Chapter 6

Cytosine-rich sequences form four stranded motifs in solution, at near physiological conditions.

Case study: c-MYC

6.1 PREFACE

DNA secondary structures, formed by pyrimidine or purine rich strands, have been shown to alter regulation in various types of diseases for e.g. in case of tumor progression, diabetes and neurological disorders like mental retardation and dystrophy (1;8) As discussed previously, secondary structure formation is primarily due to nucleotide stacking (G-quadruplex or C-tetraplex also called i-motif) sequences. C-tetraplex or i-motifs are formed by the base pairing of hemiprotonated cytosine+ and cytosine to form duplex structures that are zipped together in antiparallel orientation (3) (Figure 6.1). Such intercalation topology is enabled by stable building blocks formed by N3 protonated cytosines which can form three hydrogen bonds with another cytosine (3). The importance of i-motif structures have been studied by in vitro studies, on a 17-mer cytosine-rich fragment from human centromeric satellite III which indicated that these structures have pH dependent conformation equilibrium (4). Biological importance has been shown in case of progressive myoclonus epilepsy, where multiple dodecamer repeats implicated in the disease were observed under acidic conditions (5).

Figure 6.1: Hemiprotonated cytosine–cytosine+ base pair (left panel) and schematic showing intramolecular folding of the sequence d-CCCTCCCTTTTCCCTCCC where each thymine is shown by a circle and each cytosine by a triangle (right panel).

Another example is of oncogene c-MYC, where cytosine rich fragment, present in the nuclease hypersensitive region (NHE III1), was shown to adopt polymorphic i-motif structures. A recent study has shown that c-MYC transcription in HeLa cells is modulated by ligands which specifically bind to G-quadruplex structures in vivo (9).
However, the cytosine-rich sense strand from c-MYC NHE III has received relatively less attention. This, to a large extent, may be because in vitro formation of tetraplex requires acidic to neutral pH and there is no conclusive proof of their in vivo existence as yet. Recent reports indicated G-quadruplex as a suppressor element for many genes including c-MYC and PDGF-A and concluded that remodeling of the tetraplex structure would be a prerequisite for transcription initiation. A tetraplex structure could be envisaged on the complementary cytosine-rich strand at the c-MYC NHE, which, in principle, could play a role in both silencing and remodeling events in conjunction with the G-quadruplex moiety. Keeping this in mind, the first objective of the present study was formulated i.e. to study the thermodynamics of i-motif formation using a 31-mer cytosine-rich sense-strand from NHE of c-MYC as a case study. The preliminary results show that intramolecular tetraplex folding is accompanied by a favorable free energy change driven by favorable enthalpic factors but unfavorable entropic parameters. A closer look at the ion and proton uptake/release parameters indicates a close balance between cytosine hemiprotonation and sodium ion mediated phosphate charge screening to attain optimal stability.

Nucleoside diphosphate kinases (NdKs) are essential enzymes that catalyze the reversible transformation of (d)NTPs to (d)NDPs via a covalent phosphohistidine intermediate (as discussed in Chapter 1). Though these enzymes share high homology (43% identity between the Escherichia coli and human proteins), the enzymes are tetrameric in most prokaryotes and hexameric in eukaryotes. In humans, NM23-H1 and NM23-H2 have been observed to function as transcription regulators and also shown to nick the c-MYC and the PDGF-A promoters (6). Interestingly, extracellular secretion of NdK was reported in a number of pathogens including Pseudomonas aeruginosa, Trichenella spiralis, Vibrio cholerae, Mycobacterium bovis BCG and Mycobacterium tuberculosis. It was proposed that the secreted mycobacterial NdKs could be involved in virulence by ATP-sequestration from macrophage surface-associated P2Z receptors. M. tuberculosis (H37Rv) Ndk (mNdk) was observed to be cytotoxic to mouse macrophage cells, in an ATP-dependent P2Z receptor-mediated pathway (2). Therefore, it is anticipated that mNdk may be an important enzyme as a virulence factor in tuberculosis. In a recent study it was also shown that green fluorescent protein fused-mNdk expressed within the HeLa and COS 1 cells were localized to the nuclei of these cells and was also observed to damage chromosomal
DNA in situ (7). Also, mNdK, similar to human Ndk, was observed to cleave double strand DNA with sequence specificity within the nuclease hypersensitive element (NHE) in the c-MYC promoter. Taking all these observations into account and to study the association of mNdK with DNA the second objective of the present study was designed. The results, presented here, extends these observations by demonstrating that mNdK binds the pyrimidine-rich sense strand within the c-MYC NHE in a sequence-specific manner and induces cleavage within the strand. The results further emphasize the importance of i-motif structures in addition to G-quadruplex as potential regulators of gene transcription.

