the binding site of initiation factor eIF-2 (*9*). The sequence of RpS19 is highly conserved among nematodes, insects, vertebrates, yeast (Fig. 2), and archaebacteria (*10*), and the homologous yeast protein Rp16A is essential for viability (*11*).

Developmentally controlled ribosomal heterogeneities attributed to changes in ribosomal proteins have been identified in the cellular slime mold genera *Dictyostelium* and *Polysphondylium* (*12*). In these organisms, ribosomal switches occur at discrete stages of the life cycle. Ribosome heterogeneity was also discovered in human males, where two genes coding for RpS4 isoforms exist on the sex chromosomes, one on the Y and one on the X, suggesting that the ribosomes of human males and females differ (*13*). At different stages during the life cycle of *Plasmodium berghei* and *P. falciparum*, two structurally distinct 18S ribosomal RNA genes are expressed (*14*). In *Xenopus laevis*, two types of 5S ribosomal RNA genes exist, both of which are expressed in oocytes, but only one type of which is expressed in somatic cells (*15*).

At present, it is quite unclear why certain eukaryotic organisms make distinct populations of ribosomes at discrete stages of their life cycle. Because chromatin diminution in *A. lumbricoides* parallels germline-soma differentiation, the change in ribosomes may affect somatic cell differentiation, perhaps by controlling translation of somatic genes. Alternatively, the ribosomal heterogeneity may reflect different quantitative requirements of protein synthesis of the two cell lineages; an adult *Ascaris* female produces as many as 200,000 eggs per day (*3*), whereas somatic cells do not require such a high rate of protein synthesis.

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7. Reverse transcription with 1 μg of total muscle RNA (*16*) was performed with the RT-PCR kit (Perkin-Elmer) protocol. PCR amplification of first-strand *Ascaris rpS19S* cDNA was carried out as described [S. Hahn, S. Buratowski, P. A. Sharp, L. Guareute, *Cell* **58**, 1173 (1989)] with two degenerate oligonucleotides derived from conserved regions in the RpS19 proteins (Fig. 2): primer 1, AA(AG)GA(TC)GT(ATCG)(AG)A(TC)CA, and primer 2, CCA(TC)TC(ATCG)GG(ATCG)AC(TC)TT. Parentheses in primers indicate degenerate nucleotides.
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Inhibition of NF-κB by Sodium Salicylate and Aspirin

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The transcription factor nuclear factor–κB (NF-κB) is critical for the inducible expression of multiple cellular and viral genes involved in inflammation and infection including interleukin-1 (IL-1), IL-6, and adhesion molecules. The anti-inflammatory drugs sodium salicylate and aspirin inhibited the activation of NF-κB, which further explains the mechanism of action of these drugs. This inhibition prevented the degradation of the NF-κB inhibitor, IκB, and therefore NF-κB was retained in the cytosol. Sodium salicylate and aspirin also inhibited NF-κB–dependent transcription from the Igκ enhancer and the human immunodeficiency virus (HIV) long terminal repeat (LTR) in transfected T cells.

The salicylates, or aspirin-like drugs, are widely prescribed agents used to treat inflammation (*1*). Their effectiveness has been attributed to their ability to inhibit prostaglandin production by inhibiting the cyclooxygenase, prostaglandin H (PGH) synthase (*2*). However, doses of aspirin necessary to treat chronic inflammatory diseases are much higher than those required to inhibit prostaglandin synthesis (*1, 3*). Furthermore, whereas aspirin inhibits PGH synthase by acetylating it, salicylic acid, which lacks an acetyl group and is ineffective as a PGH synthase inhibitor, is nevertheless able to reduce inflammation at comparable doses to aspirin (*1, 3*). Salicylic acid also plays a role in transcription (*4, 5*). In plants, salicylic acid influences transcription of the pathogenesis-related (PR) genes in response to infection and injury (*4*), and in HeLa cells sodium salicylate activates the human heat shock transcription factor (HSTF) (*5*).

