haem *in vacuo* resulted in absorption shifts very similar to those observed for deoxyhaemoglobin (Fig. 4*b*). The α and β bands are replaced by a single peak at 567.5 nm, and the Soret band is shifted to 433.5 nm. The corresponding deoxyhaemoglobin peaks were at 555 and 430 nm (Fig. 4*b*). These changes were completely reversible, demonstrating that FixL* retains its ability to respond to oxygen. The extinction coefficient of monomeric oxy-FixL* at 417 nm is roughly 1.6×10^5 M^{-1} cm^{-1}, on the basis of protein assays and absorbance at 417 nm. Pyridine haemochrome assay suggests that FixL* contains one mole of haem per monomer.

FixL* is a novel haemoprotein (Fig. 4) with kinase activity (Fig. 3). We propose that FixL has at least three distinct regions (Fig. 1). The homologous region (residues 220–464) houses those functions that are common to the two-component sensors, such as ATP binding, partner recognition and phosphorylation (reviewed in refs 5–9). The hydrophobic region (residues 1–85) probably anchors FixL to the cell membrane. These residues, absent in FixL*, are unnecessary for haem binding or phosphorylation. They may stabilize the protein *in vivo* or protect the haem from oxidation. Membrane attachment may also enhance or modify the functions of FixL, or FixL may communicate with other membrane proteins. An extracytoplasmic loop could lie in the deleted hydrophobic region of FixL*. The removal of the extracytoplasmic loop from VirA, in the agrobacterial virulence system, did not eliminate the *in vivo* response to acetosyringone, indicating that the site of acetosyringone-sensing could be intramembranous or cytoplasmic[13]. The remaining region of FixL (residues 86–219) is likely to contain the haem attachment site, that is, the sensing portion of the molecule. This region is most probably on the cytoplasmic side of the membrane, because no hydrophobic stretches separate it from the enzymatic domain. Histidines are expected to have an important role in the function of this molecule, because they are commonly involved in haem coordination and are the sites of phosphorylation in several proteins homologous to FixL[10,15,16]. That FixL is a haemoprotein with kinase activity raises the question of whether the oxygenation state of the haem moiety affects its kinase activity. Experiments are in progress to answer this question and to provide evidence for a linkage between the phosphorylation events involving FixL and FixJ and signal transduction in nitrogen fixation. □

Received 12 November 1990; accepted 22 January 1991.

1. Ditta, G., Virts, E., Palomares, A. & Kim, C.-H. *J. Bact.* **169,** 3217–3223 (1987).
2. Virts, E. L., Stanfield, S. W., Helinski, D. R. & Ditta, G. S. *Proc. natn. Acad. Sci. USA* **85,** 3062–3065 (1988).
3. David, M. *et al. Cell* **54,** 671–683 (1988).
4. Nixon, B. T., Ronson, C. W. & Ausubel, F. M. *Proc. natn. Acad. Sci. USA* **83,** 7850–7854 (1986).
5. Stock, A. M. *et al. Cold Spring Harb. Symp. quant. Biol.* **53,** 49–57 (1989).
6. Stock, J. B., Ninfa, A. J. & Stock, A. M. *Microbiol. Rev.* **53,** 450–490 (1989).
7. Stock, J. B., Stock, A. M. & Mottonen, J. M. *Nature* **344,** 395–400 (1990).
8. Albright, L. M., Huala, E. & Ausubel, F. M. *A. Rev. Genet.* **23,** 311–336 (1989).
9. Gross, R., Arico, B. & Rappouli, R. *Molec. Microbiol.* **3,** 1661–1667 (1989).
10. Weiss, V. & Magasanik, B. *Proc. natn. Acad. Sci. USA* **85,** 8919–8923 (1988).
11. Forst, S., Delgado, J. & Inouye, M. *Proc. natn. Acad. Sci. USA* **86,** 6052–6056 (1989).
12. Bourret, R. B., Hess, J. F. & Simon, M. L. *Proc. natn. Acad. Sci. USA* **87,** 41–45 (1990).
13. Melchers, L. S. *et al. EMBO J.* **8,** 1919–1925 (1989).
14. Gibson, T. J. thesis, Cambridge Univ., UK (1984).
15. Hess, J. F., Bourret, R. B. & Simon, M. I. *Nature* **336,** 139–143 (1988).
16. Jin, S. *et al. J. Bact.* **172,** 525–530 (1990).

