Influence of Mg$^{2+}$ and Temperature on Formation of the Transcription Bubble*

Evgeny Zaychikov, Ludmilla Denissova, Thomas Meier, Matthias Götte, and Hermann Heumann‡

From the Max Planck Institute of Biochemistry, D-82152 Martinsried, Germany

The transcription bubble formed in the binding complex of T7A1 promoter upon *Escherichia coli* RNA polymerase was analyzed by chemical probes, namely by single-strand specific reagents, to map the unpaired bases in the bubble, and by FeEDTA, to analyze the accessibility of the DNA backbone. The latter probe could also be used as a local hydroxyl radical probe placed close to the Mg$^{2+}$-binding site in the active center. The data show that the transcription bubble consists of two parts, an Mg$^{2+}$-dependent part and an Mg$^{2+}$-independent part, both having individual transition temperatures. The data further suggest that formation of a transcription active open complex is preceded by a transition state complex having enhanced affinity for those Mg$^{2+}$ ions presumably participating in the formation of the catalytic site. Our data also suggests that the three catalytically active Mg$^{2+}$ ions in RNA polymerase are functionally not equivalent. One/two of the three Mg$^{2+}$ ions are responsible for the polymerization, the other two for enlargement of the transcription bubble.

The transcription bubble is a characteristic attribute of the eubacterial transcription-competent complex. Although no three-dimensional structural information on the transcription bubble is yet available, one can visualize it as a region in which the DNA strands are separated in order to facilitate “reading” of the sequence information for RNA synthesis. The transcription bubble is initially formed upon polymerase binding upstream of the start point of RNA synthesis (1–3). It changes its size and position when RNA synthesis proceeds, as analysis in subsequent steps of RNA synthesis between registers 11 and 20 has revealed (4). The position of the upstream lagging end of the transcription bubble remains constant in the registers 11–18, while the position of the downstream lagging end moves downstream in accordance with progress of RNA synthesis. In this way the size of the transcription bubble increases from 11 base pairs in the 11-mer complex to 18 base pairs in the 18-mer complex. If RNA synthesis proceeds beyond register 18, the transcription bubble collapses in the upstream region. As a consequence, it shrinks to its initial size of 11 base pairs. While information about the transcription bubble in the RNA synthesizing complex is rather detailed, there is no correspondingly large body of information on the transcription bubble in the binary complex.

The concept of the transcription bubble (5, 6) was developed from the finding that the transcription active complex, the open complex, is preceded by the closed complex in which the promoter DNA is assumed to be base paired (7). These two complexes are distinguished from each other by their characteristic temperature sensitivity toward heparin. The closed complex exists below 17 °C and is heparin-sensitive (8), while the open complex exists above 17 °C and is heparin-resistant. The validity of this concept was proven by showing that the temperature dependence of DNA strand separation and heparin sensitivity can be correlated (7, 9). Isomerization of the binding complex is a reaction scheme which holds good for most factor-independent promoters. This scheme had to be modified by the finding that the RNA polymerase promoter complex undergoes at least two further isomerization steps before the final transcription active open complex is formed. These transition complexes are the intermediate complex (10–12) and the Mg$^{2+}$-independent and the Mg$^{2+}$-dependent complexes (13).

Transition state complexes have been defined by kinetic studies in which concentrations of the reactants, temperature, and ionic strength were varied (10, 13). It is plausible that by lowering the temperature, transition state complexes can be “frozen” and accumulated. However, correspondence between time-dependent and temperature-dependent isomerization steps has not been convincingly demonstrated. Having these reservations in mind, chemical probing studies at different temperatures can provide useful information on structural changes of transition state complexes. It has been shown that RNA polymerase has contact with the promoter in the closed complex from base position −53 to −4 (14–16). In the intermediate complex the protected region is extended downstream to position +21 (14, 15).

Probing of the A1 promoter using FeEDTA-generated hydroxyl radicals has revealed that closed and intermediate complexes differ not only in size but also in the kind of interaction. In the closed complex the polymerase faces one side of the DNA (14, 15), a result which is in line with neutron solution studies (17). In the intermediate complex, domains of RNA polymerase wrap around the DNA in the region between base position −15 and +18 covering both DNA strands and including the starting point of RNA synthesis (14, 15), a finding which is in line with information obtained from electron microscope studies (18). The footprint of the open complex visible above 30 °C does not differ from that of the intermediate complex except for a region of the template strand at base position −1 which shows enhanced reactivity toward FeEDTA. It has been suggested by Schickor (14) that this hyper-reactivity reflects enhanced accessibility of the template strand due to DNA strand separation. Record and colleagues (16) found a similar effect in the Mg$^{2+}$ containing complex and explained it by a charge effect. We show here that the enhanced cleavage is due to interaction

* This work was supported by the Deutsche Forschungsgemeinschaft. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: Max Planck Institute of Biochemistry, Am Klopferspitz 18A, D-82152 Martinsried, Germany. Tel.: 49-89-8578-2216; Fax: 49-89-8578-2822; E-mail: heumann@lns.biochem.mpg.de.
of the FeEDTA probe with the RNA polymerase promoter complex. FeEDTA acts as local hydroxyl radical source, presumably by binding at or near the site occupied by the catalytically active Mg$^{2+}$, similar to the recent observation with Fe$^{2+}$ (19).