NF-κB is an inducible eukaryotic transcription factor of the *rel* family (*6*). It exists in an inactive form in the cytoplasm of most cells where it is bound to an inhibitory protein, IκB. NF-κB is activated in response to a number of stimulants including bacterial lipopolysaccharide (LPS), double-stranded RNA, phorbol esters, IL-1, and tumor necrosis factor–α (TNF-α) (*6*). Stimulation triggers the release of NF-κB from IκB, resulting in the translocation of NF-κB from the cytoplasm to the nucleus where NF-κB binds to DNA and regulates transcription of specific genes. Most of the genes known to be activated by NF-κB

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are involved in the immune and inflammatory responses. These include cytokines such as IL-1, IL-6, IL-8, interferon β (IFN-β), and TNF-α (6) and the cell adhesion molecules endothelial leukocyte adhesion molecule–1 (ELAM-1), intercellular adhesion molecule–1 (ICAM-1), and vascular cell adhesion molecule–1 (VCAM-1) (6–8). In addition, a number of viruses, including HIV-1, exploit NF-κB for their replication (6, 9).

Because salicylates are anti-inflammatory drugs that also appear to affect transcription, we examined the effect of sodium salicylate on the activity of NF-κB. We used the human Jurkat T cell line and the mouse pre–B cell line, PD31, for our experiments. We treated PD31 cells with varying concentrations of sodium salicylate in the absence or presence of the NF-κB activator, LPS (Fig. 1A). Whole-cell extracts were made and subjected to electrophoretic mobility-shift assays with a κB site DNA probe. Extracts from LPS-induced cells had more active NF-κB available to bind to the κB probe relative to extracts of untreated cells. Sodium salicylate inhibited the activation of NF-κB by LPS in a dose-dependent manner but did not alter the gel-shift profile of unstimulated cells. In contrast and in confirmation of another report (5), 20 mM sodium salicylate activated DNA binding of HSTF. In the same extracts, an uninducible, constitutive transcription factor, Oct-1, was unaffected by the presence of sodium salicylate, which suggests that this drug does not affect transcription factors in a general manner. The activation of NF-κB by a different inducer [phorbol 12-myristate 13-acetate (PMA) plus phytohemagglutinin (PHA)] in the Jurkat human T cell line was similarly inhibited by sodium salicylate (Fig. 1B). When a whole-cell extract from activated Jurkat cells was incubated with sodium salicylate in vitro (Fig. 1C), the gel-shift assay was unaltered, which further eliminates the possibility that sodium salicylate interferes with DNA binding in our assay.

We examined the concentration of NF-κB in our samples to determine whether the lack of DNA binding in our inhibited extracts was the result of a lack of NF-κB in these extracts. Treatment of extracts with the detergent deoxycholate (DOC) disrupts the association of NF-κB with IκB, which allows the detection of inactive NF-κB. Treatment with DOC of salicylate-treated extracts revealed as much DNA binding activity in the inhibited as in the control sample (Fig. 2A). This indicated that NF-κB was present but not active in salicylate-treated cells. Furthermore, the amount of the p50 subunit of NF-κB was not appreciably different in extracts of control, activated, and 20 mM sodium salicylate–inhibited Jurkat cells (Fig. 2B).

Because NF-κB was present in inhibited extracts in a DOC-inducible form, we surmised that the salicylate was preventing the release of NF-κB from IκB after treatment with inducers. The phosphorylation and degradation of IκBα is necessary for the activation of NF-κB and its subsequent appearance in the nucleus; therefore, on activation of NF-κB, IκB concentrations decrease (6, 10). We examined the amount of IκBα protein in induced cells that were treated with sodium salicylate. The amount of IκBα protein in activated cells was reduced relative to the control; however, IκB concentrations in the sodium salicylate–inhibited extract were the same as in control cells (Fig. 2B). This result strongly suggests that sodium salicylate inhibits the activation of NF-κB by interfering with a pathway that leads to the phosphorylation or degradation (or both) of IκB.

