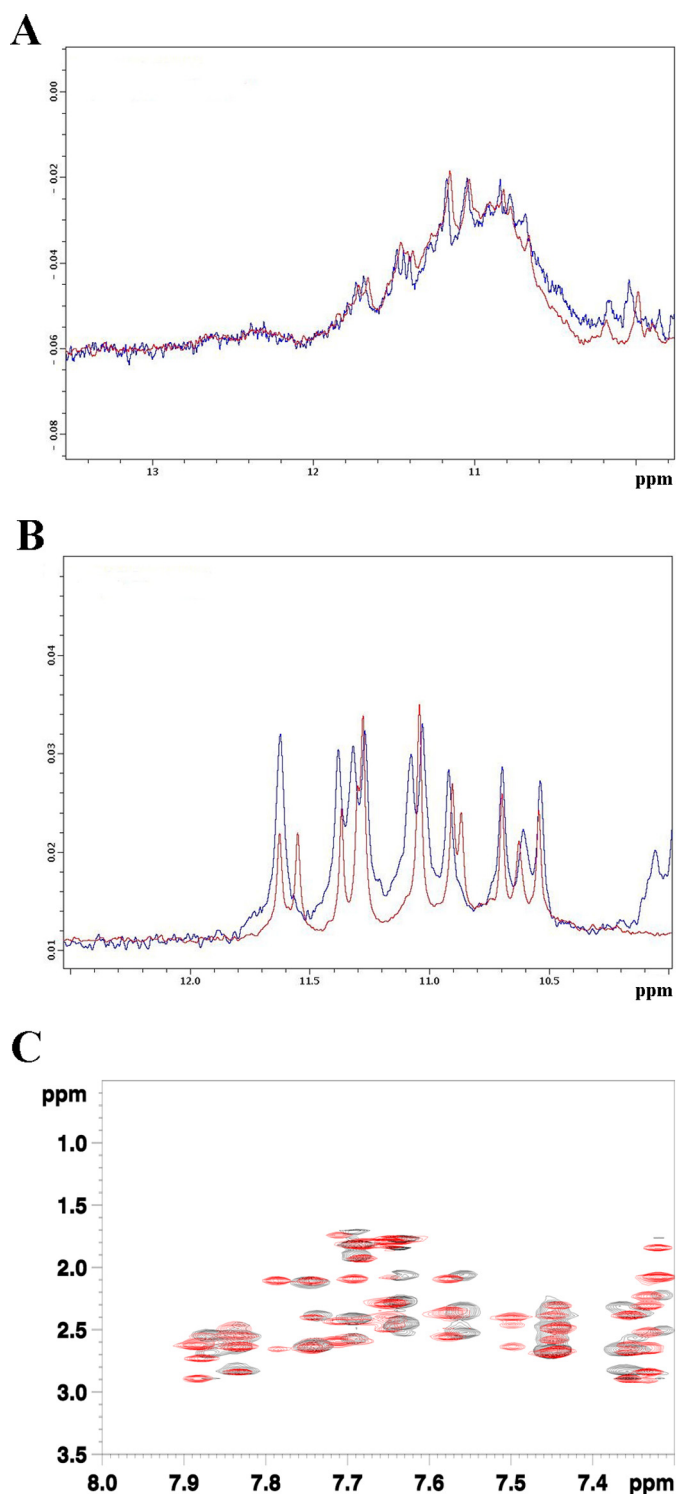
FIGURE 2. **Analysis of Pu27 and Pu24I G-quadruplex conformations.** *A,* superimposition of the one-dimensional ^{1}H NMR spectra of Pu27 in the free state (*blue*) and bound (*red*) to NPM1-C70. Both spectra were acquired at 700 MHz and at 290 K. *B,* superimposition of the one-dimensional ^{1}H NMR spectra of Pu24I in the free state (*blue*) and bound (*red*) to NPM1-C70. Both spectra were acquired at 700 MHz and 290 K. *C,* details of the superimposition between ω_1-^{13}C-filtered, ω_2-^{13}C-filtered NOESY experiments in a two-dimensional plane (^{1}H-^{1}H plane) of Pu24I·NPM1-C70 complex (*black*) and a classical two-dimensional NOESY of Pu24I in its free state (*red*). Both spectra were acquired at 700 MHz and 290 K.

