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Polymerase Arrest at the λP_R Promoter during Transcription Initiation*

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During transcription initiation by *Escherichia coli* RNA polymerase, a fraction of the homogeneous enzyme population has been kinetically shown to form two types of nonproductive complexes at some promoters: moribund complexes, which produce only abortive transcripts, and fully inactive ternary complexes (Kubori, T., and Shimamoto, N. (1996) *J. Mol. Biol.* 256, 449–457). Here we report biochemical isolation of the complexes arrested at the λP_R promoter and an analysis of their structure by DNA and protein footprintings. We found that the isolated promoter-arrested complexes retain a stoichiometric amount of σ^{70} subunit. Exonuclease III footprints of the arrested complexes are backtracked compared with that of the binary complex, and $KMnO_4$ footprinting reveals a decrease in the melting of DNA in the promoter region. Protein footprints of the retained σ^{70} have shown a more exposed conformation in region 3, compared with binary complexes. This feature is similar to that of the complexes arrested in inactive state during transcription elongation, indicating the existence of a common inactivating mechanism during transcription initiation and elongation. The possible involvement of the promoter arrest in transcriptional regulation is discussed.

Transcription initiation has been conventionally supposed to consist of a sequential multistep reaction, starting with formation of a binary preinitiation complex and ending after clearance of the RNA polymerase from the promoter (1). A strict interpretation of this model predicts that 1 mol of polymerase-promoter complex synthesizes a stoichiometric amount of full-length transcript in a single-round transcription reaction. Results from the studies of the λP_R and *lacUV5* promoters, however, contradict this prediction; the amount of full-length transcripts synthesized was significantly less than stoichiometric (2). Moreover, kinetic analyses have suggested the existence of inactivation during transcription initiation and a relationship between the inactivation and abortive synthesis (2–5), an iterative synthesis and release of short transcripts that has been generally observed from most promoters (6–11).

A plausible model of initiation at such promoters proposes two mutually exclusive pathways: a productive pathway leading to the synthesis of full-length transcripts and a dead-end pathway in which enzyme is likely to be arrested at these promoters (2–5). During initiation from a modified λP_R promoter, λP_RAL, a homogeneous preparation of holoenzyme generates different complexes in these different pathways (3). Two types of transcription complexes can be kinetically distinguished in the dead-end pathway (2–4). The moribund complexes keep producing short transcripts for more than 20 min, even after the completion of synthesis of all full-length transcripts. They are therefore the major source of abortive transcripts at these promoters (3–5), although it is still unclear whether small amounts of abortive transcripts are synthesized in the productive pathway or not. Moribund complexes cannot escape from the abortive cycle to make full-length transcripts; rather, they slowly convert into the second type of arrested complexes. The second type still retains a transcript of abortive size but has no detectable elongating activity, thus constituting the "dead-end" of the pathway. This complex is thus tentatively called the dead-end complex here.[1]

It is important to know what structural differences exist between the complexes in these two different pathways. We here report physical separation of initiation complexes arrested at the promoter and analysis of their structures by DNA and protein footprinting. The complexes arrested at the promoter share a common structural feature with arrested complexes formed during transcription elongation, irrespective of the presence of σ^{70}, suggesting the existence of a common basic mechanism of inactivation during different stages of transcription.

EXPERIMENTAL PROCEDURES

Immobilized templates were prepared essentially as described earlier (12) and used as before (2–5), except that 0.025% Tween 20 was included in the transcription buffer to reduce nonspecific adsorption of proteins to the beads. In exonuclease III footprinting experiments, 35 nM holoenzyme was preincubated at 37 °C with 12 nM ^{32}P-labeled λP_RAL73 DNA for 10 min in T buffer (3), and then 40 μg/ml heparin was added to trap free enzyme originated from nonspecific complex. Substrates (GTP, CTP, and UTP) were added 15 s later, if necessary, and 40 units of exonuclease III (Toyobo, Tokyo) were added at different time points. After 4 min of digestion, the reaction was stopped by phenol/chloroform/isoamyl alcohol. DNA was precipitated with ethanol and loaded onto an 8% sequencing gel. The reactions with $KMnO_4$ were carried out for 1 min. All of the other reagents and methods, including $KMnO_4$ footprinting (5) and protein footprinting of σ^{70} (12), have been described elsewhere (2, 3).