ACKNOWLEDGEMENTS. We thank P. Saltman and B. Dyke for discussions, comments on the manuscript and assistance with the spectroscopy; P. Geidushek for comments on the manuscript; and M. Adams and G. Anders for help with immunizations. This work was supported by the NSF. M.G.-G. is supported by a Fellowship from the NIH.

Single-site enzymatic cleavage of yeast genomic DNA mediated by triple helix formation

Scott A. Strobel & Peter B. Dervan*

Division of Chemistry and Chemical Engineering,
California Institute of Technology, Pasadena,
California 91125, USA

PHYSICAL mapping of chromosomes would be facilitated by methods of breaking large DNA into manageable fragments, or cutting uniquely at genetic markers of interest. Key issues in the design of sequence-specific DNA cleaving reagents are the specificity of binding, the generalizability of the recognition motif, and the cleavage yield. Oligonucleotide-directed triple helix formation is a generalizable motif for specific binding to sequences longer than 12 base pairs within DNA of high complexity[1–3]. Studies with plasmid DNA show that triple helix formation can limit the operational specificity of restriction enzymes to endonuclease recognition sequences that overlap oligonucleotide-binding sites[4,5]. Triple helix formation, followed by methylase protection, triple helix-disruption, and restriction endonuclease digestion produces near quantitative cleavage at the single overlapping triple helix–endonuclease site[4,5]. As a demonstration that this technique may be applicable to the orchestrated cleavage of large genomic DNA, we report the near quantitative single-site enzymatic cleavage of the *Saccharomyces cerevisiae* genome mediated by triple helix formation. The 340-kilobase yeast chromosome III was cut uniquely at an overlapping homopurine–*Eco*RI target site 27 base pairs long to produce two expected cleavage products of 110 and 230 kilobases. No cleavage of any other chromosome was detected. The potential generalizability of this technique, which is capable of near quantitative cleavage at a single site in at least 14 megabase pairs of DNA, could enable selected regions of chromosomal DNA to be isolated without extensive screening of genomic libraries.

Chemical reagents and biological methods that infrequently cleave double helical DNA are being developed for genomic mapping[1–12]. Recently, Szybalski and coworkers reported an 'Achilles heel cleavage' technique that limits restriction enzyme cleavage to an overlapping *lac* repressor–endonuclease site[11,12]. Protein-mediated Achilles heel cleavage may, however, not be readily generalized to unique cleavage at selected genetic markers owing to the paucity of applicable DNA-binding proteins relative to the number of sites in megabase genomic DNA. To achieve orchestrated cleavage at selected genetic markers in human DNA by this approach may require the protein-binding sequence to be artificially inserted into the chromosome at precise locations. A more general method for recognition of endogenous DNA sequences might be a chemical approach based on oligonucleotide-directed triple helix formation[1]. Pyrimidine oligonucleotides bind specifically to purine sequences in duplex DNA to form a local triple-helix structure[1–5,13,14]. The generalizable code for triple-helix specificity is derived from thymine (T) binding to adenine–thymine base pairs (T·AT base triplet)[15] and N3 protonated cytosine (C+) binding to guanine–cytosine base pairs (C+GC base triplet)[16,17]. Higher affinity oligonucleotides can be obtained by substituting 5-bromouracil for thymine and 5-methylcytosine for cytosine[18]. Moreover, the number of potential target sequences amenable to recognition by the triple helix motif can be extended to some mixed purine-pyrimidine sequences[19,20]. By replacing the *lac* repressor protein in the Achilles heel technique with triple-helix-mediated protection, one combines the strengths of the two approaches; a general chemical method for recognition of DNA sequences in the range of 15–20 base pairs (bp) with the cleavage efficiency of restriction enzymes (Fig. 1).

To demonstrate single-site enzymatic cleavage of the yeast genome by this technique, a sequence containing an overlapping 24-bp purine tract and 6-bp *Eco*RI site was inserted proximal to the *LEU2* (ref. 21) gene on the short arm of chromosome

* To whom correspondence should be addressed.