C70 interacts with Pu24I mainly via hydrophilic residues located in helices H1 and H2 and exposed on the same side of the protein (Fig. 3, *B* and *C*). Interestingly, a few residues

located on helix H3 also display chemical shift variations (Fig. 3, *B* and *C*). Because the latter are buried and interact with helices H1 and H2, we conclude that a strong coupling is present among the three helices.

In the protein·Pu24I complex, no chemical shift variations were detected for residues belonging to the NPM1-C70 N-terminal unstructured segment, indicating that this tail is not directly involved in the complex (Fig. 3*B*). This was confirmed by analysis of the ^{15}N heteronuclear relaxation data in the complex where the N-terminal tail is still characterized by high values for heteronuclear ^{15}N-^{1}H R_1 (Fig. 3*D*) and low values for heteronuclear ^{15}N-^{1}H R_2 (Fig. 3*E*) and heteronuclear NOEs (Fig. 3*F*), consistent with a natively unstructured state. Conversely, the folded C-terminal region of NPM1-C70 shows ^{15}N-^{1}H R_1 and ^{15}N-^{1}H R_2 values typical of a complex of 16-kDa molecular mass (in agreement with the sum of the molecular weights of NPM1-C70 and Pu24I) and positive values for heteronuclear ^{15}N{^{1}H} NOE values (Fig. 3, *D–F*). Both results suggest that the complex between NPM1-C70 and Pu24I is stable, consistent with the high affinity observed previously for the Pu27 oligonucleotide (11). The relaxation data analysis also pointed out that the heteronuclear ^{15}N{^{1}H} NOE values increase in the folded region of NPM1-C70, indicating an increasing rigidity for the three-helix bundle of NPM1-C70 upon binding of Pu24I (Fig. 3*F*).

Experimentally Restrained Molecular Docking of the NPM1-C70·Pu24I complex—To gain additional information on the structure of the complex, ^{13}C-filtered NOESY experiments were performed. Six intermolecular NOEs were detected between NPM1-C70 and Pu24I in the complex (Table 2). An example of assignment of NOE cross-peaks is shown in Fig. 4*A*. They involve Lys and Asn residues of the protein (whose side chains are free to rotate during the subsequent docking calculation) and sugar backbone protons on Pu24I. These distance restraints, together with the chemical shift perturbations defined as AIRs (also listed in Table 2), were used to calculate a structural model of the NPM1-C70·Pu24I complex within the data-driven HADDOCK docking program (see "Experimental "Procedures" for details about the protocol used). Importantly, HADDOCK calculations identified a single cluster of docking poses. In particular, 184 final complex structures were obtained at the end of the procedure with an root mean square deviation from the lowest energy solution of 1.5 ± 0.9 Å. As an example, the 20 lowest energy docking poses are reported in Fig. 4*B*.

