2.1 MATERIALS

Trizma base [tris(hydroxymethyl)aminomethane], xylene cyanol, N,N’bis acrylamide, EDTA, Magnesium chloride, MTT (3-(4,5-dimethylthiazolyl-2)-2), Foetal calf serum (FCS), diethyl pyrocarbonate (DEPC), Sodium cacodylate, Agarose, Ethidium bromide, Sodium dodecyl sulphate, Boric acid, Proteinase K, Ampicillin, Kanamycin and Isopropyl β-D-thiogalactopyranoside (IPTG) were from Sigma or Amersham Biosciences, USA. Phenylmethyl sulphonyl fluoride (PMSF), Dithiothreitol (DTT), Bromophenol blue and BCA-1 kit were purchased from Sigma, USA.

Restriction enzymes for DNA manipulations, T4 DNA Ligase, T4 Polynucleotide kinase, S1 Nuclease, Taq polymerase and DNase were obtained from MBI Fermentas Life Sciences (Germany) or New England Biolabs Inc., USA. Plasmid DNA isolation and purifications kits were obtained from Amersham Biosciences, (USA) or QIAGEN (Germany).

Hydrochloric acid, Sodium bicarbonate, and sodium hydroxide pellets were procured from Emek, Germany. DNA isolation kit from agarose gel was from Biological industries, Israel. RNA isolation kit was obtained from SIGMA, USA. cDNA synthesis kit was from Applied biosystems (US) or from Qiagen (Germany). SYBR green and Taqman master mixes for realtime assay were from Applied biosystems (ABI), US. Microarray cDNA and RNA labeling kits, cDNA synthesis for microarrays, DNA/RNA purification kits for array were from Roche Applied sciences (USA). Luciferase vectors were from Promega, Luciferase Assay reagents were purchased from Clontech or from Promega, Medium components for the cell culture experiments, DMEM and OPTI-MEM were from Gibco. Tryptone, Yeast extract, Agar powder etc. were purchased from Difco laboratories (USA) or Hi-Media Ltd. (India). All other chemicals and reagents were purchased from Merck Ltd. (India). All reagents used were of analytical grade.

2.2 BUFFERS AND REAGENTS

In preparing various buffers and solutions autoclaved double-distilled water was used. Volumes of solutions were adjusted after complete dissolution of component(s). Solutions which were not autoclavable were filter sterilized.
### Materials and Methods

<table>
<thead>
<tr>
<th>Material</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acrylamide, 30%</td>
<td>Acrylamide 29.2g, Bis-acrylamide 0.8g was dissolved in 100ml of distilled water</td>
</tr>
<tr>
<td>SDS, 10%</td>
<td>10g of SDS in 100ml distilled water</td>
</tr>
<tr>
<td>APS, 10%</td>
<td>Ammonium persulphate 0.5g in 5ml of distilled water (prepared fresh)</td>
</tr>
<tr>
<td>SDS loading buffer, 2X</td>
<td>0.5M Tris.HCl (pH 6.8) 1.25ml, 10% SDS 2.0ml, glycerol 1.2ml, bromophenol blue 0.1%, B-Mercaptoethanol 2.5%/v by MilliQ water (Total volume made upto 5ml)</td>
</tr>
<tr>
<td>SDS PAGE Electrode buffer</td>
<td>Glycine 14.4g, Trizma base 3.0g, 10%SDS (Total volume made upto 1 liters)</td>
</tr>
<tr>
<td>Staining solution</td>
<td>1% Coomassie Brilliant BlueR-250, prepared in destaining solution.</td>
</tr>
<tr>
<td>Destaining solution</td>
<td>Methanol:Acetic acid:Water, 35:10:55</td>
</tr>
<tr>
<td>1M Tris HCL, pH 8.0</td>
<td>121.4g of Trizma base dissolved in distilled water, pH adjusted to 8.0 using 6N HCl (Volume made upto 1 liters with distilled water)</td>
</tr>
<tr>
<td>0.5m EDTA, pH 8.0</td>
<td>186.1 g of disodium salt of EDTA , in 500ml distilled water, pH adjusted to 8.0 with 1M NaOH</td>
</tr>
<tr>
<td>Ethidium bromide</td>
<td>10mg/ml solution in distilled water</td>
</tr>
<tr>
<td>50X Tris-acetate buffer (TAE)</td>
<td>242g of Trizma base dissolved in 57.1 ml of glacial acetic acid and 100ml of 0.5M EDTA(ph8.0) (Volume made upto 1 liters)</td>
</tr>
<tr>
<td>10X DNA loading buffer</td>
<td>2.5%Bromophenol Blue, 2.5% Xylene cynol, 25% glycerol</td>
</tr>
<tr>
<td>Sodium phosphate buffer (PBS)</td>
<td>137mM NaCl, 2.7mM KCl, 4.3mM Na2HPO4, 7H2O, 1.4mM KH2PO4(pH 7.4)</td>
</tr>
<tr>
<td>S1 Nuclease stop buffer</td>
<td>70% formamide, 57 mM EDTA (pH 7.5)</td>
</tr>
<tr>
<td>S1 Nuclease loading dye</td>
<td>Blue dextran : formamide (1:4)</td>
</tr>
<tr>
<td>5X RNA loading buffer</td>
<td>16μl saturated aqueous bromophenol blue solution, 80μl 500mM EDTA (pH8.0), 720 μl 37% (12.3mM) Formaldehyde, 2ml 100%</td>
</tr>
</tbody>
</table>
10x Formaldehyde Gel buffer