6.2 RESULTS

6.2.1 Confirmation of i-motif formation

Presence of a folded oligonucleotide conformation under the in vitro conditions employed in this study and its stability as a function of pH were examined by CD and UV spectroscopy and non-denaturing PAGE. The sequence of the oligonucleotide used here is, 5'CCCCACCTTCCCCACCCTCCCCACCCTCCCC3'. Figure 6.2A shows UV scans at different pH. Increase in pH shows hyperchromism above pH 5.5 at the observed maxima of 267 nm and decrease in absorbance at 295 nm. Inset, Figure 6.2A depicts the pH profile of UV scans at 267nm and indicating an overall pKa of 6.4 ± 0.1. The presence of characteristic positive (287nm) and negative (254 nm) maxima in CD spectra (Figure 6.2B) indicates formation of hemiprotonated cytosine–cytosine+ base-paired tetrad motif by the 31-mer cytosine-rich DNA fragment from c-MYC control region. A pH profile plotted by measuring change in ellipticity of the positive band at 287 nm (inset, Figure 6.2B) shows conformational integrity in the pH range 4.5–6.0 while further increase in pH resulted in unfolding to an unstructured single strand conformation. A transition mid-point was evaluated at pH 6.8 ± 0.2 from this plot. Figure 6.3 shows the electrophoretic mobility of the oligonucleotide under native conditions at different pH. Additionally the two bands were observed, one with higher mobility in the pH range 5.3–7.0 (lanes 2, 4, and 5) while a retarded band at pH 8.0 (lane 7). A 31-mer dT oligonucleotide was used as control in each case.

6.2.2 Thermodynamics of i-motif folding

Based on the above results, to study thermodynamic stability of 31-mer MYC oligonucleotide, three pH were selected; pH 5.3, 5.6, and 6.1. Figure 6.4 shows mole
fraction (α) of folded motif observed by thermal denaturation at 260nm at various pH and ionic strength. Transitions between the two states were observed to be highly cooperative. Table 6.1 summarizes the evaluated Tm. The melting temperature was maximum at pH 5.3 and 20mM sodium chloride, which is near the pKa of cytosine (4.8 in low-salt buffer). Temperature dependent CD spectroscopy was used to further confirm these findings from UV melting experiments. Free energy of tetraplex formation was evaluated, where both enthalpy ΔH⁰ and entropy ΔS⁰ are assumed to be independent of temperature, using the Gibbs equation. ΔG⁰ = -RT ln(K) = ΔH⁰ - TΔS⁰ whereby ln(K) = -(ΔH⁰/R)1/T + (∆S⁰/R). Equilibrium constant K was expressed as K = α/(1 - α) for an intramolecular equilibrium, where α is the mole fraction of folded oligonucleotide determined at each temperature from denaturation profiles. Plot of ln(K) against 1/T (Figure 6.5) gives a straight line under the two-state transition approximation, whose slope (-ΔH⁰/R) and y-intercept (∆S⁰/R) were used to evaluate enthalpy and entropy of transition, respectively. Table 6.2 summarizes representative thermodynamic parameters.

Table 6.1:  Tm at different pH and sodium chloride concentrations determined from thermal denaturation (UV melting) at 260nm.