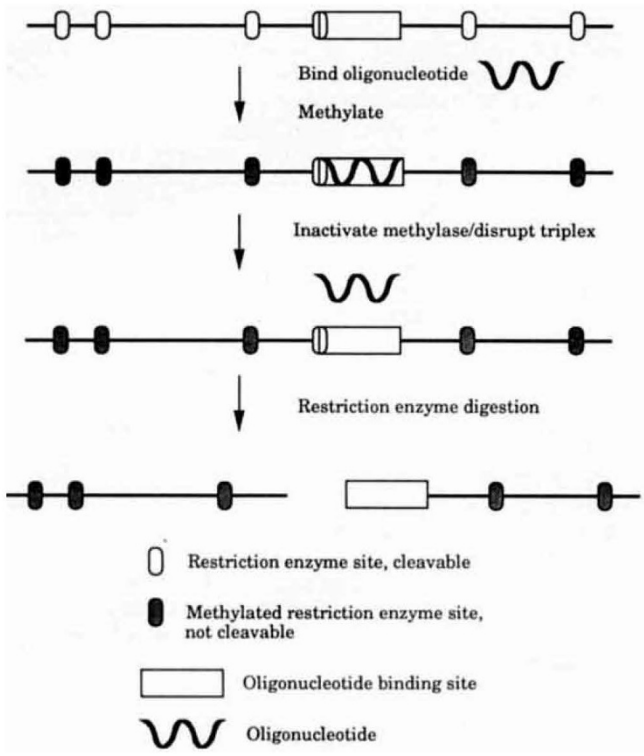


FIG. 1 General scheme for single-site enzymatic cleavage of genomic DNA by oligonucleotide-directed triple helix formation. Chromosomal DNA is equilibrated with an oligonucleotide in a methylase compatible buffer containing polycation. *Eco*RI methylase which methylates the central adenines of the sequence 5'-GAATTC-3' and renders the sequence resistant to cleavage by *Eco*RI restriction endonuclease, is added and allowed to proceed to completion. The methylase is inactivated and the triple helix is disrupted at 55 °C in a high-pH buffer containing detergent. After washing extensively, the chromosomal DNA is re-equilibrated in restriction enzyme buffer and cut to completion with *Eco*RI restriction endonuclease. The cleavage products are separated by pulsed-field gel electrophoresis and efficiencies quantitated by Southern blotting.

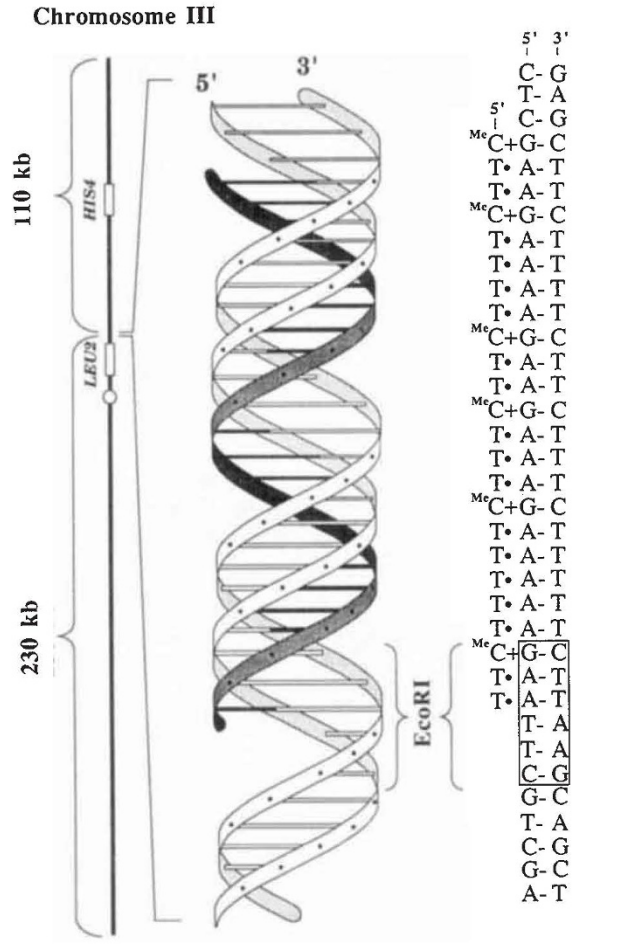
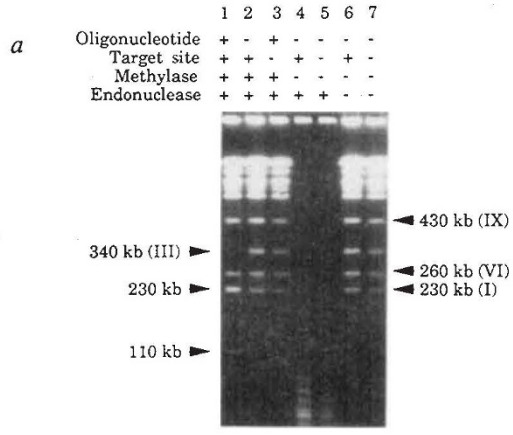


