Acknowledgment. We thank John Schellman and Hong Qian, University of Oregon, for discussion and for use of their computer program for the Lifson–Roig theory. This research was supported by NIH Grant GM 31475. J. M. Scholtz is an NIH fellow (GM 13451). We thank Leland V. Miller of the NIH Clinical Mass Spectrometry Resource, University of Colorado, supported by Grant RR 01152, for FAB mass spectra and Robert J. Binard for amino acid analysis.

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Template-Directed Interference Footprinting of Protein–Guanine Contacts in DNA

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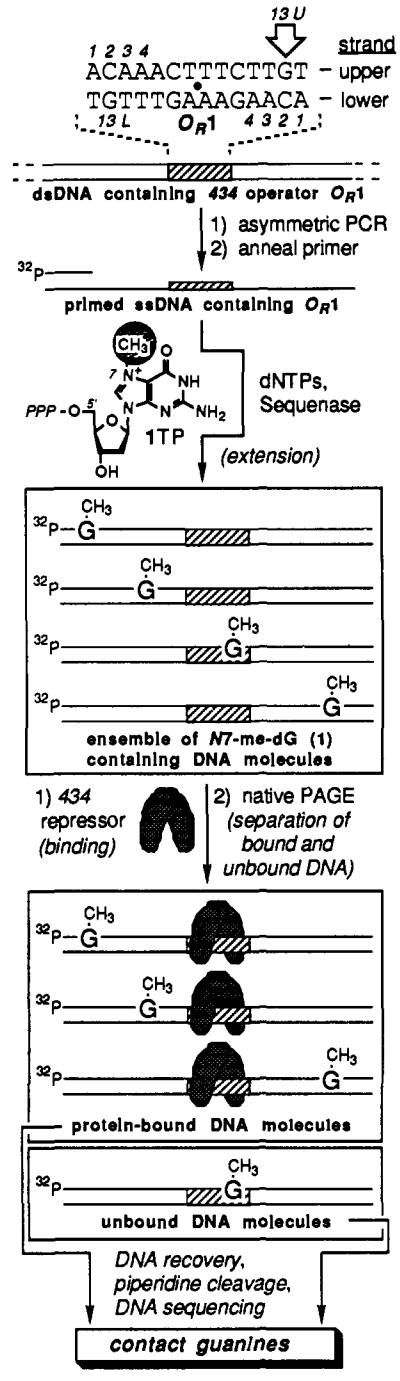
Matched contacts between amino acid residues and DNA bases are important for sequence discrimination by DNA binding proteins.[1] DNA base contacts are thus the subject of chemical methods that probe molecular recognition in protein-DNA complexes.[2-4] These *interference footprinting* methods involve treating DNA with reagents that produce lesions, which are then assayed site-specifically for interference of protein binding; interfering sites are interpreted as contact bases and *vice versa*. Current methods are limited by a common problem: the difficulty of carrying out selective chemical modification of DNA to produce defined lesions that do not significantly alter secondary structure. We report an alternative approach—template-directed interference (TDI) footprinting—that achieves these objectives by enzymatically incorporating base analogues into DNA. Below, we demonstrate the use of TDI footprinting in the analysis of contacts between the *434* repressor protein and guanine (G) residues of the *434* O_R1 operator (binding site).[5]

For TDI footprinting of guanine (TDI-G footprinting), we chose the analogue N^7-methyl-2′-deoxyguanosine (**1**, Scheme I),[6] in which the N^7-methyl group is the interfering function.[2] A radiolabeled primer, annealed to single-stranded DNA[7] containing O_R1, was extended enzymatically by using the four natural 2′-deoxynucleoside 5′-triphosphates (dNTPs) plus the 5′-triphosphate of **1** (1TP) in an amount sufficient to afford approximately one random incorporation per extension.[9] The resulting ensemble of G-methylated DNA molecules was incubated with *434* repressor[10] over a range of protein concentrations,[11] and the protein-bound DNA molecules were separated electrophoretically from unbound DNA.[12] The DNA samples were recovered,

Scheme I



cleaved at residues **1** with piperidine,[2] and electrophoresed on a DNA sequencing gel.[9]