<table>
<thead>
<tr>
<th>pH</th>
<th>20mM</th>
<th>50mM</th>
<th>70mM</th>
<th>100mM</th>
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<tr>
<td>5.3</td>
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<td>64.0</td>
<td>63.0</td>
<td>63.0</td>
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<tr>
<td>5.6</td>
<td>58.5</td>
<td>58.5</td>
<td>57.0</td>
<td>57.0</td>
</tr>
<tr>
<td>6.1</td>
<td>53.0</td>
<td>52.5</td>
<td>52.5</td>
<td>51.0</td>
</tr>
</tbody>
</table>

Table 6.2:  Thermodynamic profiles (at 20ºC) and release of protons and counter ions upon folding at 20mM sodium chloride as a function of pH.

<table>
<thead>
<tr>
<th>pH</th>
<th>ΔG (kcal mol⁻¹)</th>
<th>ΔH (kcal mol⁻¹)</th>
<th>ΔS (kcal mol⁻¹ K⁻¹)</th>
<th>Δn_H⁺ (mol mol⁻¹)</th>
<th>Δn_Na⁺ (mol mol⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.30</td>
<td>-10.36</td>
<td>-75.99</td>
<td>-0.224</td>
<td>-2.31 ± 0.54</td>
<td>-1.05 ± 0.16</td>
</tr>
<tr>
<td>5.60</td>
<td>-8.88</td>
<td>-77.01</td>
<td>-0.232</td>
<td>-2.45 ± 0.58</td>
<td>-0.50 ± 0.24</td>
</tr>
<tr>
<td>6.10</td>
<td>-8.69</td>
<td>-83.90</td>
<td>-0.257</td>
<td>-2.76 ± 0.65</td>
<td>-0.58 ± 0.26</td>
</tr>
</tbody>
</table>
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Table 6.3: Thermodynamic profiles (at 20°C) and release of protons upon folding at pH 5.3 as a function of sodium chloride.

<table>
<thead>
<tr>
<th>NaCl (mM)</th>
<th>ΔG (kcal mol⁻¹)</th>
<th>ΔH (kcal mol⁻¹)</th>
<th>ΔS (kcal mol⁻¹K⁻¹)</th>
<th>Δn_{Na⁺} (mol mol⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>-10.36</td>
<td>-75.99</td>
<td>-0.224</td>
<td>-2.30 ± 0.54</td>
</tr>
<tr>
<td>50</td>
<td>-8.54</td>
<td>-66.14</td>
<td>-0.196</td>
<td>-1.77 ± 0.17</td>
</tr>
<tr>
<td>70</td>
<td>-9.09</td>
<td>-71.58</td>
<td>-0.213</td>
<td>-1.80 ± 0.42</td>
</tr>
<tr>
<td>100</td>
<td>-8.60</td>
<td>-67.92</td>
<td>-0.202</td>
<td>-1.89 ± 0.23</td>
</tr>
</tbody>
</table>

Figure 6.2: UV–Vis and CD spectroscopy of the cytosine-rich oligonucleotide from c-MYC promoter at various pH. (A) UV–Vis spectra of 0.55 µM oligonucleotide, arrow shows trend with increasing pH. Inset: Absorbance at 267 nm is plotted against pH; (B) Circular Dichroism spectra of 2.18 µM oligonucleotide, arrow shows trend as pH is increased. Inset: profile of ellipticity at 287 nm versus pH. All spectra were recorded at 20°C in 10mM sodium cacodylate buffer at pH ranging from 4.5 to 8.1.
Figure 6.3: Non-denaturing polyacrylamide gel electrophoresis of cytosine rich 31-mer DNA from promoter site of human c-MYC at different pH in Robinson–Britton buffer. Lanes 1, 3, 6, and 8 show dT31 and lanes 2, 4, 5, and 7 the 31-mer c-MYC oligonucleotide.
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Figure 6.4: Effect of pH and ionic strength. UV melting profiles observed at pH 5.3 (solid square), 5.6 (up triangle), and 6.1 (solid circle) in 20mM sodium chloride (upper panel) and 20mM (solid square), 50mM (circle), 70mM (up triangle), and 100 mM (cross) sodium chloride at pH 5.3 (lower panel). All thermal denaturation profiles were generated at 260 nm with 1–3 µM oligonucleotide in 10mM sodium acetate buffer by plotting mole fraction (α) of folded oligonucleotide as a function of temperature.
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Figure 6.5: Thermodynamic analysis. (A) Plot of lnKp versus 1/T at pH 5.3 (up triangle), 5.6 (down triangle), and 6.1 (solid square) in 10mM sodium acetate and 20mM sodium chloride; (B) plot of lnKp versus 1/T at 20mM (solid square), 50mM (up triangle), 70mM (down triangle), and 100mM (cross) sodium chloride concentration in 10mM sodium acetate, pH 5.3. Plots were fitted as described in Materials and method.
6.2.3 **Effect of single nucleotide substitution on i-tetraplex formation**