FIG. 2 Left, genetic map of *S. cerevisiae* chromosome III. The locations of the *HIS4* and *LEU2* loci (boxes), the centromere (circle), and the triple helix–*Eco*RI target site are indicated. The expected sizes of the cleavage products are shown. Right, schematic diagram of the triple helix complex overlapping the *Eco*RI restriction–methylation site. The pyrimidine oligonucleotide is bound in the major groove parallel to the purine strand of the DNA duplex, and covers half the *Eco*RI site.



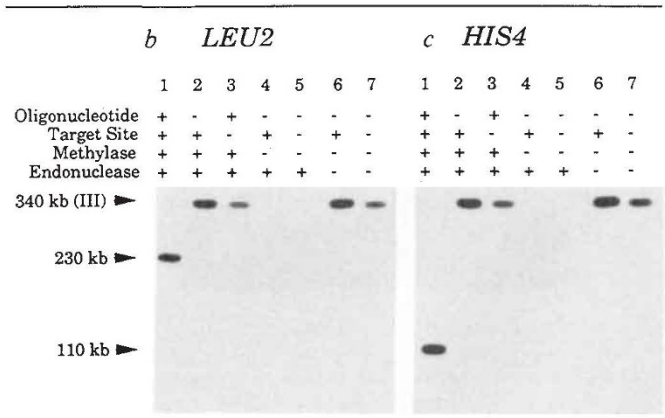


FIG. 3 Single-site enzymatic cleavage of yeast genomic DNA with reagents indicated above figure. 1, Yeast chromosomal DNA embedded in low-melting-point agarose (~50 μl) was washed twice in 1.0 ml of 100 mM NaCl, 100 mM Tris-HCl, 10 mM EDTA, 2 mM spermine, pH 7.6 for 10 min, decanted, and overlayed with 150 μl of the same buffer. Oligonucleotide was added to 1 μM final concentration and incubated 8 h at 22 °C. 2, Bovine serum albumin (100 μg ml^{-1}), *S*-adenosylmethionine (160 μM) and *Eco*RI methylase (80 units) were added to the overlay and incubated with shaking for 3 h. 3, The triple helix was destabilized and the methylase was simultaneously inactivated at 55 °C in 1.0 ml of 1% lauryl sarcosyl, 100 mM Tris-HCl pH 9.5, 10 mM EDTA for 30 min. The oligonucleotide and detergent were then removed with 4 × 10 min washes (1.0 ml) with 10 mM Tris-HCl pH 9.5, 10 mM EDTA. 4, The agarose plug was washed twice with 1.0 ml 2 × potassium glutamate restriction enzyme buffer (200 mM potassium glutamate, 50 mM Tris-acetate, pH 7.6, 20 mM magnesium acetate, 100 μg ml^{-1} BSA, 1 mM 2-mercaptoethanol), decanted, overlayed with 150 μl of buffer and the DNA digested to completion with 20 U *Eco*RI restriction endonuclease for 6 h at 22 °C. After digestion the enzyme was heat-inactivated at 55 °C for 10 min and the DNA loaded onto a 1% agarose, 0.5 × TBE pulsed-field gel at 14 °C, 200 V. Switch times were ramped from 10–40 s for 18 h followed by ramping from 60–90 s for 6 h. Products were visualized by ethidium bromide staining (*a*) and Southern blotting with *LEU2* (*b*) and *HIS4* (*c*) chromosome markers. Cleavage efficiencies were measured using storage phospho imaging plates with a Molecular Dynamics 400S Phospholmager.

III (Fig. 2) (ref. 3). Oligonucleotides designed to form triple helix complexes that overlap half of the *Eco*RI recognition site were synthesized with CT, ^{Me}CT or ^{Me}C^{Br}U nucleotides[18]. The genetic map of yeast chromosome III (refs 22, 23) and affinity cleaving data[3] indicate that cleavage at the target site should produce two fragments 110 ± 10 and 230 ± 10 kilobases (kb) in size (Fig. 2).

