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# Template-Directed Interference Footprinting of Protein–Phosphate Contacts in DNA

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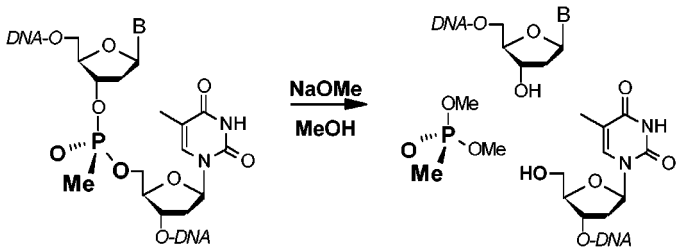
## ABSTRACT




**We have developed a method for interference footprinting of contacted phosphates in protein–DNA complexes. Template-directed enzymatic polymerization using a synthetic triphosphate analogue (αMe-dTTP) generates a product having a modified internucleotide linkage, which perturbs protein–phosphate contacts. We found that treatment of the methylphosphonodiester-substituted extension product under nonaqueous conditions ($MeO^-$/MeOH) led to the formation of a single cleavage product at each T residue but to two cleavage products when treated under the standard aqueous piperidine cleavage protocol.**

Proteins contact DNA through a combination of interactions with the heterocyclic bases and the deoxyribose-phosphate backbone.[1] Although protein−phosphate contacts do not generally appear to play a significant role in DNA sequence discrimination, they often make a dominant contribution to the overall energetics of protein−DNA recognition.[2] High-resolution structures of protein−DNA complexes reveal not only that phosphate contacts are extensive[3] but also that their pattern is a highly characteristic feature of each complex. Determination of electrostatic interactions with the DNA backbone can therefore yield valuable insights into the structural and energetic character of protein−DNA interfaces; this provides impetus for the development of methods that enable routine phosphate contact analysis. Here we report a chemically based method for interference footprinting of contacted phosphates in protein−DNA complexes.

The most widely employed technique for mapping contact phosphates, ethylation interference footprinting, relies on treatment of DNA with a chemical reagent (ethylnitrosourea) that ethylates the anionic phosphodiester oxygens.[4] The resulting ethylphosphotriesters are less able than the corresponding phosphates to participate in hydrogen bonding and Coulombic interactions with a protein; hence they interfere

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(3) Mandel-Gutfreund, Y.; Schueler, O.; Margalit, H. *J. Mol. Biol.* **1995**, *253*, 370−382.

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with binding of the protein when present at a position that is contacted. Although this method has proven valuable in certain instances, its drawbacks have discouraged routine use. Among the practical difficulties are capriciousness in the extent and selectivity of ethylation and the requirement of handling ethylnitrosourea, a toxic, mutagenic, and potentially explosive reagent. More fundamental concerns regarding interpretation originate from the fact that ethyl phosphotriesters are generated as a diastereomeric mixture and are a sterically unconservative replacement for a phosphodiester.

We reasoned that the foregoing limitations might be overcome by designing an alternative phosphodiester analogue that would (i) obviate the need to use dangerous reagents by being incorporated into DNA enzymatically, (ii) exist as a single stereoisomer, (iii) resemble a phosphodiester sterically but differ electrostatically. Finally (iv) the analogue should undergo selective chemical scission leading to the production of a single detectable DNA cleavage product. The method we have developed to fulfill these criteria is an extension of template-directed interference (TDI) footprinting, a design-based approach for determining base contacts in protein−DNA complexes.[5]

The key chemical components of our system (TDI-p footprinting) are 2′-deoxynucleoside 5′-triphosphate analogues having one of the two nonbridging α-phosphate oxygen atoms replaced by a methyl group (for example, αMe-dTTP, Figure 1). Enzymatic DNA polymerization using

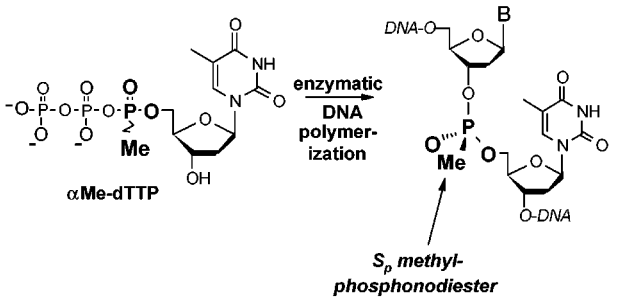


**Figure 1.** Schematic representation of the analogue incorporation step of TDI-p footprinting. αMe-dTTP is the synthetic triphosphate analogue used as substrate for template-directed enzymatic polymerization. The atoms highlighted in bold are incorporated stereospecifically into DNA resulting in the formation of a methylphosphonodiester linkage.