The stability of folded geometry under in vitro conditions was studied as a function of pH using CD spectroscopy of the oligodeoxynucleotides; Wt, M2, and M3. Figure 6.6 shows CD scans at different pH for M3 where a characteristic positive (287 nm) peak and negative (254 nm) peak, with approximately half the amplitude, were observed. Similar spectral profiles were obtained for M2 and M3 also. Respective pH profiles were plotted by measuring change in ellipticity of the positive band at 287nm (inset). It showed that the tetraplex fold was largely retained in the pH range 4.5–5.5 while further increase in pH resulted in unfolding to an unstructured single strand conformation. The CD results were further confirmed by non-denaturing PAGE (Figure 6.6). The mobility of all the oligodeoxynucleotides was higher than the 31-mer dT or unfolded Wt (at pH 8, lane 14) between pH 5.5 and 7 indicating intramolecular folding. The existence of predominantly folded conformations at pH 7.0 was somewhat surprising based on the mid-transition points evaluated from CD and may be due to the different experimental protocols.

6.2.4 **S1 nuclease digestion confirms two conformations**

To distinguish between various polymorphic forms of C-tetraplex structures which could be present at the same time, S1 nuclease digestion experiments were performed. Considering the dynamic equilibrium between the species, it was necessary to find digestion conditions such that cleavage patterns specific to predominant species could be distinguished. pH 5.3 and 7.0 were observed to give reasonably distinct cleavage patterns. Figure 6.7a shows the 5'-fluorescein-labeled bands after S1 nuclease digestion, and Figure 6.7b shows the respective intensities of each band; the intensities were compared in bar graph based on their peak areas (Figure 6.7c). At pH 7.0, S1 nuclease induced three major cleavages showing the 5’-end labeled bands B, C, and F (as marked on the right margin of the gel, Figure 6.7a) of about 24-, 16-, and 5-mer size (as indicated by size markers, with an error of (1 base), respectively, and one minor band E (6-mer). At pH 5.3, in addition to the presence of bands because of the structure predominant at neutral pH, band D was observed, which appears to be specific to the conformation at acidic pH (Figure 6.7c). It was clearly observed that the species at neutral pH was more S1- nuclease-sensitive than the one at a lower pH.
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**Figure 6.6:** Non-denaturing polyacrylamide gel electrophoresis of cytosine-rich 31-mer DNA from promoter site of human c-myc. Wild-type (Wt), mutants (M2, M3) and control (dT31) were observed at different pH in Robinson–Britton buffer.
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Figure 6.7: Two tetraplex conformations observed by S1 nuclease digestion. Denaturing gel electrophoresis was done following S1 nuclease digestion of the 5'-fluorescein-labeled 31-mer oligonucleotide at pH 5.3 and 7.0. Band intensities were used to compare the cleavage pattern generated under the different conditions. (a) CCD image of fluorescent denaturing PAGE after S1 digestion. Untreated 31-mer sequence (lane 1); S1 treated at pH 7.0 for 5 min (lane 2) and 10 min (lane 3); and S1 treated at pH 5.3 for 5 min (lane 4) and 10 min (lane 5). The bands are labelled on the right margin. (b) Electrophoretogram showing cleavage profile at the indicated pH after 10 min of digestion; peak labels correspond to bands shown in a. Peak areas indicate the intensity of the fluorescent bands. (c) Bar graph comparing peak intensities of individual bands based on their respective peak area after 10 min of S1 treatment at pH 7 (dark bars) and pH 5.3 (light bars). A total of 6.5 µM oligonucleotide was digested in 0.01 M Tris-HCl (pH 7.0) or 0.01 M sodium acetate (pH 5.3), 0.05 M NaCl, 1 mM ZnCl2, and 5 units of S1 nuclease at 37 °C and separated on a 4.5% polyacrylamide gel by electrophoresis at 55°C and 27 V/cm. Values in bar graph are averaged from three independent experiments.
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6.2.5 mNdK cleaves pyrimidine-rich strand from c-MYC promoter