Glycerol, 3084μl formamide, 4ml 10X Formaldehyde agarose gel buffer, make volume upto 10ml with RNase free water

200 mM 3-[N-morpholino]propanesulfonic acid (MOPS) (free acid), 50 mM sodium acetate, 10 mM EDTA, pH to 7.0 with NaOH

Trypan Blue

0.4% Trypan blue in PBS

MTT

5mg/ml MTT in PBS

DEPC water

0.01% in MilliQ water

Cell lysis buffer (4X)(Cl buffer)

1.28M Sucrose, 40mM Tris-HCl (pH 7.5), 20mM MgCl\textsubscript{2}, 4% Triton X-100, Final volume make up with water, autoclaved and stored at 4°C until further use.

Nuclear lysis buffer

10mM Tris-HCl (pH 7.5), 400mM NaCl, 2mM Na\textsubscript{2}EDTA (pH 8.0), Final volume make up with water, autoclaved and stored at room temperature until further use.

TE buffer

10mM Tris (pH 8.0), 1mM EDTA (pH 8.0), Autoclaved and stored at 4°C until further use.

2.3 METHODS

2.3.1 Competent cell preparation and transformation (E. coli strains; DH5\textalpha and BL21)

The E. coli culture was allowed to grow overnight and 1 ml of overnight culture was inoculated in fresh 100 ml LB medium at 37°C with shaking at 200 rpm for 2 h. For preparation of high transformation efficiency cells, cells were grown to 0.5 OD\textsubscript{566} and chilled in ice before spinning. The cells were then harvested by centrifugation at 5000 rpm for 10 min at 4°C and supernatant was discarded. Cell pellet was suspended in equal volume of prechilled autoclaved Milli-Q water and centrifuged at 6500 rpm for 10 min. Again the cell pellet was suspended in equal volume of autoclaved prechilled 10% glycerol prepared Milli-Q water. The cells were centrifuged at 7000 rpm for 10 min. The cell pellet was suspended in 1/10<sup>6</sup> volume of autoclaved prechilled 10% glycerol. The cells were again centrifuged at 7000 rpm for 10 min and finally suspended in 500 μl of autoclaved 10% glycerol and 50 μl aliquots of the competent...
cells were prepared and stored at -80°C for future use. The transformation efficiency was checked by transforming cells with 50 ng DNA.

2.3.2 Overexpression of mycobacterial nucleoside diphosphate kinase

Nucleoside diphosphate kinase coding plasmid was transformed in BL-21 cells. A single colony was inoculated in 5ml LB media and allowed to grow overnight. 1 ml inoculum from overnight grown culture was added to 1 liter of YT media. IPTG to induce the expression of protein was added to the media as the OD reaches 0.5 followed by harvesting of cells after 3-4 hours. Pelleted cells were lysed using sonication. The GST-tagged protein was then purified using glutathione columns. The isolated protein was run on a SDS-PAGE to check for the purity of the protein (Figure 2.1).

