18. R. Sen and D. Baltimore, *Cell* **47**, 921 (1986).
19. C. Hunt and R. I. Morimoto, *Proc. Natl. Acad. Sci. U.S.A.* **82**, 6455 (1985).
20. E. Schreiber, P. Matthias, M. M. Müller, W. Schaffner, *Nucleic Acids Res.* **17**, 6419 (1989).
21. DEAE-dextran transfection was performed as described [F. Ausubel *et al.*, Eds., *Current Protocols in Molecular Biology* (Greene and Wiley-Interscience, New York, 1991), suppl. 14, 9.2.4] with the following modifications: Cells were incubated with DEAE-dextran at a final concentration of 500 μg/ml for 15 min before addition of chloroquine to 100 μM for 45 min. No dimethyl sulfoxide shock was performed. RPMI with 10% fetal calf serum was used for all manipulations.
22. We thank C. Rivard for her expert technical help. We also thank K. Saksella for gifts of the pfLuc and pBIIXLuc plasmids; M. Feinberg for gifts of the HIV wild-type and mutant reporter plasmids; and R. Flavell, J. Pober, D. Schatz, and A. Horwich for reviewing our manuscript. E.K. was supported by a predoctoral training grant to the Department of Cell Biology. This work was supported by NIH grant RO1 AI 33443-01A1 to S.G. and the Howard Hughes Medical Institute.

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High-Specificity DNA Cleavage Agent: Design and Application to Kilobase and Megabase DNA Substrates

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Strategies to cleave double-stranded DNA at specific DNA sites longer than those of restriction endonucleases (longer than 8 base pairs) have applications in chromosome mapping, chromosome cloning, and chromosome sequencing—provided that the strategies yield high DNA-cleavage efficiency and high DNA-cleavage specificity. In this report, the DNA-cleaving moiety copper:*o*-phenanthroline was attached to the sequence-specific DNA binding protein catabolite activator protein (CAP) at an amino acid that, because of a difference in DNA bending, is close to DNA in the specific CAP-DNA complex but is not close to DNA in the nonspecific CAP-DNA complex. The resulting CAP derivative, OP^{26}CAP, cleaved kilobase and megabase DNA substrates at a 22–base pair consensus DNA site with high efficiency and exhibited no detectable nonspecific DNA-cleavage activity.

Artificial DNA cleavage agents able to cleave double-stranded DNA at specific DNA sites longer than 8 base pairs (bp) can be constructed by covalently attaching a DNA-cleaving moiety to a sequence-specific DNA binding molecule having a DNA site longer than 8 bp (*1*, *2*). However, such agents have exhibited significant nonspecific DNA-cleavage activity (that is, DNA-cleavage activity at nonspecific, random-sequence DNA sites). In this report, we describe an approach to construct agents that cleave DNA when bound at the specific DNA site but do not cleave DNA when bound at nonspecific DNA sites.

Escherichia coli CAP is a structurally, biochemically, and genetically characterized sequence-specific DNA binding and DNA bending protein (*3*). CAP binds as a dimer of two identical subunits to a 22-bp, two-fold-symmetric DNA site: 5′-AAATGTGATCTAGATCACATTT-3′ (*4*, *5*). CAP bends DNA in the specific CAP-DNA complex to an angle of ~90° (*6*, *7*). As a result of the DNA bend, amino acids 24 to 26 and 89 to 91 on the "sides" of the CAP dimer are close to DNA in the specific CAP-DNA complex. Biophysical data indicate that, in contrast to the situation in the specific CAP-DNA complex, CAP does not sharply bend DNA in the nonspecific CAP-DNA complex (*8*).

We reasoned that it would be possible to exploit the difference in DNA bending in the specific CAP-DNA complex compared with the nonspecific CAP-DNA complex to construct an artificial DNA cleavage agent able to cut DNA in the specific complex but not able to cut DNA in the nonspecific complex. Specifically, we reasoned that attachment of a DNA-cleaving moiety to CAP at an amino acid located on the "sides" of each subunit of CAP would result in a CAP derivative that (i) would place the DNA-cleaving moiety close to DNA in the specific CAP-DNA complex and, thus, would cut DNA in the specific CAP-DNA complex (Fig. 1A), but (ii) would place the DNA-cleaving moiety far from DNA in the nonspecific CAP-DNA complex and, thus, would not cut DNA in the nonspecific CAP-DNA complex (Fig. 1B). Here we have constructed and characterized a CAP derivative having the DNA-cleaving moiety copper:*o*-phenanthroline (*11*) incorporated through a six-atom linker at amino acid 26 of each subunit of the CAP dimer: [(((copper:*o*-phenanthrolin-5-yl) carbamoylmethyl) carbamoylmethyl)-Cys26;Ser178]CAP, referred to as copper:OP^{26}CAP.

