Passage of RNA Polymerase from Open Complex to Elongation Mode at the *Escherichia coli lacUV5* Promoter: Nucleolytic Hypersensitivity as a Probe for Complex Conformational Changes†

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ABSTRACT: In transcriptionally active complexes between RNA polymerase and promoters, the center of the melted region is hyperreactive to the nucleolytic activity of the cuprous complex of 1,10-phenanthroline (OP-Cu). *In the first part* of this work, using synthetic oligonucleotides and exploiting gel retardation assays, I demonstrate that DNA unpairing is not the only determinant of this hyperreactivity. Polymerase binding is directly implicated, presumably participating in the stabilization of an intermediate required for the cutting. *In the second part* of the work, I show that, from fine analysis of the nucleolytic pattern of *lacUV5* promoter DNA towards OP-Cu and Phe OP-Cu, it is possible to locate polymerase and to characterize its contacts at any time during the early stages of transcription. This analysis provides a description of the passage from the "open complex" to the elongation mode in terms of, first, release of the upstream contacts, and second, loss of σ subunit. Occupancy of the overlapping promoter, P2, has a positive effect on the escape of polymerase from abortive cycling. The involvement of σ and β subunits in the reactivity pattern is discussed with respect to previous cross-linking studies.

Conformational variability of DNA has been shown to play a major role in its interaction with proteins [reviewed in Travers (1989)]. In particular, the kinetics of the formation of transcriptionally active complexes in *Escherichia coli* depends on the local conformation of promoters, which can be dictated by primary sequence per se or induced by torsional stress or protein binding [reviewed in McClure (1985) and Travers (1987)]. These complexes result from multiple conformational changes of DNA and RNA polymerase and are characterized by the unpairing of bases just upstream from the transcription start. Depending on the primary sequence of the promoter, the overall architecture varies (Chan et al., 1990), as well as the ability to pass into the efficient elongation mode. In some cases, such as *lacUV5* promoter, polymerase–DNA contacts, which stabilize the formation of the "open" complex, compete with the formation of new interactions, necessary for the efficient synthesis of RNA chain (Carpoussis & Gralla, 1980, 1985). This competition has been proposed to result in a "stressed" intermediate (Straney & Crothers, 1987), leading to cycling and abortive initiation (Carpoussis & Gralla, 1980, 1985).

Although enzymatic and chemical probes have revealed the conformational features of stable "open" complexes (Carpoussis & Gralla, 1985; Straney & Crothers, 1987; Siebenlist et al., 1980; Kirkegaard et al., 1983; Buckle & Buc, 1989) and of elongating ternary complexes (Krummel & Chamberlin, 1989; Arndt & Chamberlin, 1990; Schickor et al., 1990), little is known about the mechanism of the molecular rearrangements occurring at promoters during the initiation steps. The nucleolytic activity of the cuprous complex of 1,10-phenanthroline (OP-Cu)[1] [reviewed in Sigman and Spassky(1989)] is a unique tool: on one hand, it specifically detects the conserved −10 box of "naked" promoters (Sigman et al., 1985), and on the other hand, it gives rise, when the promoter is actively engaged in priming transcription, to hypersensitive sites in the unpaired region (Spassky & Sigman, 1985). A correlation has been observed between the respective disappearance and appearance of each of these reactivities, demonstrating the fundamental importance of an altered helical conformation in the promoter melting process, as well as the necessity of the precise location of this defect in the overall structural organization of promoters (Spassky et al., 1988). Furthermore, during the steady-state synthesis of the tetranucleotide initiator of the RNA chain at the *lacUV5* promoter, the hyperreactivity is shifted by two bases (Spassky, 1986). In this study, using also this promoter, I present experiments demonstrating that "open" complex OP-Cu hypersensitivity requires RNA polymerase holoenzyme as a necessary determinant and that the position of the reactivity depends on the overall interactions between polymerase and promoter. Furthermore, I show the complementarity of both OP-Cu and Phe OP-Cu (Thederhan et al., 1989) probes in the analysis of the changes in the interactions between polymerase and promoter during the early stages of mRNA synthesis. These changes are, first, the breakage of the upstream constraints, which appears as early as the formation of the sixth or the seventh bond in the nascent RNA, and second, the release of σ subunit. Strikingly, at high polymerase concentration, occupancy of both the P1 and P2 promoters is possible as early as the addition of the third base. As previously observed (Thederhan et al., 1990), during the synthesis of the 10-mer RNA, OP-Cu hyperreactivity is lost but a new reactivity toward Phe OP-Cu appears. The involvement of polymerase subunits in these different signals will be discussed in the light of previous cross-linking (Simpson, 1979; Chenchick et al., 1981; Park et al., 1982a,b) and photoaffinity labeling studies (Bowser & Hanna, 1991).

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[1] Abbreviations: OP-Cu, nucleolytic activity of the cuprous complex of 1,10-phenanthroline; Phe OP-Cu, nucleolytic activity of the cuprous complex of the 5-phenyl derivative of 1,10-phenanthroline.

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I      3'  ACGAAGGCCGAGC  ATATTACACACC  TTAACACTCG  5'
              -20           -10     -1        +10

II     5'  TGCTTCCGGCTCG  TATAATGTGTGG  AATTGTGAGC  3'
       3'  ACGAAGGCCGAGC  ATATTACACACC  TTAACACTCG  5'
              -20           -10     -1        +10

                          CCCCCCCCCCCC
III    5'  TGCTTCCGGCTCG                AATTGTGAGC  3'
       3'  ACGAAGGCCGAGC  ATATTACACACC  TTAACACTCG  5'
              -20           -10     -1        +10
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FIGURE 1: Conformations of the −25 to +10 template strand UV5 promoter sequence oligonucleotides.

MATERIALS AND METHODS

(*a*) *Materials.* The following enzymes and chemicals are obtained commercially and used without further purification: T4 polynucleotide kinase (Pharmacia), calf intestine alkaline phosphatase (BRL), 1,10-phenanthroline and its 5-phenyl derivative (Sigma), mercaptopropionic acid (MPA) (Aldrich), [γ-^{32}P]ATP (Amersham), and NTPs (Pharmacia).

(*b*) *RNA Polymerase and DNA. E. coli* RNA polymerase was purified according to the modified procedure of Burgess and Jendrisak (1975), described in Lowe et al. (1979). The concentration of enzyme was calculated from absorption measurements.