As shown in Fig. 5*A*, the interaction surface involves one side of the three-helix bundle, the interacting residues all being located on helices H1 and H2, with a buried area of 1358.6 ± 92.2 Å^2. Based on the assignment of the intermolecular NOEs (Fig. 4*A* and Table 2), the interaction involves side chains of NPM1-C70 residues Lys[250], Lys[257], Asn[270], and Asn[274]. On the Pu24I side, intermolecular NOEs indicate the involvement of protons of the backbone (mainly the deoxyribose ring) of nucleotides T1, G11, A12, G13, G15, and G23. From the structure model analysis, a linear stretch of nucleotides (from G11 to G16) located on one side of the G-quadruplex scaffold (Fig. 5*A*) intercalates into a groove formed by helices H1 and H2 (Fig. 5*B*). Interestingly, this stretch contributes to the formation of each of the three stacked guanine tetrads in the G-quadruplex
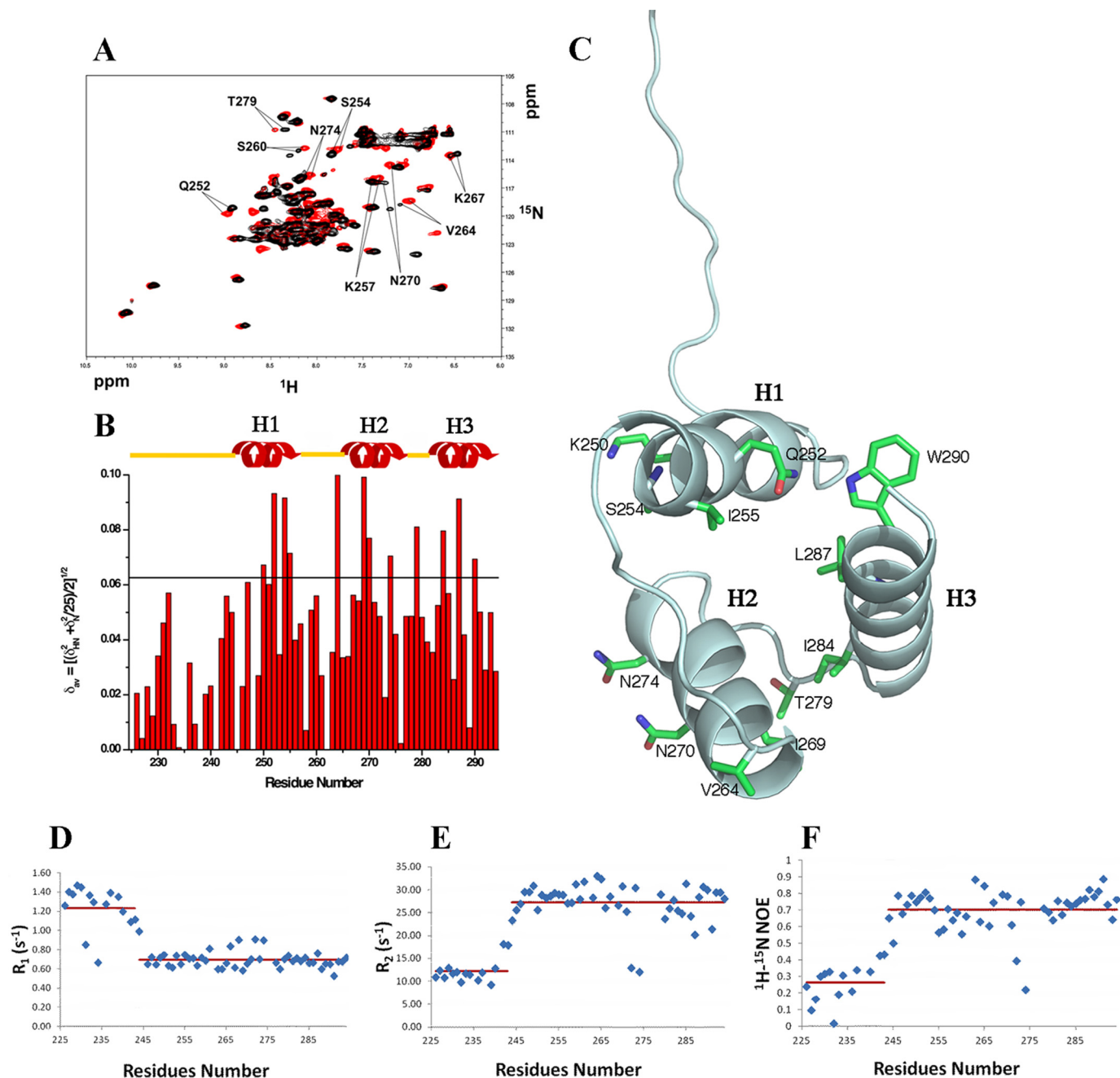
FIGURE 3. **Interaction of NPM1-C70 with the Pu24I G-quadruplex.** *A*, ^{15}N HSQC spectra of the protein before (*black*) and after addition of stoichiometric amounts of unlabeled Pu24I (*red*). Representative chemical shift variations are labeled, indicating relevant residues. *B*, chemical shift variations cluster in the three-helix bundle, whereas they are not found at the N-terminal 225–242 segment. The *horizontal black line* indicates the average chemical shift variation plus one standard deviation upon Pu24I addition. *C*, residues experiencing chemical shift variations higher than the average plus one standard deviation are highlighted on the structure of the protein. Residues belonging to helices H1 and H2 are solvent-exposed. A few hydrophobic residues belonging to helix H3 are also affected, indicating coupling between the helices upon Pu24I binding. Heteronuclear [15]N-[1]H R_1 (*D*) and heteronuclear [15]N-[1]H R_2 values (*E*) for NPM1-C70 in complex with Pu24I indicate that the N-terminal region flanking the three-helix bundle remains unstructured after Pu24I binding. *F*, the increase of heteronuclear [15]N{[1]H} NOE values in the three-helix bundle upon Pu24I binding (see Fig. 1*D* for comparison) suggests increased rigidity.