To construct copper:OP^{26}CAP, we used a three-step procedure consisting of (i) introduction of a unique solvent-accessible cysteine residue at position 26 of CAP, (ii) cysteine-specific chemical modification, and (iii) metallation (Fig. 2). In step (i), we used site-directed mutagenesis to replace the preexisting solvent-accessible cysteine residue at position 178 with serine and to replace the lysine residue at position 26 with cysteine (*12*). In step (ii), we reacted the resulting CAP derivative with 5-iodoacetylglycylamino-*o*-phenanthroline (*15*), under conditions that resulted in highly efficient and selective derivatization of solvent-accessible cysteine (*16*–*18*). In step (iii), we reacted the resulting CAP derivative with copper(II) in aqueous buffer.

To determine whether copper:OP^{26}CAP is able to cleave DNA, we performed DNA-cleaving experiments with a 7.2-kb DNA substrate containing a single consensus DNA site for CAP [linearized genomic DNA of bacteriophage M13mp2-*lacP1*(*ICAP*)] and with a 48-kb DNA substrate containing a single consensus DNA site for CAP [genomic DNA of bacteriophage λ*i434plac5-P1*(*ICAP*)]. For each DNA substrate, we incubated the DNA substrate with copper:OP^{26}CAP, adenosine 3′,5-monophosphate (cAMP) (the allosteric effector required for site-specific DNA binding by CAP), and reducing agent for 6 hours at 37°C, and we analyzed the reaction products by agarose gel electrophoresis followed by ethidium bromide staining (*20*). With each DNA substrate, copper:OP^{26}CAP cleaved the DNA substrate at the consensus DNA site for CAP, yielding two product DNA fragments with the expected lengths (Fig. 3). The reaction was highly efficient; the reaction proceeded to ≥90% completion. In addition, the reaction was highly specific; there was no detectable nonspecific cleavage of either the DNA substrate or the product DNA fragments. The DNA-cleavage efficiency and DNA-cleavage specificity substantially exceeded those of other artificial DNA cleavage agents (*1*, *2*)—including a CAP derivative having copper:*o*-phenanthroline incorporated at an amino acid within the helix-turn-helix DNA binding motif of CAP (*2*)—and were comparable to those of multistep DNA-alkylation–DNA-cleavage procedures (*23*). Control experiments established that the reaction absolutely required copper:OP^{26}CAP, cAMP, and reducing agent. Additional control experiments, performed with 7.2- and 48-kb DNA substrates lacking DNA sites for CAP [genomic DNA of bacteriophage M13mp2-*lacP1*(−66C;−57G) (*24*) and λ*i434plac5-P1*(−66C;−57G) (*22*)], established that the reaction absolutely required that the DNA substrate contain a DNA site for CAP.

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To determine whether copper:OP^{26}CAP is able to yield single-site, specific cleavage of a megabase DNA substrate, we performed DNA-cleaving experiments with a 4.7-Mb DNA substrate containing a single consensus DNA site for CAP [genomic DNA of *E. coli* strain XAE4000: Δ*lacproX111 argEam metB ara rpoB nal Δcrp45 strA fnr 1 zci*::Tn10 Su2 λ *i434plac5-P1*(*ICAP*) (*25*)]. We prepared agarose-embedded DNA substrate, performed single digests with restriction endonuclease Not I or double digests with restriction endonuclease Not I and copper:OP^{26}CAP, and analyzed the products by pulsed-field agarose gel electrophoresis followed by Southern (DNA) blot hybridization (*26*). The DNA substrate was cleaved by copper:OP^{26}CAP at the consensus DNA site for CAP, yielding two Not I–copper:OP^{26}CAP product DNA fragments with the expected lengths (Fig. 4). The reaction was highly efficient, proceeding to ≥70% completion. In addition, the reaction was highly specific, with no detectable nonspecific DNA cleavage. On the basis of the observed DNA-cleavage efficiency and DNA-cleavage specificity, together with the length of the DNA substrate, we infer that copper:OP^{26}CAP has a ≥10^6-fold preference for cleavage at the specific DNA site compared with a nonspecific DNA site. We conclude that copper:OP^{26}CAP has promise for use as a practical tool for chromosome mapping and chromosome cloning.