Oligonucleotides were synthesized on an automatic synthesizer (Pharmacia) and purified on denaturing 20% polyacrylamide gels as described in Sambrook et al. (1989). Oligonucleotide I (see Figure 1) was labeled radioactively at the 5′ end, using T4 polynucleotide kinase (Maxam & Gilbert, 1980), then mixed with the complementary strand (duplex II) or with another only partly complementary oligonucleotide (duplex III), and hybridized by cooling slowly from 70 °C to room temperature. Samples were electrophoresed on a nondenaturing 5% polyacrylamide gel and the corresponding duplexes were cut out of the gel and extracted. Polymerase (250 nM) and radioactive duplex (4 nM) are mixed using standard buffer (see below), and the polymerase–DNA complex was separated from noncomplexed DNA by electrophoresis on a 4% nondenaturing gel and then digested directly in the gel slice, as previously described (Kuwabara & Sigman, 1987).

The DNA fragment covering the *lacUV5* promoter from −140 to +63 was isolated from plasmids as described in Schaeffer et al. (1982) and 5′-end-labeled as above. Uniquely end-labeled fragment was generated by subsequent cleavage with *Pvu*II, which cuts the fragment at −123.

(*c*) *Digestion Experiments of Promoter during the First Elongation Steps.* Standard conditions for open complex formation were 4 nM of the 203-bp DNA fragment, 25 nM (low) or 250 nM (high) concentration of *E. coli* RNA polymerase holoenzyme, 100 mM KCl, 40 mM Tris, pH 8, 10 mM $MgCl_2$, 0.1 mM DTT, and 0.1 mM EDTA. Various nucleotide mixtures, using the efficient primer ApA (500 μM) (McClure, 1980), were added to the "preformed open complex": standard concentrations, except when indicated, are ApA, 500 μM; UTP, GTP, and ATP, each 100 μM; and OMeGTP or OMeCTP, 50 μM.

Footprinting with OP-Cu and 5-Phe OP-Cu (Sigma) was done as described in Spassky and Sigman (1985) and Thederhan et al. (1990), respectively. Digestion was terminated by the addition of a stop solution (final concentration 0.3 M sodium acetate, 1 mM EDTA, and 100 mg/mL tRNA), followed by phenol extraction and ethanol precipitation. Analysis was performed on 8% polyacrylamide sequencing gels, followed by autoradiography. Calibrations were made from sequencing reactions, performed on the same labeled fragments according to Maxam and Gilbert (1980). After autoradiography, the relative amount of radioactivity in each band of the gel was measured by densitometric analysis, performed with a LKB Model 2222-010 densitometer.

The calibrations shown in the figures were deduced by running sequence reactions on the same gel and are numbered with respect to the start point for the P1 transcription start.

RESULTS

DNA Melting Is Not the Only Determinant Governing the Hyperreactivity of Transcriptionally Competent Complexes to OP-Cu. The first experiment, designed to identify factors that participate in the reactivity of open complex, was based on earlier results focusing on the DNA fragment length independence of the OP-Cu cutting kinetics (Yoon et al., 1988). I first compared the OP-Cu nucleolytic sensitivity of a 35-mer oligonucleotide, carrying the template strand UV5 promoter sequence from residues −25 to +10, in various conformational situations (Figure 1): the single-stranded oligonucleotide (oligo I), the oligonucleotide paired with the complementary sequence (oligo II), and the oligonucleotide hybridized with an oligonucleotide complementary only from −25 to −13 and from +1 to +10, containing 12 cytosines from positions −12 to −1 (oligo III).

The OP-Cu digestion pattern of oligo II was compared, in a control experiment, with that of the same sequence in a 186-base-pair DNA restriction fragment (Figure 2, profile A). Similar patterns are observed in both cases; in particular, positions −13 to −10 exhibit identical hypersensitivity. These positions are not reactive in the single-stranded oligonucleotide (oligo I), which displays uniform weak OP-Cu sensitivity (Figure 2, profile B). Strikingly, the digestion of the unpaired region of the heteroduplex III results in weak sensitivity only at the center; at both edges, the reactivity increases, particularly around positions −12 to −8 (Figure 2, profile B). It is interesting to notice that these positions are just slightly shifted with respect to reactive positions of the double-stranded structure (Figure 2, profile C).

In the second part of the experiment, polymerase (250 nM) was incubated in the presence of either oligo II or oligo III (4 nM). Polymerase failed to bind to homoduplex (oligo II), but, as probed by its retarded migration on a nondenaturing polyacrylamide gel (data not shown), 40% of the heteroduplex (oligo III) was complexed with polymerase. OP-Cu digestion of polymerase–DNA complex was performed directly on the excised gel slice. Polymerase binding results in a dramatic change of the OP-Cu reactivity pattern: disappearance of the naked DNA reactivity and very prominent reactivity at position −3 (see Figure 3, lanes 4 and 5). For comparison, the OP-Cu reactivity changes resulting from the binding of polymerase to the 186-base-pair UV5 promoter fragment are shown in lanes 1 and 2 of the same figure and the densitometric profiles of lanes 2 and 5 are compared in Figure 3C.

This experiment provides the evidence that the only opening of one helix turn bases upstream from the transcription start does not induce hyperreactivity inside the unpaired region. Interestingly, polymerase can bind to the 35-mer heteroduplex, and, as in the case of the "open" complex, this binding causes OP-Cu sensitivity amplification and shift in the interior of the "bubble". This result suggests that, in both cases, identical local conformation, resulting from the interaction of polymerase with the unpaired DNA, is responsible for the stabilization of the cuprous complex of *o*-phenanthroline. The position of the hyperreactivity would reflect the position of the enzyme with respect to the template, as a function of the overall interactions between the enzyme and DNA. This