Mycobacterium NdK-induced sequence-specific double strand cleavage of the c-MYC NHE was observed previously. Incubation of single strand 50-labeled P1 (10 nM) with mNdK also resulted in a cleaved product (Figure 6.8B). The amount of cleaved P1 increased as mNdK concentration was increased from 10 nM to 2 mM (Figure 6.8). Nondenaturing electrophoresis conditions were used to detect any protein–oligonucleotide complex formation; however, bands with retarded electrophoretic mobility were not observed. No cleavage was observed in the absence of mNdK (lane 2) or when the non-specific oligonucleotide d(T31) (lane 1) was incubated with 2 mM mNdK. To study the cleavage pattern of the oligonucleotide, induced by the protein, three single base mutations (Figure 6.8A) were incorporated to disrupt the potential structured form of oligonucleotide. Mutant 1(M1) and mutant 2 (M3) showed a similar pattern of cleavage (band 1) as wild type oligonucleotide (P1). In case of M2, an additional cleaved product (band 2) was also observed but with relatively less amount. However, the intensity of band 2 was increased in case of mutant 3 (M3).

6.3 DISCUSSION

The results presented, in this part of the work indicates that cytosine rich elements in the genome, though relatively unexplored, also have the potential to form a folded structure. This was studied using cytosine rich sequence taken from the nuclease hypersensitive region (NHE III) of c-MYC gene. The observed hypochromism at 297 nm (Figure 6.2A), indicating cytosine deprotonation, and concomitant unfolding (observed by CD) with increasing pH (Figure 6.2B) reflects decreasing stability of the tetrad assembly and reduced protonation on cytosines to be concurrent at higher pH. This suggests participation of hemiprotonated cytosine – cytosine + base pairs in the folded state. To further explore intramolecular folding, PAGE was done. In Figure 6.3, two additional bands were observed, one with higher mobility in the pH range 5.3–7.0 while a retarded band at pH 8.0. The band with higher mobility may be attributed to the folded i-motif and thus is consistent with the UV and CD results suggesting the presence of an i-motif at neutral pH. A small difference in mobility of the unfolded c-MYC sequence (lane 7) is observed with respect to dT31, which could be due to the high cytosine content of the c-MYC sequence.
A

P1: 5’CCCCACCTTCCCCACCCTCCCCACCCTCCCC3’
M1: 5’CCCCACCTTCTACCCTCCCCACCCTCCCC3’
M2: 5’CCCCACCTTCCCCACCCTCCCACCCTCCCC3’
M3: 5’CCCCACCTTCCCCACCCTCCCCACCCTCCC3’

B

P1 + mNdK (nM)

![Cleaved P1](image)
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Figure 6.8: Autoradiograph of cleavage profile of cytosine rich fragment (P1) of c-MYC (B) and three mutants (C), as induced by mNdK. Sequences of oligonucleotides used for EMSA studies are shown in (B).
It is interesting to note that the slight Electrophoretic mobility difference observed between pH 6 and pH 7 (lanes 4 and 5) may be due to variation in folding geometry at these pHs. It was also observed that stability of the folded motif decreases at higher pH (Figure 6.4), at all ionic strengths, up to 100mM sodium chloride as exemplified by decreasing melting temperatures. This is in accordance with earlier reports on stability of intercalated DNA. On the other hand, increasing ionic strength, from 20 to 100mM, at any pH had a moderate effect on stability with the maximal change (about 3.5°C) observed at pH 5.3 while changes at other pH were marginal (1.5°C at pH 5.6 and 2°C at pH 6.1). The slight destabilization observed at higher ionic strength is unlike normal B-DNA and may indicate a partial disruption of the charge balance between the cytosine+ core and the negatively charged sugar–phosphate backbone by cation mediated phosphate charge screening.