scaffold. By analyzing the 20 lowest energy docking solutions, it appears that residues Lys[250], Lys[257], Asn[270], and Asn[274] always participate in the formation of salt bridges or hydrogen bonds with backbone phosphates belonging to the G11–G16 linear stretch. In addition, residues Lys[267] and Cys[275] are always found at the interface where they contact phosphate groups in the G11–G16 stretch in all 20 lowest energy docking poses.

DISCUSSION

The propensity of NPM1 to interact with nucleic acids appears to be crucial for several functions that this protein plays both in the nucleoli and in the cytoplasm where a small fraction of the protein is always present due to continuous shuttling back and forth from the nucleus. For instance, a great deal of data show that NPM1 controls both ribosome assembly and

TABLE 2

Intermolecular NOEs and AIRs used for HADDOCK docking calculations

Gua, guanine.

Intermolecular NOEs

NPM1-C70 atoms	Pu24I atoms	Distance (Å)
Lys 250-HG3/HG2	G13-H3'	4.01
Lys 250-HE2/HE3	G11-H5'	5.83
Lys 257-HD2/HD3	A12-H1'	5.53
Lys 257-HD3/HD2	T1-H72	4.03
Asn 270-HD22/HD21	G15-H5"	4.52
Asn 274-HD22/HD21	G23-H21	3.12

Ambiguous Interaction Restraints

Active Residues of NPM1-C70	Active Residues of Pu24I
Glu245	Gua5
Lys248	Gua13
Lys250	Gua17
Gln252	Gua20
Ser254	
Ile255	
Lys257	
Val264	
Phe268	
Ile269	
Asn270	
Asn274	
Phe276	
Thr279	
Ile284	
Leu287	
Trp290	

FIGURE 4. **Intermolecular NOEs and HADDOCK calculations.** *A*, an example of NOE cross-peak assignment from ω_1-^{13}C-edited, ω_2-^{13}C-filtered NOESY experiments in a two-dimensional plane (^{1}H-^{1}H plane) of the NPM1-C70·Pu24I complex acquired on a ^{13}C,^{15}N-labeled NPM1-C70·unlabeled Pu24I sample. *B*, the 20 lowest energy complex structures obtained by NMR data-restrained molecular docking calculations using the HADDOCK protocol. NPM1-C70 is represented in *blue*, and Pu24I is represented in *green*.

transport (9). Recently, it was also shown that NPM1 is selectively deposited on the mRNA body during polyadenylation, suggesting a putative role in a variety of post-transcriptional events, including splicing (45). A role of NPM1 in the control of gene transcription has been also suggested either through its association with several transcription factors at gene promoters (9) or through its direct interaction with the G-rich sequence found at the *SOD2* gene promoter (10). We further showed that the latter region is folded as a G-quadruplex at least *in vitro* and that G-quadruplexes are bound by NPM1-C70 with high affinity (11).

Based on these premises, we analyzed the three-dimensional structure of NPM1-C70 bound to G-quadruplexes. Among several oligonucleotides tested, we focused our attention on the interaction of NPM1-C70 with the G-quadruplex at the NHE$_{III}$ region of the c-*MYC* promoter because this is the DNA sequence bound with the highest affinity (11) whose structure is known (40).

