vals from no egg to small egg or an increase in egg size from small to medium or medium to large. A change from large to no egg was interpreted as a successful laying event and given an equivalent score. Total egg state changes were divided by the number of females in the unit to yield a per capita fecundity index. This index was used instead of the number of eggs laid per female because a number of females began and finished the experiment with partially developed eggs.

18. In a 19th hangar, four small sections of wall (1.75 m by 2.5 m) were isolated with Fluon and aluminum floor barriers. Each unit was equipped with four small (20 cm by 15 cm) refuges. Two refuges were positioned 1.5 m off the floor and two were 0.75 m high, with one water dish centered in the middle of the four. Each unit contained one light mounted on the wall 2.1 m high. The region around the light was narrowed to about 1 m^2 with strips of Fluon. The position of all four units together made observation from one large blind possible, with the observer 2 m from the light. Eight geckos were placed in each unit, and two units could be watched simultaneously. Geckos were marked and painted as described in (*12*). Positions of all individuals were noted at 15-min intervals and scored as follows: 0 = on light, 1 = within 20 cm above or below, 2 = within 20 cm on the sides in the shadows, 3 = 20 to 50 cm, 4 = 50 cm to 1.2 m, and 5 = >1.2 m or not visible. These marks were measured and drawn on the walls around the light to facilitate accuracy. During the initial phase, species were isolated (units A and B with *L. lugubris* and units C and D with *H. frenatus*) and geckos were allowed to acclimatize to the light and surroundings. After 20 days, including 9 days of data gathering, the four least phototaxic *L. lugubris* in A and B were replaced with the four most phototaxic *H. frenatus* from C and D, respectively (two males, two females). The *H. frenatus* immediately adjusted and moved toward the light in their new enclosures. Positions were again scored at 15-min intervals for 6 days after the switch. During these observations (which spanned the month of March) lights were only turned on when data were being recorded (daily from 1900 to 2200).

19. Alpha individuals were defined as those that never retreated from an encounter with another individual of the same species in the enclosure, and all encounters culminated with the retreat of one of the participants. Before species were combined, there were two alpha individuals for each species (two enclosures each). After the species were combined there were four alphas of each species (four enclosures), with only two focal enclosures reported here. Intraspecific and interspecific interactions were recorded in the combined treatments. The frequencies of the pairwise interactions were: *L. lugubris–L. lugubris*, 160 in 30 hours of observation (5.4 per hour); *H. frenatus–H. frenatus*, 25 in 16 hours (1.6 per hour); and *H. frenatus–L. lugubris*, 39 in 12 hours (3.25 per hour). In approximately half of the *H. frenatus–L. lugubris* interactions, a dominant *L. lugubris* attacked an *H. frenatus*, and in the other half, a subordinate *L. lugubris* retreated from a nonaggressive *H. frenatus*. The approaches of *H. frenatus* were termed nonaggressive when they continued on the same path unaffected by the retreating *L. lugubris*.

20. We wish to thank the staff at Barbers Point and the U S. Navy in general for their cooperation and for granting permission for the use of the hangars (Real Estate license N6274291RP00164). Supported by NSF grant BSR-9107739 (T.J.C.).

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Two Open Complexes and a Requirement for Mg^{2+} to Open the λP_R Transcription Start Site

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Potassium permanganate ($KMnO_4$) footprinting in the absence and presence of magnesium (Mg^{2+}) at the λP_R promoter identified two different open complexes with *Escherichia coli* $E\sigma^{70}$ RNA polymerase (designated RP_{o1} and RP_{o2}). The single-stranded region in RP_{o1} (formed in the absence of Mg^{2+}) was at most 12 bases long, whereas that in RP_{o2} (formed in the presence of Mg^{2+}) spanned at least 14 bases. Only in RP_{o2} did the single-stranded region extend to the start point of transcription (+1, +2). These results provide a structural basis for the requirement for uptake of Mg^{2+} in the formation of RP_{o2} from RP_{o1}, as deduced from kinetic studies at this promoter.