To define the positions within the DNA site at which DNA cleavage occurs, we performed single-nucleotide–resolution DNA-cleaving experiments with a 40-bp DNA fragment containing a consensus DNA site for CAP. We end-labeled the DNA substrate with ^{32}P on the top DNA strand or bottom DNA strand, perfomed copper:OP^{26}CAP digests, and analyzed the results by denaturing polyacrylamide gel electrophoresis followed by autoradiography (*27*). The results show that DNA cleavage occurs primarily at one nucleotide on each DNA strand: nucleotide 1 on the top DNA strand and nucleotide 22 on the bottom DNA strand (Fig. 5). These nucleotides occupy twofold-symmetry–related positions within the DNA site. The results suggest that a large fraction of product DNA fragments have 21-nucleotide 5′-overhanging ends. The presence of long 5′-overhanging ends is likely to facilitate practical applications involving product DNA–fragment capture and product DNA–fragment cloning [see (*28*)]. The positions of the nucleotides at which DNA cleavage occurs are in excellent agreement with the model for the structure of the copper:OP^{26}CAP-DNA complex (Fig. 1A). There is a one-to-one correspondence between the nucleotides at which DNA cleavage occurs and the nucleotides predicted to be closest to the copper:*o*-phenanthroline moiety in the model (Fig. 1A). This correspondence supports the view that the favorable properties of copper:

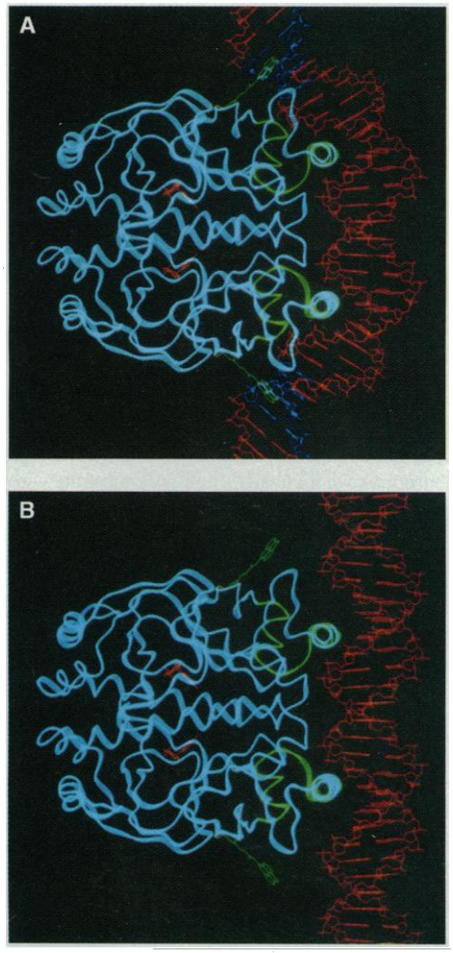

Fig. 1. (**A**) Model for the structure of the specific copper:OP^{26}CAP-DNA complex. The ((copper:phenanthrolin-5-yl)carbamoylmethyl)carbamoylmethyl moiety of each subunit of copper:OP^{26}CAP is illustrated in green. The nucleotides at which DNA cleavage occurs are illustrated in dark blue. (**B**) Model for the structure of the nonspecific copper:OP^{26}CAP-DNA complex. The models in (A) and (B) were based on, respectively, the crystallographic structure of the specific CAP-DNA complex (*7*) and the model of Weber and Steitz for the structure of the nonspecific CAP-DNA complex (*30*). Crystallographic coordinates for the "closed" subunit of CAP were from the Brookhaven Protein Data Bank (*31*). Crystallographic coordinates for copper:*o*-phenanthroline were from the Cambridge Structural Database (*32*). Model coordinates for the (carbamoylmethyl)carbamoylmethyl linker and for DNA were generated with the programs INSIGHT (Biosym Technologies) and DNA FIT MAN (*33*).

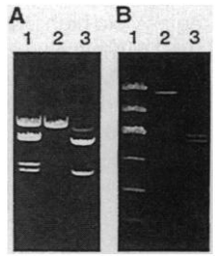

Fig. 3. DNA-cleaving experiments with kilobase DNA substrates containing single consensus DNA sites for CAP. (**A**) The 7.2-kb DNA substrate [Sna BI–linearized replicative-form genomic DNA of M13mp2-*lacP1*(*ICAP*); DNA site at position 4847]. (**B**) The 48-kb DNA substrate [genomic DNA of λ*i434plac5-P1*(*ICAP*); DNA site at position 22,593]. Lanes 1, markers [7.2, 5.9, 5.0, 2.2, and 1.3 kb in (A) and 49, 34, 23, 15, 9.5, and 6.5 kb in (B)]; lanes 2, DNA substrate; lanes 3, copper:OP^{26}CAP digest of DNA substrate.