The structure of NPM1-C70 alone and bound to the DNA fragment was investigated by a combination of NMR data and docking calculations guided by experimental restraints. As shown in Fig. 5, NPM1-C70 binds the Pu24I G-quadruplex through a specific surface at the interface between helices H1 and H2. Several positively charged and polar residues establish interactions mainly with phosphate groups of a linear stretch of nucleotides that fits the small groove at the H1-H2 interface.
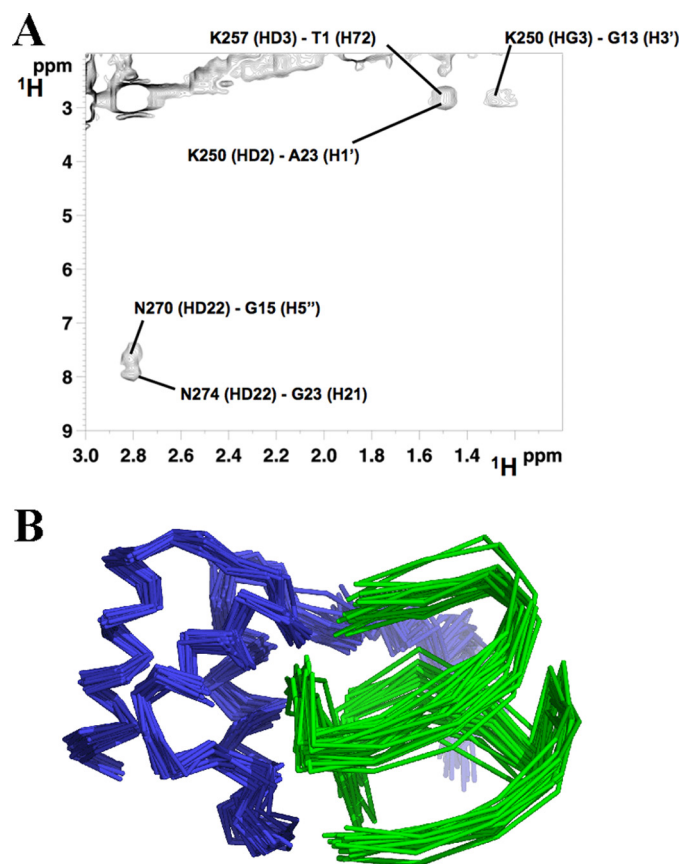
Interestingly, this stretch contributes to the formation of the main G-quadruplex scaffold, whereas the interactions with nucleotides belonging to the connecting loops appear marginal. This may explain why NPM1 recognizes with comparable affinity several G-quadruplexes that differ in loop length and distribution (11).

Among the NPM1-C70 residues found at the interface with the Pu24I G-quadruplex, Lys[257] and Lys[267] are acetylated *in vivo* by p300 and deacetylated by SIRT1 (46). NPM1 acetylation results in dislocation of the protein from the nucleoli to the nucleoplasm where NPM1 interacts with transcriptionally active RNA polymerase II. Our finding that these two residues are at the interface with Pu24I DNA suggests that loss of nucleolar localization may be due to impaired DNA binding at the nucleoli coupled to acetylation.

Interestingly, we also found from our docking simulations that Cys[275] is always located at the center of the surface buried by Pu24I. Cys[275] is targeted and alkylated by the natural antitumoral compound (+)-avrainvillamide (47). Treatment of LNCaP or T-47D cells with (+)-avrainvillamide leads to an increase in cellular p53 concentrations and promotes apoptosis. It is therefore conceivable that these effects may be linked to (+)-avrainvillamide-mediated impairment of NPM1 nucleic acid binding efficiency.
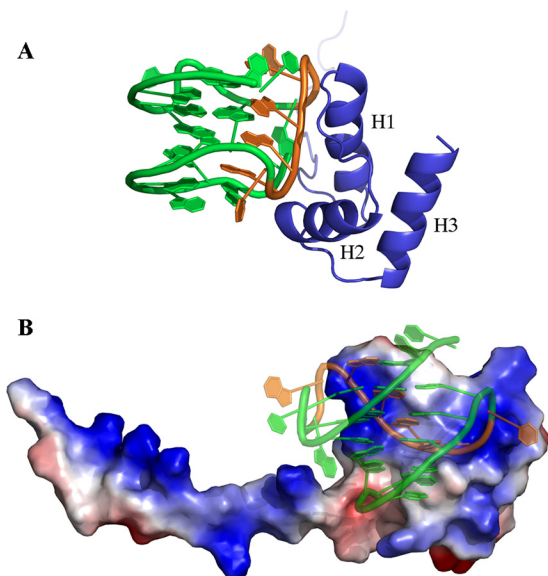
A