Resolution of total yeast chromosomal DNA by pulsed-field gel electrophoresis[24,25] revealed that chromosome III was cut exclusively at the target site when a ^{Me}CT oligonucleotide was used for triple helix formation at pH 7.6 (Fig. 3, lane 1). No cleavage was detected on any other chromosomes under these conditions nor was cleavage observed in the absence of oligonucleotide (lane 2) or in a yeast strain lacking the target sequence (lane 3). The expected 110-kb product was visualized with ethidium bromide staining and confirmed by Southern blotting with a *HIS4* (ref. 26) marker (Fig. 3*c*). The 230-kb product comigrated with chromosome I, but was detected by Southern blotting with a *LEU2* (ref. 24) marker (Fig. 3*b*). The cleavage efficiency was 94 ± 2%. Similar efficiencies were seen with a CT oligonucleotide up to pH 7.4 and ^{Me}CT and ^{Me}C^{Br}U oligonucleotides past pH 7.8 (Fig. 4). The cleavage efficiency with all oligonucleotides was gradually reduced with longer methylation times, suggesting that the oligonucleotide dissociation rate might be the limiting factor for efficiency in this system[27].

The specificity of triple helix formation has been shown to be pH-dependent[1-3]. By lowering the pH, sequences of near but imperfect similarity can be bound and cleaved[1-3]. In agreement with this observation, secondary cleavage sites were revealed as a function of oligonucleotide composition and pH (Fig. 4). Cleavage at the secondary site was preferentially reduced by longer methylation times, suggesting differential oligonucleotide dissociation rates between the primary and secondary target sites. Cleavage at all secondary sites could be eliminated at a threshold pH for each oligonucleotide.

Whereas affinity cleaving using oligonucleotide-EDTA·Fe identifies all sites of oligonucleotide binding[1-3], triple-helix-mediated endonuclease cleavage exposes only those sequences that also partially overlap a restriction site[4,5]. The sequence requirement of a methylation–restriction site increases the cleavage specificity but reduces the number of available sites. To partially overcome this limitation, other commercially available methylation and restriction enzyme pairs with homopurine half sites were tested on plasmid DNA. In addition to *Taq*I (ref. 4) and *Eco*RI (ref. 5), single-site protection of plasmid DNA was possible with *Msp*I, *Hpa*II and *Alu*I methylases. *Bam*HI, *Hae*III and dam methylases could potentially be used, but remain untested.

The generalizability of triple helix-mediated enzymatic cleavage affords high specificity that can, in principle, be customized to unique genetic markers without artificial insertion of a target sequence. The use of degenerate oligonucleotides in this technique to rapidly screen genetic markers for overlapping triple-helix methylation–restriction sites could make it possible to cut chromosomal DNA uniquely and efficiently at endogenous sites with minimal sequence information (S.A.S. and P.B.D., unpublished observations). □

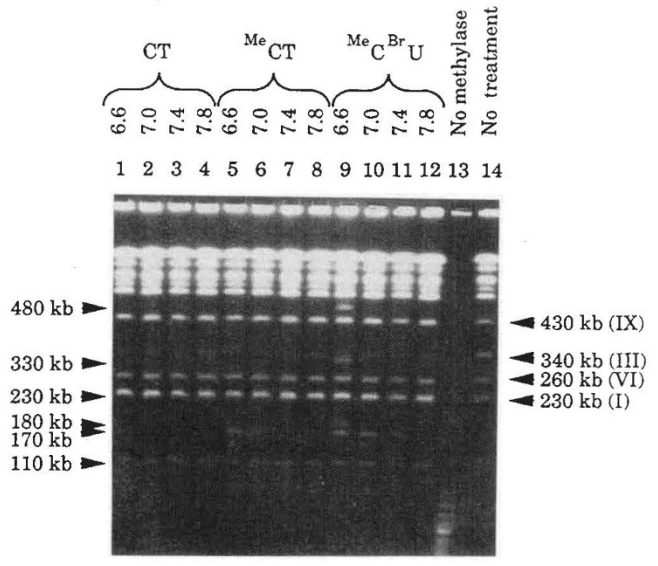


FIG. 4 Triple-helix-mediated enzymatic cleavage of the yeast genome as a function of oligonucleotide composition and pH. Lanes 1–12, reactions on a yeast strain containing the triple helix target site with oligonucleotides (CT, ^{Me}CT and ^{Me}C^{Br}U) and pH values (6.6, 7.0, 7.4 and 7.8) as indicated above figure. Methylation time was 4.5 h. 170-kb and 650-kb (unresolved) secondary cleavage products were observed with both ^{Me}C substituted oligonucleotides at or below pH 7.4. The cleavage site can be assigned to chromosome II (820 kb) by two-dimensional pulsed-field gel electrophoresis in which the DNA was triple helix-protected and methylated before the first dimension and restriction enzyme-digested before the second dimension (data not shown). Additional secondary cleavage sites were observed with the ^{Me}C^{Br}U oligonucleotide at pH 6.6 (lane 9). The 180- and 480-kb products were assigned to chromosome XI (660 kb), and the 330 and 780 kb (unresolved) products were assigned to the VII/XV doublet (1,100 kb) by the same method (data not shown).