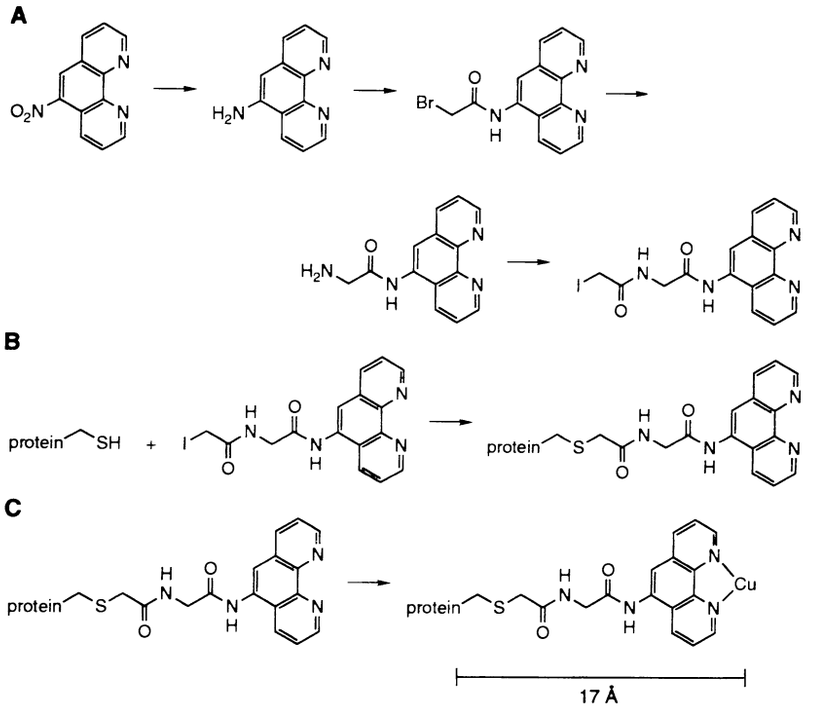

Fig. 2. (**A**) Synthesis of 5-iodoacetylglycylamino-*o*-phenanthroline (*15*). (**B**) Reaction of [Cys26; Ser178]CAP with 5-iodoacetylglycylamino-*o*-phenanthroline to yield OP^{26}CAP. (**C**) Reaction of OP^{26}CAP with copper(II) to yield copper:OP^{26}CAP.

OP^{26}CAP result from successful design—not chance.

In summary, copper:OP^{26}CAP exhibits high DNA-cleavage efficiency and high DNA-cleavage specificity. In important respects, the strategy used in this work to obtain high DNA-cleavage specificity—that is, use of a difference in DNA bending in specific and nonspecific protein-DNA complexes to position DNA-cleaving groups close to DNA only in the specific protein-DNA complex—mimics a strategy used by restriction endonucleases. For characterized restriction endonucleases Eco RI and Eco RV, a critical component of DNA-cleavage specificity is differential DNA bending and DNA twisting in the specific enzyme-DNA complex and concomitant positioning of active-site residues close to DNA only in the specific enzyme-DNA complex (*29*).

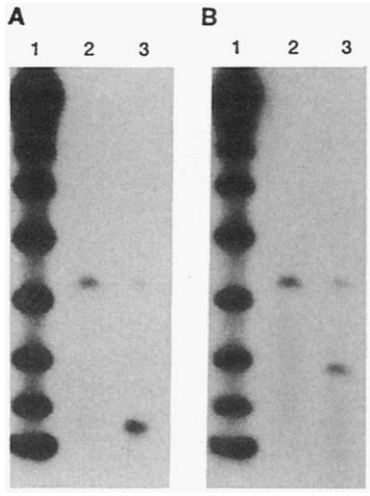

Fig. 4. DNA-cleaving experiments with a 4.7-Mb DNA substrate containing a single consensus DNA site for CAP [genomic DNA of *E. coli* strain XAE4000; *ΔlacproX111 argEam metB ara rpoB nal Δcrp45 strA fnr1 zci*::Tn10 Su2 λ*i434plac5-P1(lCAP)*; DNA site at position ~65,000 of the Not I–Not I DNA fragment bearing the integrated λ*i434plac5-P1(lCAP)* prophage (*25*)]. (**A**) Southern hybridization with a probe corresponding to the left arm of integrated λ*i434plac5-P1(lCAP)* prophage. (**B**) Southern hybridization with a probe corresponding to the right arm of integrated λ*i434plac5-P1(lCAP)* prophage. Lanes 1, markers (λ-concatemers, 49-kb steps; New England Biolabs); lanes 2, Not I digest of DNA substrate; lanes 3, Not I–copper:OP^{26}CAP double digest of DNA substrate.