A previous study concerned Mg$^{2+}$-dependence of the transcription bubble of λPr, a promoter which in order to form a transcription active complex, requires supercoiling of the promoter DNA. Our study focuses on the Mg$^{2+}$ effect on the linear promoter A1. Using this promoter, Escherichia coli RNA polymerase forms a transcription active complex at 37°C with promoter DNA in the relaxed state. In this study three different probes for analysis of unpaired thymidines, adenines, and cytidines were applied, allowing us to determine the size of the transcription bubble precisely within 1 base pair.

**EXPERIMENTAL PROCEDURES**

RNA polymerase was isolated from E. coli as described in Ref. 17. Promoter fragment A1–130 containing the sequence of the T7A1 promoter between −69 and +61 was prepared as described (20). The 5’-end labeling and 3’-end labeling was performed as described in Ref. 14. The radioactivity of the preparations was typically 1 μCi/pmol. For some experiments promoter fragment A1–220 (representing A1 promoter sequence between −96 and +123) was used. End labeling was performed by filling the BamHI termini with deoxy-[α-32P]dATP, subsequent removal of the short fragments near one of the termini with either HaeIII (non-template strand labeling) or AluI (template strand labeling), and final purification of the end-labeled promoter fragment with QIAGEN PCR purification kit. Sequencing ladders were obtained using piperidine.

**Formation of Binary Complexes**

A1 promoter fragment (1 μg of A1–130 or 2 μg of A1–220 containing typically 10⁶ cpm of the corresponding radioactive labeling fragment) and 12 μg of RNA polymerase were incubated for 5 min at 37°C in 50 μl of 8 mM Hepes, pH 8, containing 6 mM MgCl₂. The reaction mixture was then dialyzed for 1–2 h against proper buffer using the floating membrane dialysis bag. For some experiments promoter fragment A1–220 (representing A1 promoter sequence between −96 and +123) was used. End labeling was performed by filling the BamHI termini with deoxy-[α-32P]dATP, subsequent removal of the short fragments near one of the termini with either HaeIII (non-template strand labeling) or AluI (template strand labeling), and final purification of the end-labeled promoter fragment with QIAGEN PCR purification kit. Sequencing ladders were obtained using piperidine.

**Hydroxyl Radical Footprinting using FeEDTA**

Hydroxyl radical cleavage was conducted in a similar way as described in Ref. 9. Three drops, 1.2 μl each, of 2 mM (NH₄)₂Fe(SO₄)₂·4 mM EDTA, 5% H₂O₂, and 0.1 mM dithiothreitol were separately placed onto the inner wall of a Eppendorf test tube. The cleavage was started by simultaneous mixing of them with 10 μl of binary complex formed as described above. Cleavage was performed for 4 min at 37°C. The reaction mixture was then quickly passed through a nitrocellulose filter (13-mm Sartorius) and washed with 200 μl of 8 mM Hepes, 50 mM NaCl in order to get rid of free DNA. The DNA was eluted from the filter using a 100-μl solution containing 1% SDS, 0.3 mM sodium acetate, and 0.1 mM/mg carrier DNA (15 min at 37°C) and precipitated with 300 μl of ethanol. The pellet was dissolved in 80% formamide containing 0.02% of bromophenol and xylene cyanol. The solution was heated for 2 min at 95°C and applied on a 8% sequencing gel. As a control, free DNA was dissolved in 90°C, added 10 μl of 50 mM imidazole, 0.6 mM NaAc, pH 7, 0.1 mg/ml carrier DNA and with precipitation with 60 μl of ethanol.

**Thymidines Using Dimethyl Sulfate and Hydrazine (Hz)—Adenines Using Diethyl Pyrocarbonate—Cytidines Using Dimethyl Sulfate and Hydrazine (Hz)—**

The reaction mixture was incubated for 2 min at 37°C. The methylation was stopped by addition of an equal volume of 0.1 mM imidazole, 0.6 mM NaAc, containing 0.1 mg/ml carrier DNA. The DNA was precipitated with 60 μl of ethanol and dissolved in 20 μl of water. Subsequently 10 μl of 1-butanol and 20 μl of hydrazine were added in order to cleave the methylated cytosines. The reaction mixture was incubated at 0°C for 5 min. After precipitation with ethanol, the DNA was subjected to piperidine treatment.

**Piperidine Treatment**

The pellet was dissolved in 90°C of 10% piperidine and incubated at 90°C for 20 min. Ten μl of 5 μl LiCl was then added and DNA precipitated with 300 μl of ethanol. The precipitate was dissolved in 5–10 μl of 80% formamide containing 0.02% of bromophenol and xylene cyanol.

**Gel Electrophoresis**

The samples were heated at 90°C for 2 min, chilled on ice, and then applied on 22 × 50-cm (wedge thickness from 0.2 to 0.4-mm) slab of 6 or 8% polyacrylamide gel containing 8 μm urea and 50 μm TBE buffer. Gels were run at 55 W for 1–1.5 h using heating plates (55°C). The gels were incubated for 15 min in 10% acetic acid, then washed twice with water (total washing time: 1 h), dried, and exposed to x-ray film (3 μ). For quantification, gels were exposed to Fuji Imager plate BAS HIS, which was scanned with BAS-1000 PhosphorImager. Scans were processed with MacBas software.