We also examined the effect of sodium salicylate on the expression of reporter genes that are controlled by κB sites. Jurkat cells were transiently transfected with a plasmid that contained two κB sites from the Ig-κ enhancer upstream of a luciferase reporter gene. Two days after transfection, cells were activated with PMA and PHA in the presence or absence of several nonsteroidal anti-inflammatory drugs (NSAIDs) and tested for luciferase activity (Fig. 3A). The concentrations of these drugs were established on the basis of the plasma concentration of drug attained in the clinical treatment of inflammation. Aspirin and sodium salicylate are generally prescribed at 1 to 3 mM plasma concentration, whereas acetaminophen and indomethacin are prescribed at a much lower concentration (3, 11). Aspirin and sodium salicylate each effectively inhibited transcription from these reporters. However, neither acetaminophen, which is a poor PGH synthase inhibitor and a poor anti-inflammatory drug (11),

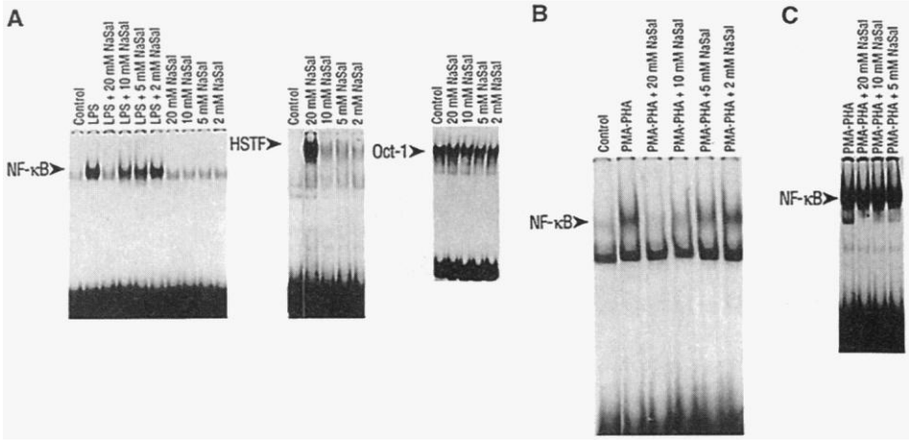

Fig. 1. Effect of sodium salicylate on DNA binding activity of NF-κB, HSTF, and Oct-1 transcription factors. (**A**) Dose-response of sodium salicylate on PD31 cells activated with LPS. Whole-cell extracts were prepared as described (*17*) and subjected to electrophoretic mobility-shift assay (EMSA) (*18*) with a ^{32}P-labeled oligonucleotide probe to the Igκ-κB site (*6*), the heat shock element (*19*), or the Oct-1 binding site (Promega). Cells were either unstimulated (control) or stimulated as indicated with LPS at 100 ng/ml for 30 min in the presence or absence of the indicated concentration of sodium salicylate (NaSal). (**B**) Dose-response of sodium salicylate on Jurkat cell extracts activated with PMA and PHA. Nuclear extracts were prepared as described (*20*) and subjected to EMSA with the κB probe as in (A). Cells were stimulated with PMA (25 ng/ml) and PHA (2 μg/ml) for 45 min in the presence or absence of the indicated concentrations of sodium salicylate. Control cells were neither stimulated nor treated with sodium salicylate. (**C**) In vitro DNA binding of extracts in the presence of sodium salicylate. Jurkat cells were activated with PMA (25 ng/ml) and PHA (2 μg/ml) for 60 min and then lysed as described (*20*). Nuclear extracts were either left untreated or treated in vitro with the indicated concentrations of sodium salicylate and analyzed by EMSA with the κB oligonucleotide probe as in (A).