αMe-dTTP as a substrate[6] generates the $S_p$-configurated[7] methylphosphonodiester internucleotide linkage (Figure 1) on the 5′-side of T residues. Experiments using synthetic, monosubstituted oligonucleotides indicated that methylphosphonodiesters can interfere with phosphate contacts,[8] and other data clearly indicate that this linkage is labile toward aqueous base.[9]

To test TDI-p footprinting,[10] we generated a 183 bp single-stranded DNA template containing the consensus binding site for the nuclear factor of activated T cells (NFAT), a protein that acts as a master regulator of T cell proliferation.[11] This template was annealed to a radiolabeled primer, from which extension was catalyzed by HIV reverse transcriptase in the presence of αMe-dTTP and the four naturally occurring 2′-deoxynucleotide 5′-triphosphates (dNTPs). High-resolution gel electrophoretic analysis of the extension mixtures and the products of their cleavage with 1 M aqueous piperidine revealed that an αMe-dNTP:dTTP ratio of 4:1 afforded complete extension, with roughly one methylphosphonodiester substitution per DNA molecule, randomly distributed throughout the DNA pool. However, we found that each T position in the sequence was represented by two cleavage products (Figure 2A), which presumably results from DNA backbone scission on either side of the phosphonodiester (cleavage of either bond ***a*** or ***b*** in Figure 2B); such product mixtures have the highly undesirable effects of complicating the interference analysis and decreasing its sensitivity.[12]

Noting a report that a simple dinucleoside methylphosphonodiester appeared to undergo tandem displacement (at both bonds ***a*** and ***b***, Figure 2C) when treated with $MeO^-$/MeOH,[13] we treated the extension product with 0.5 M NaOMe in anhydrous MeOH and now observed a single band at each T position (Figure 2A). We believe that this modified cleavage procedure will be generally applicable to other labile

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(7) Terminal deoxynucleotidyl transferase has been shown to incorporate only the $S_p$ diastereomer of the methylphosphonodiester into DNA,[6a] from which it can be inferred that the enzyme utilizes only the $R_p$ diastereomer of αMe-dTTP. The stereochemical specificity of terminal deoxynucleotidyl transferase for analogues having modifications at the α-phosphorus is like that of all DNA polymerases tested thus far (Lesnikowski, Z. J. *Bioorg. Chem.* **1993**, *21*, 127−155). Although the stereochemical specificity of HIV reverse transcriptase (RT) for α-modified triphosphate analogues has not been reported, the recently determined X-ray crystal structure of an RT/primer−template/nucleotide complex (Huang, H.; Chopra, R.; Verdine, G. L.; Harrison, S. C. *Science* **1998**, *282*, 1669−1675) shows that the enzyme binds the nucleotide in a way that is nearly identical to that of polymerases that have been analyzed stereochemically.

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(9) Sinha, N. D.; Grossbruchhaus, V.; Köster, H. *Tetrahedron Lett.* **1983**, *24*, 877−880.

(10) A scheme depicting the overall procedure for TDI-p footprinting, accompanied by a detailed experimental procedure, is available in the Supporting Information or can be obtained via the Internet at the URL http://glviris.harvard.edu.

(11) (a) Crabtree, G. R.; Clipstone, N. A. *Annu. Rev. Biochem.* **1994**, *63*, 1045−1083. (b) Rao, A.; Luo, C.; Hogan, P. G. *Annu. Rev. Immunol.* **1997**, *15*, 707−747.

(12) Ethylation interference footprints also show mixtures of products resulting from 5′- and 3′-cleavage of the ethylphosphotriester by hydroxide ion. The major cleavage pathway, however, involves cleavage of the ethyl substituent (with liberation of ethanol), a process that regenerates the original phosphodiester. The dominance with which this cleavage pathway operates greatly reduces the sensitivity of ethylation interference footprints by erasing most of the information-bearing adducts.