Contact analysis of the O_R1 upper strand (cf. Scheme I) is presented in Figure 1. Sanger sequencing[13] lanes define the O_R1 region, as indicated. The bands in the TDI-G footprint lane *Cn* reveal all sites at which analogue **1** was incorporated; its 1:1 correspondence with the Sanger G-lane demonstrates that the analogue base-pairs only as G. In contrast to the clean DNA modification effected by incorporation of **1**, alkylation of DNA by dimethyl sulfate (DMS lane) produces numerous cleavable

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(6) Hendler, S.; Fürer, E.; Srinivasan, P. R. *Biochemistry* **1970**, *9*, 4141. Commercially available from Sigma.
(7) The 233 base pair (bp) template DNA was obtained by asymmetric PCR (APCR)[8] using a plasmid donor having O_R1 cloned into the polylinker.[9]
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(9) Detailed experimental descriptions are deposited in the supplementary material or may be obtained directly from the authors by FAX (617-495-8755).
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(11) Concentration variation allows one in principle to distinguish the effect of modifying each guanine on the K_d of the protein–DNA complex.
(12) Chodish, L. A. In *Current Protocols in Molecular Biology*; Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Smith, J. A., Seidman, J. G., Struhl, K., Eds.; John Wiley & Sons: New York, 1989; Vol. 2, pp 12.2.1–12.2.10.
(13) Sanger sequencing was carried out by using the same ^{32}P end labeled, primed ssDNA used in TDI-G footprinting (Scheme I). Sanger, F.; Nicklen, S.; Coulson, A. R. *Proc. Natl. Acad. Sci. U.S.A.* **1977**, *74*, 5463.

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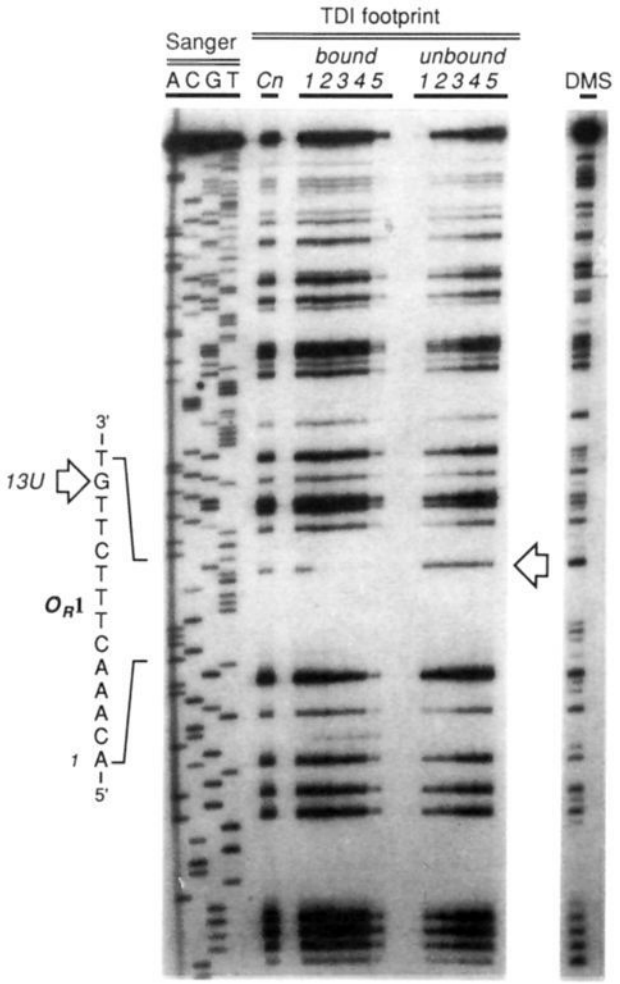

Figure 1. TDI-G footprinting of the *434* operator O_R1 upper strand. O_R1 is contained within a larger DNA fragment; the region corresponding to O_R1 is denoted on the far left. The leftmost set of four lanes is a Sanger sequence of the DNA fragment. The TDI-G footprinting lanes are as follows: *Cn* (*control*), analogue-containing DNA not incubated with *434* repressor (entire G-methylated ensemble of Figure 1); *bound* and *unbound*, protein-bound and unbound analogue-containing DNA, with protein concentration increasing from right to left in each (lane *1*, 1×10^{-6} M, lane *2*, 1×10^{-7}; lane *3*, 2×10^{-8}; lane *4*, 1×10^{-8}; lane *5*, 2×10^{-9}. The concentration of specific DNA used in the binding experiment was $\sim 1 \times 10^{-8}$ M. The rightmost (DMS) lane shows cleavages resulting from DMS treatment of the same DNA fragment.[2,15] Arrow denotes position of G-13U. Piperidine-cleaved DNA molecules (TDI-G and DMS) migrate faster (lower in the gel) than the corresponding dideoxy-terminated (Sanger) molecules because they have one less 3′-nucleoside unit.