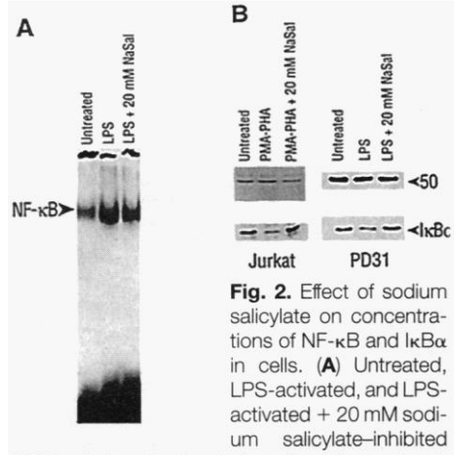

Fig. 2. Effect of sodium salicylate on concentrations of NF-κB and IκBα in cells. (**A**) Untreated, LPS-activated, and LPS-activated + 20 mM sodium salicylate–inhibited PD31 whole-cell extracts from Fig. 1A were treated with the detergent DOC to reveal NF-κB masked by the cytoplasmic inhibitor IκB. (**B**) Immunoblot of p50 and IκBα concentrations in sodium salicylate–treated Jurkat and PD31 cells. Activated and sodium salicylate–inhibited whole-cell extracts from Fig. 1, A and B, were probed with polyclonal antibodies to p50 or IκBα as indicated.

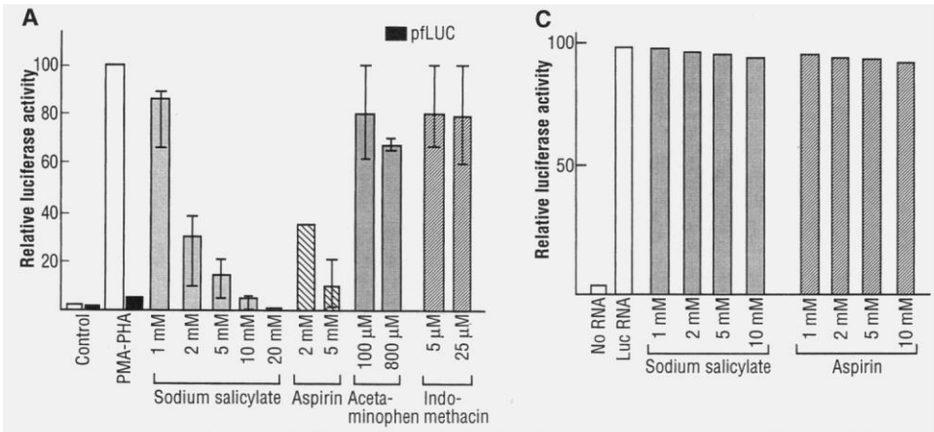

Fig. 3. (**A**) Effect of nonsteroidal anti-inflammatory drugs (NSAIDs) on NF-κB–dependent transcription. Jurkat cells were transfected by a DEAE-dextran method (*21*) with 5 μg of the luciferase reporter plasmid pBIIXLUC containing two Igκ-κB sites driving a luciferase reporter gene or the pfLUC plasmid that is identical to pBIIXLuc but lacks the κB sites. Forty-two hours after transfection, the cells were induced with PMA (25 ng/ml) and PHA (2 μg/ml) with or without the indicated NSAIDs. After 2 hours of induction, the cells were lysed and assayed for luciferase activity according to manufacturer's instructions (Promega). The columns indicate the average of multiple experiments, and the error bars indicate the highest and lowest values obtained for any one condition. (**B**) Effect of sodium salicylate and aspirin on an in vitro transcription assay. In vitro transcription was assayed with the use of a HeLa nuclear extract from a template containing the cytomegalovirus early promoter. The linearized template yields a run-off transcript of 432 nucleotides. (**C**) Effect of sodium salicylate and aspirin in an in vitro translation assay. A plasmid containing the luciferase gene expressed from a T7 promoter was transcribed and translated in a coupled rabbit reticulocyte TnT system (Promega) with or without the indicated concentrations of drugs. After a 90-min incubation at 30°C, the translated luciferase was assayed for activity.