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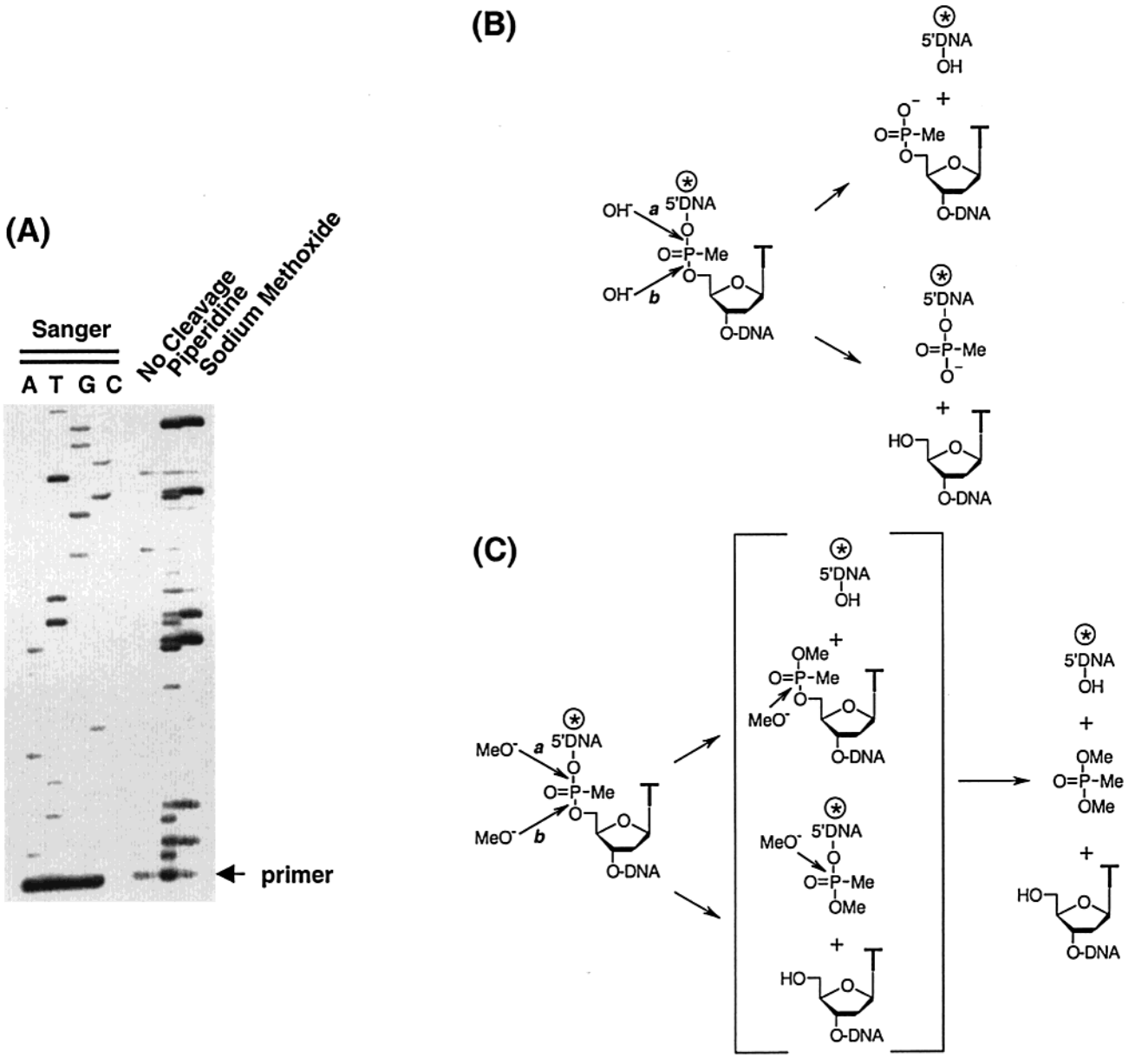


**Figure 2.** Products of cleavage of methylphosphonodiester linkages in DNA under aqueous (1 M aqueous piperidine) versus nonaqueous (0.5 M NaOMe/in anhydrous MeOH) conditions. (A) High-resolution polyacrylamide gel electrophoresis analysis of 5′-radiolabeled DNA bearing statistically distributed methylphosphonate linkages cleaved under either aqueous (piperidine) or nonaqueous (sodium methoxide) conditions. The uncleaved control lane is blank, except for minor premature termination products, because the full-length extension product migrates in the upper position of the gel, which is not shown. Authentic Sanger dideoxy sequencing lanes are shown on the left. Note the 1:1 correspondence between the T Sanger lane and the bands in the base-cleaved DNA samples on the right; the latter migrate somewhat faster (lower in the gel) because they possess one fewer nucleotide unit. (B) Reaction pathway under aqueous conditions, generating two methylphosphomonoester products, which migrate differently on an electrophoretic gel. Circled asterisk denotes the location of the 5′-$^{32}$P label; only those products that contain this label are detected by autoradiography. (C) Reaction pathway under nonaqueous conditions. The two methylphosphonodiester products react further to give a single cleaved DNA species.

backbone modifications, such as phosphotriesters and *S*-alkylphosphorothioates, greatly improving methods based thereon.[4,14]

Having established efficient cleavage conditions, we then incubated the pool of randomly phosphonodiester-modified DNA with varying concentrations of human NFATp[15] and separated protein-bound from unbound fractions using native gel electrophoresis (refer to Supporting Information). Following recovery of the two fractions from the gel, we treated