Received 5 October; accepted 20 December 1990.

1. Moser, H. E. & Dervan, P. B. *Science* **238,** 645–650 (1987).
2. Strobel, S. A., Moser, H. E. & Dervan, P. B. *J. Am. chem. Soc.* **110,** 7927–7929 (1988).
3. Strobel, S. A. & Dervan, P. B. *Science* **249,** 73–75 (1990).
4. Maher, L. J., Wold, B. & Dervan, P. B. *Science* **245,** 725–730 (1989).
5. Hanvey, J. C., Shimizu, M. & Wells, R. D. *Nucleic Acids Res.* **18,** 157–161 (1990).
6. Barlow, D. P. & Lehrach, H. *Trends Genet.* **3,** 167–171 (1987).
7. Wenzlau, J. M., Saldanha, R. J., Butow, R. A. & Perlman, P. S. *Cell* **56,** 421–430 (1989).
8. Delahodde, A. *et al. Cell* **56,** 431–441 (1989).
9. Patel, Y., Van Cott, E., Wilson, G. G. & McClelland, M. *Nucleic Acids Res.* **18,** 1603–1607 (1990).
10. Weil, M. D. & McClelland, M. *Proc. natn Acad. Sci. U.S.A.* **86,** 51–55 (1989).
11. Koob, M., Grimes, E. & Szybalski, W. *Science* **241,** 1084–1086 (1988).
12. Koob, M. & Szybalski, W. *Science* **250,** 271–273 (1990).
13. Praseuth, D. *et al. Proc. natn Acad. Sci. U.S.A.* **85,** 1349–1353 (1988).
14. Lyamichev, V. I. *et al. Nucleic Acids Res.* **16,** 2165–2178 (1988).
15. Felsenfeld, G., Davies, D. R. & Rich, A. *J. Am. chem. Soc.* **79,** 2023–2024 (1957).
16. Lipsett, M. N. *J. biol. Chem.* **239,** 1256–1260 (1964).
17. Rajagopal, P. & Feigon, J. *Nature* **339,** 637–640 (1989).
18. Povsic, T. J. & Dervan, P. B. *J. Am. chem. Soc.* **111,** 3059–3061 (1989).
19. Griffin, L. C. & Dervan, P. B. *Science* **245,** 967–971 (1989).
20. Horne, D. A. & Dervan, P. B. *J. Am. chem. Soc.* **112,** 2435–2437 (1990).
21. Andreadis, A., Hsu, Y. P., Kohlhaw, G. B. & Schimmel, P. *Cell* **31,** 319–325 (1982).
22. Mortimer, R. K. & Schild, D. *Microbiol. Rev.* **49,** 181–212 (1985).
23. Carle, G. F. & Olson, M. V. *Proc. natn Acad. Sci. U.S.A.* **82,** 3756–3760 (1985).
24. Schwartz, D. C. & Cantor, C. R. *Cell* **37,** 67–75 (1984).
25. Carle, G. F. & Olson, M. V. *Nucleic Acids Res.* **12,** 5647–5664 (1984).
26. Keesey, J. K., Bigelis, R. & Fink, G. R. *J. biol. Chem.* **254,** 7427–7433 (1979).
27. Maher, L. J., Dervan, P. B. & Wold, B. *Biochemistry* **29,** 8820–8826 (1990).

ACKNOWLEDGEMENTS. We thank J. Hanish for helpful discussions. The work was supported by the National Institutes of Health and the Howard Hughes Medical Institute (predoctoral fellowship to S.A.S.).

ERRATUM

Meteoritic silicon carbide: pristine material from carbon stars

Roy S. Lewis, Sachiko Amari & Edward Anders

Nature **348,** 293–302 (1990)

IN this article, the second affiliation for Edward Anders was omitted. In addition to the Chicago address, he is attached to the Physikalisches Institut der Universität, CH-3012 Bern, Switzerland.