![SDS-PAGE (12%) showing mNDK expression. Lanes 1-3 indicates overexpressed protein after IPTG induction (20μl, 10 μl and uninduced cell lysate respectively), Lane 4 show purified protein (17kda) after GST-tag removal, Lane 5 show GSH resin with remaining GST tag on it, while last lane contains low molecular weight marker (size is represented in kilodaltons)]

2.3.4 Agarose gel electrophoresis

Agarose gel electrophoresis was performed as described previously (Sambrook and Russell, 2001). DNA fragments of size >400 bp were resolved on 1% agarose gel. The gels were electrophoresed in 1X TAE buffer containing 0.5 μg/ml ethidium bromide.
2.3.5 Elution of DNA from agarose gel

DNA fragment used for cloning and labeling reactions were purified by using DNA isolation kit (Cat no. 20-200-300) using manufacturers protocol (Biological Industries, Israel). The eluted DNA fragments were kept at -20°C until used.

2.3.6 DNA isolation

Genomic DNA was isolated from the peripheral blood leukocytes of the patients, family members and control individuals using a modified salting out procedure (2;3). Briefly, 8-10ml blood was obtained from individuals using ACD Vacutainers (BD Biosciences, San Jose, CA, USA). Four volumes of ice-cold C1 buffer (1X) was added to disrupt the integrity of the cell membrane. The suspension was mixed by inverting the tube several times and incubated on ice for 10 minutes till the solution became translucent. Subsequently, the nuclei were pelleted by centrifugation at 2500 rpm for 15 min at 4°C. The supernatant was discarded and the nuclear pellet was washed again with 20ml of ice-cold 1X C1 buffer. 12 ml of nuclear lysis buffer, 0.8ml of 10% SDS and 50µl proteinase-K (20 µg/µl) was added and the pellet resuspended by brief vortexing. After incubation at 65°C for 2-3 hrs, the digested proteins were precipitated with the addition of 4 ml of 6M NaCl. After centrifugation for 15 min at 2500 rpm, the supernatant was transferred to another tube. Two volumes of absolute ethanol (room temperature) was then added slowly to the supernatant to spool the DNA (Miller et al., 1988). The spooled DNA was then washed twice with 70% ethanol, air-dried and dissolved in TE buffer by incubation at 65°C for 2-3 hrs. The quantity of DNA was determined by making appropriate dilutions to determine the OD at 260 nm and 280 nm. The DNA quality was assessed by measuring the 260 nm/280 nm ratio. To determine the integrity of genomic DNA, 2µl of the resuspended DNA was electrophoresed on a 0.8% agarose gel (Sigma, St. Louis, USA), stained with ethidium bromide (Sigma, St. Louis, USA). The stock solution of DNA was diluted to the concentration of 50ng/µl and used for further genotyping experiments. The stock solutions were stored at -20°C until further use.

2.3.7 Sequence analysis and primer design

Minimal functional promoter regions sequences for the genes: recombination activating gene1 (RAG1) and thymidine kinase1 (TK1), to be cloned, were retrieved
from the Eukaryotic Promoter database (http://www.epd.isb-sib.ch/). EPD Gene ID are as follows, for RAG1: EP64001 and for TK1: EP25035. The Primers used for Cloning are shown in Table 2.1. The Primers used for quantitative RT-PCR were designed using Primer express software (Applied Biosystems, USA). The primers were designed in the exonic regions, which were common among different transcripts (Table 5.2). The messenger RNA sequences for multiple transcripts were obtained from ensembl (http://www.ensembl.org/index.html).

### 2.3.8 RNA isolation

Total RNA was isolated using Trizol kit (Sigma) according to manufacturer protocol. Briefly, cells were washed twice with 1X PBS and then the cells were lysed in 1 ml Trizol reagent per well of a 6-well plate. 200 ul of chloroform is added per ml of Trizol used, solution is vortexed for proper mixing of the two and left at room temperature undisturbed for 2-5 minutes. Spin at 10,000 rpm for 15 minutes. RNA is isolated from the upper aqueous layer with isopropanol (500 ul per ml of Trizol). RNA is washed with 75% ethanol (chilled) and dissolved after air drying in DEPC treated water.

