TEMPLATE DIRECTED INTERFERENCE FOOTPRINTING OF PROTEIN-PHOSPHATE CONTACTS IN DNA

Origène Nyanguile[‡] and Gregory L. Verdine*

Department of Chemistry and Chemical Biology, Harvard University, 12 Oxford Street, Cambridge, Massachusetts 02138, USA.

E-mail: verdine@chemistry.harvard.edu.

Supporting Information:

Materials and Methods

Materials. 2'-deoxythymidine was purchased from Peninsula Laboratories, Inc. Methylphosphonic acid, 1,3-dicyclohexylcarbodiimide and Sodium methoxide were from Aldrich. Pyridine was from Alfa Aesar. Tributylammonium pyrophosphate was from Sigma. Naturally occuring dNTPs, thermostable inorganic pyrophosphatase, and T4 polynucleotide kinase were from New England Biolabs, Inc. Sequenase 2.0 and the Sequenase 2.0 kit were from United States Biochemical (Cleveland, OH). [γ-^{33}P]ATP and [γ-^{32}P]ATP were from New England Nuclear. NFATp (residues 396-678, with an 18-residue N-terminal His_6-tag) was overexpressed in *Escherichia coli* and purified on a nickel-chelate column as described.[1] HIV reverse transcriptase a generous gift of H. Huang.[2]

Synthesis of 5'-O-(methylphosphonyl)-2'-deoxythymidine. For this synthesis, we employed a modified version of literature procedures.[3] Briefly, methylphosphonic acid (82 mg, 0.84 mmol) and 2'-deoxythymidine (204 mg, 0.84 mmol) were dissolved in 3.2 mL of freshly distilled pyridine. 1,3-Dicyclohexylcarbodiimide (347.5 mg, 1.68

mmol) was added and the reaction mixture was stirred at 45°C for 2 hrs. Water (93.5 mL) was added, the precipitate was filtered off, washed extensively with water (80mL) and the filtrate was freeze-dried. The residue was dissolved in water (100 mL) and 50 mL of the resulting solution was loaded on an anion-exchange column (DEAE Sepharose fast flow 21 x 1.5 cm) equilibrated with water. The column was washed with 100 mL of water and elution was carried out with a 340 mL gradient from 0 to 0.3M ammonium bicarbonate, with 10 mL fractions been collected. Fractions 6-9 were combined and freeze-dried. The yield was estimated to be 0.4 mmol (47%) by spectrophotometric analysis using the reported coefficient (λ_{max} = 267nm, ε = 9600) for the related compound 2'-deoxythymidine-5'-phosphate. ^{1}H-NMR (400 MHz) (D_2O): δ 1.27 (3H, d): 1.73 (3H, s, CH_3): 2.15-2.35 (2H, m, H2'); 3.86 2H, br s, H5'); 3.4 (1H, br s, H4'); 4.39 (1H, br s, H3'); 6.16 (1H, t, H1'); 7.54 (1H, s, H6). ^{31}P-NMR (500 MHz) (D_2O/ 85% H_3PO_4 ext) : δ 24.96 (s). MS (FAB, negative mode): 319 (M-1). HPLC retention time = 11.7 min.

Synthesis of 5'-O-(α-methylphosphonyl)-β,γ-pyrophosphoryl-2'-deoxy-thymidine.[3] Carbonyldiimidazole (38.24 mg, 0.224 mmol) was added to a suspension of 5'-O-(methylphosphonyl)-2'-deoxythymidine (15.12 mg, 47.2 μmol) in dry DMF and the resulting solution was stirred at room temperature for 2 hrs. A solution of 10% methanol in DMF was added (153 μL) to quench the excess carbonyldiimidazole. After 30 min at room temperature 300 μl of 0.8M bis-(tri-n-butylammonium)pyrophosphate (0.24 mmol) in dry DMF was added slowly and the mixture was stirred overnight at room temperature. The solution was centrifuged 2 min at 14'000 rpm and the supernatant was diluted with 40 mL of 10 mM ammonium bicarbonate pH 7.45 and loaded on DEAE column previously equilibrated. The column was washed with 200 mL 10 mM ammonium bicarbonate and the elution was carried out with a 340 mL gradient from 10 mM to 250 mM ammonium bicarbonate with 12 mL fractions been collected. Fractions 16-18 were combined and freeze-dried. The residue was repurified

by medium-pressure reverse phase chromatography on a PepRPC column (Pharmacia Biotech 5x50 mm) in isochratic mode with water as an eluent. The earliest eluting peak was collected and lyophilized to dryness. The residue was dissolved in water, aliquoted into several fractions and freeze-dried. The residue was stored at -80°C. The yield was estimated to be 629 μmol (18%) by spectrophotometric analysis using the reported extinction coefficient (λ_{max} = 267 nm, ε = 9600) for the related compound 2'-deoxythymidine-5'-triphosphate.[4] ^{1}H-NMR (400 MHz) (D_2O): δ(ppm) 1.60 and 1.64 (3H, 2d, P-CH_3); 1.74 (3H, s, CH_3); 2.22-2.24 (2H, m, H2'): 3.98-4.08 (1H, m, H5'); 4.40-4.47 and 4.48-4.57 (1H, 2m, H3'); 6.12-6.23 (1H, m, H'); 7.40 and 7.41 (1H, 2s, H6). ^{31}P-NMR (500 MHz) (D_2O/85%H_3PO_4ext): δ 23.71 and 23.45 (2d, P-α); -9.82 (m, P-β); -24.99 (m, P-γ). MS (FAB, negative mode) 478 (M-2).