**Temperature Profile of the Activity**

Binary complexes were prepared at different temperatures in the presence of MgCl₂, as described above. One μl of "initiation mixture" (1 mM ApUpC + 1 mM GTP + 1 mM CTP + 0.1 mM ATP + ~100 nCi [α-32P]dATP) was added to 10 μl of the binary complex and incubated for 5 min at the same temperature (initial experiments have shown that the kinetics is linear for at least 10 min). The reaction was stopped by addition of 10 μl of 20 mM EDTA in formamide, and 5 μl was analyzed on 20% sequencing gel. The gel was visualized by PhosphorImager, and the bands corresponding to 20-mer product were quantified.

**RESULTS**

E. coli RNA polymerase was incubated at 37°C with DNA containing the sequence of the strong promoter A1 of the phage T7, as described under "Experimental Procedures." The complex was subjected to analysis with two kinds of probes, namely single strand-specific reagents in order to map the transcription bubble, and FeEDTA as a hydroxyl radical source in order to map the RNA polymerase on the DNA. The DNA was subsequently analyzed on a sequencing gel to determine the cleavage sites.

**Mg$^{2+}$-dependent Changes of the Transcription Bubble—**

Three types of single strand-specific reagents were applied, namely (a) dimethyl sulfate, which methylates cytosines at the N-3 position, thus activating them toward hydrazine (Hz) (23); (b) OsO₄, which oxidizes thymidines at the C5-C6 double bond (24); (c) diethyl pyrocarbonate, which attacks purines with
obtained at the non-template strand visualized by labeling of the 5' terminus of the promoter fragment. Positions of modified residues are indicated.

Two sets of experiments were performed with the radioactive label either in the template or in the non-template strand, each consisting of three samples, namely DNA (a) without RNA polymerase as reference, (b) in the complex with RNA polymerase without Mg$^{2+}$, and (c) in the complex with RNA polymerase and Mg$^{2+}$. Each of these samples was treated with OsO$_4$, diethyl pyrocarbonate, and dimethyl sulfate/H$_2$O, then cleaved at the modified nucleotides with piperidine and analyzed on a sequencing gel, as described under “Experimental Procedures.” Since dimethyl sulfate modifies guanines with high yield, probing by dimethyl sulfate or H$_2$O alone was included as a control experiment.

Fig. 1 shows the results obtained at the template strand (Fig. 1A) and the non-template strand (Fig. 1B). The accessible bases indicated in Fig. 1, A and B, are shown in a schematic representation in Fig. 2. Accessibility is defined here as enhanced reactivity of a base upon polymerase binding. A base pair is considered as being part of the transcription bubble if one of the two complementary bases is accessible.

Based on the above definition, the pattern obtained by each reagent provided an upper and a lower limit of the transcription bubble at both ends. By combining the results from all three reagents, the bubble could be located accurately within 1 base pair. Fig. 2 shows that the upstream lagging end of the bubble is situated at base position -12 and the downstream lagging end at base position -1, if the incubation of the complex was performed in the absence of Mg$^{2+}$.

Comparison of the accessibility patterns with and without Mg$^{2+}$ (Fig. 2) shows that Mg$^{2+}$ has two effects. In the presence of Mg$^{2+}$, the transcription bubble is enlarged downstream to base position +2 and the interaction between RNA polymerase and DNA is enhanced, as indicated by a reduced modification of some bases. Thus A(-4) and A(-6) in the non-template strand (Fig. 1B) are protected in the Mg$^{2+}$ containing complex and modified in the Mg$^{2+}$ free complex. The thymidine at base position -5 in the non-template strand shows a similar, although less pronounced, effect to that observed with the adenines mentioned above. T(-8) is moderately accessible for OsO$_4$ in the Mg$^{2+}$ free complex and not accessible in the Mg$^{2+}$ containing complex. There is an exception to this scheme. Cytidine at position -3 in the template strand is inaccessible without Mg$^{2+}$ and moderately accessible if Mg$^{2+}$ is added.

Guanine is the only base for which no probe has been suggested which would allow a differentiation between paired or
unpaired state. Dimethyl sulfate is assumed to methylate guanines irrespective of whether they are base paired or not. Our probing studies using dimethyl sulfate alone show that this is not correct. Dimethyl sulfate has a preference for guanines in the single stranded region, as indicated by the improved cleavage of G(−3) in the non-template strand and G(−1) and G(−2) in the template strand, as the patterns in Fig. 1, A and B, show.

G(−9) in the template strand deviates from the pattern described above in that it is protected, while the complementary C(−9) is accessible. This might be due to a similar effect to that observed with A(−4) and A(−6). These bases are fully or partly protected, although the complementary base is accessible, reflecting perhaps improved interaction with RNA polymerase. G(−5) at the template strand is not affected at all by RNA polymerase binding, either in the presence or absence of Mg2+.

It is interesting to note that some of the guanines located outside of the transcription bubble show differences in the accessibility depending on whether Mg2+ is present or not. G(−13) and G(−14) in the non-template strand are protected, indicating strong interaction of these bases with RNA polymerase. It is surprising that G(±12) in the template strand is enhanced, although this base is not part of the transcription bubble according to the criteria developed above. We assume that this is due to distortion, perhaps bending of the DNA.