### 2.3.9 Formaldehyde agarose gel electrophoresis of RNA

Formaldehyde agarose gel (1.2% agarose) was made by mixing 1.2g agarose in 10 ml 10x Formaldehyde agarose gel buffer. The volume was adjusted with RNase-free water to 100 ml. Mixture was heated to melt agarose and was cooled to 65°C in a water bath. 1.8 ml of 37% (12.3 M) formaldehyde and 1 µl of a 10 mg/ml ethidium bromide stock solution was added to the gel mix. Prior to running the gel, equilibrate in 1x Formaldehyde agarose gel running buffer for at least 30 min. Casted gel as well during the run, the gel was covered with an aluminum foil to protect formaldehyde from light, which is light sensitive.

### 2.3.10 Quantification of RNA quality

Quality of RNA was assessed using spectrometry (260/280 nm ratio) and also by Agilent Bioanalyzer. Formaldehyde gel was additionally run to measure the integrity of the RNA (Figure 2.2A, B). RNA with 260/280 ratio in the range 1.5-2.0 was used for further experiments, in case of microarray experiments the RNA ratio more than
1.8 was used. RNA Integrity Number was also used for quality assessment using Bioanalyzer in case of RNA used for Microarrays (Suitable range ~8.0 to 9.5).

![Figure 2.2: The above figure represents the quality and integrity of RNA used in all the experiments.](image)

2.3.11 Real-time reverse transcription polymerase chain reaction (qRT-PCR)

Two step real-time reverse transcription polymerase chain reaction (RT-PCR) was performed to validate the microarray results as determined by SAM analysis. First strand cDNA synthesis was done using 2.0 μg of total RNA with the help of Omniscript RT kit (QIAGEN) or cDNA archive kit (Applied Biosystems), according to the manufacturer’s instructions in presence of 1uM Oligo (dT) primers (Ambion) and 1uM random hexamers at 37° C for 1 h according to the manufacturer’s instructions (Ambion). Relative quantitative real-time RT-PCR was subsequently performed with an Applied Biosystems 7900 system and SYBR Green Master Mix (Applied Biosystems, Foster City, CA). For each gene, real-time PCR was performed in duplicate wells on cDNA generated from the reverse transcription of 2ug of total RNA, in a 5 μl reaction. Negative controls for each gene included no template (water) and no reverse transcriptase. The PCR amplification consisted of 10 min denaturation at 95°C, followed by 40 cycles of amplification (15 s at 95°C, 60 s at 60°C) according to the manufacturer’s instructions (Applied Biosystems). A standard curve was derived from serial dilutions with an external cDNA obtained from Hela S3 cells or A549 cells for each gene. Relative concentrations were expressed in arbitrary units. Reactions for standard curve were done in 10ul reaction, in duplicates. After amplification, amplicons were melted and the resulting dissociation curve assessed to
ensure a single product. Relative quantification of each of the genes of interest was performed against a housekeeping gene, β-2-microglobulin (B2M), according to Relative Standard Curve Method as per the manufacturer’s instructions (Applied Biosystems). Several commonly employed housekeeping genes were assessed. B2M was found to remain stable in expression in both untreated and 100 uM TMPyP4 treated cells.

2.3.12 MTT assay

The cells were grown in 96 well plates in presence of compounds to be tested for either 24 hrs or 48 hrs. After the required time-period the cells were washed twice with PBS.
REGULATORY SIGNIFICANCE OF POTENTIAL G-QUADRUPLEX MOTIFS (PG4)

> cDNA microarray and hybridization

cDNA Microarrays representing 19200 single spotted clones of human genes that represent about 7029 unique genes, from University Health Network Microarray Centre (UHN, Canada) were used in the present study. 10μg of RNA was isolated from treated (100μM TMPyP4 for 48hrs) and untreated HeLa S3 cells and A549 cells using TRIZOL reagent (Sigma) as per manufacturer protocol. The RNA was converted to cDNA using Microarray cDNA Synthesis Kit (ROCHE), as per manufacturer protocol. Purified cDNA was labeled with either Cy5 or Cy3 dyes (Amersham Biosciences) with the help of RNA target synthesis kit T7 (Roche) as per manufacturer’s protocol. The untreated control samples were labeled with Cy3 and the treated samples were labeled with Cy5. Labeling was reversed in case of dye swap reactions. Labeled product was purified with Microarray Target Purification Kit (Roche) as per manufacturer’s protocol. For each cell-line four replicate experiments were done, consisting of two standard replicate arrays and two dye swap arrays. The labeled cRNA, both Cy3 and Cy5 labeled, were pooled together and precipitated with ammonium acetate and the dried pellet dissolved in 18MΩ RNAse free water (Sigma). The labeled product was loaded onto the slides and the hybridization done in presence of Dig Easy hybridization buffer (Roche), 10 mg/ml salmon testis DNA (Sigma) and 10 mg/ml yeast tRNA (Sigma), at 37 °C for 16hrs. Slides were washed three times (15 minutes each) with 1X SSC and 0.1% SDS at 50 °C with occasional swirifng, followed by three washes with 1X SSC at room temperature (15 minutes each). Final two washes were done with 0.1X SSC for 15 minutes, each. The slides were dried and scanned at 10 um resolution with GenePix 4000A Microarray Scanner (Molecular Devices), using lasers for both Cy3 and Cy5 dyes.