The formation of an open complex between RNA polymerase (RNAP) and promoter DNA involves passage through a series of intermediate complexes that involve conformational changes in both RNAP and DNA (*1, 2*). Kinetic-mechanistic (*3*) and structural (*4–6*) characterizations demonstrated that RNAP (R) and promoter (P) form an initial specific closed complex at the promoter (RP_{c1}), an intermediate closed complex (RP_{c2}) that involves a major conformational change in RNAP, and an open complex (RP_o) in which the DNA in the vicinity of the start site is single-stranded. No studies to date have probed the steps of the process of DNA strand opening. It is unknown if opening is a sequential or an all-or-nothing process and if sequential, which regions are opened early and which are opened late. Chemical probing of RNAP-λP_R open complexes with $KMnO_4$ in the presence and absence of Mg^{2+} provides some answers to these questions.

On the basis of the effects of $MgCl_2$ on the dissociation rate constant of open complexes at the λP_R promoter in the absence of initiating nucleotides, we (*1*) deduced that Mg^{2+} is required for the formation of an initiation-competent open complex designated RP_{o2} to distinguish it from the open complex that exists in the absence of Mg^{2+} (RP_{o1}). We proposed (*1*) a mechanism (Eq. 1) in which Mg^{2+} has a specific stoichiometric role in the isomerization of RP_{o1} to RP_{o2}:

$$R + P \rightleftarrows RP_{c1} \rightleftarrows RP_{c2} \rightleftarrows RP_{o1} \rightleftarrows RP_{o2} \quad (1)$$

(6 Mg^{2+} (or 12 Na^+) on the left; 4 Mg^{2+} (or 8 Na^+) on the right; 3 Mg^{2+} above RP_{o1})

According to this mechanism, RP_{o1} is converted to RP_{o2} upon uptake of approximately three Mg^{2+} cations, presumably at specific sites on RNAP. In contrast, Na^+ or Mg^{2+} (or other cations, if present) function nonspecifically as cation competitors for RP_{c1} and RP_{o1}.

$KMnO_4$ preferentially oxidizes unpaired or distorted pyrimidines in DNA (T >> C) (*7*) and has been used to detect single-stranded regions of DNA in RNAP-DNA complexes (*8–10*). To characterize the two open complexes at the λP_R promoter deduced from kinetic studies, we probed the accessibility of pyrimidines in these complexes to oxidation by $KMnO_4$ in the presence and absence of Mg^{2+}. The $KMnO_4$ reactivity of the top and bottom strands is shown in Fig. 1, A and B, respectively, and summarized in Fig. 1C. Radioanalytic imaging (*11*) provides a quantitative comparison of the reactivities of pyrimidines to $KMnO_4$ in the presence and absence of Mg^{2+} (Fig. 1D).

RNAP formed open complexes on supercoiled plasmid DNA at the λP_R promoter from 4° to 37°C both in the presence and in the absence of 10 mM Mg^{2+} (Fig. 1). However, Mg^{2+} enhanced $KMnO_4$ modification at many positions, especially at both boundaries of the open region where the ratio of $KMnO_4$ reactivity in the presence of Mg^{2+} to that in the absence of Mg^{2+} was in the range of 3 to 8 (Fig. 1D). Positions +1T and +2T, unreactive or weakly reactive with $KMnO_4$ in the absence of Mg^{2+}, became accessible to reaction with $KMnO_4$ in the Mg^{2+}-induced open complex. In a similar manner, a large enhancement of $KMnO_4$ reactivity of −11T and −12C was observed in the presence of Mg^{2+}. In contrast, enhancement was more modest in the central part of the open region (positions −3T, −4T, −8T, −9T, and −10T) where the ratio of $KMnO_4$ reactivity in the presence of Mg^{2+} to that in the absence of Mg^{2+} was ≤3 (*12*).

Together, these data indicate a significant difference in the open complex in the presence and absence of Mg^{2+}. In the absence of Mg^{2+}, the extent of strand