Preparation of Single-Stranded Template DNA. The single-stranded template employed primers and vectors reported for use with the polymerase chain reaction/immobilized metal affinity chromatography (PCR-IMAC) procedure.[5] One H_6 (6-histaminylpurine)-tagged primer (ref. 6, primer **1b**) and one unmodified primer (ref. 6, primer **2a**) were used to amplify a 183 base pair segment of the plasmid pUC18-mARRE2.[4] This plasmid was derived from the commercial cloning vector pUC18 by inserting a segment of the murine interleukin-2 enhancer into the BamH1 site. We took advantage of the difference in electrophoretic mobility of H_6-tagged DNA strands relative to their complementary strands generated by PCR to accomplish the purification of the two individual single strands, to be used as templates for enzymatic extension. Amplification was carried out in 100 μL reactions (10 tubes), each containing 1 μg of pUC18-mARRE2 as template in 20 mM Tris-HCl (pH8.8), 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.1% Triton X-100, 0.25 mM (each) dATP, dCTP, dGTP and dTTP, 2 units of Vent^R DNA polymerase, 100 pmol (each) of primer **1b** and primer **2a**.[6] The 10 PCR mixtures were combined into 2 fractions, each containing 500 μL. After phenol-chloroform extraction and ethanol precipitation, the duplex DNA was dissolved in 10 mM Tris pH8 (70 μL) and diluted with formamide (70 μL). The DNA was denatured for 3 min at 90°C and loaded directly onto a 6% polyacrylamide denaturing gel. The single stranded templates were visualized by UV shadowing and excised from gel using a razor blade. The gel slices were electroeluted into 130 μL of 10M ammonium acetate using a V-channel apparatus (Harvard BioLabs) with 0.5x TBE [45 mM Tris-borate (pH8), 0.5 mM EDTA] as running buffer. The DNA samples were precipitated directly with 1μL of *E. coli* tRNA 20 mg/mL (Boehringer Mannheim) and 3 volumes of 100% ethanol. The tubes were chilled overnight at -20°C and microcentrifuged for 20 min at 14,000 rpm. The supernatant was removed and the pellet was washed with 3 volumes of 70% aqueous ethanol. After removal of the

ethanol solution, the pellet was air-dried and dissolved in 50 μL 10 mM Tris (pH8). The DNA concentration of the untagged DNA single strand was quantified by UV spectrophotometry (5.21 μM, 26%) and adjusted to 1μM with 10 mM Tris (pH8).

End Labeling of Primer. For primer extension of the single-stranded template**,** primer **3**[6] was radiolabelled at the 5'-end as follows. 2μL of primer **3** (5 pmol) was combined with 1 μL of PNK buffer (70 mM Tris-HCl (pH7.6), 10 mM $MgCl_2$, 5 mM DTT), 6 μL of [γ-^{32}P]ATP (6000 Ci/mmol, 10 mCi/mL), and 10 units of T4 polynucleotide kinase. After 30 min at 37°C, the labeling reaction was stopped with 2 μL 0.5M EDTA and diluted by the addition of 388 μL 10 mM Tris (pH 8). The kinase was removed by extraction with 400 μL phenol:chloroform:isoamylalcohol (25:25:1). The DNA was precipitated with 1 μL of *E. coli* tRNA 20 mg/mL, 40 μL 3M sodium acetate (pH 5) and 1 mL of cold ethanol, followed by washings as described above. The pellet was air-dried and dissolved in 100 μL 10 mM Tris (pH 8).

Primer Extension. 16.25 μL of the untagged DNA single stranded template prepared above (40 fmol/μl) was annealed with 10.8 μl of [γ^{32}P]-labeled primer **3** by heating the resulting mixture 2 min at 70°C and by cooling slowly to room temperature. The 66 μL extension reaction contained 36 mM Tris.HCl (pH 7.4), 3.6 mM $MgCl_2$, 18 mM KCl, 18 mM NaCl, 3.6% glycerol, 18 μM (each) dATP, dCTP and dGTP, 3.64 μM dTTP, 14.55 μM αMe-dTTP, 105 nM HIV reverse transcriptase and 1.2 units of thermostable inorganic pyrophosphatase. The reaction mixtures was incubated for 70 min at 40°C and the extended product was precipitated directly by the addition of sodium acetate 3M (8 μL), *E. coli* tRNA 4 mg/mL (3 μL) and cold ethanol (240 μL). After centrifugation and washing as described above, the pellet was air-dried.