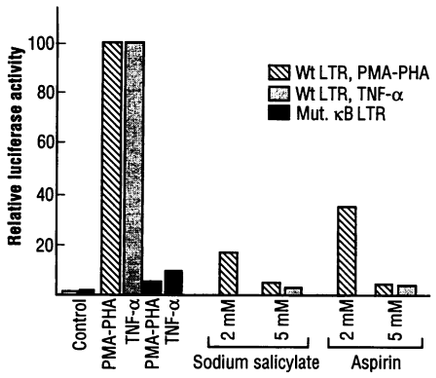

Fig. 4. Effect of sodium salicylate on the expression of the HIV LTR in Jurkat cells stimulated with PMA and PHA or TNF-α. Jurkat cells were transfected with luciferase reporter plasmids containing the wild-type (Wt) HIV LTR or a mutant (Mut.) HIV LTR in which both κB sites had been mutated. Thirty-two hours after transfection, the cells were stimulated for 2 to 3 hours with PMA (25 ng/ml) and PHA (2 μg/ml) or TNF-α (*10*).

nor indomethacin, which is an active PGH synthase inhibitor and an effective anti-inflammatory drug (*3*, *11*), had a substantial effect on the expression of the reporter gene, even at concentrations significantly higher than those administered clinically (*12*). This inhibition is probably not the result of interference of the transcriptional machinery because transcription was unaffected in vitro by the presence of sodium salicylate and aspirin at varying concentrations (Fig. 3B). Furthermore, results obtained in HeLa cells suggest that overall RNA polymerase II activity is not inhibited by treatment with concentrations of salicylate ranging from 1 to 20 mM (*5*).

We also tested whether sodium salicylate affected translation or luciferase enzyme activity in vitro. A plasmid containing a luciferase gene cloned downstream of a T3 RNA polymerase promoter was transcribed and translated in vitro with varying concentrations of sodium salicylate in a transcription- and translation-coupled rabbit reticulocyte lysate system. Sodium salicylate did not inhibit the production or the activity of luciferase in this assay, which indicates that neither translation nor luciferase enzyme function was affected by this drug (Fig. 3C).

The HIV-1 LTR contains two inducible NF-κB sites (*9*). Treatment of chronically infected cells with agents that lead to the activation of NF-κB also leads to an increase in the level of viral replication (*13*). We determined whether salicylate would inhibit transcription from a native HIV-1 promoter in T cells (Fig. 4). Jurkat cells were transiently transfected with a luciferase reporter construct containing the HIV-1 LTR. Aspirin and sodium salicylate both effectively inhibited the induced, NF-κB–dependent transcription from this reporter construct.

The ability of salicylic acid to influence prostaglandin-independent signaling processes is well documented (*3*, *4*, *14*). The finding that NF-κB activity is inhibited by sodium salicylate and aspirin functionally links an important transcription factor with the high-dose effect of salicylates. Because NF-κB is pivotal in the cytokine network, even partial inhibition of NF-κB by sodium salicylate could have a substantial effect on inflammation. Although we only observe effects on NF-κB at relatively high concentrations of salicylates, such high concentrations are maintained in the plasma for treatment of chronic inflammatory states like arthritis (*1*, *11*). Furthermore, the local condition and pH of inflamed areas such as joints may favor the uptake and concentration of salicylates (*15*). Thus, the inhibition of NF-κB may be enhanced at these sites. We have also shown that at high doses, sodium salicylate and aspirin can inhibit κB-dependent transcription from the HIV-1 LTR in Jurkat T cells. This transcriptional inhibition may be relevant to the clinical trial conducted on HIV-positive patients with high doses of aspirin (*16*).

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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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