> Microarray data filtering and analysis

Array images were scanned using GenePix 4000A Microarray Scanner (Molecular Devices), using both green and red lasers. The 16 bit TIFF images were preprocessed and quantified using Gene Pix Pro 6.0 (Molecular Devices). Data normalization was performed using Acuity 4.0 (Molecular Devices). Ratio based normalization was done
for all slides. Data were expressed as the log2 ratio of the samples and the reference, for each spot on the array. Data was filtered to include only those elements which contained only a small percentage (<3) of saturated pixels, were not flagged bad or found absent (flags >= 0), had relatively uniform intensity and uniform background (Rgn R2 (635/532) >= 0.6) and were detectable above background (SNR >= 3). Analyzable spots in at least three of four biological replicates performed were retrieved for downstream analysis using Significance Analysis of Microarrays (SAM 2.21, Excel Add-In, Stanford (5)) under the conditions of one class response and 100 permutations, with data input parameters kept at the default values. The results from SAM analysis include a SAM score and false discovery rate (q-value). Differentially expressed genes were retrieved at a cut-off of around 20% False Discovery Rate (FDR).

Quantitative real time RT-PCR

Two step real-time reverse transcription polymerase chain reaction (RT-PCR) was performed to validate the microarray results as determined by SAM analysis. RNA was isolated using Trizol reagent (Sigma) and first strand cDNA synthesis was done using 2.0 µg of total RNA with the help of High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) at 37 °C for 2 hrs. Relative quantitative real-time RT-PCR was subsequently performed with an Applied Biosystems 7900 system and SYBR Green Master Mix (Applied Biosystems), as per manufacturer’s protocol. Reverse and forward primer sequences for the genes of interest were designed using Primer Express software (Materials). For each gene, real-time PCR was performed in duplicate wells on cDNA generated from the reverse transcription of 2 µg of total RNA, in a 10 µl reaction. Negative controls for each gene included no template (water) and no reverse transcriptase. The PCR amplification consisted of 10 min denaturation at 95°C, followed by 40 cycles of amplification (15 s at 95 °C, 60 s at 60 °C). A standard curve was derived from serial dilutions with an external cDNA obtained from HeLa S3 or A549 cells for each gene. Relative concentrations were expressed in arbitrary units. After amplification, amplicons were melted and the resulting dissociation curve assessed to ensure a single product. Relative quantification of each of the genes of interest was performed against a housekeeping gene, β-2-microglobulin (B2M), according to Relative Standard Curve Method as described previously (6). Several commonly employed housekeeping genes were
assessed; B2M was found to remain stable in expression in both untreated and 100 μM TMPyP4 treated cells.

- **G4 DNA motif searching, genomic mapping**

Potential G4 motif (PG4) sequences were mapped to the promoters of all differential expressed genes. PG4 sequences were searched with a program written using Perl. A general pattern: \( G_n-NL_1-G_n-NL_2-G_n-NL_3-G_n \), where \( G \) is guanine and \( N \) is any nucleotide including \( G \). The number of guanines constituting the stem of PG4 DNA is given by \( n \). \( n \) was varied from 2 to 5 while the loop length was varied from 1 to 7. The same program was rerun with cytosine instead of guanine to identify motifs on the \( - \) strand and appropriately corrected for orientation before mapping their position in the context of genes.