Analysis of the Transcription Bubble at Different Temperatures—The mapping studies on the transcription bubble in the previous paragraph allowed differentiation between two parts of the transcription bubble, namely an Mg2+-independent part reaching from base position −12 to −1 and an Mg2+-dependent part reaching from base position +1 to +2. In order to analyze whether the two parts show different melting behaviors, the Mg2+ containing complex was analyzed in the temperature range from 4 to 37 °C. For the purpose of this analysis, it was sufficient to probe only the accessibility of thymidines, since the Mg2+-dependent part of the transcription bubble comprising the bases at position +1 and +2 (see Fig. 2) consists of AT pairs only.

The binding complex was formed in the presence of Mg2+, as described above, and subjected to treatment with KMnO4 or OsO4 at the temperatures indicated. The resulting accessibility patterns obtained at the non-template strand and the template strand are shown in Fig. 3A and Fig. 4A, respectively.

The thymidine patterns obtained at different temperatures were quantified and the resulting intensities were plotted, in pairs only. In order to analyze whether charge or shielding effects, especially in the presence of Mg2+, influence the cleavage pattern, we compared the patterns obtained with KMnO4 and OsO4. Both reagents attack the same positions of thymidines, but have different charges and sizes. Probing of the thymidines of the non-template strand using both probes (Fig. 3A) revealed that the pattern is qualitatively the same but differs quantitatively. When KMnO4 is used, the yield of cleavage is generally higher, and moreover cleavage of T(−12) in the template strand is more pronounced. Otherwise no difference was observed between the two reagents with respect to temperature or Mg2+ dependence of the transcription bubble.

For comparison purposes, the transition temperatures of the Mg2+-dependent and the Mg2+-independent part of the transcription bubble of another eubacterial RNA polymerase, Thermotoga maritima, a thermophilic organism, was included in Fig. 4C. The data were taken from a previously published study (26) on the Mg2+-dependent and Mg2+-independent part having characteristic transition temperatures is the same in both systems (26). Of course, according to the thermophilic nature of T. maritima the transition temperatures are shifted to higher values, as shown in Table I.

In order to analyze whether charge or shielding effects, especially in the presence of Mg2+, influence the cleavage pattern, we compared the patterns obtained with KMnO4 and OsO4. Both reagents attack the same positions of thymidines, but have different charges and sizes. Probing of the thymidines of the non-template strand using both probes (Fig. 3A) revealed that the pattern is qualitatively the same but differs quantitatively. When KMnO4 is used, the yield of cleavage is generally higher, and moreover cleavage of T(−12) in the template strand is more pronounced. Otherwise no difference was observed between the two reagents with respect to temperature or Mg2+ dependence of the transcription bubble.

For comparison purposes, the transition temperatures of the Mg2+-dependent and the Mg2+-independent part of the transcription bubble of another eubacterial RNA polymerase, Thermotoga maritima, a thermophilic organism, was included in Fig. 4C. The data were taken from a previously published study (26) on the Mg2+-dependent and Mg2+-independent part having characteristic transition temperatures is the same in both systems (26). Of course, according to the thermophilic nature of T. maritima the transition temperatures are shifted to higher values, as shown in Table I.

Probing of the Binding Complex using FeEDTA-generated Hydroxyl Radicals—The binding complex of RNA polymerase and the A1 promoter is formed as described in the previous paragraph with and without Mg2+ and subjected to footprint-
ing analysis using FeEDTA-generated hydroxyl radicals (14, 27, 28), as described under “Experimental Procedures.” Fig. 5 shows the patterns obtained on both strands.

The probing pattern of the Mg$^{2+}$-free binding complex shows, besides the footprint of RNA polymerase, an enhanced cleavage at position $+1$ in the template strand, previously discovered by Schickor et al. (14). This hyper-reactive region is located within a stretch of fully protected DNA reaching from base position $+13$ to $+13$. This enhanced cleavage is also observed, although less pronounced, in the Mg$^{2+}$-containing complex. We were interested in finding out the origin of this hyper-reactivity, especially since its temperature behavior (14) is similar to that observed with T($+1$) in the above described Mg$^{2+}$-containing complex.

In order to understand the reason for the enhanced cleavage of the template strand around position $+1$, it is worth recalling how the hydroxyl radicals were generated, namely according to the Fenton reaction by reduction of H$_2$O$_2$ with Fe$^{2+}$. The resulting Fe$^{3+}$ is reduced by dithiothreitol back to Fe$^{2+}$, starting a new cycle. EDTA is necessary in order to prevent Fe$^{2+}$ binding to DNA (27). A prerequisite for obtaining a footprint which reflects the contact sites between RNA polymerase and DNA is spatial independence of the hydroxyl radical source and the probed DNA molecule (14, 27). This is achieved if the hydroxyl radicals stem from molecules freely diffusing in solution. An enhancement of the cleavage beyond that expected for free DNA could indicate activation of sugars by interaction with the enzyme or by a distortion of DNA (e.g. due to bending). An alternative explanation could be an increase of the local concentration of hydroxyl radicals due to binding of the hydroxyl radical source, similar to what was reported for, e.g. OpCuphenanthroline (29). In order to determine which of these possibilities is correct, other OH radicals generating systems, namely KOONO and Fe$^{2+}$, were applied and compared with the FeEDTA footprints.