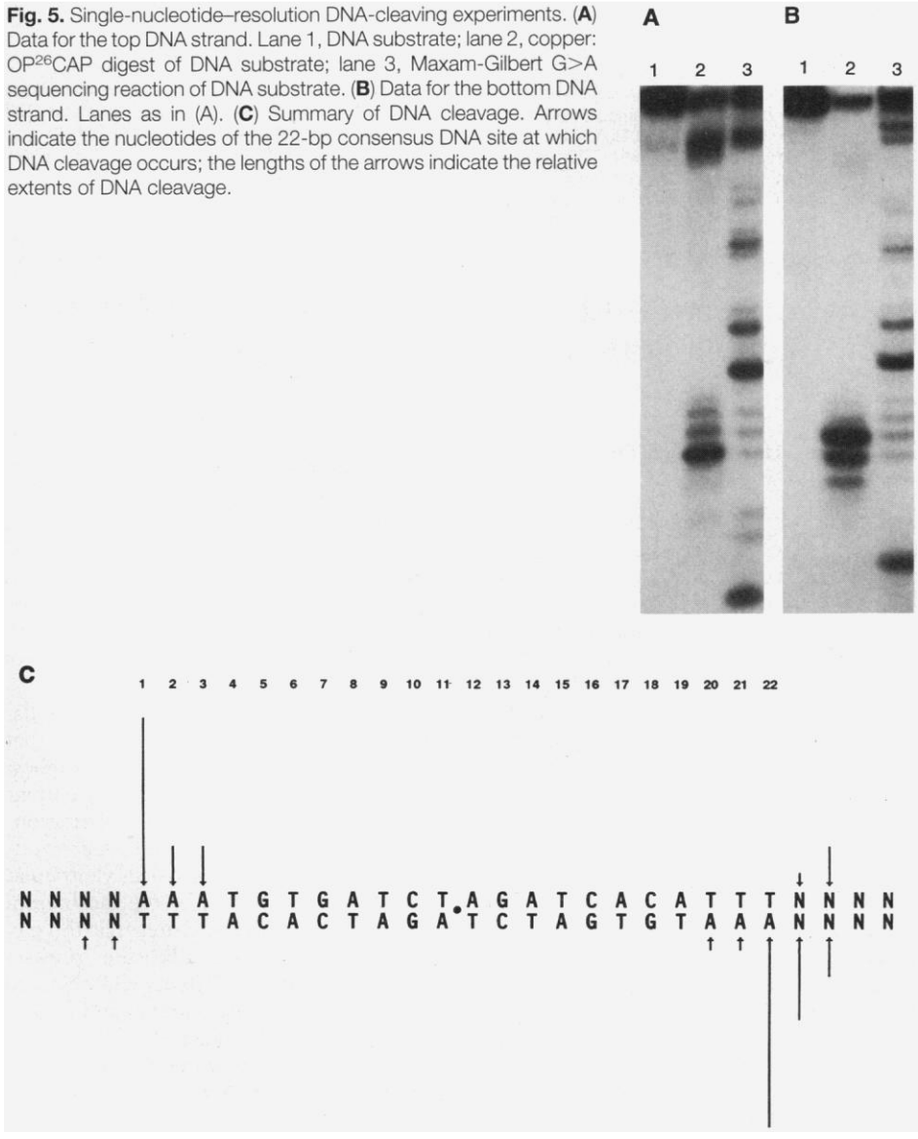

Fig. 5. Single-nucleotide–resolution DNA-cleaving experiments. (**A**) Data for the top DNA strand. Lane 1, DNA substrate; lane 2, copper:OP^{26}CAP digest of DNA substrate; lane 3, Maxam-Gilbert G>A sequencing reaction of DNA substrate. (**B**) Data for the bottom DNA strand. Lanes as in (A). (**C**) Summary of DNA cleavage. Arrows indicate the nucleotides of the 22-bp consensus DNA site at which DNA cleavage occurs; the lengths of the arrows indicate the relative extents of DNA cleavage.