RESULTS

RNA Polymerase Arrested at the λP_RAL Promoter Produces Only Abortive Products and Retains σ^{70}—In order to isolate

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[1] These complexes retaining various lengths of short transcripts have been denoted as inactivated complexes in our previous publications (2–5). We rename them dead-end complexes because they make the arrested pathway dead-end. However, "dead-end complexes" have first been documented in Ref. 16 as inactive elongation complexes formed from different promoters. The relationship between these inactive initiation and elongation complexes is not known.

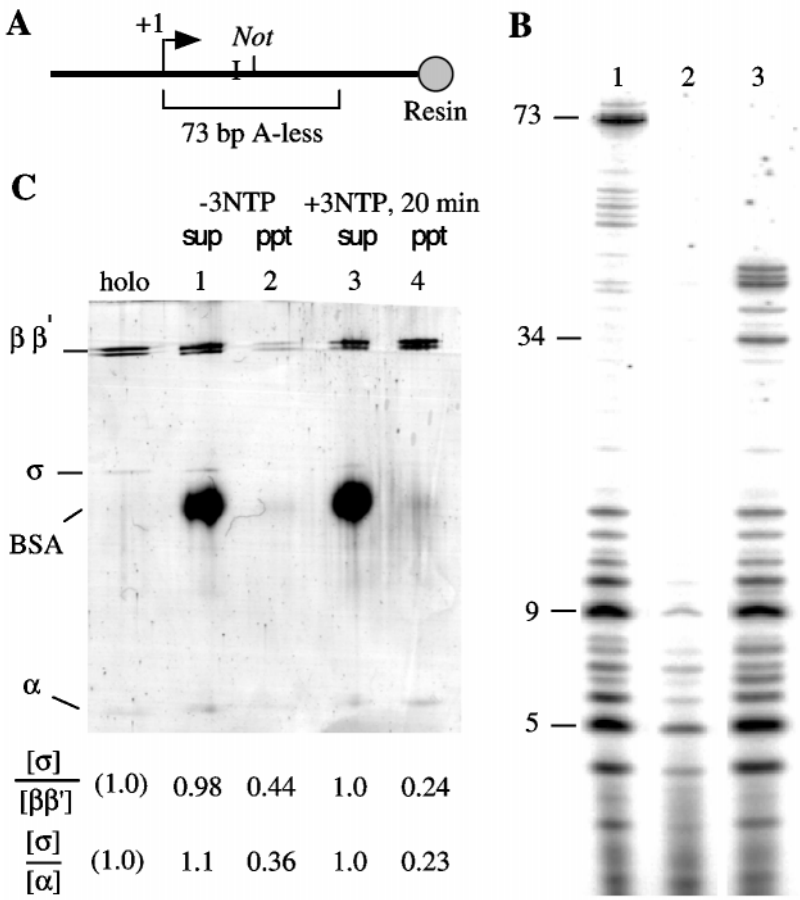

FIG. 1. **Isolation of complexes arrested at the λP_RAL promoter.** *A*, schematic representation of λP_RAL73 template immobilized at the downstream end. The transcription start site is shown by an *arrow*. The *Not*I site is approximately at the center of the template. *Not*I digestion releases the promoter-containing fragment, while the promoter-distal fragment remains bound to the resin. *B*, transcription of immobilized λP_RAL73 template. Binary complex was formed by adding 60 nM holoenzyme to 20 nM template in 20 μl, and excess enzyme was removed by washing and a brief centrifugation. Transcription was started by adding substrate solution to give 5 μM [$^{32}\gamma$-P]GTP, 0.1 mM CTP, and 0.1 mM UTP. Templates used were uncut DNA (*lanes 1* and *2*) or DNA digested with *Not*I (*lane 3*). After 20 min of transcription with unlabeled substrates, DNA was digested with *Not*I, the promoter-proximal fragment was isolated, and the labeled substrate was added (*lane 2*). *C*, composition of proteins bound to *Not*I fragments. Washed binary complexes were incubated for 20 min without substrates (*lanes 1* and *2*) or with substrates (*lanes 3* and *4*) and washed once. The mixture was then digested with *Not*I for 3 min, and DNA fragments were separated by a brief centrifugation. Proteins in the supernatant (*sup*) and precipitate (*ppt*) fractions were analyzed by 7.5% SDS-polyacrylamide gel electrophoresis. The gel was stained with silver nitrate, and proteins were quantitated using calibration curves obtained from bands of various amounts of holoenzyme in the same gel. Among these standards, only one lane is shown (*holo*). Bovine serum albumin (*BSA*) was present in the solution of *Not*I. The molar amount of α-subunit is expressed as α dimer in the molar ratio of σ^{70}. Six independent measurements showed 0.97 ± 0.09 and 0.98 ± 0.05 mol of σ^{70} subunit per core enzyme exists in the binary complex (*lane 1*) and the complex arrested (*lane 3*), respectively.