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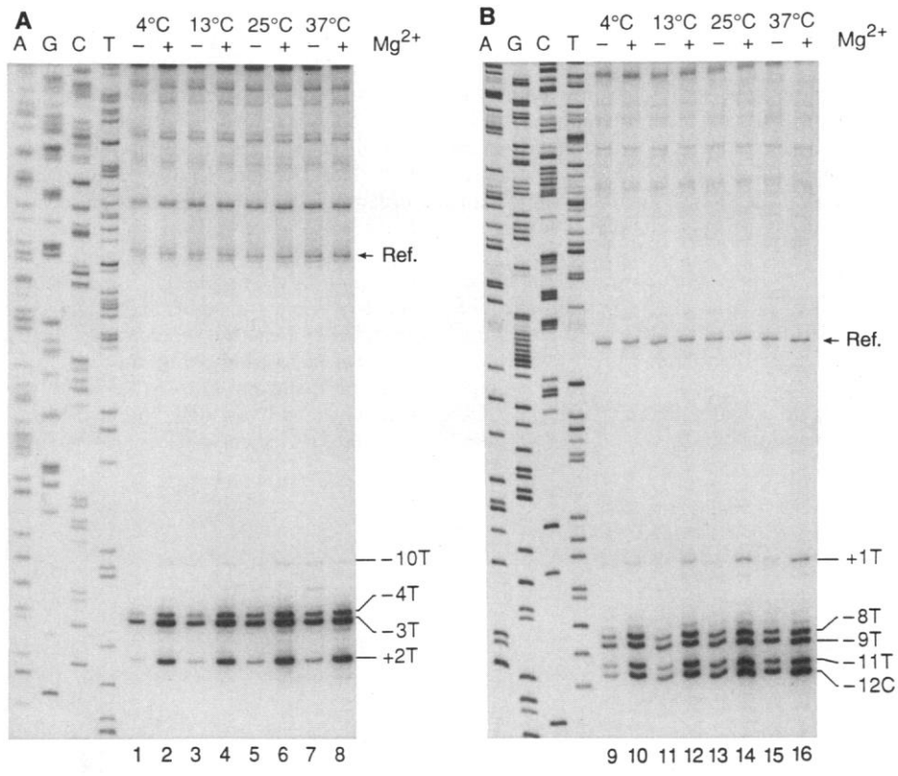