- **Tissue specific enrichment analysis of differentially expressed genes in microarrays**

Enriched expression of genes which had significantly responded in microarrays was evaluated from the list of expressed genes (S) for each tissue in 79 normal human tissues (4). Both up-regulated genes and down-regulated were considered for the analysis. \( z \)-score of each gene-set was evaluated with respect to the rest of the genes in S (control) for each tissue using the Mann-Whitney Test, which measures enrichment in terms of number of standard deviations away from the mean of control set. Similar analysis was done for evaluation of enrichment of expression in 12 cancer tissues for the genes whose expression was decreased in presence of TMPyP4, in array.

- **Pathway mapping**

To elucidate the different pathways that may be primary responsive to TMPyP4 treatment via G4 formation, it is extremely useful to analyze the relationship of the functions and the actual expression levels of mRNA of different genes in the context of G4 formation and it behaving as a transcription regulator. All the genes which were differentially expressed after TMPyP4 treatment were mapped onto different biological pathways using ArrayXPath. ArrayXPath (http://www.snuib.org/software/ArrayXPath/) is a web-based service for mapping and visualizing microarray gene-expression data for integrated biological pathway
resources (1). When one inputs gene-expression clusters, ArrayXPath produces a list of the best matching pathways for each cluster. All the responded genes were divided into two clusters: Cluster1: Up-regulated genes (851) and Cluster2: Down-regulated genes (314). Fisher's exact test was applied to evaluate the statistical significance of the association between a cluster and a pathway. Enriched pathways with a p-value <0.05 were considered significant.
Chapter 2

IN VIVO PROBING OF QUADRUPLEX MOTIFS: CLONING AND EXPRESSION ASSAYS

➢ Plasmid used for cloning

The promoter less pGL2 basic vector and SV40 promoter harbouring pGL3 luciferase control vectors were obtained from Promega. The plasmids were isolated as described above.

➢ Polymerase chain reaction

Promoter regions of respective genes were amplified from human genomic DNA using Polymerase chain reaction (PCR), using previously described methods. The list of oligonucleotides and nucleotide sequence used for various PCR reactions are described in Table 2.1. All PCR reactions were performed using Taq polymerase (MBI, Fermentas). Fwd and Rev denote the forward and reverse primer. Restriction sites used for cloning PCR product are shown in bold letters.

Table 2.1: Primers used for cloning

<table>
<thead>
<tr>
<th>S.No.</th>
<th>Gene Name</th>
<th>Primer/Plasmid ID</th>
<th>Sequence (5’-3’*)</th>
<th>Amplified Fragment Length</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Recombination activating gene1 (RAG1)</td>
<td>RAG1fwd</td>
<td>CTAAAGACTCGAGTGGCAGCTGGA</td>
<td>~225bp</td>
</tr>
<tr>
<td></td>
<td></td>
<td>RAG1rev</td>
<td>GTTGAAGATGTCTAAGCTTTGGTTGA</td>
<td></td>
</tr>
<tr>
<td>2.</td>
<td>Thymidine Kinase1 (TK1)</td>
<td>TK1fwd</td>
<td>AAATCTCCCCTCGAGTCAGCGG</td>
<td>~186 bp</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TK1rev</td>
<td>AGCTCATTAAGCTCCGGAAGATTC</td>
<td></td>
</tr>
<tr>
<td>3.</td>
<td>c-MYC</td>
<td>Del 4</td>
<td>This plasmid was a gift from Prof. Bert Vogelstein (Howard Hughes Medical Institute, MD)</td>
<td>~850 bp (around TSS)</td>
</tr>
</tbody>
</table>

*Forward and Reverse primers have incorporated restriction enzyme sites for XhoI (CTCGAG) and Hind III (AAGCTT) respectively.

All the PCRs were performed using 1X Taq polymerase buffer (10mM Tris, pH 9.0; 50mM KCl, 0.01% gelatin), 10pmole each of forward and reverse primers, 1.5 or 2.0 mM MgCl2 (condition standardized for each amplicon), 250μM deoxyribonucleotide triphosphates (dNTPs) and 0.03units/μl of Taq DNA polymerase (Bangalore Genie, India). The PCR thermo-cycling conditions, generally involved an initial denaturation step at 94°C for 5 minutes (min), followed by 25/35 cycles (25 cycles for GeneScan
and 35 cycles for SNapShot and Sequencing) of 30sec denaturation at 94°C, 30 sec annealing at primer-pair specific temperature (Tm), extension at 72°C for 1min per Kb of the amplicon; followed by a final extension at 72°C for 10 min and final cooling at 4°C. The PCR product was resolved on 1% agarose gel and purified by GFXTM PCR and gel band purification kit as described above.