REFERENCES AND NOTES

1. C.-H. B. Chen and D. S. Sigman, *Science* **237**, 1197 (1987); S. Strobel, H. Moser, P. Dervan, *J. Am. Chem. Soc.* **110**, 7927 (1988); J.-C. Francois *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **86**, 9702 (1989); D. Corey, D. Pei, P. Schultz, *J. Am. Chem. Soc.* **111**, 8523 (1989); S. A. Strobel and P. B. Dervan, *Science* **249**, 73 (1990); D. Pei and P. Schultz, *J. Am. Chem. Soc.* **112**, 4579 (1990); D. Pei, D. Corey, P. Schultz, *Proc. Natl. Acad. Sci. U.S.A.* **87**, 9858 (1990); D. Pei and P. Schultz, *J. Am. Chem. Soc.* **113**, 9398 (1991); C.-H. Chen, M. Gorin, D. Sigman, *Proc. Natl. Acad. Sci. U.S.A.* **90**, 4206 (1993); C. Sutton, A. Mazumder, C.-H. Chen, D. Sigman, *Biochemistry* **32**, 4225 (1993).
2. R. Ebright, Y. Ebright, P. S. Pendergrast, A. Gunasekera, *Proc. Natl. Acad. Sci. U.S.A.* **87**, 2882 (1990).
3. A. Kolb, S. Busby, H. Buc, S. Adhya, *Annu. Rev. Biochem.* **62**, 749 (1993).
4. O. Berg and P. von Hippel, *J. Mol. Biol.* **200**, 709 (1988).
5. R. Ebright, Y. Ebright, A. Gunasekera, *Nucleic Acids Res.* **17**, 10295 (1989).
6. H.-W. Wu and D. Crothers, *Nature* **308**, 509 (1984).
7. S. C. Schultz, G. C. Shields, T. A. Steitz, *Science* **253**, 1001 (1991).
8. Whereas the thermodynamically defined DNA site size for specific CAP-DNA complex formation is 28 to 34 bp (*9*)—reflecting extended CAP-DNA contacts due to DNA bending (*9*)—the thermodynamically defined DNA site size for nonspecific CAP-DNA complex formation is only 13 to 15 bp (*10*), inconsistent with substantial DNA bending. [The diameter of the CAP dimer is ~55 Å (*7*); the length of an unbent 28- to 34-bp DNA segment is ~95 to ~115 Å; the length of an unbent 13- to 15-bp DNA segment is ~44 to ~51 Å.]
9. H.-N. Liu-Johnson, M. Gartenberg, D. Crothers, *Cell* **47**, 995 (1986); A. Gunasekera, Y. Ebright, R. Ebright, *J. Biol. Chem.* **267**, 14713 (1992).
10. S. Saxe and A. Revzin, *Biochemistry* **18**, 255 (1979); M. Takahashi, B. Blazy, A. Baudras, *Nucleic Acids Res.* **7**, 1699 (1979).
11. D. Sigman, *Acc. Chem. Res.* **19**, 180 (1986).
12. Plasmid pXZCRP26C; 178S encodes [Cys26;Ser178]-CAP; plasmid pXZCRP26C;178S was constructed from plasmid pXZCRP178S (*13*) by site-directed mutagenesis (*14*). [Cys26;Ser178]CAP was prepared as described for CAP (*13*).
13. X. Zhang, A. Gunasekera, Y. Ebright, R. Ebright, *J. Biomol. Struct. Dyn.* **9**, 463 (1991).
14. T. Kunkel, K. Bebenek, J. McClary, *Methods Enzymol.* **204**, 125 (1991).
15. Z. Shang *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **91**, 118 (1994).
16. Reaction mixtures (500 μl) contained 5.8 nmol [Cys26;Ser178]CAP, 350 nmol 5-iodoacetylglycylamino-*o*-phenanthroline (*15*), 20 mM tris-HCl (pH 8.0), 200 mM KCl, 0.1 mM EDTA, 5% glycerol, and 1.7% dimethylformamide. Reactions were carried out for 3 hours at 23°C, and then for 15 hours at 4°C. OP^{26}CAP was purified by chromatography on Bio-Gel P-6DG (Bio-Rad) and stored at −70°C in 20 mM tris-HCl (pH 8.0), 200 mM KCl, and 5% glycerol. All