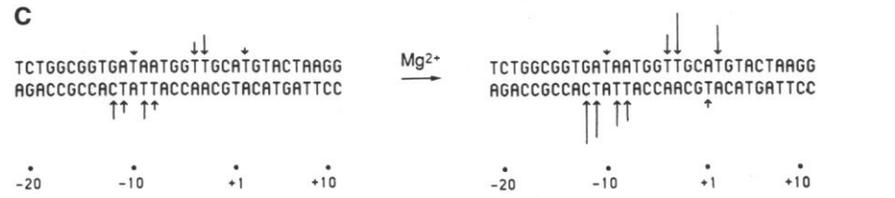

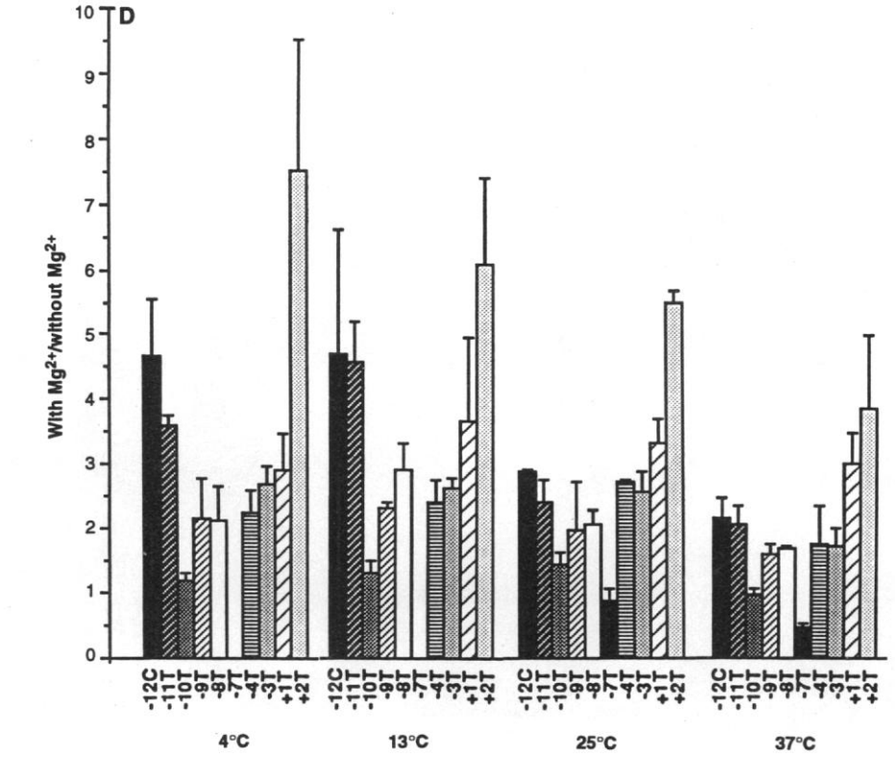

Fig. 1. Probing of RNAP ($E\sigma^{70}$)-λP_R promoter complexes on supercoiled plasmid DNA with $KMnO_4$. (**A**) Top strand. (**B**) Bottom strand. $KMnO_4$ probing was performed on complexes formed in the absence (−) (odd-numbered lanes) or presence (+) (even-numbered lanes) of 10 mM $MgCl_2$ at the temperatures indicated (*11*). The DNA sequence shown to the left in each panel was obtained with dideoxy DNA sequencing (*24*). The bases that exhibited hyperreactivity to $KMnO_4$ are indicated at the right of each autoradiograph, numbered relative to the start of transcription. To permit a quantitative comparison between lanes, we included the reference band, indicated by an arrow (Ref.) (*11*). (**C**) Effect of the addition of Mg^{2+} on $KMnO_4$ reactivities of bases in the RNAP-λP_R complexes at 25°C. The arrows indicate reactive bases, and the length of the arrows represents the degree of reactivity to $KMnO_4$. (**D**) Quantification of the enhanced reactivity of the open region in Mg^{2+} on supercoiled DNA. The relative $KMnO_4$ reactivity (with Mg^{2+}/without Mg^{2+}) represents the degree of $KMnO_4$ reactivity of a base in the presence of Mg^{2+} relative to that in the absence of Mg^{2+}. The intensities of the bands in (A) and (B) were quantified by radioanalytic imaging with a Betascope Model 603 (Betagen) and normalized to the reference band for each lane. The bases that show hyperreactivity to $KMnO_4$ are shown on the *x* axis at each different temperature. Each bar is an average value obtained from three independent experiments, and error bars indicate the standard deviation.

opening was no greater than approximately 12 bp (at most from −12 to −1). (Because reactivity of $KMnO_4$ with C is intrinsically low, we were unable to determine whether positions −2 and −1 were open or closed). Positions −11T and −12C reacted with $KMnO_4$ in the absence of Mg^{2+}, but the greatly enhanced reactivity in the presence of Mg^{2+} indicates that these positions either were less denatured or, if denatured, interacted more strongly with RNAP in the absence of Mg^{2+} (*12*). Thus, the extent of opening in the complex formed in the absence of Mg^{2+} (RP_{o1}) may be only 8 to 10 bp.

In the complex formed in the presence of Mg^{2+} (RP_{o2}), at least 14 bp were open (from −12 to at least +2). The maximum upstream and downstream limits of strand opening were probably −12C and +3C, respectively, because −13T and +4T were not reactive. Only in RP_{o2} were the bases of the start site (+1 and +2) open for initiation of transcription. The location and extent of the single-stranded region of RP_{o2} at λP_R were similar to open complexes formed in Mg^{2+} at other promoters, including T7 A3 (*13*), *merR* (*9*), and *lac* UV5 (*4, 8, 14, 15*). The differences in the RP_{o1} and

RP_{o2} footprints at λP_R suggest that strand opening propagates from the center toward both extremes of the opened region.

Because other promoters, including *lac* UV5 (*5*, *15*), T7 A1 (*16*), and *tac* (*15*), form predominantly closed complexes at low temperatures (4° to 13°C), it was unexpected that RNAP formed significant amounts of both open complexes at temperatures as low as 4°C at λP_R. However, these previous studies were performed on linear templates. To examine the possible effect of negative supercoiling on the temperature dependence of formation of RP_{o1} and RP_{o2}, we repeated the $KMnO_4$ reactivity studies on λP_R in linear plasmid DNA (Fig. 2). The relative $KMnO_4$ reactivities at most positions are similar on linear and supercoiled DNA at 13° to 37°C (Figs. 1D and 2). At 4°C on linear DNA, a significant reduction in the extent of formation of both open complexes (especially RP_{o1}) was observed (as estimated from band intensities) (*17*). Negative supercoiling therefore drives formation of RP_{o2} (in the presence of Mg^{2+}) and of RP_{o1} (in the absence of Mg^{2+}) at 4°C. At higher temperatures, formation of the appropriate open complex occurs without the additional driving force provided by negative supercoiling. Neither negative supercoiling nor an increase in temperature (from 4° to 37°C) is by itself sufficient to convert RP_{o1} to RP_{o2}; Mg^{2+} is required for this conversion (*18*).