lesions at non-G positions,[14] which are primarily the products of adenine methylation.[15] *Thus, by incorporating an analogue into DNA, we have achieved a base-selective modification that could not be accomplished by treatment of DNA with reagents.*

Incorporation of **1** at a contact G-site causes an increase in the concentration of protein required to bind that DNA molecule (i.e., K_d increases) as compared with that required to bind unmodified DNA or DNA containing **1** in non-contact positions. This interference can be seen in the *bound* lanes of a TDI-G footprint (Figure 1) as a band that disappears at higher protein concentration (disappears more quickly moving from left to right) than

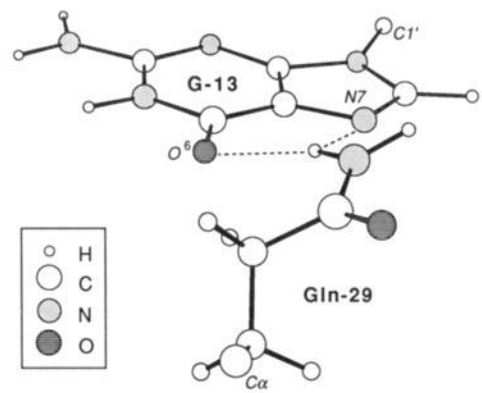

Figure 2. Hydrogen-bonding interaction (dashed lines) between the amide NH of Gln-29 in *434* repressor and the carbonyl oxygen (O^6) and imidazole nitrogen (N^7) of G-13.[5,17] This interaction is disrupted by attachment of a methyl group at N^7, resulting in loss of sequence-specific protein–DNA binding.

the majority of the bands in the *bound* lanes. In the *unbound* lanes, interference is seen as a band that appears at higher protein concentration (appears more quickly moving from left to right) than the majority of the bands in the *unbound* lanes.[16] This behavior is observed at a single position, G-13U in O_R1 (arrow). This base is so critical to site recognition by *434* repressor that substitution of G-13U (and the symmetry-related G-13L;[17] data not shown) by **1** virtually abolishes sequence-specific protein binding.[18] Inspection of the X-ray crystallographic structure of the *434* repressor–O_R1 complex indicates that G-13 hydrogen bonds with *434* Gln-29 (Figure 2).[5] Interference was not observed elsewhere, even at noncontact G residues within the O_R1 lower strand. This agreement with crystallographic data confirms the ability of TDI-G footprinting to generate reliable contact information.

In this report, we have shown that a nucleotide analogue can be incorporated enzymatically into DNA and used to determine protein–DNA base contacts. TDI footprinting has significant advantages over previous methods: (i) it eliminates the need to handle the carcinogen DMS; (ii) a single kind of lesion is produced, the structure of the modified base is known, and the level of modification is easily controlled; (iii) while this study has addressed only major-groove G-contacts, the TDI footprinting approach should be extendable to include major- and minor-groove-contact analysis of all four DNA bases;[19] (iv) this synthesis-based approach affords great control over the nature of DNA modification, which in turn allows one to minimize perturbation of DNA secondary structure;[20] and (v) TDI footprinting probes the thermodynamic value of base contacts and thus complements X-ray or NMR analysis.

Acknowledgment. We thank M. Ptashne for providing the *434* overproducer, S. Harrison for the crystallographic coordinates of the *434* repressor–O_R1 complex, and M. Terranova for assistance in protein purification. G.L.V. is an Alfred P. Sloan Fellow, a Searle Scholar, and an Eli Lilly Fellow. K.C.H. was supported

(14) For the DMS lane, the same ^{32}P end labeled, primed ssDNA used in TDI-G footprinting (Scheme I) was extended using only the four natural dNTPs (no **1**TP). This duplex DNA was then treated with DMS and piperidine-cleaved.[2,9]

(15) Methylation of adenines by DMS produces the minor-groove adduct N^3-methyl-dA, which is of limited usefulness in footprinting protein–DNA complexes.