B

FIGURE 5. **Structural model of the NPM1-C70·Pu24I complex.** *A*, ribbon representation of the lowest energy model showing helices H1 and H2 of NPM1-C70 approaching the G-quadruplex "laterally" and interacting with a specific segment of the backbone (in *orange*). *B*, NPM1-C70 is represented with its electrostatic surface (*blue* for positive and *red* for negative), whereas Pu24I is shown in ribbon representation. The Pu24I structure is shown in *transparency* to highlight the small positively charged groove in between helices H1 and H2 that accommodates a stretch of Pu24I nucleotides (G11–G16; colored in *orange*). The long unstructured tail is also positively charged and may play a role in long range electrostatic interactions with the approaching oligonucleotide.

The N-terminal tail of the NPM1-C70 construct, although necessary for high affinity binding of any DNA tested (11), is natively unstructured both in the isolated domain and in the complex with Pu24I. This finding parallels previous observations in other systems. For instance, the affinity of the cAMP-responsive element-binding protein KID domain for the KIX domain of the cAMP-responsive element-binding protein-binding protein is significantly reduced when an unstructured portion of the domain that does not participate directly to the complex contact surface is deleted (48). Similarly, the interaction of splicing factor 1 with the large subunit of the U2 small nuclear RNA auxiliary factor (U2AF^{65}) is affected by flanking unstructured regions that do not physically contact the partner (49). This phenomenon, which is more frequent than previously anticipated, has been termed "flanking fuzziness" (50). Furthermore, it was shown recently that a large fraction of transcription factors are characterized by the presence of unstructured regions that flank the DNA-binding domain at one or both ends and that these regions may impact the affinity for specific or nonspecific DNA sequences (51).

What could the role of this unstructured N-terminal tail of NPM1-C70 be? Although this issue is still under investigation, it is conceivable that the presence of an unstructured segment adjacent to the interacting domain may provide a larger platform for long range electrostatic interactions or even transient physical contacts that facilitate the fine tuning of binding (see Fig. 5*B*) (50). This is supported by the observation that two concomitant Lys to Ala substitutions (Lys^{229}-Lys^{230}) in the unstructured segment of NPM1-C70 result in a dramatic decrease of global affinity (11).

Furthermore, exposed unstructured regions may be modified by post-translational modifications, driving regulatory changes. Several residues in the unstructured segment may be modified, such as Lys^{229} and Lys^{230} that are among the lysines acetylated by p300 and deacetylated by SIRT1 (46). A number of putative phosphorylation sites are also present in the tail, including Ser^{227}, Thr^{234}, Thr^{237}, and Ser^{242}; among these, the phosphorylations of Ser^{227} by PKC and of Thr^{234}-Thr^{237} by the Cdk1·cyclinB complex were experimentally validated (52, 53). Both acetylation and phosphorylation may therefore interfere with NPM1 nucleic acid binding and play a role in NPM1 activity and trafficking throughout the cell cycle.

Leukemia-associated NPM1 mutations cause dramatic destabilization up to total unfolding of the terminal three-helix bundle that is responsible for the aberrant cytosolic translocation of the protein. Scaloni *et al.* (6, 7) showed that a malleable "native-like" structure that accelerates folding is retained in the denatured state of the wild-type NPM1-C53 three-helix bundle and involves helices H2 and H3 (H3 is also the site of leukemic mutations), whereas helix H1 is totally unfolded in the denatured state. A possible strategy to rationally target NPM1 for the treatment of this type of leukemia might be that of developing a drug able to stabilize in the leukemic variant a native-like structure by altering through binding the folding-unfolding equilibrium in favor of the native state. Our folding studies suggested that such a drug should preferentially target helix H1. Because we have shown here that G-quadruplex DNA specifically binds a region in between helices H1 and H2 of NPM1-C70, we may attempt to rationally design aptamers or other smaller molecules that by mimicking the binding properties of G-quadruplexes to NPM1 C-terminal domain might stabilize a native-like state in the leukemic variant.

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