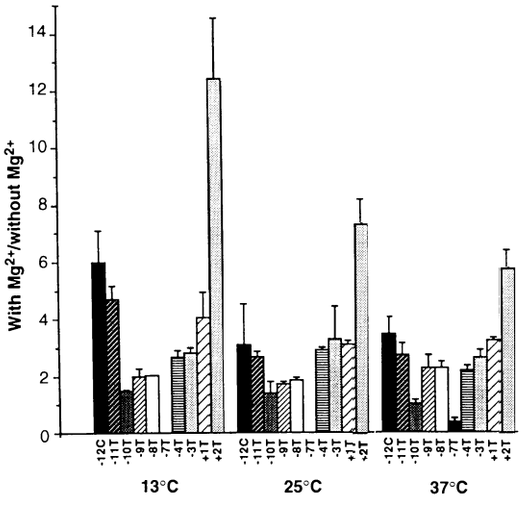

Fig. 2. Relative $KMnO_4$ reactivity as a function of temperature (13° to 37°C) on linear plasmid DNA (see legend to Fig. 1D).

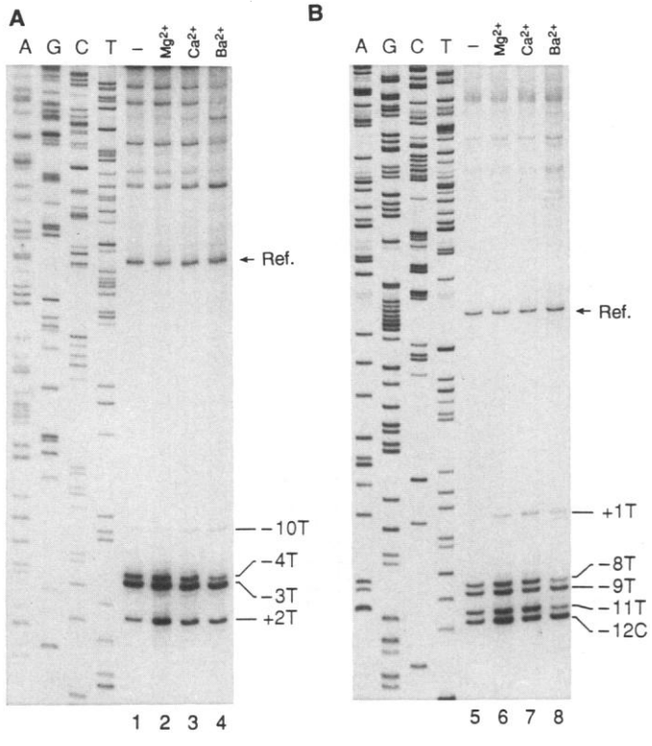

Fig. 3. Dependence of the formation of the open complex (RP_{o2}) on the nature of the divalent cation. (**A**) Top strand. (**B**) Bottom strand. RNAP was incubated with λP_R promoter DNA for 15 min at 25°C in the presence of 10 mM $MgCl_2$ (lanes 2 and 6); 10 mM $CaCl_2$ (lanes 3 and 7); or 10 mM $BaCl_2$ (lanes 4 and 8); or in the absence (–) of any divalent cation (lanes 1 and 5) followed by treatment with $KMnO_4$ (*11*). Ref. is as in Fig. 1.

Mg^{2+} and Ca^{2+} are the two major intracellular inorganic divalent cations in biological systems. Specific requirements exist for each of these ions in a wide variety of cellular processes. We therefore investigated whether the alkaline earth ions Ca^{2+} and Ba^{2+} were capable of inducing the transition of RP_{o1} to RP_{o2} at 25°C on supercoiled DNA (Fig. 3). Cation-specific differences in the relative and absolute intensities of individual bands were observed. The effects of Ca^{2+} on enhancement of $KMnO_4$ reactivity at positions +1T, +2T, −11T, and −12C were approximately 100%, 30%, 60%, and 30% of the effects of Mg^{2+}, respectively. Ba^{2+} showed much smaller effects, with only +1T exhibiting approximately 50% of the reactivity enhancement of Mg^{2+}. The order of effectiveness of these cations in inducing the transition from RP_{o1} to RP_{o2} was $Mg^{2+} > Ca^{2+} >> Ba^{2+}$. This specificity for Mg^{2+} suggests that Mg^{2+} ions occupy specific binding sites and that these sites interact less strongly with Ca^{2+} and much less strongly with Ba^{2+}. The free Mg^{2+} concentration in vivo was estimated to be in the range of 1 to 4 mM (*19*), a concentration comparable to that used in $KMnO_4$ probing. Although the biological implications of our observations are unknown because the cytoplasmic Mg^{2+} concentration is thought to be stable throughout growth, our data provide evidence that the Mg^{2+} ion has the potential to serve as a regulatory factor in the formation of the initiation-competent open complex RP_{o2}.