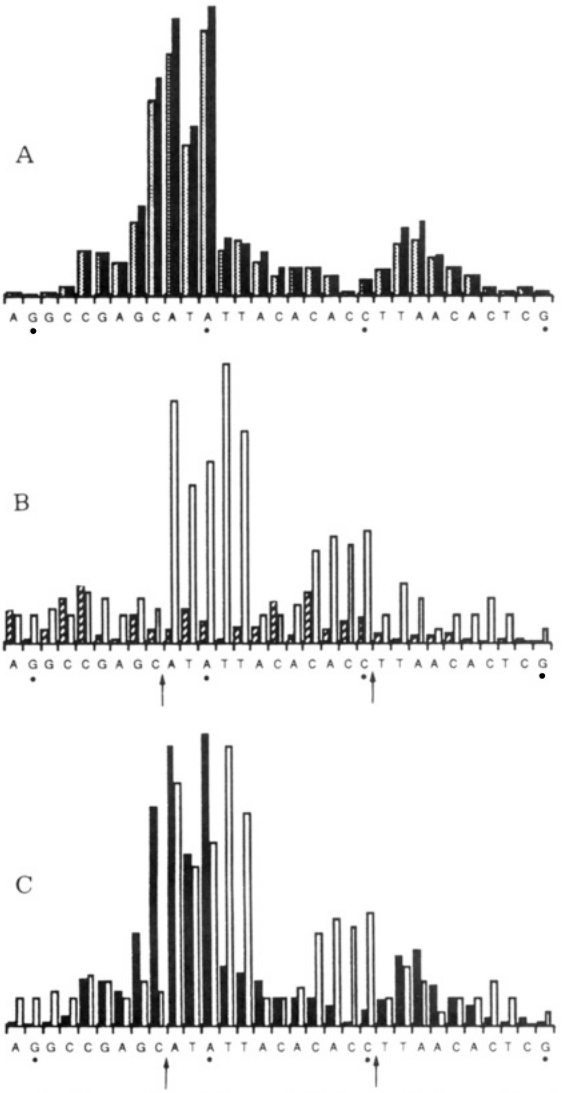

FIGURE 2: Comparison of the nucleolytic sensitivity profiles of the −25 to +10 template strand UV5 promoter sequence. Profiles of the patterns of the nucleolytic attack of the −25 to +10 template strand UV5 promoter sequence are compared in the different conformations: (A) oligo II (black columns)/186-bp DNA restriction fragment (shaded columns); (B) oligo I (hatched columns)/oligo III (white columns); (C) oligo II (black columns)/oligo III (white columns). Arrows indicate the boundaries of the artificially unpaired region.

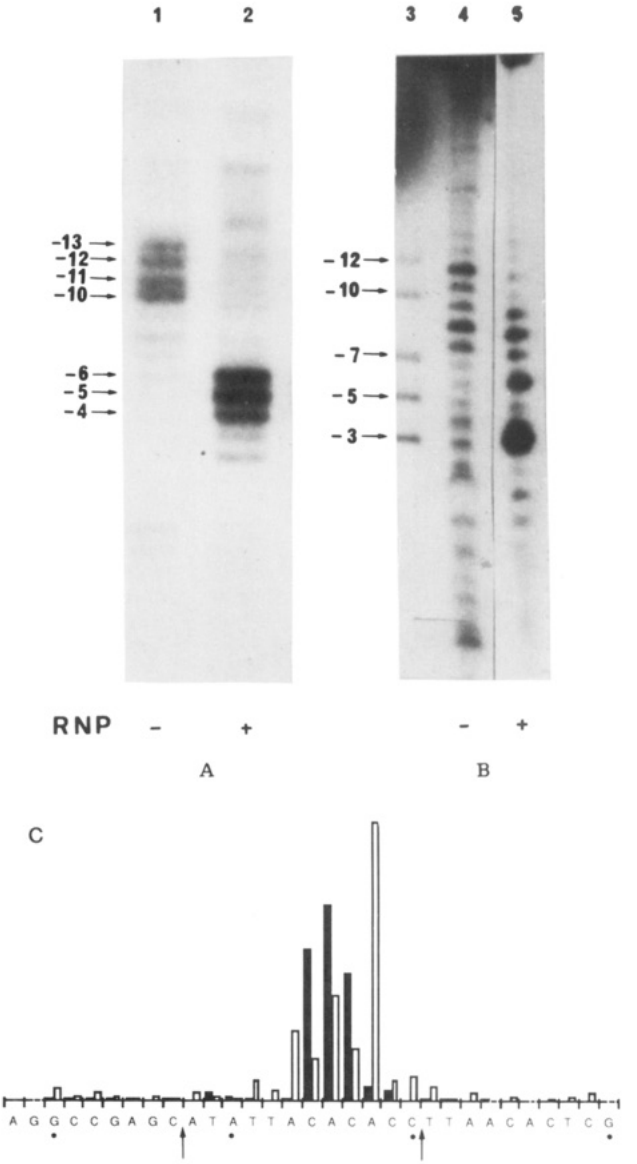

FIGURE 3: OP-Cu digestion patterns of DNA bare or polymerase complexed. (A) Autoradiogram of an 8% sequencing gel on which has been analyzed the result of the nucleolytic attack of the 5′-end-labeled template strand UV5 promoter, naked (lane 1) or polymerase complexed (lane 2). (B) Autoradiogram of a 20% sequencing gel on which has been analyzed the result of the nucleolytic attack of the "artificial bubble", bare (lane 4) or polymerase complexed (lane 5), performed directly on the excised retardation gel slice. Lane 3 is an A + G Maxam and Gilbert sequencing reaction. (C) Comparison of the densitometric profiles of lanes 2 (black columns) and 5 (white columns) of the autoradiogram.

hypothesis, strengthened by results of the second part of this study, will be discussed later.

Analysis of the Nucleolytic Sensitivity of lacUV5 Promoter during the First Steps of the Initiation of Transcription. It was of interest to analyze the hypersensitivity of promoter DNA during the first steps of the initiation of transcription. Preformed open complex between RNA polymerase and the 186-base-pair *lacUV5* promoter fragment was incubated with the different nucleotide combinations noted in Table I: using the primer ApA and the RNA chain terminators OMe-GTP or OMe-CTP, the synthesis can be sequentially restricted to short RNA chains from four up to 10 bases long. After each incubation, the complex was submitted to nucleolytic reactions, and the synthesized RNA was checked on a 25% acrylamide gel. In addition to OP-Cu nuclease, I used the 5-phenyl derivative of OP-Cu, Phe OP-Cu, which has been shown to display the same conformational specificity as OP-Cu but a weak efficiency of cleavage with naked promoter and much stronger cleavage with polymerase/promoter complex (Thederhan et al., 1990). Furthermore, in order to analyze the nucleolytic patterns of complexes not only during but also after the end of RNA steady-state synthesis, the concentration

Table I

3'..... T T A A C A C T C G C C T A T T G .....5' template strand
+1 +10 +16

(1) ApA + UTP	4 mer: ApApUpU
(2) ApA + UTP + OMeGTP	5 mer: ApApUpUpG
(3) ApA + UTP + GTP	7 mer: ApApUpUpGpUpG
(4) ApA + UTP + GTP + ATP	9 mer: ApApUpUpGpUpGpApG
(5)ApA + UTP + GTP + ATP + OMeCTP	10 mer: ApApUpUpGpUpGpApGpC

(4) possible misincorporation leading to a read through until the 16 mer (Carpoussis & Gralla, 1985) ApApUpUpGpUpGpApGp X pGpGpApUpApA
+10

of nucleotides and the incubation time have been varied. In some cases, heparin was added to prevent reinitiation. Two sets of experiments, using low (25 nM) or high (250 nM) RNA polymerase concentration, have been performed.