(16) *Unbound* fractions in interference footprints typically have a high background. Nonetheless, interference can readily be seen by comparing bands of similar maximum intensity; for example, compare G-13U with the band immediately above.

(17) *434* repressor binds as a dimer to the pseudodyad-symmetric O_R1 to form a complex that is approximately C_2 symmetric (dot in Scheme I sequence denotes axis of symmetry).[5] G-13U and G-13L contact symmetry-related Gln-29 residues in the two protamers.

(18) This is apparent by the lack of a band at that position in the *bound* lanes in all lanes but lane *1*, which used a protein concentration above the K_d of *434* repressor for nonoperator DNA sequences.

(19) We have demonstrated the feasibility of TDI-C footprinting using the analogue 5-aza-2′-deoxycytidine 5′-triphosphate. Hayashibara, K. C.; Verdine, G. L., manuscript in preparation.

(20) Although the precise effect of analogue **1** on DNA secondary structure is not known, recent experiments in this laboratory suggest that it is modest, decreasing the T_m of a duplex oligonucleotide by ~ 5 °C. Ezaz-Nikpay, K.; Verdine, G. L., manuscript in preparation.

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Supplementary Material Available: Detailed experimental procedures for TDI-G footprinting and autoradiogram of nondenaturing PAGE gel used in separation of protein-bound from unbound DNA (8 pages). Ordering information is given on any current masthead page.

Radical Reactions of Vinyl Epoxides via Radical Translocations by a Novel 1,5-*n*-Bu_3Sn Group or a 1,5-Hydrogen-Atom Transfer

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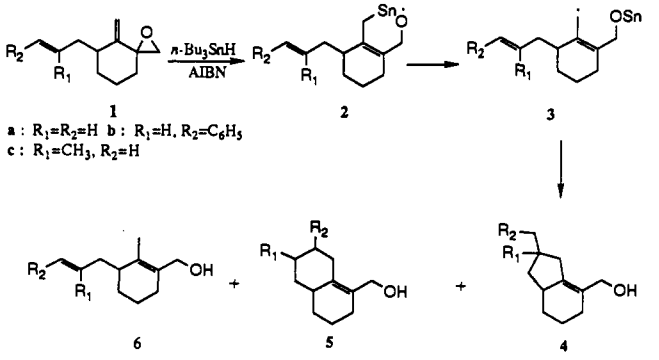
Radical reactions of vinyl epoxides have received little attention[1] despite their synthetic usefulness,[2] and we are unaware of any studies of *n*-Bu_3Sn radical addition to vinyl epoxides, which serves as a starting point for the generation of allylic or alkyl radicals via translocation of radical sites.[3] We wish to report novel sequential radical reactions of vinyl epoxides utilizing 1,5-*n*-Bu_3Sn group or 1,5-hydrogen atom transfer from carbon to oxygen, depending on the structural nature of vinyl epoxides.

Although very little is known on 1,5-transfers of heteroatoms bearing d orbitals[3] such as organosilicon[4] and organotin groups,[5] the ease of a 1,5-*n*-Bu_3Sn group transfer to an alkoxy radical is anticipated on the basis of three factors. First, the C–Sn bond is much weaker than the C–H bond and an alkoxy radical would abstract a 1,5-*n*-Bu_3Sn group rather than a hydrogen atom. Second, 1,5-*n*-Bu_3Sn abstraction should be facile because of the presence of the α-vinyl group. Third, a favorable geometry for 1,5-*n*-Bu_3Sn transfer is realized with **2**. The reaction of the vinyl exo epoxide **1** with *n*-Bu_3SnH occurred smoothly, yielding initially the alkoxy radical **2** bearing allyltin moiety. 1,5-*n*-Bu_3Sn transfer in **2** proceeded rapidly and cleanly, yielding the allylic radical **3** which underwent cyclization as shown in Scheme I.

The radical reaction of a vinyl exo epoxide[6] was carried out by the addition of a 0.05 M benzene solution of *n*-Bu_3SnH (1.2 equiv) and AIBN (0.1 equiv) by a syringe pump for 3 h to a 0.025 M refluxing benzene solution of a vinyl epoxide and an additional stirring for 1 h (method A). As shown in Table I, cyclization reactions depend largely on the nature of substituents.[7,8] The

Scheme I[a]



[a] Sn = *n*-Bu_3Sn.