These structural studies of the effect of Mg^{2+} on λP_R open complex formation are consistent with our kinetic studies (*1*) and with the proposed mechanism shown in Eq. 1, in which RP_{o1} converts to RP_{o2} upon uptake of approximately three specifically bound Mg^{2+} ions. This specific role of Mg^{2+} differs profoundly from its nonspecific roles as a competitor with RNAP for interaction with DNA phosphates and as a stabilizer of double-stranded DNA against thermal denaturation (*1*, *20*). Possibly Mg^{2+} forms ionic bridges between RNAP anionic side chains and the phosphate groups of DNA or acts as an allosteric effector of a conformational change in RNAP, which facilitates full-strand separation in the initiation-competent open complex. Mg^{2+} is required for transcription initiation at this promoter not only as a cofactor for the binding of nucleotide substrates as Mg^{2+}–nucleoside triphosphate complexes (*21*) but also to open the promoter DNA at the transcription start site.

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A synthetic λP_R fragment (−60 to +20 with respect to the transcription start site) was cloned into the Hpa I–Sal I sites of pBend5 (*22*), and the fragment containing the Eco RI–Hind III promoter was then cloned into pBluescript II SK (Stratagene) to generate plasmid pBR81. The λP_R-RNAP complexes were preformed for 15 min at the temperatures indicated (Fig. 1) with or without 10 mM $MgCl_2$ in 40-μl reactions containing 1 pmol of pBR81 DNA in 10 mM K Hepes (pH 7.5), 50 mM NaCl, 0.1 mM EDTA, 1 mM dithiothreitol, bovine serum albumin (100 μg/ml), and 87 nM RNA polymerase (*1*). $KMnO_4$ was added to a final concentration of 10 mM and incubated for 2 min, and the reactions were quenched with 10 μl of 3.5 M β-mercaptoethanol and 1.4 M sodium acetate. The samples were divided for probing modifications on each strand, 0.1 μg of λP_R plasmid DNA linearized with either Xba I at −68 or Sal I at +26 was added to generate a reference band for quantitative comparison, and the samples were phenolchloroform-extracted and precipitated with ethanol. DNA was then denatured with 0.2 M NaOH, neutralized, and precipitated with ethanol. A 17-residue primer [5.4 pmol of 5′-AATACGACTCACTATAG-3′ (for the top strand)] or 5′-TCGAGGGATCCTCTAGA-3′ (for the bottom strand) was annealed in 40 mM tris HCl (pH 7.5), 20 mM $MgCl_2$, and 50 mM NaCl at 65°C for 2 min with slow cooling to room temperature. The first three G residues of each primer-extension product were labeled by incubation for 5 min at 37°C with 1 U of Bst DNA Polymerase I (Bio-Rad) (*23*), 10 μCi of [α-^{32}P]deoxyguanosine triphosphate (dGTP), and either 0.6 μM deoxycytidine triphosphate (dCTP) or thymidine triphosphate (TTP). Deoxyadenosine triphosphate, dCTP, dGTP, and TTP (100 μM each) were added, and the reactions were incubated for 10 min at 65°C, then brought to a final concentration of 20 mM EDTA and 2 M ammonium acetate, precipitated with ethanol, washed with 70% ethanol, dried, counted (Cerenkov), and resuspended in 80% formamide, 10 mM NaOH, 0.1% xylene cyanol (Sigma), and 0.1% bromophenol blue. The samples were heated at 85°C for 3 min, and equivalent counts per minute from each were electrophoresed on an 8% acrylamide and 8 M urea sequencing gel in 0.5× TBE (45 mM tris HCl, 45 mM borate, 1 mM EDTA). The ratios of $KMnO_4$ reactivities in the presence and absence of Mg^{2+} at individual positions in the open region of the promoter are directly proportional to the relative extents of opening-accessibility of these positions if the intrinsic rate constant and the local [MnO_4^-] are unaffected by the addition of 10 mM Mg^{2+}. Mg^{2+} may increase the local concentration of MnO_4^- near the surface of the DNA polyanion and therefore account for part or all of the modest increases in $KMnO_4$ reactivity at many positions in the core of the open region. Such an effect cannot also explain the larger increases in reactivity at the boundaries of the open region. In addition, the observation that the $KMnO_4$ reactivity of −10T is approximately the same in the presence and absence of Mg^{2+} suggests that both the intrinsic rate constant and local [MnO_4^-] are unaffected by Mg^{2+}.
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17. The intrinsic $KMnO_4$ reactivities of T and C appear to be essentially independent of temperature over the range examined. Small differences, if any, could not be quantified because no internal control band was present.
18. $KMnO_4$ reactivity ratios at +1T, +2T, −11T, and −12C decrease with increasing temperature on both supercoiled (Fig. 1D) and linear DNA (Fig. 2) because $KMnO_4$ reactivity at these flanking positions in RP_{o1} increases with increasing temperature. This effect may result from the transient opening of these flanking bases in RP_{o1} by thermal fluctuations or from a small extent of conversion of RP_{o1} to RP_{o2} with increasing temperature in the absence of Mg^{2+}. [The extent of any thermally induced conversion of RP_{o1} to RP_{o2} appears small because the increase in $KMnO_4$ reactivity at +1T and +2T upon addition of 10 mM Mg^{2+} (Fig. 1, A and B) is large even at 25° and 37°C.]
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25. We gratefully acknowledge the many significant contributions of R. Gourse and C. A. Gross to this work. We also thank P. Richey, P. Schlax, D. Frank, and M. P. Kladde for discussions and comments on the manuscript, the referees for constructive criticisms, and S. Aiello for assistance in its preparation. Supported by NIH grants GM23467 and GM37048.