solutions were treated with Chelex-100 (Bio-Rad) to remove trace metals.
17. Titration with 5,5′-dithiobis(nitrobenzoic acid) (*13*) showed that $OP^{26}CAP$ contained <0.1 mol of accessible cysteine per mole of subunit, indicating that derivatization was at least 90% complete.
18. Nitrocellulose filter binding assays (*5*) indicated that $OP^{26}CAP$ retained high specific DNA binding affinity (a dissociation constant, K_d, of 4.2 ± 0.7 nM). Electrophoretic mobility-shift DNA bending experiments (*19*) indicated that $OP^{26}CAP$ retained nearly full DNA bending activity (DNA bend angle = 116 ± 2°).
19. Y. Zhou, X. Zhang, R. Ebright, *Proc. Natl. Acad. Sci. U.S.A.* **90**, 6081 (1993).
20. Bacteriophage M13mp2-*lacP1*(*ICAP*) was described in (*21*), and λ*i434plac5-P1*(*ICAP*) was constructed according to the procedure of (*22*). DNA-cleaving reactions (110 μl) contained 1 nM DNA substrate, 200 nM $OP^{26}CAP$ (added in five equal amounts at 0, 45, 90, 135, and 180 min), 2.0 μM Cu(II)SO_4, 2.5 mM mercaptopropionic acid, 0.2 mM cAMP, 10 mM MOPS-NaOH (pH 7.3), 200 mM NaCl, bovine serum albumin (50 μg/ml), and 2.2% ethanol. Reactions were initiated by addition of mercaptopropionic acid and proceeded for 6 hours at 37°C. Reactions were terminated by the addition of 2,9-dimethyl-*o*-phenanthroline to 3.0 mM, then phenol-extracted, chloroform-extracted, and ethanol-precipitated. Products in experiments with the 7.2-kb DNA substrate were analyzed by electrophoresis through 0.8% agarose, 1 μM ethidium bromide slab gels; products in experiments with the 48-kb DNA substrate were analyzed by pulsed-field electrophoresis (CHEF; 1.5 V/cm²; switch time = 2 s; 30 hours at 16°C) through 1% chromosomal-grade agarose slab gels (Stratagene), followed by ethidium bromide staining.
21. X. Zhang, Y. Zhou, R. Ebright, *J. Biol. Chem.* **267**, 8136 (1992).
22. R. Ebright, A. Gunasekera, X. Zhang, T. Kunkel, J. Krakow, *Nucleic Acids Res.* **18**, 1457 (1990); X. Zhang and R. Ebright, *Proc. Natl. Acad. Sci. U.S.A.* **87**, 4717 (1990).
23. M. Koob, E. Grimes, W. Szybalski, *Science* **241**, 1084 (1988); M. Koob and W. Szybalski, *ibid.* **250**, 271 (1990); S. Strobel and P. Dervan, *Nature* **350**, 172 (1991); S. A. Strobel, L. A. Douchette-Stamm, L. Riba, D. E. Housman, P. B. Dervan, *Science* **254**, 1639 (1991); L. Ferrin and R. D. Camerini-Otero, *ibid.*, p. 1494; M. Koob, A. Burkiewicz, J. Kur, W. Szybalski, *Nucleic Acids Res.* **20**, 5831 (1992); T. Povsic, S. Strobel, P. Dervan, *J. Am. Chem. Soc.* **114**, 5934 (1992).
24. R. Ebright *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **84**, 6083 (1987).
25. Strain XAE4000 was constructed by integration of λ*i434plac5-P1*(*ICAP*) at the λ*att* site [procedure in (*22*)]. There are no consensus DNA sites for CAP in wild-type *E. coli* (*3*). DNA sites for CAP in wild-type *E. coli* have a minimum of five differences from the consensus DNA site and exhibit one to three orders of magnitude lower affinities (*5*).
26. Agarose plugs containing genomic DNA of strain XAE4000 were prepared with InCert agarose (FMC BioProducts) and the procedure of the manufacturer with two modifications: (i) proteinase-K digestion was for 72 hours, and (ii) residual low molecular weight contaminants were removed by pulsed-field electrophoresis (TAFE in a matrix of 1% chromosomal-grade agarose; 1.5 V/cm²; switch time = 15 s; 12 hours at 4°C) followed by equilibration in 500 μl of 10 mM tris-HCl (pH 7.9), 150 mM NaCl, 10 mM $MgCl_2$, and 0.01% Triton X-100. Digestions with Not I (500 μl; 14 hours at 37°C) contained an agarose plug with DNA substrate, 30 U Not I (New England Biolabs), 50 mM tris-HCl (pH 7.9), 100 mM NaCl, 10 mM $MgCl_2$, and 1 mM dithiothreitol. Digestions with copper:$OP^{26}CAP$ (200 μl; 3 hours at 37°C) contained an agarose plug with DNA substrate, 120 nM $OP^{26}CAP$ (added in two equal amounts at 0 and 1.5 hours), 0.2 mM cAMP, 1 μM Cu(II)SO_4, 2.5 mM mercaptopropionic acid, 10 mM MOPS-NaOH (pH 7.3), and 200 mM NaCl. The copper:$OP^{26}CAP$ digestions were initiated by addition of mercaptopropionic acid and were terminated by transfer of agarose plugs to 500 μl of reaction buffer plus 3 mM 2,9-dimethyl-*o*-phenanthroline at 4°C. Reaction products were analyzed by pulsed-field electrophoresis (TAFE; 1.5 V/cm²; switch time = 15 s; 12 hours at 4°C) through 1% chromosomal-grade agarose slab gels, followed by transfer to Nytran (Schleicher & Schuell) and Southern hybridization with ^{32}P-labeled probes corresponding to the left and right arms of integrated λ*i434plac5-P1*(*ICAP*) prophage [prepared by nick-translation of Xba I–digested λ*i434plac5-P1*(*ICAP*)].
27. Reactions were performed essentially as in (*20*). The DNA substrate was 40-bp synthetic DNA fragment ICAP 5′ end-labeled with ^{32}P (*5*). The $OP^{26}CAP$ concentration was 80 nM (added in a single amount). Products were analyzed by denaturing gel electrophoresis through 20% polyacrylamide, 8.3 M urea slab gels, followed by autoradiography.
28. C. Kang and M. Inouye, *Biotechniques* **15**, 659 (1993).
29. Y. Kim, J. C. Grable, R. Love, P. J. Greene, J. M. Rosenberg, *Science* **249**, 1307 (1990); D. R. Lesser, M. R. Kurpiewski, L. Jen-Jacobson, *ibid.* **250**, 776 (1990); F. Winkler, *Curr. Opin. Struct. Biol.* **2**, 93 (1992); F. Winkler *et al.*, *EMBO J.* **12**, 1781 (1993); I. B. Vipond and S. Halford, *Mol. Microbiol.* **9**, 225 (1993).
30. I. Weber and T. Steitz, *Nucleic Acids Res.* **12**, 8475 (1984).
31. ———, *J. Mol. Biol.* **198**, 311 (1987). Accession code is 3GAP.
32. L. Battaglia, A. Bonamartini Corradi, G. Marcotrigiano, G. Pellacani, *Acta Cryst.* **B33**, 3886 (1977). Accession code is ACGLCU.
33. DNA FIT MAN; J. Warwicker, Yale University.
34. We thank D. Axelrod and J. Messing for access to a pulsed-field gel electrophoresis apparatus. This work was supported by NIH grant GM41376, a Searle Scholar Award, and a Boehringer Mannheim GmbH research contract to R.H.E.