complexes arrested at the promoter, we used a template DNA, λP_RAL73, which has a λP_R promoter and a 73-base pair A-less initially transcribed sequence incorporating a *Not*I site, as indicated (Fig. 1*A*). The template was immobilized at its downstream end, so that the promoter-containing fragment could be separated from the promoter-distal fragment by *Not*I digestion and a brief centrifugation (12). The template was preincubated with a saturating concentration of holoenzyme for 10 min at 37 °C, and the excess enzyme was removed by washing (3). Transcription was then initiated with substrates containing no ATP.

Transcription from this template DNA produced abortive transcripts up to 13 nucleotides in length and a 73-mer stalled product (Fig. 1*B*, *lane 1*), while the template digested with *Not*I produced run-off transcripts around 34-mer (*lane 3*). Under these conditions, the synthesis of the full-length transcripts is completed within 5 min (2–5). In the experiment shown in *lane 2*, transcription was first carried out with unlabeled substrates for 20 min, and template was then digested *in situ* with *Not*I. Finally, the promoter-containing fragment was separated from the immobilized fragment by centrifugation and collected. After adding substrates containing the labeled initiating nucleotide, [γ-^{32}P]GTP, this material was found to produce exclusively abortive transcripts, proving that the collected promoter DNA fragment contains no productive complexes. Therefore, moribund complexes, which have previously been defined kinetically (3), were recovered on the promoter-containing fragment. Taking into account the efficiency of recovery of the complexes during isolation, the abortive synthesis was reduced by 75–85%. This is consistent with the previous observation of a slow conversion of moribund complexes into dead-end complexes (3).

Next we analyzed the subunit content of the enzymes bound to the promoter-proximal and promoter-distal DNA fragments (Fig. 1*C*). In the absence of transcription, the enzyme bound to the promoter contained a stoichiometric amount of σ^{70} (*lane 1*). The proteins seen in *lane 2* were nonspecifically adsorbed to the resin, as judged from the experiment by using DNA-free resin (not shown). After 20 min of transcription, the supernatant (promoter-proximal) fraction contained enzyme with a stoichiometric amount of σ^{70} (*lane 3*), proving that the complexes arrested at the promoter still retained σ^{70}. The σ^{70} content of

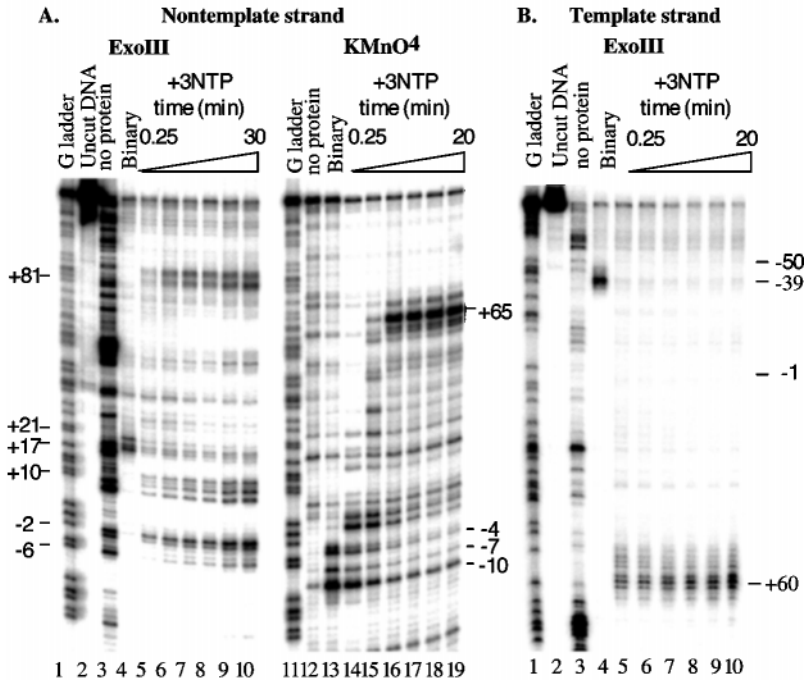

FIG. 2. **Exonuclease III and $KMnO_4$ footprinting of transcription complexes.** After the addition of 5 μM GTP, 0.1 mM CTP, and 0.1 mM UTP (*+3NTP*), footprinting reagents were added at different time points: 0.25, 1, 5, 10, 15, and 30 min (*ExoIII* in *A*) or 0.25, 1, 5, 10, 15, and 20 min ($KMnO_4$ in *A* and *B*).

the enzyme stalled at +73 was very small (*lane 4*), consistent with the expected loss of σ^{70} from the elongation complexes. According to kinetic analysis (3), these arrested complexes are a mixture of moribund complexes still engaged in abortive cycles and dead-end complexes that retain short transcripts but lack elongation activities.