(A) Reactivity Patterns at Low Polymerase Concentration. Low (25 nM) polymerase concentration allows only the

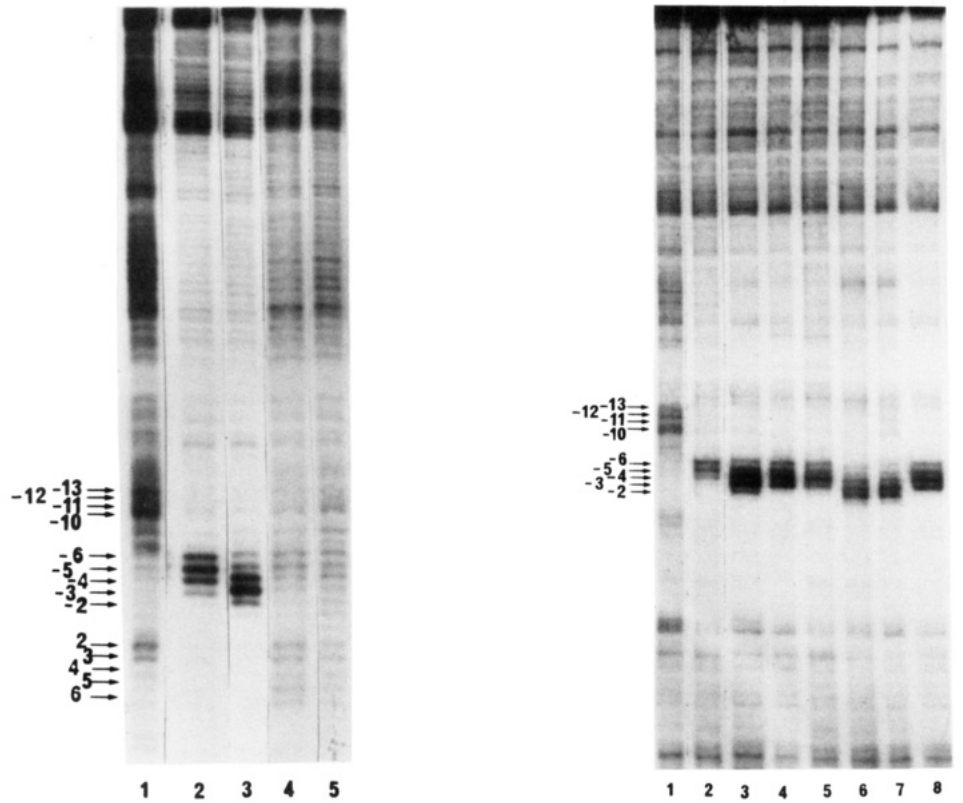

FIGURE 4: Nucleolytic sensitivity of template strand UV5 promoter fragment during the first steps of the initiation of transcription: low polymerase concentration. (Left) Autoradiogram of an 8% sequencing gel showing the OP-Cu digestion patterns of the 5′-end-labeled template strand UV5 promoter; DNA alone (4 nM) (lane 1), incubated 10 min with RNA polymerase (25 nM) (lane 2) and then 10 min with ApA + UTP (lane 3) or with ApA + UTP + GTP + ATP (lane 4) or with ApA + UTP + GTP + ATP + OMeCTP (lane 5). (Right) Autoradiogram of an 8% sequencing gel showing the OP-Cu digestion patterns of the 5′-end-labeled template strand UV5 promoter; DNA alone (4 nM) (lane 1), incubated 10 min with RNA polymerase (25 nM) (lane 2) and then with ApA + UTP for 30, 60, and 80 min (respectively lanes 3, 4, and 5) or with ApA + UTP + OMeGTP for 10, 30, and 60 min (respectively lanes 6, 7, and 8).

occupancy of the promoter P1 (Buc & McClure, 1985). The OP-Cu nucleolytic pattern of the promoter during the steady-state synthesis of 4-mers to 10-mers of RNA (see Table I) is shown in the left panel of Figure 4: the cleavage positions are shifted two bases downstream during the synthesis of 4-mers with respect to the reactivity of the open complex (lanes 1–3) and are not significantly changed (shown in Figure 4, right panel) during 5-mer synthesis; in contrast, weak and scattered reactivity is observed from positions –13 to +6 (lanes 4 and 5) during the next synthesis steps.

Increasing the incubation time of the polymerase–promoter complex with nucleotide combinations 1 and 2 (see Table I) progressively reverses the effect, the reactivity shifting up toward "open" complex position (Figure 4, right panel). As probed by the concomitant stop of the RNA steady-state synthesis, this effect has to be correlated with the gradual depletion of substrates (data not shown). Similar results are obtained if heparin is added concomitantly to the nucleotides.

Scattering of the reactivity is observed as early as the addition of the nucleotide combination 3, which induces the synthesis of 7-mer RNA (see Table I). This step has been analyzed in detail by using the activity of Phe OP-Cu, which amplified the nucleolytic signal exhibited by the active complex with respect to the weak reactivity of naked DNA (Figure 5, left panel; compare lanes 1 and 2). Fixed concentrations of ApA (500 μM) and UTP (100 μM) and then different concentrations of GTP (from 20 to 200 μM) were added to the preformed open complex. The resulting nucleolytic pattern extends from positions –6 to +4, the distribution of the reactivity depending on both the GTP concentration and the incubation time [Figure 5, left and right (a–c)]. The intensity of bands at positions +2 to +4 increases with the incubation time as the intensity of upstream bands decreases. The plot of the intensity of bands +3 and +4 as a function of incubation time for four GTP concentrations shows that increasing GTP affects the rate of appearance of bands at +3 and +4 as well as their intensity (Figure 5d). In addition, the analysis of the reactivity pattern at a given GTP concentration, as a function of time, reveals two clusters of bands: cluster I, spanning from –6 to –1, and cluster II, from +2 to +4. The relative intensity of the bands of cluster II does not change in time, in contrast with that of cluster I (shown, as an example, for 20 μM GTP in lanes 12–14 of Figure 5, left panel).