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Molecular Mapping and Detoxification of the Lipid A Binding Site by Synthetic Peptides

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Endotoxin [lipopolysaccharide (LPS)], the major antigen of the outer membrane of Gram-negative bacteria, consists of a variable-size carbohydrate chain that is covalently linked to *N,O*-acylated β-1,6-D-glucosamine disaccharide 1,4′-bisphosphate (lipid A). The toxic activity of LPS resides in the lipid A structure. The structural features of synthetic peptides that bind to lipid A with high affinity, detoxify LPS in vitro, and prevent LPS-induced cytokine release and lethality in vivo were defined. The binding thermodynamics were comparable to that of an antigen-antibody reaction. Such synthetic peptides may provide a strategy for prophylaxis and treatment of LPS-mediated diseases.

Bacterial LPS produces a plethora of biological effects in mammals via its lipid A moiety, which is the conserved hydrophobic region (*1*). The toxic activity of LPS is related to the induction of tumor necrosis factor (TNF) release after LPS binds to macrophages and monocytes, with the consequent activation of the cytokine cascade that involves interleukin-1 (IL-1) and IL-6 (*2, 3*). Mortality from endotoxicosis consequent to sepsis is 20 to 60% (*4*). LPS has also been proposed as a potent activator of human immunodeficiency virus type 1 (HIV-1) expression in monocytes and macrophages (*5*) and as the agent that produces the symptoms characteristic of acute graft-versus-host disease (GVHD) (*6*).

Polymyxin B (PmB), a cationic cyclic peptide antibiotic, inhibits the biological effects of LPS through its high-affinity binding to lipid A (*7*). The effects that are inhibited by PmB include the Shwartzman reaction (*8*), B-II cell mitogenicity (*9*), TNF and IL-1 release from macrophages and monocytes (*10, 11*), anticomplementary activity (*12*), pyrogenic reaction in rabbits (*13*), and lethality in mice (*8, 14*). The structural features of PmB that permit binding to and detoxification of lipid A are unknown, and its use for treatment of septic shock (*10*) is limited by its high toxicity (*8, 14*). Accordingly, we have designed synthetic peptides (comprised of L-amino ac-

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