Interference Binding Assay. 0.1 pmol of the extended template was incubated for 30 min at room temperature with various amount of NFATp in 20 mM Hepes•NaOH (pH 7.5), 100 mM NaCl, 1mM dTT, 10% (w/v) glycerol, 10 μg/mL BSA. The bound and unbound protein-DNA complexes were resolved on a 5% non-denaturing

polyacrylamide gel in 1 x TBE. The gel was Saran-wrapped and exposed 15 hrs on a BIOMAX film (Kodak) with an intensifying screen. Bands were visualized by autoradiography and excised from the gel (Figure 5). The gel slices were electroeluted into 130 μL of 10 mM ammonium acetate as described above.

Cleavage and Sequencing Procedure. The pellet obtained after ethanol precipitation was dissolved in water (50 μL) and evaporated. The residue was incubated 40 min at 60°C in 50 μL of 0.5M sodium methoxide in dry methanol. Neutralization and precipitation were carried out simultaneously by addition of 20 μL acetic acid 1.9M and 13 μL of methanol. The solution was stored overnight at -20°C and microcentrifuged 20 min at 14,000 rpm. After removal of the supernatant, the pellet was washed with 70 μL 70% aqueous ethanol. The pellet was air-dried, dissolved in 5 μL 10 mM Tris-HCl (pH8) and 4 μL loading dye containing 95% formamide, 20 mM EDTA, 0.05% bromophenol blue and 0.05% xylene cyanole FF. Samples were heated at 70°C for 2 min, chilled on ice and loaded onto a 7% polyacrylamide sequencing gel. The gel was visualized by autoradiography. The generation of an authentic sample by Sanger sequencing made use of the Sequenase 2.0 kit (United States Biochemicals) according to the manufacturer's protocol. A volume of 2.5 μL of annealed [^{33}P]primer/template mixture was used in each dideoxy termination reaction.

For the piperidine cleavage reaction (Figure 4) 0.2 pmol of [^{33}P]extended template was dissolved in 50 μL of 1M aqueous piperidine. This solution was heated at 90°C for 30 min, cooled to room temperature, and microcentifuged briefly. Samples were evaporated, twice resuspended in 30 μL of water, evaporated, and then dissolved in 5μL 10 mM Tris-HCl (pH 8) and 4 μL loading dye. Samples were loaded onto a 15% polyacrylamide sequencing gel.

Figure Captions

Figure S1. Schematic representation of the TDI-p phosphate footprinting procedure. Primed ssDNA, the annealed duplex consisting of labeled primer **3** and the H6-untagged single stranded DNA that contains the murine IL2 enhancer. PPase, thermostable inorganic pyrophosphatase used to prevent the accumulation of inhibitory pyrophosphate in extension reactions. T_{Me}, thymidyl methylphosphonodiester linkage.

Figure S2. Native PAGE electrophoresis after incubation of various concentration of NFATp with the mARRE2 extended template containing statistically distributed methylphosphonodiester linkages. T_{Me}, the methylphosphonodiester extension product that has not been incubated with NFATp. (a) The bound and unbound fractions are indicated by an arrow. Concentration of NFATp: (lane 1, 2.5×10^{-8} M; lane 2, 5×10^{-8}; lane 3, 7.5×10^{-8}; lane 4, 1×10^{-7}; lane 5, 1.5×10^{-7}). Concentration of DNA: 5×10^{-9} M. (b) Quantitation: the radioactivities of the bound and unbound fractions as determined by scintillation counting before methanolate solvolysis.

Footnotes

1) McCaffrey, P. G.; Luo, C.; Kerppola, T. K.; Jain, J.; Badalian, T. M.; Ho, A. M.; Burgeon, E.; Lane, W. S.; Lambert, J. N.; Curran, T.; Verdine, G. L.; Rao, A.; Hogan, P. G. *Science* **1993**, *262*, 750-754.

2) Huang, H.; Chopra, R.; Verdine, G. L.; Harrison, S. C. *Science* **1998**, *282*, 1669-75.

3) Higuchi, H.; Endo, T.; Kaji, A. *Biochemistry* **1990**, *29*, 8747-53.

4) Maniatis, T.; Fritsch, E. F.; Sambrook, J. *Molecular Cloning: A Laboratory Manual*; Cold Spring Harbor Laboratory Press: Cold Spring Harbor, NY, 1982.

5) Min, C.; Verdine, G. L. *Nucleic Acids Res* **1996**, *24*, 3806-10.

6) Primer **1b** = 5'-d(HHHHHHCGCCAGGGTTTTCCCAGTCACGAC)-3', primer **2a** = primer 5'-dCGCCAGGGTTT-TCCCAGTCACGAC)-3', in which H = N6-histaminyl purine (see Ref. 5). Primer **3** = 5'-d(AACAGCTATGACCATG)-3'.