(*B*) *Relation between the Nucleolytic Sensitivity of P1 Promoter and the Occupancy of P2 Promoter.* Similar experiments have been performed, at higher polymerase concentration (250 nM), in order to allow the occupancy of the overlapping promoter P2 (Spassky et al., 1984). As shown in Figure 6, similar DNA nucleolytic patterns as at low polymerase concentration are observed during the steady-state synthesis of the tetra- or pentanucleotide (lanes 1–4 and 9–12). In contrast, the digestion pattern of the complex synthesizing the 7-mer RNA displays three well-separated clusters of bands in place of two: the two clusters previously observed, cluster I at positions –13 to –1 or –6 to –1 with OP-Cu or Phe OP-Cu, respectively, and cluster II at positions +2 to +6, and also a third cluster at positions –25 to –28 (lanes 5 and 13). These last hyperreactive positions, located a half helix turn upstream from the P2 transcription start, at position –22, have been previously shown to be a probe of the occupancy of the promoter P2, the kinetics of their appearance being concomitant with the kinetics of the formation of the transcriptionally active P2 complex (Amouyal & Buc, 1987;

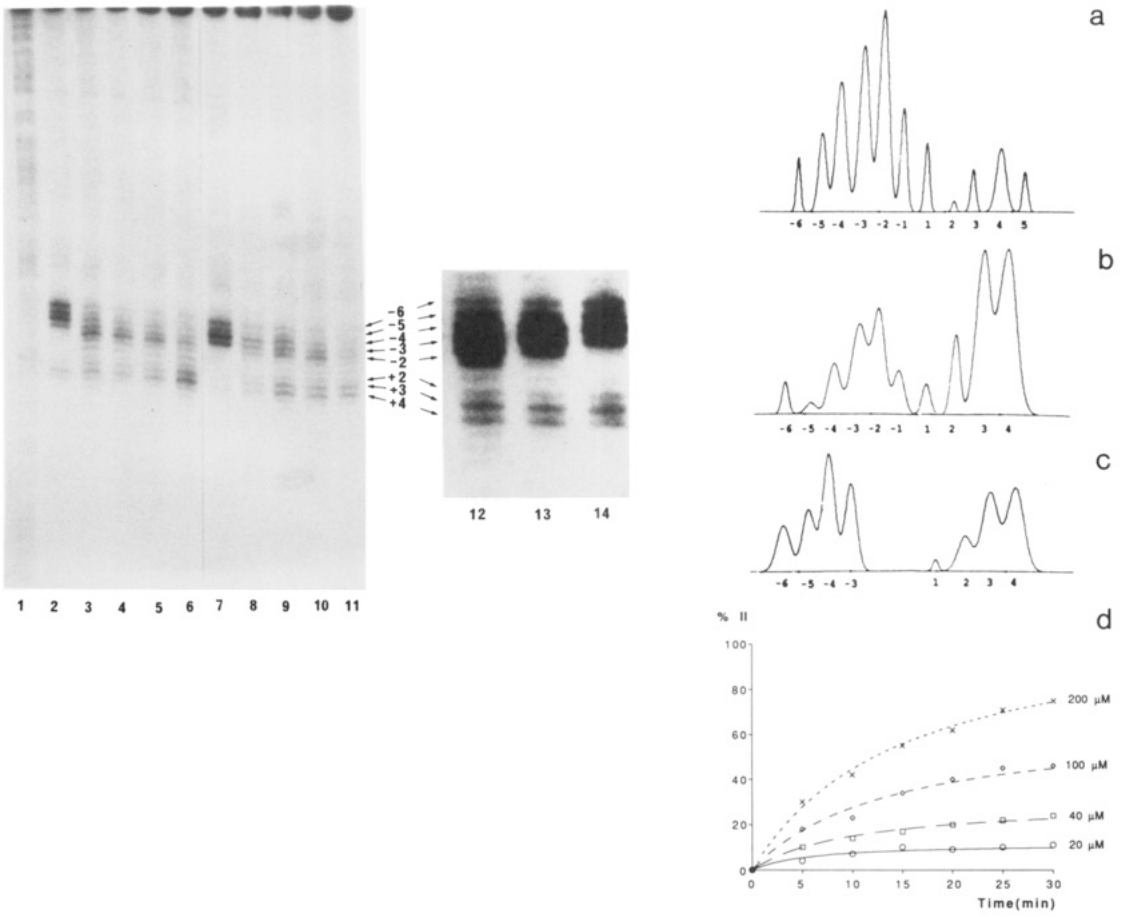

FIGURE 5: Analysis of the scattering of the reactivity. (Left) Autoradiogram of an 8% sequencing gel showing the Phe OP-Cu digestion patterns of the 5′-end-labeled template strand UV5 promoter; DNA alone (4 nM) (lane 1), incubated 10 min with RNA polymerase (25 nM) (lanes 2 and 7) and then respectively 10 or 30 min with ApA + UTP + various GTP concentrations: 20 μM, lanes 3 and 8; 40 μM, lanes 4 and 9; 100 μM, lanes 5 and 10, or 200 μM, lanes 6 and 11. Lanes 12, 13, and 14 show the results of the same experiments, using a GTP concentration of 20 μM and an incubation time of respectively 30, 60 and 80 min. (Right, a–c) Densitometer tracing of autoradiogram in left panel: (a) lane 3, (b) lane 6, and (c) lane 14. (Right, d) Time course, at various GTP concentrations, of the relative intensity of bands +3 and +4 (cluster II) with respect to the total intensity of bands from –6 to +4 at 25 nM polymerase concentration. Values have been determined from the densitometric scans, normalized against that of nonhydrolyzed DNA, which has been quantitated by scanning the corresponding band after very short exposure times. Linearity of the response of the film has been verified by scanning after various exposure times, in order to not exceed maximum optical density of 1.2.