➢ Restriction digestion of DNA

The restriction enzyme digestions of DNA were carried out at specified temperature, as per manufacturer’s recommendations. The analytical digestions were carried out in a reaction volume of 30 µl. 2% agarose gel was run to analyze the restricted DNA.

➢ Ligation of DNA termini

Ligations reactions were carried out in a volume of 10 µl at 16°C in water bath for 12-16 h or at 37°C for 3-4 hrs. The reaction mixture contained appropriate molar concentration of backbone and insert, 1X ligase buffer containing 1mM ATP and 1U T4 ligase. Two microlitre of ligation reaction was used for transformation of 50 µl E. coli competent cells and selected on LB agar medium supplemented with appropriate antibiotics.

➢ Screening of clones

The plasmids after ligation were transformed in E.Coli, DH5α. Plasmids were isolated following the manufacturer protocol by using plasmid isolation kit (Qiagen miniprep kit). Two approaches were followed for screening the colonies: 1. Restriction digestion of the plasmid with the enzymes used for cloning (Hind III and Xho I); 2. Sequencing the plasmids in all the colonies (commercially done by TCGA, Delhi).

➢ Eukaryotic cell culture

Three different cell-lines were used in the present study; human cervix cancer cells, HeLa S3; human lung carcinoma cells, A549 and lung squamous cell carcinoma cells, H520. These cell lines were procured from NCCS Pune, INDIA. Cells were grown in DMEM complete medium with antibiotics. The cells were cultured in test plates whenever experiments were done.
Transfections and luciferase assays

Transfections were done using Lipofectamine2000™ (Invitrogen) according to the following protocol: Lipofectamine: Plasmid ratio used was 3:1 for approximately 5-7 Lakh cells (~80% confluency in a 6-well plate (Griener, Germany)). The transfections were done in absence or presence of TMPyP4 (10, 50, 100μM concentrations) and protein was isolated using 1X Cell Lysis Buffer, provided with the Luciferase kit (Promega) at 24 hrs or at 48hrs timepoint. Prior to transfections and cell lysis the cells were washed twice with 1X PBS (phosphate buffer saline). Luciferase counts were normalized using total protein concentrations. Luciferase counts were monitored in a luminometer.
ANALYSIS OF QUADRUPLEX PRESENCE IN PROMOTERS OF CANCER VERSUS NON-CANCER GENES

Selection of genes involved in oncogenesis

To explore the functional relevance of quadruplex in the regulatory regions of oncogenes, cancer related genes were selected as sample set, taken from a published study which reports meta-analysis of cancer microarray data for the identification of common transcriptional profiles of neoplastic transformation and progression. There were 67 genes which showed consistent differential over-expression across 12 different cancer tissues. The −1 kb to +100 bases proximal promoter regions of these genes were downloaded from ‘Promoter database’ (http://rulai.cshl.edu/cgi-bin/TRED/). Annotated promoters of only 62 genes were available. As a control set, randomly 100 genes not shown to be associated with cancer were selected.

Search for orthologous genes with conserved G4 DNA

To identify the orthologs of human genes in mouse and rat, promoter database, Transcription regulatory element database (TRED) was used. The −1 kb to +100 bases of promoter regions (with reference to TSS) of orthologously conserved genes, were extracted and searched for G4 DNA motif sequences as per the search strategy discussed above. The promoter regions were first mapped for G4 motifs and then aligned using T-COFFEE (Version_5.05) [http://www.tcoffee.org]. The one or more motifs occurring in ±100 bases of G4 sequence were considered as conserved motifs.