(i) Hypercleavage Around the Start Point Is due to Bound

---

**Figure 4.** Temperature dependence of the thymidines accessibility in the template strand. The lanes show the accessibility patterns of thymidines in the temperature interval between 0 and 45°C, as indicated, by applying KMnO$_4$. All other conditions are the same, as described in the legend to Fig. 3 for the non-template strand. The plot in C shows a quantification of the accessibility studies on the template strand using the same promoter A1 but upon binding of polymerase from *T. maritima*, a thermophilic organism. The data were taken from Ref. 26.

**Table I**

Transition temperatures ($T_{1/2}$) and free energy ($\Delta H$) for unpaired thymidines in the Mg$^{2+}$-containing complex

|          | $T_{1/2}$ | $\Delta H$ |
|----------|----------|------------|
| *E. coli* |          |            |
| Template strand | |          |
| T(+1)    | 29.4 ± 1.0 | 122 ± 17   |
| T(−6)    | 15.6 ± 1.5 | 105 ± 23   |
| T(−10) + T(−12) | 11.3 ± 1.5 | 119 ± 25   |
| Non-template strand | |          |
| T(−11)   | 10.6 ± 3.0 | 151 ± 61   |
| T(−7)    | 11.4 ± 0.5 | 255 ± 34   |
| T(+2)    | 22.4 ± 0.8 | 188 ± 34   |
| Initial RNA synthesis activity | 32.4 ± 0.9 | 107        |

| *T. maritima* |          |            |
| Template strand | |          |
| T(−10) + T(−12) | 45 ± 1    | 240 ± 30   |
| T(−4) + T(−6)   | 70 ± 5    | 140 ± 80   |

---

**Figure 5.** Accessibility patterns obtained with the binary complex by applying FeEDTA-generated hydroxyl radicals. Lanes indicated $-\text{RNAP}$ show the pattern of DNA only, the lane indicated $+\text{MgCl}_2$ shows the pattern of the complex formed with A1–130 promoter fragment in the presence of Mg$^{2+}$, the lane indicated by $-\text{MgCl}_2$ shows the pattern of the complex formed without Mg$^{2+}$. Left panel, pattern of the non-template strand visualized by labeling of its 5’-terminus; right panel, pattern of the template strand visualized by labeling of its 3’-terminus. The base positions are indicated by arrows. They were determined by a sequence ladders (A + G and A + C).
**FeEDTA**—It has previously been reported that potassium peroxonitrite (KOONO) produces hydroxyl radicals as freely diffusing molecules (22, 30) and can thus be used as an alternative to FeEDTA. We used KOONO in order to decide whether a component of the Fenton reaction is responsible for the observed hyper-reactivity. We subjected the binding complex formed with and without Mg$^{2+}$ to KOONO treatment (Fig. 6, A, lane 5, and B, lanes 3 and 4). The patterns obtained by using both hydroxyl radical generating reagents is essentially the same with exception of the hyper-sensitive spot. We conclude from this finding that a component of the Fenton reaction, namely FeEDTA, acts as local hydroxyl radical source. In order to determine which part of the pattern can be attributed to hydroxyl radicals stemming from freely diffusing FeEDTA and which part to those stemming from bound FeEDTA, the Mg$^{2+}$-free binding complex was subjected to FeEDTA treatment in the presence of glycerol. Glycerol is a hydroxyl radical scavenger which can absorb diffusing hydroxyl radicals (28). Lane 3 of Fig. 6A shows the pattern obtained. As expected, the typical RNA polymerase footprint is blurred, because the hydroxyl radicals generated in solution are captured by glycerol. On the other hand, the hyper-sensitive spot remains unchanged, as comparison with the glycerol free complex in Fig. 6A, lane 2, indicates. From this finding we conclude that the hyper-reactive spot generated by a hydroxyl radical source specifically bound to the binding complex is shielded against access by glycerol.

(ii) **FeEDTA Competes with Mg$^{2+}$ for a Binding Site in the Transcription Complex near Base Position −1**—As seen at Fig. 5, Mg$^{2+}$ ions specifically inhibit the hyper-cleavage of the template strand centered at base position −1, while the footprint outside the hyper-sensitive part remains unchanged.

Fig. 7 shows the dependence of the hyper-reactivity on the Mg$^{2+}$ concentration in the range of 0–10 mM. In the absence of Mg$^{2+}$, the cleavage intensity exceeds that of free DNA by a factor of 10. The hyper-reactivity decreases with increasing Mg$^{2+}$ concentration, indicating displacement of FeEDTA by Mg$^{2+}$. But whether the displacement is competitive or non-competitive is not clear from this curve. The cleavage intensity decreases to 50% at a Mg$^{2+}$ concentration of about 0.5 mM comparable with the applied FeEDTA concentration, indicating that both Mg$^{2+}$ and FeEDTA have an affinity constant of the same order of magnitude.