Spassky et al., 1988). Thus, unexpectly, the overlapping P2 promoter can be occupied as soon as P1 polymerase forms the sixth or the seventh bond. At the same time, the P1 –10 region becomes again accessible to the nucleolytic activity of OP-Cu [Figure 6 (left), lane 5].

The reactivity of cluster II is strengthened and extended downstream during the synthesis of the 9-mers at the expense of cluster I and concomitantly a gradual protection of the OP-Cu cutting from +11 to +24 appears [Figure 6 (left), lanes 6 and 14]. During the synthesis of the 10-mer RNA, specific OP-Cu reactivity diappears, but new reactivity toward Phe OP-Cu, cluster IV, appears at positions +11 and +12 (lane 7; Thederhan et al., 1989). It is interesting to note, in relation with the readthrough synthesis observed in similar conditions by Carpoussis and Gralla (1985), that two weak bands are observed at positions +18 and +19, when the mixture 4 is added to the "open" complex (see lane 14).

The appearance of bands of clusters II and III is parallel. Figure 7 (top) shows the time course of appearance of the bands in clusters II and III in an experiment using 100 μM GTP, where open complex formation results from the addition of 250 nM polymerase. Figure 7 (bottom) shows that the kinetic fit is found, at every time, located above at high with respect to low polymerase concentration.

DISCUSSION

Results of the first experiment of this study demonstrate, without ambiguity, that the unpairing of DNA is not the sole determining factor of the hyperreactivity observed in the center of the single-stranded part of the active polymerase–promoter complex, at the *lacUV5* promoter. The comparison between the OP-Cu digestion patterns of a 35-base oligonucleotide UV5 template strand sequence in a number of situations shows that reactivity is weak inside the "bubble" but increases at each edge, the intensity depending on the primary sequence. This increase of reactivity at the junctions between single- and double-stranded regions points to a correlation between the OP-Cu sensitivity and a defect of the helical stacking and strengthens the proposition according to which the reactivity of positions –13 to –10 of naked promoter reveals intrinsic local distortion or deformability of DNA (Spassky et al., 1988).

Polymerase does not bind to the 35-mer homoduplex but binds to the same size heteroduplex. Footprinting studies of the open complex have shown that RNA polymerase extends upstream from the transcription start over approximately 50 base pairs in length on the *lacUV5* promoter. Present results demonstrate that contacts upstream from position –25 are not absolutely required in stabilizing the complex between polymerase and unpaired DNA, but that they must be used as a source of energy in the nucleation of base-pair unwinding.