Mapping of transcription factor binding sites to G4 DNA

A ±100 bases flanking a PG4 DNA was used for mapping transcription factor binding sites, Based on earlier reports it was predicted that a transcription factor binding site occurring within a window size of 100 bases may be more relevant as a regulatory signal as compared to a site farther away from a G4 DNA. Mapping of all the known transcription factor binding sites onto the promoters (C and NC genes) was done using ‘Matrix Search for Transcription Factor Binding Sites’ MATCH™ (www.gene-regulation.com) under condition ‘to minimize false positives’.
CYTOSINE-RICH SEQUENCES FORMS FOUR STRANDED MOTIFS IN SOLUTION, AT NEAR PHYSIOLOGICAL CONDITIONS. CASE STUDY: c-MYC

Circular dichroism spectroscopy

The 31-mer oligonucleotide d(CCC CAC CTT CCC CAC CCTCCC CAC CCT CCC C) from promoter site of c-MYC corresponding to bases 2181–2211 of the locus was obtained from Sigma Genosys. Single-strand oligonucleotide concentration was determined using molar extinction coefficient (ε260nm=7913 M⁻¹cm⁻¹). All CD measurements were performed on Jasco Spectropolarimeter (model J 715) equipped with a thermostat controlled cell holder and the cell path length was 1 cm. pH titrations were performed at 20 °C by addition of NaOH directly to 2 mL of 10 mM sodium cacodylate pH 4.8, in a cuvette and monitoring pH using an Orion microelectrode. For temperature dependent CD scan, temperature was increased at a heating rate of 0.25°C/min and DNA samples were equilibrated for 5 min prior to each scan. CD spectra were recorded from 220 to 400 nm with an averaging time of 3 s. All experiments were done with 2.5 – 4.0 μM oligonucleotide in 10mM sodium acetate (pH 5.3, 5.6, and 6.1).

UV-Visible spectroscopy

UV-Visible thermal scans were performed on a Cary 400 (Varian) spectrophotometer equipped with Hitachi SPR-10 thermoprogrammer and temperature probe. UV spectra were recorded from 220 to 400 nm, with an averaging time of 3 s, in 10mM sodium acetate. Heating rates were fixed at 0.5°C/min. Thermal denaturation was recorded at 260 nm with a heating rate of 0.5°C/min. Samples were annealed and degassed, before each experiment, by heating the sample cuvette at 95°C for 5 min and then slowly cooling to room temperature. Each sample was allowed to stabilize for 5 min at the initial temperature of each heating-cooling cycle. Mole fraction of the folded oligonucleotide (α) was evaluated as ΔA/ΔAₘₐₓ, where ΔA is the change in absorbance at 260 nm at any temperature and ΔAₘₐₓ is the maximum change recorded at the highest temperature observed.
Chapter 2

Non-denaturing polyacrylamide gel electrophoresis

Non-denaturing gel electrophoresis experiments were performed with the 31-mer oligonucleotide 5'-end labeled with T4 Polynucleotide kinase (New England Biolabs) and [γ-32P] ATP. Free ATP was removed by gel exclusion (column) chromatography. All reactions had 10nM Probe (or dT31), 5'-end labeled with [γ-32P] ATP. Reactions were performed in 50mM Tris-HCl, pH 7.0 (or pH 7.4), 1mM (or 20 mM) MgCl2 and 75mM NaCl for 10 min at 37°C in the presence of 1mM single strand poly(dA) as non-specific competitor. Radiolabeled oligonucleotide after heating at 95°C for 5 min followed by incubation at room temperature for 30 min in the respective buffer were loaded on a non-denaturing 20% polyacrylamide gel. Electrophoresis was performed in a thermo-stated apparatus (SE 600, Hoefer Scientific) run at 4 °C for 16 h at 70 V (about 5 V/cm). Gels were vacuum-dried and analyzed on a phosphorimager (Fujifilm 1800).

SI nuclease digestion

Enzymatic digestions were performed at two different pH; 7.0 and 5.3. Reaction mixtures contained 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 SI Nuclease (US Biochemicals). The template, 5'-fluorescein-labeled oligomer (6.5 µM in 15µM of reaction mixture) was incubated overnight at 4 °C in respective buffers before initiating digestion at 37°C by adding SI nuclease and Zn2+. Digestions were stopped by removing 5 µL aliquots after 5 and 10 min, adding them to 4µL of stop buffer, and freezing. Frozen samples were added to loading buffer and heated at 95 °C for 1 min prior to loading on a 4.5% polyacrylamide gel and electrophoresed (90 min in 1X TBE running buffer) at 55 °C and 27 V/cm using the 3100 Genetic Analyzer (from Applied Biosystems). Fluorescent size markers were loaded along with the sample as internal standards. Bands were scanned by a CCD application integrated with the instrument.
2.4 BIBLIOGRAPHY