It appears that hyper-cleavage near the −1 position cannot be suppressed fully even at the highest MgCl$_2$ concentration. This fact along with the finding that the KOONO-generated hydroxyl radical pattern shows no hyper-cleavage either in the absence or presence of Mg$^{2+}$ (Fig. 6B, lanes 3 and 4) suggests that residual cleavage in the presence of Mg$^{2+}$ is also caused by FeEDTA, indicating that FeEDTA can bind but with reduced affinity even in excess of Mg$^{2+}$. This result is in line with a finding by Craig et al. (16) on the AP$_r$, who showed slightly enhanced cleavage near the −1 position in the pre-
FeEDTA Footprint—A striking difference between the

DISCUSSION

Formation of the Transcription Bubble—To map the transcription bubble, we have applied three different probes which modify unpaired thymines, adenines, and cytidines. Until now there has been no adequate procedure for probing unpaired guanines. We have filled this gap by showing that dimethyl sulfate cleaves guanines located within the transcription bubble more efficiently than those located outside. The observation that dimethyl sulfate has a preference toward guanines in single stranded regions is in line with previous dimethyl sulfate methylation studies on the transcription bubble in the ternary complex (4). Analysis of results obtained from methylation studies on the binary complex using the AP promoter by Cowing et al. (15) also coincides with our view.

Using these different probes, the transcription bubble was located between base position −12 to −1 in the Mg2+ free complex. In the Mg2+ containing complex the transcription bubble is enlarged further downstream to base position +2 encompassing the starting point of RNA synthesis (Fig. 2).

The necessity for opening the +1 position is obvious, because the sequence information required for initiation of RNA synthesis would otherwise not be readable. Mg2+-dependent enlargement of the transcription bubble including the +1 position seems to be a requirement for open complex formation of all eubacterial promoters. This enlargement was observed previously in the AP, promoter, but only if the promoter DNA was supercoiled (31), and also in a RNA polymerase promoter complex of a thermophilic eubacteria, T. maritima (26). The Mg2+-dependent enlargement was observed in this system at the physiological temperature of this organism which is 80 °C.

Mapping of the transcription bubble was complicated due to the finding that there are several base pairs of which only one of both complementary bases was modified. Such a base pair was considered as open. But this finding also shows that other effects, such as shielding due to close interaction with the protein can prevent modification and can obscure the disruption of base pairs. Differentiation between the two effects was facilitated by using the surplus information obtained by probing the single strandedness of all four bases.

In order to judge the conclusiveness of results from single stranded probing studies, it is worth recalling the different modifications due to treatment by the single strand-specific reagents. Only dimethyl sulfate/H2 probes cytidines directly involved in Watson-Crick base pairing. All other single strand-specific reagents modify positions of the corresponding bases which do not directly participate in base pairing. The suggested single strand specificity of these reagents is a phenomenological finding for which there is no obvious explanation. Analysis of a DNA model suggests that for example, the C5-C6 bond of thymines becomes accessible for OsO4 only if the stacking interaction is disrupted. Such rather drastic conformational change can be induced by, e.g. breakage of the hydrogen bonding between the base pairs.

Among the bases participating in formation of the transcription bubble most adenines are protected despite accessibility of the complementary thymidine, indicating close contact between protein and adenines. This effect is even more pronounced in the Mg2+ containing complex, suggesting that the adenines are the target sites for opening of the DNA strands.

Additional information about protein-DNA contacts are provided by footprinting studies using FeEDTA-generated hydroxyl radicals. This reagent cleaves the sugar moiety (28) of the bases. Comparison of the results from footprinting studies and single strand-specific probing studies show that the transcription bubble is located at the upstream end of a DNA stretch which is fully protected. This region reaches from base position −12 to +16 at the non-template strand and from base position −13 to +14 at the template strand. The fully protected region is interrupted by a window of accessibility at base positions −9, −10 in the non-template strand and by a window of enhanced cleavage around position −1 in the template strand.

We speculate that the contacts with the sugar phosphate moiety hold the DNA in the proper position, while the contacts with the base moiety, especially of adenines, are required to turn the two strands against each other leading to base pair disruption.

Analysis of the Hyper-reactive Spot at Position −1 in the FeEDTA Footprint—A striking difference between the
FeEDTA footprint of the Mg$^{2+}$-free and the Mg$^{2+}$ containing complex is the hyper-sensitive spot in the template strand at position −1. While Schickor et al. (14) suggested that the observed effect reflects an enhanced accessibility of the DNA due to a conformational change of the DNA, our results rule out this possibility and suggest that the enhancement is due to hydroxyl radicals generated by a FeEDTA molecule bound to polymerase and thus acting as a local hydroxyl radical source. Analysis of the origin of the hyper-reactivity was complicated by the fact that FeEDTA can act as a hydroxyl radical source in solution which generates the footprinting pattern of RNA polymerase and can as well act as bound hydroxyl radical source which generates the hyper-sensitive spot. It was possible to differentiate between the two effects by using glycerol as scavenger of FeEDTA-generated hydroxyl radicals in solution (28) and using peroxonitrite (KOONO) as an alternative hydroxyl radical source instead of FeEDTA.

KOONO is assumed to produce hydroxyl radicals directly upon protonation of the OONO$^{-}$ anion as freely diffusing reagent. The footprint with KOONO has the same appearance as that obtained with FeEDTA, except for the hyper-sensitive spot. This observation supports the view that the hyper-cleavage observed with FeEDTA is due to binding of FeEDTA acting as local hydroxyl radical source. Glycerol has the opposite effect. It suppresses the RNA polymerase footprint but leaves unchanged the hyper-sensitive spot, suggesting that hydroxyl radical generated by bound FeEDTA are not accessible for glycerol, probably due to steric hindrance.