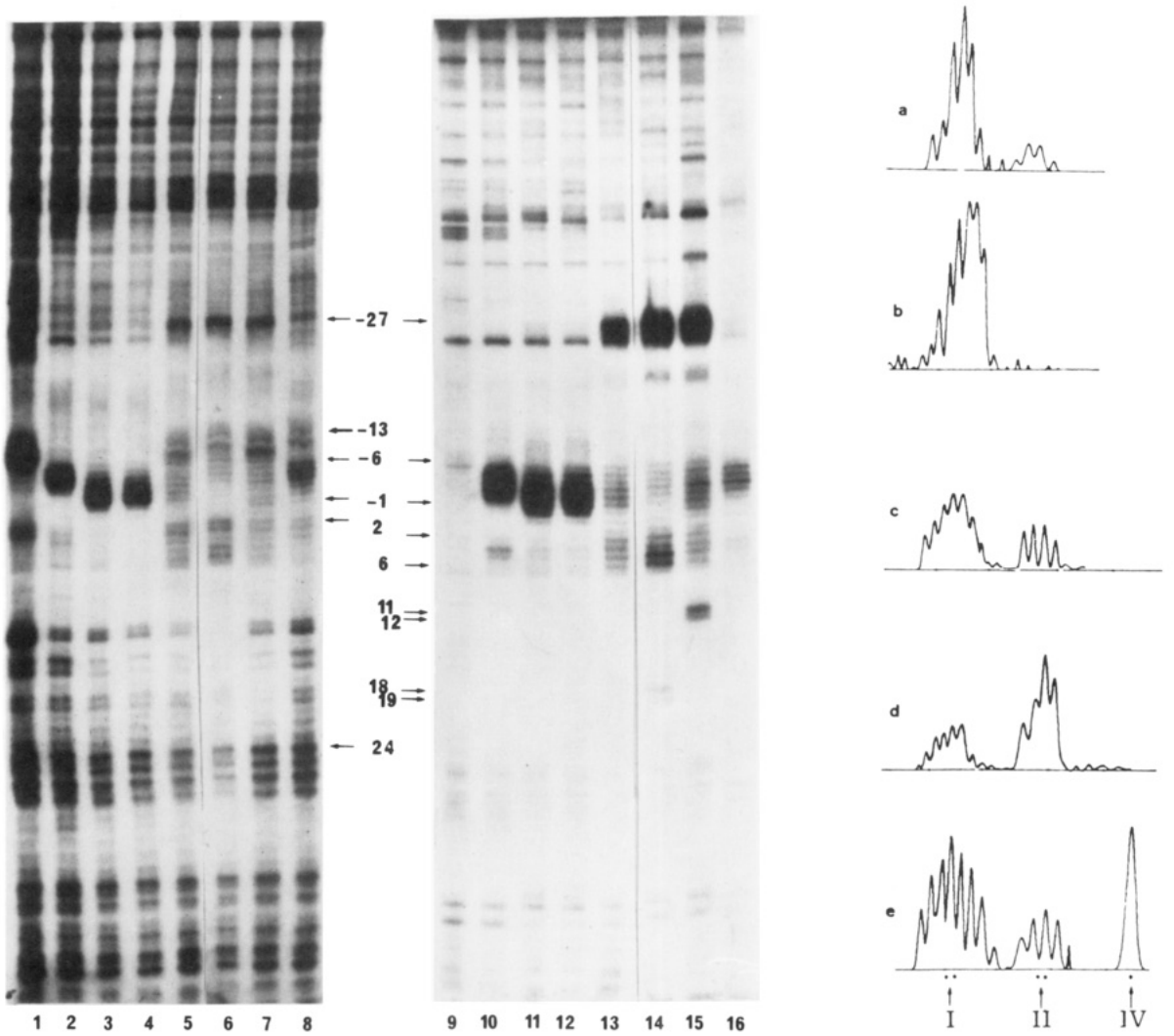

FIGURE 6: Simultaneous occupancy of the overlapping *lac* P1 and P2 promoters at high polymerase concentration. (Left) Comparison of the OP-Cu (lanes 1–8) and Phe OP-Cu (lanes 9–16) nucleolytic digestion of polymerase/promoter complexes during the first elongation steps. The experiment was carried out using identical procedures and nucleotide concentrations as in Figure 4, except with a concentration of polymerase of 250 nM. The autoradiogram shows the result of nucleolytic attack of DNA alone (4 nM) (lanes 1 and 9), incubated 20 min with polymerase (lanes 2 and 10) and then 30 min with ApA + UTP (lanes 3 and 11), with ApA + UTP + OMeGTP (lanes 4 and 12), with ApA + UTP + GTP (lanes 5 and 13), with ApA + UTP + GTP + ATP (lanes 6 and 14), with ApA + UTP + GTP + ATP + OMeCTP (lanes 7 and 15), and with ApA + UTP + GTP + ATP + CTP (lanes 8 and 16). (Right) Densitometer tracings of autoradiogram in left panel: (a) lane 10), (b) lane 11, (c) lane 13, (d) lane 14, and (e) lane 15.

The complex between polymerase and heteroduplex exhibits a local OP-Cu hypersensitivity in the unpaired region, as the promoter open complex. Thus, this hyperreactivity appears as a probe of a particular conformation, which arises from a specific interaction between polymerase and unpaired promoter sequence DNA. The comparison between the magnitude of the OP-Cu and Phe OP-Cu sensitivity of DNA, alone or bound to RNA polymerase, also argues in favor of the fact that the morphological features of each of these structures are different: naked DNA is much less reactive to Phe OP-Cu than to OP-Cu. In contrast, polymerase–promoter complexes are much more reactive to Phe OP-Cu than to OP-Cu, suggesting that the bulky phenyl group, which most likely hinders the entry of the metallic complex inside the minor groove (Thederhan et al., 1989), helps the stabilization of the metallic compound by the polymerase–promoter complex (Thederhan et al., 1990). The differences between both reactivity patterns, polymerase–"artificial bubble" and polymerase–promoter (downstream shift and concentration in a discrete site), might be due first to a slight difference in the location of the boundaries of the respective unpaired region (Spassky, 1986; Buckle & Buc 1989) and second to the absence of the strain energy due to polymerase–promoter upstream interactions (Straney & Crothers, 1987). The displacement of the position of hyperreactivity, observed during the first steps of the mRNA synthesis in parallel with the progressive loss of upstream interactions (see below), supports this interpretation.

Taking advantage of the respective features of both OP-Cu and Phe OP-Cu reactivities, I analyzed the passage from the open to the elongation complex: on one hand, using OP-Cu as a footprinting agent, it is possible to accurately determine polymerase DNA protected regions; on the other hand, the magnitude of the Phe OP-Cu hyperreactivity allows identification of low concentrations of polymerase–promoter active complexes and thus differentiation of populations of molecules engaged in the same time, in different ways.

Notice, first of all, that the common OP-Cu and Phe OP-Cu hypersensitivity is observed only until the 9-mer RNA synthesis step, strongly suggesting that it involves the holoenzyme, since σ has been shown to be released, in the case of *lacUV5* promoter, during the 10-mer RNA synthesis step (Kolb et al., 1988). From the analysis of the digestion pattern with time after the addition of ribonucleotide mixtures, it is possible to associate each step of the formation of the first few phosphodiester bonds with specific hypersensitive sites along

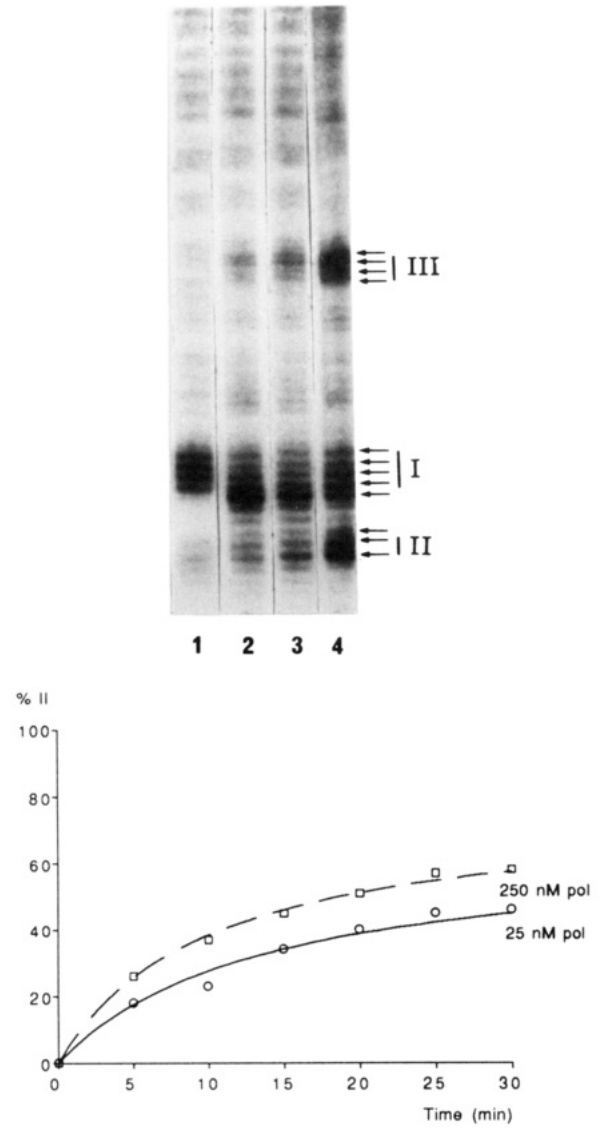

FIGURE 7: Correlation between the occupancy of the promoter P2 and the intensity of the cluster II. (Top) Autoradiogram of an 8% sequencing gel analysis of the Phe OP-Cu digestion products of polymerase promoter complex (250 nM/4 nM) (lane 1) incubated with ApA + UTP + GTP for 10 (lane 2), 20 (lane 3), or 50 min (lane 4). (Bottom) Time course experiments, at 25 and 250 nM polymerase concentration and using 100 μM GTP, of the relative intensity of bands +3 and +4 with respect to the total intensity of bands from –6 to +4.

the template. The steady-state abortive synthesis is associated with hypersensitivity sites, slightly shifted down with respect to the open complex position (Figure 4, left panel) (Spassky, 1986). At the end of the synthesis, the reactivity returns to the position corresponding to the open complex (Figure 4, right panel), even in the presence of heparin. No significant change in the OP-Cu footprint is observed. In agreement with the previous model (Carpoussis & Gralla, 1985), the simplest interpretation of such results is that the slight shift of the reactivity reflects a transient pause site of polymerase during its "cycling without template release" movement, which can no longer be reached when the lack of substrates abolishes the reiterative synthesis. Note that the whole reactivity is shifted down at once, indicating that this position corresponds very likely to the rate-limiting step in the cycling process [see Figure 4 (left), lanes 1–3].