Scheme II

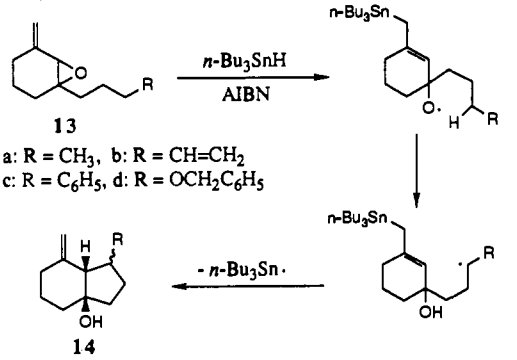
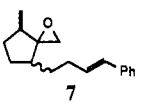
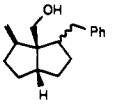
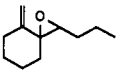
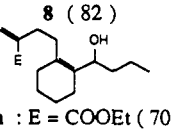
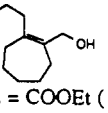
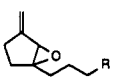
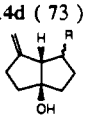


Table I. Radical Reactions of Vinyl Epoxides[a]

vinyl epoxide	method	product (yield, %)[b]
1a	A	**4a** (12), **5a** (50), **6a** (24)
1b	A	**4b** (89)
1c	A	**5c** (84)
 **7**	A	 **8** (82)
 **9**	B[c] B[d]	 **10a** : E = COOEt (70) **10b** : = CN (86)
 **11**	B[c] B[d]	 **12a** : E = COOEt (63) **12b** : = CN (87)
13a : R = CH_3	C	**14a** (60)
13b : = $CH{=}CH_2$	C	**14b** (76)
13c : = C_6H_5	C	**14c** (82)
13d : = $OCH_2C_6H_5$	C	**14d** (73)
		
15a : R = C_6H_5	C	**16a** (60)
15b : = $OCH_2C_6H_5$	C	**16b** (58)

[a] The ratio of diastereomers is shown in ref 24. [b] The yield refers to the isolated yield and was not optimized. [c,d] Allylations were done with 2-carbethoxy- and 2-cyanoallyltri-*n*-butylstannane, respectively.

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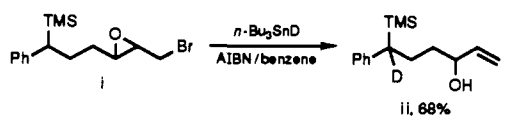
(2) For recent reports, see: Trost, B. M.; Sudhakar, A. R. *J. Am. Chem. Soc.* **1988**, *110*, 7933. Oshima, M.; Yamazaki, H.; Shimizu, I.; Nisar, M.; Tsuji, J. *J. Am. Chem. Soc.* **1989**, *111*, 6280 and references cited therein.

(3) Reviews: Beckwith, A. L. J.; Ingold, K. U. *Rearrangements in Ground and Excited States*; de Mayo, P., Ed.; Academic Press: New York, 1980; Vol. 1, Chapter 4. Freidlina, R. Kh.; Terent'ev, A. B. *Adv. Free Radical Chem.* **1980**, *6*, 1.

(4) As far as we are aware, 1,5-TMS transfer has not been reported. According to our preliminary experiment (i → ii), 1,5-TMS transfer from benzylic carbon to oxygen did not occur, indicative of a strong preference for 1,5-H transfer over 1,5-TMS transfer, probably due to the stabilizing effect of the TMS group (Miura, K.; Oshima, K.; Utimoto, K. *Tetrahedron Lett.* **1989**, *30*, 4413). A similar phenomenon has been recently observed by Curran (Snieckus, V.; Cuevas, J.-C.; Sloan, C. P.; Liu, H.; Curran, D. P. *J. Am. Chem. Soc.* **1990**, *112*, 896).



(5) For one example of 1,5-*n*-Bu_3Sn transfer from enoxyl oxygen to alkoxy oxygen, see: Davies, A. G.; Tse, M.-W. *J. Organomet. Chem.* **1978**, *155*, 25.

(6) Vinyl exo epoxides (**1a**,**1b**,**1c**) were prepared from cyclohexanone in seven steps (LDA, allyl bromide/LDA, HCHO/MsCl–pyridine, DBU/$NaBH_4$–$CeCl_3$/*t*-BuOOH, $VO(acac)_2$/Swern/$KOC(CH_3)_3$, Ph_3PCH_3Br, toluene, reflux).

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