It was previously shown using locally generated OpCu-hydroxyl radicals that, provided there are no shielding effects, hydroxyl radicals have a range of about 15 Å (32). Our data showed that the bases ranging from base position −5 to +3 are affected, which is in line with the proposed range of locally generated hydroxyl radicals.

It was reported recently that Fe$^{2+}$ can act as local hydroxyl radical source replacing Mg$^{2+}$ (19). We rule out the possibility that FeEDTA dissociates and that enzyme-bound Fe$^{2+}$ generates the hydroxyl radical, since the binding constant of the Fe$^{2+}$-EDTA equilibrium is about 9 orders of magnitude higher than that of the Fe$^{2+}$-RNA polymerase equilibrium. Replacement of Mg$^{2+}$ by Fe$^{2+}$ is feasible due to the same positive charge of the two reagents, it is less feasible for FeEDTA which is negatively charged. Despite this charge difference, the binding site of the two reagents at the polymerase promoter complex must be adjacent but is not identical, as indicated by the similarity of the hydroxyl radical cleavage patterns obtained by the two reagents.

Role of Mg$^{2+}$ Ions at the Active Site—Crystallographic analysis of different template-dependent polymerases (33) indicates that two or three Mg$^{2+}$ ions are required to form the active site for nucleic acid polymerization. These Mg$^{2+}$ ions are bound by chelating with aspartates (or glutamates) belonging to a sequence motif which is conserved in all nucleic acid polymerases (34, 35). Comparison of the different polymerase structures known today shows that the distance geometry of the Mg$^{2+}$ ions and the aspartate residues in the active site is the most conserved structural detail.

There are indications that the polymerization active site of E. coli RNA polymerase is assembled in a similar way as in the other polymerases: kinetic studies using E. coli RNA polymerase showed that uptake of three Mg$^{2+}$ ions is required for formation of a transcription active complex (13); site-specific hydroxyl radical probing by Fe$^{2+}$ shows cleavages around the suggested Mg$^{2+}$ pocket (19); the ψ subunit of E. coli RNA polymerase contains the preserved sequence motive with three aspartates which is assumed to participate in formation of the binding pocket of Mg$^{2+}$ (19).

If the aspartates are replaced, the RNA polymerase (DDD mutant) is unable to synthesize RNA, but still retains promoter binding specificity and the capacity to form the Mg$^{2+}$-dependent enlarged form of the transcription bubble (19). We conclude from these findings that the three Mg$^{2+}$ ions participating in formation of the active site fulfill different functions. That Mg$^{2+}$ ions which are coordinated by the aspartates facilitates the polymerization activity. The other Mg$^{2+}$ ions facilitate enlargement of the transcription bubble. These conclusions derived from functional studies using wild type and mutant RNA polymerase are supported by our site-specific hydroxyl radical cleavage studies of the binding complex using Fe$^{2+}$ and FeEDTA.

Using wild type RNA polymerase both probes generate similar patterns suggesting that they have binding sites which are adjacent but not identical. Both probes can be displaced by Mg$^{2+}$ indicating that the sites are identical with, or close to, at least one of the putative Mg$^{2+}$-binding sites in wild type RNA polymerase. But they differ with respect of their affinity to the polymerase promoter complexes formed with mutant enzyme (DDD), where Mg$^{2+}$-chelating aspartates are replaced by alamines. While Fe$^{2+}$ does not bind to the DDD mutant RNA polymerase (19), FeEDTA does. These findings support the view mentioned above, in that the two probes monitor different subsets of Mg$^{2+}$ ions.

Temperature-dependent Change of the Size of the Transcription Bubble—Temperature-dependent analysis of thymidines shows that size and position of the transcription bubble in the Mg$^{2+}$-free complex is the same as that in the Mg$^{2+}$ containing complex at low temperature reaching from base position −12 to −1. Increasing the temperature of the Mg$^{2+}$ containing complex leads to enlargement of the transcription bubble downstream by 2 base pairs. The two parts of the transcription bubble, namely the Mg$^{2+}$-independent part at base position −12 and −1 and the Mg$^{2+}$-independent part encompassing base positions +1 and +2, melt at different transition temperatures, the Mg$^{2+}$-independent part at $T_m = 10–15 \degree C$ and the Mg$^{2+}$-independent part at a temperature more than 10 °C higher. If one accepts that lowering the temperature leads to freezing of transition state complexes, our finding show that formation of the transcription active open complex is preceded by formation of a complex which has the appearance of a Mg$^{2+}$ free complex. A similar suggestion was made previously by Record for the supercoiled AP$^-$ promoter (13, 31).

It is interesting to note that temperature course of the hypercleavage reaction in the FeEDTA footprint is the same as the modification reaction of T(+1) by KMnO$_4$ having a transition temperature of 30 °C. However, the first effect is observed in the Mg$^{2+}$ free complexes, while the second one in the Mg$^{2+}$ containing complex. We suggest from this finding that the transition at 30 °C reflects a conformational change of the polymerase promoter complex which does not require Mg$^{2+}$, but enhances the affinity for Mg$^{2+}$. We suggest further that the bound Mg$^{2+}$ facilitates then the apparent Mg$^{2+}$ dependent effects, such as enlargement of the transcription bubble and enhancement of the contacts between bases of the transcription bubble and RNA polymerase.