As early as the formation of the sixth phosphodiester bond, reactivity is no longer gathered into three or four positions but is scattered from –6 to +4 (see Figure 5). The analysis shows the clear differentiation of a new cluster of bands, cluster II (positions +3 and +4), beside the previous cluster I (–6 to –1). The stability of these new bands and their association with the occupancy of the promoter P2, as well as the accessibility of the "–10 box" of the promoter P1 by the nucleolytic activity of OP-Cu (see Figure 6), indicate that they have to be correlated with polymerase no longer engaged in the cycling process because of the release of the upstream interactions, which stretch polymerase to the upstream part of the promoter. The correlation of the decrease of the intensity of the cluster I and the increase of that of the cluster II support this hypothesis.

Thus, the first cluster would correspond to cycling abortive polymerase molecules and the second cluster would reveal "escaped" holoenzyme molecules. As previously observed (Carpoussis & Gralla, 1980), the escape of polymerase is found to be easier at high nucleotide concentrations. Furthermore, at high concentrations of RNA polymerase, as early as polymerase has synthesized the 6-mer RNA from the promoter P1, the occupancy of the overlapping promoter P2 is possible. This is an unexpected result, since, to date, the binding of polymerase to both promoters was thought to be mutually exclusive. We found, in addition, that the occupancy of P2 is coupled with an increase of the amount of productive polymerase molecules [reactivity II in Figure 7 (top)]. All the lactose promoter mutations that affect P2 activity in vitro have been found to also alter P1 *lac* regulation in vivo, suggested that the RNA polymerase–P2 interaction is important in the regulation of lactose expression (Peterson & Reznikoff, 1985), but, to date, studies have failed to find a P2 possible functional role (Yu & Reznikoff, 1985; Gralla, 1985). No P2 productive initiation in vivo could be detected (Peterson & Reznikoff, 1985). Present findings suggest a way by which P2 promoter could play a positive role on the expression of lactose operon, not directly but by favoring P1 efficient elongation versus abortive initiation; this would be similar to the influence of the CRP–CyAMP complex in the escape of polymerase in the case of promoter *malT* (Menendez et al., 1987). However, the effect found here is small, and further studies, using various experimental conditions, are necessary to determine if the productive versus abortive initiation from P1 could be, "in vivo", affected by the occupancy of P2.

Results presented here together with previous data support the direct involvement of σ subunit in the single-stranded region of Cu hypersensitivity. First, cross-linking studies have shown direct association of σ with the promoter, in the unwound region of the open complex, at positions –5 and –3 (21–24). Second, genetic results have suggested direct contacts between σ and the –10 region of promoter during the formation of the open complex (Doi & Wang, 1986), and OP-Cu reactivity has been found to appear as early as the first contact of polymerase with promoter in the –10 region, followed by the shift to position –5 during the formation of the open complex (Spassky et al., 1988). Third, the σ region implicated in DNA contacts has been proposed to be the conserved subregion of σ, rich in aromatic amino acids (Hellman & Chamberlin 1988), which would explain the enhancement of the reactivity toward the phenyl derivative of phenanthroline by aromatic ring-stacking stabilization. In addition, recent photoaffinity labelling results (Bowser & Hanna, 1991) have shown that a crucial change in the positioning of σ with respect to the 3′ end of the nascent RNA occurs after the 5-mer RNA synthesis. That is in good agreement with the "jump" of the reactivity we have observed just between the synthesis of the 5-mer and the 6-mer, at the same time as the loss of upstream constraints. Note also that positions –37 and –35 and –18 and –17 have also been found associated with σ (Simpson, 1979; Chenchick

et al., 1981; Park et al., 1982a,b) and that the release of σ from core enzyme is dependent on upstream promoter sequences (Schimamoto et al., 1986; Stackhouse et al., 1989). Two other sites of the active complex exhibit sensitivity only toward Phe OP-Cu: positions +5 and +6 at the edge of the melted region of the transcriptionally active complex and positions +11 and +12 during the synthesis of the 10-mer RNA. A very weak reactivity is also observed at positions +18 and +19 during the synthesis of the 9-mer RNA, which by analogy with the +11 and +12 bands could be related to a pause site of polymerase at position +16, in agreement with the readthrough synthesis which has previously been shown to occur, in the absence of CTP, from misincorporation of position +10 (Carpoussis & Gralla, 1985). These cutting sites exhibit common features: two bands occurring on both strands of DNA (data not shown), specificity of the 5-phenyl-substituted phenanthroline metallic compound, and at least 1 order of magnitude less than the previous ones. It is therefore proposed that the corresponding sites exhibit the same morphology and involve the core enzyme in place of the holoenzyme. The β subunit could be directly involved in this reactivity, since cross-linking studies have shown its association with promoter at positions +3 and +5 in the open complex (Simpson, 1979; Chenchick et al., 1981; Park et al., 1982a,b).

Thus, using the combination of OP-Cu and Phe OP-Cu nucleolytic activities, it is possible to discern polymerase molecules involved in the abortive or efficient transcription process, during the passage from the open to the stable elongation complex. Using the *lacUV5* promoter, we have shown that this passage occurs by a two-step process: first progressive release of upstream restraints, as early as the sixth bond formation, which appears as the rate-limiting step, directly depending on the strength of upstream promoter–polymerase interactions, likely due, at least in part, to DNA–σ interaction, and then the loss of σ only during 10-mer synthesis. Studies are in progress, by using mutants, to specify the determinants of this regulation. Abortive initiation is very likely an important regulation step of the initiation process for both prokaryotic and eukaryotic RNA polymerase (Luse & Jacob, 1987), and the OP-Cu hypersensitivity signal, which has been recently shown to be associated with the active complex DNA–RNA polymerase II conformation (Buratowski et al., 1991), will be very useful tool in these studies.

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