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Oscillations of Membrane Current and Excitability Driven by Metabolic Oscillations in Heart Cells

Brian O'Rourke, Brian M. Ramza, Eduardo Marban*

Periodic changes in membrane ionic current linked to intrinsic oscillations of energy metabolism were identified in guinea pig cardiomyocytes. Metabolic stress initiated cyclical activation of adenosine triphosphate–sensitive potassium current and concomitant suppression of depolarization-evoked intracellular calcium transients. The oscillations in membrane current and excitation-contraction coupling were linked to oscillations in the oxidation state of pyridine nucleotides but were not driven by pacemaker currents or alterations in the concentration of cytosolic calcium. Interventions that altered the rate of glucose metabolism modulated the oscillations, suggesting that the rhythms originated at the level of glycolysis. The energy-driven oscillations in potassium currents produced cyclical changes in the cardiac action potential and thus may contribute to the genesis of arrhythmias during metabolic compromise.

Periodic rhythms underlie biological processes as diverse and fundamental as neuronal firing, secretion, and muscle contraction. Cellular oscillators have been broadly categorized by the location of the primary rhythm generator, either at the surface membrane or at an intracellular site (*1*). Extracellular and intracellular inputs modulate the activity of both types of oscillator, and interactions between the two may be necessary for a maintained response (*2*). Descriptions of several oscillatory biochemical pathways have lent support to the notion that single rate-controlling enzymes possess regulatory properties capable of supporting stable nonequilibrium oscillatory states under defined conditions (*3*). One such system is the glycolytic oscillator, which is primarily controlled by phosphofructokinase (*4*). The ubiquitous distribution of glycolytic enzymes has motivated proposals that the oscillator could mediate various oscillatory physiological behaviors, including slow waves of contraction in smooth muscle (*5*), bursting electrical activity in *Aplysia* neurons (*6*), and insulin release from the β islet cells of the pancreas (*7*).

Our results describe a metabolic oscillator revealed by substrate deprivation that drives oscillations in the adenosine triphosphate (ATP)–sensitive potassium current and in the amplitude of depolarization-evoked increases in intracellular Ca^{2+} concentration (Ca^{2+} transients) in ventricular myocytes. Examination of the mechanism of this phenomenon indicates that the glycolytic oscillator may be the primary rhythm generator underlying the observed changes in membrane currents and excitation-contraction coupling.

Membrane current and fluorescence were measured in guinea pig ventricular myocytes equilibrated with intracellular

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