It is observed that folding at 20°C is accompanied by favorable free energy (Figure6.5) terms resulting from characteristic compensation of favorable enthalpy and unfavorable entropy terms. Favorable enthalpy, in case of C+ intercalated geometry, reflects electrostatic stabilization as a result of C+-C base pairing and favorable interactions between the protonated bases and the sugar–phosphates. Unfavorable entropy arises largely out of loss of translational and rotational degrees of freedom by DNA monomers and counter ions. Inspection of Table 6.2 indicates that the formation of the folded state is most favored at pH 5.30. This is consistent with the fact that major contribution towards formation of folded molecules is from hemiprotonated cytosines and hence maximal stabilization is expected near the pKa of cytosine, which is -4.8 at 20mM sodium chloride. In this context, it is interesting to consider that a pH dependent equilibrium has been observed for the i-motif formed by the human centromeric satellite III sequence where respective topologies could be stabilized by an interloop T–T pair at pH below 4.6 and an intraloop A–T pair above pH 4.6[11]. In this context, it may be noted that the calculated proton uptake difference is only -0.45mol mol-1 (Table 6.2) of folded molecules instead of expected 1.5 mol. Thus, we believe that the enthalpic gain could be partially due to additional protonation at higher pH and associated favorable electrostatic interactions between protonated bases and the backbone. Unfavorable entropy at increased pH is most probably due to higher proton uptake at pH 5.6 and 6.1 (Table 6.2). As shown in Table 6.3, folding at 20mM is favored over other ionic strengths. Enthalpically folding is favored at 20mM and characteristically is also the most disfavored reaction entropically with the highest
negative entropy change in comparison to the other higher ionic strengths examined. Unfavorable entropy at 20mM salt is consistent with the higher uptake of protons.

To study the effect of mutations the stability of i-motif, CD spectroscopy was employed. In case of mutants, M2 and M3, a positive peak at 287nm and a negative peak at 254 nm characteristics of i-motif were observed. At high temperatures the observed CD spectra are indicative of the unstructured single strand. Observed transition mid points varied from pH 6.0 ± 0.2 (for Wt), 5.8 ± 0.2 (M3) to 5.7 ± 0.2 (M2), indicating that the oligodeoxynucleotides with single nucleotide mutations needed lower pH for tetraplex stability, which could be due to differences in intercalation topology whereby smaller number of C–C+ base pairs are involved in the stem in case of the mutants. S1 nuclease cleavage pattern (figure 6.7) indicates that folded motifs with intervening single-nucleotide stretches are present at neutral pH, which is consistent with CD results and supports tetraplex formation. All of the three major bands (B, C, and F) appear to be due to cleavage within the loops. This is consistent with a tetraplex structure with eight C-C+ base pairs. Band D, at pH 5.3, could result from cleavage within the two-nucleotide loop formed by a tetraplex with six C-C+ bonds in the stem. A relatively higher amount of band F was also formed at pH 5.3, suggesting a cleavage within the overhang. Lower sensitivity to S1 nuclease at lower pH as compared to neutral pH, accompanied by the resistance of the folded state at a lower pH toward S1 nuclease cleavage is emphasized by the fact that S1 nuclease activity is optimal at a lower pH (4.0-4.3) and hence may indicate an intercalation topology with limited exposed single-strand segments.

Previous reports indicated that human Ndk binds to NHE III region of c-MYC promoter and also mNdk localizes to the nucleus of HeLa cells. To further explore the potential of mNdk binding to a structured form of DNA, electrophoretic mobility shift assays were done. Figure 6.8 show interaction of mNdk with 31mer cytosine rich element (used in previous studies). Though a retarded band of bound protein was not observed in the gel but at the lower end of the gel substantial amount of cleaved product was seen. The difference of cleavage patterns between the wild typr oligonucleotide and the mutants could be attributed to difference in the binding of protein to the DNA. Results obtained suggest that DNA-damage within c-MYC NHE is induced by sequence specific single strand DNA binding. The mNdk nuclear localization and nuclease activity together indicates the potential role of this protein in tuberculosis infection by *M. tuberculosis*. 

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