Acknowledgments—We thank Anne Whelan and Peter Frings for valuable discussion during preparation of the manuscript.

REFERENCES
1. Siebenlist, U. (1979) Nature 279, 651–652
2. Wang, J. C., Jacobsen, J. H., and Sausier, J.-M. (1977) Nucleic Acids Res. 4, 1225–1241
3. Gamper, H. B., and Hearst, J. E. (1982) Cell 29, 81–90

1 A. Mustaev, personal communication.
4. Zaychikov, E., Denissova, L., and Heumann, H. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 1739–1743
5. von Hippel, P. H., Bear, D. G., Winter, R. B., and Berg, O. G. (1982) in Promoters: Structure and Function (Rodriguez, R. L., and Chamberlin, M. J., eds) pp. 3–31, Praeger, New York
6. Yager, T. D., and von Hippel, P. H. (1987) in Escherichia coli and Salmonella thphimurium (Neidhardt, F. C., Ingraham, J. L., Brooks, L. K., Magasanik, B., Schaechter, M., and Umbarger, H. E., eds) pp. 1241–1275, American Society for Microbiology, Washington
7. Chamberlin, M. J. (1976) in RNA Polymerase (Chamberlin, M. J., and Losick, R., eds) pp. 159–192, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
8. Walter, G., Zililig, W., Palm, P., and Fuchs, E. (1967) Eur. J. Biochem. 3, 194–201
9. Metzger, W., Schickor, P., and Heumann, H. (1989) EMBO J. 8, 2745–2754
10. Buc, H., and McClure, W. R. (1985) Biochemistry 24, 2712–2723
11. Straney, D. C., and Crothers, D. M. (1987) J. Mol. Biol. 195, 267–278
12. Straney, D. C., and Crothers, D. M. (1987) J. Mol. Biol. 195, 279–292
13. Suh, W. C., Leirino, S., and Record, M. T. (1992) Biochemistry 31, 7815–7825
14. Schickor, P., Metzger, W., Werel, W., Lederer, H., and Heumann, H. (1990) EMBO J. 9, 2215–2220
15. Cowing, D. W., Mecas, J., Record, M. T., Jr., and Gross, C. A. (1989) J. Mol. Biol. 210, 521–530
16. Craig, M. L., Suh, W.-Ch., and Record, T. M. (1995) Biochemistry 34, 15624–15632
17. Heumann, H., Lederer, H., Baer, G., May, R. P., Kjems, J. K., and Crespi, H. L. (1986) J. Mol. Biol. 210, 115–125
18. Darst, S. A., Kuhbale, E. W., and Kornberg, R. D. (1989) Nature 340, 730–732
19. Zaychikov, E., Martin, E., Denissova, L., Kozlov, M., Markovtsov, V., Kashlev, M., Heumann, H., Nikiforov, V., Goldfarb, A., and Mustaev, A. (1996) Science 273, 107–109
20. Heumann, H., Lederer, H., Kammerer, W., Palm, P., Metzger, W., and Baer, G. (1987) Biochim. Biophys. Acta 909, 126–132
21. Maniatis, T., Fritsch, E. F., and Sambrook, J. (1982) Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
22. Goette, M., Marquet, R., Joel, C., Anderson, V. E., Keith, G., Gross, H. J., Ehresmann, B., and Heumann, H. (1996) FEBS Lett. 390, 226–228
23. Kirkegaard, K., Buc, H., Spassky, A., and Wang, J. C. (1983) Proc. Natl. Acad. Sci. U. S. A. 80, 2544–2548
24. Palecek, E. (1992) Methods Enzymol. 212, 139–155
25. Buckle, M., and Buc, H. (1989) Biochemistry 28, 4388–4396
26. Meier, T., Schikor, P., Wedel, A., Cellai, L., and Heumann, H. (1995) Nucleic Acids Res. 23, 988–994
27. Tullius, T. D., and Dombroski, B. A. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 5469–5473
28. Tullius, T. D. (1987) Trends Biochem. Sci. 12, 297–300
29. Thederahn, T., Spassky, A., Kuwabara, M. D., and Sigman, D. S. (1990) Biochem. Biophys. Res. Commun. 168, 756–762
30. King, P. A., Jamison, E., Strahs, D., Anderson, V. E., and Brenowitz, M. (1993) Nucleic Acids Res. 22, 2473–2478
31. Suh, W. C., Ross, W., and Record, M. T. (1993) Science 259, 358–361
32. Hermann, T., and Heumann, H. (1995) RNA 1, 1009–1017
33. Joyce, C. M., and Steitz, T. A. (1994) Annu. Rev. Biochem. 63, 777–822
34. Kamer, G., and Argos, P. (1984) Nucleic Acids. Res. 12 7269–7282
35. Delarue, M., Poch, O., Tordo, N., Moras, D., and Argos, P. (1990) Protein Eng. 3, 461–467
36. Meier, T. (1995) Comparative Studies on the Mechanism of Transcription Initiation in Thermophilic and Mesophilic Bacteria Ph. D. Thesis, Ludwig-Maximilian University, München