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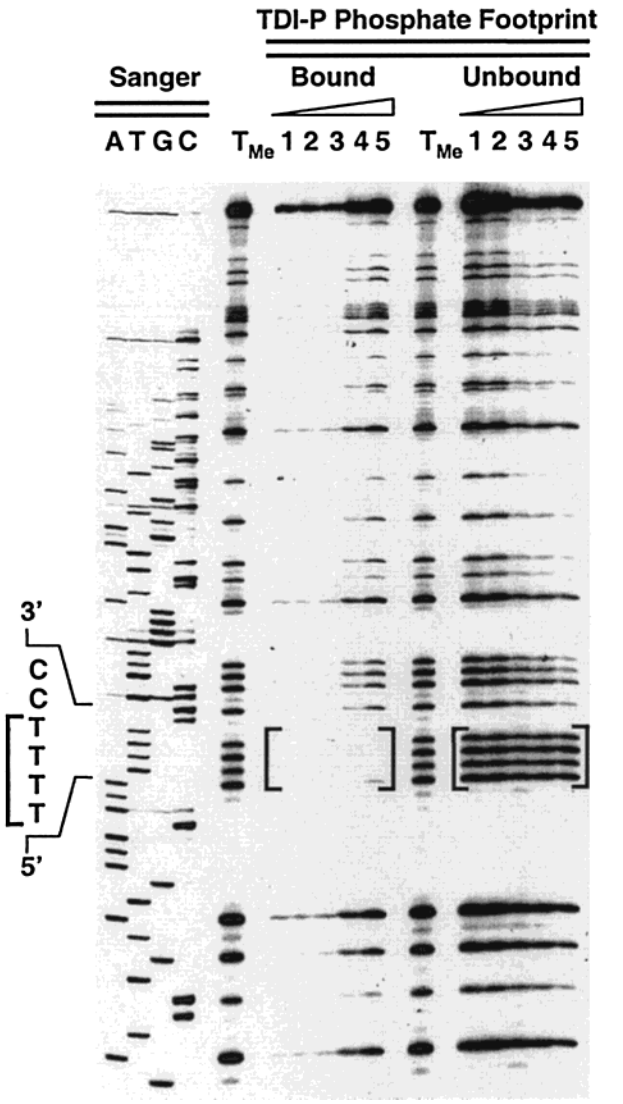


**Figure 3.** TDI-p footprinting of the NFATp−mARRE2 complex. The sequence of the noncoding strand of the consensus NFATp recognition site[16] is shown alongside the gel. The four lanes at the far left (A, T, G, and C) are an authentic Sanger (dideoxy) sequence of the template. The remainder of the gel shows T-cleavage ladders generated after NaOMe/MeOH treatment. Lanes 1−5 represent the cleavage reaction of the respective bound and unbound fraction after incubation with NFATp and native gel electrophoresis (see Supporting Information): lane 1, $2.5 \times 10^{-8}$ M NFATp; lane 2, $5 \times 10^{-8}$; lane 3, $7.5 \times 10^{-8}$; lane 4, $1 \times 10^{-7}$; lane 5, $1.5 \times 10^{-7}$. Concentration of DNA: $5 \times 10^{-9}$ M. A bracket indicates positions at which interference is evident. The two lanes denoted $T_{Me}$ are duplicate controls in which the extended template was cleaved without incubation with NFATp.

them in parallel with NaOMe/MeOH and resolved the cleavage products on a DNA-sequencing gel (Figure 3).

In a TDI footprint,[5] interference is evident as those bands that are weak or missing in the bound fractions while being correspondingly enriched in the unbound fractions (enrichment is most clearly evident at high protein concentrations, when most of the DNA molecules containing noninterfering substitutions have been shifted to the bound fraction). On the basis of these criteria, we observe interference of NFATp binding at four phosphates in the noncoding strand of the consensus site, namely those 5′ to the four T residues; interference was not observed at any other T position. Inspection of the solution structure of human NFATc (an NFATp isoform) bound to DNA[17] reveals that the protein contacts these four phosphates and no others on the noncoding strand. Closer inspection of these contacts reveals that interference of NFATp binding caused by methyl phosphonodiester substitution occurs regardless of whether the protein more closely approaches the pro-*R* or pro-*S* phosphate oxyanion.

Here we have reported a new design-based method suitable for the routine analysis of phosphate contacts in protein−DNA complexes. Although the example shown applies only to phosphate contacts 5′ to thymine, straightforward extension of this procedure using the three other αMe-dNTPs[18] should enable mapping of all such backbone interactions.

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**Supporting Information Available:** Detailed description of experimental procedures and 2 figures describing (i) the TDI-p phosphate footprinting procedure and (ii) native PAGE separation of protein-bound and unbound fractions. This material is available free of charge via the Internet at http://pubs.acs.org.

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