DNA Footprints of the Complexes Arrested at the λP_R Promoter—Next we investigated the structure of moribund and the dead-end complexes on the λP_RAL73 DNA using exonuclease III and $KMnO_4$ footprintings. In the absence of transcription, exonuclease III digestion formed footprints of the binary complex near +18 on the nontemplate strand (Fig. 2*A*, *lane 4*) and close to −39 on the template strand (Fig. 2*B*, *lane 4*). Transcription was initiated by adding substrates, excluding ATP, to the binary complex. Footprinting agents were then added at different time points. In the presence of RNA synthesis, the exonuclease III footprints moved to approximately +80 on the nontemplate strand and near +60 on the template. These boundaries represent the elongation complex, stalled at +73 due to the exclusion of ATP.

In the promoter region, another complex was detected. It was noted that exonuclease III cleavage boundaries between −6 and +10 on the nontemplate strand appeared at later time points (Fig. 2*A*, *lanes 5–10*). These footprints are backtracked by 8–24 base pairs compared with the binary complex (*lane 4*). The scattered downstream edges of these footprints indicate the existence of a heterogeneous population of moribund and dead-end complexes, which might reflect the heterogeneity in the transcripts retained by these complexes. Alternatively, the observed backtracked footprints may not indicate the real positions of the inactivated complexes. Instead, the footprints may present a weak physical block against exonuclease III, which may gradually push them upstream.

The promoter arrest is composed of a slow and rapid phases. The amounts of abortive transcripts increase in time but gradually level off in this condition. The exponential time course has a rate constant of 0.11 min^{-1} (Fig. 3*A*), and this has been assigned to the rate constant of the formation of the dead-end complex (3). The disappearance of a species incorporating the initiating nucleotide, presumably open binary complex, was measured by a pulse-labeling technique (3–5) and has a rate constant of 0.30 min^{-1} (Fig. 3*A*). The time course of appearance of the backtracked footprints contains a slow rising phase and sometimes a rapid rising phase (Fig. 3*A*), and the rate constants of these phases agree with those found in the kinetics of the promoter arrest. Therefore, these backtracked footprints are likely to belong to the two types of complexes arrested at the promoter, moribund complexes and the dead-end complexes. The smaller contribution of the rapid component suggests that moribund complexes are less efficiently footprinted, presumably due to their dissociation during the exonuclease digestion.

Surprisingly, no footprints of these complexes were observed on the template strand in the relevant positions (between −50 and −39 in *lanes 5–10* of Fig. 2*B*). This suggests either that this strand is more exposed in these complexes or that the complexes dissociated when attacked from upstream by the nuclease. In any case, the interaction with the template strand in two arrested complexes is significantly different from that in the binary complex.

Footprinting with $KMnO_4$ detects the DNA melting in transcription complexes. Here, the thymine residues at −10, −7, −4, and −3 normally showed permanganate sensitivity in open binary complex (Fig. 2*A*, *lane 13*). The sensitivities of these thymine residues gradually reduced with time after the addition of substrates, while a new "bubble" appeared around +65 (*lanes 14–19*).

In contrast to exonuclease III footprinting, $KMnO_4$ footprinting traced the movement of productive complexes better as distinct bubbles: −10 to +4 at 15 s (*lane 14*), very scattered between +2 and +65 at 1 min (*lane 15*), and mostly around +65 after 5 min (*lanes 16–19*). The time courses of bubble collapse in $KMnO_4$ footprinting at −10 to −3 are similar to the disappearance of open complex, the fast reaction with the rate constant of 0.30 min^{-1}. The level of footprinting at −10 to −3 was still significantly above the background at 10 min (compare *lanes 12* and *16*). Therefore, moribund complex is likely to be partially open, and dead-end complex is less open.

Protein Footprinting of Retained σ^{70} in the Complexes Arrested at the λP_R Promoter—The change in protein conformations upon the promoter arrest was investigated by protein footprinting of σ^{70} whose mutations in the conserved region 3.1

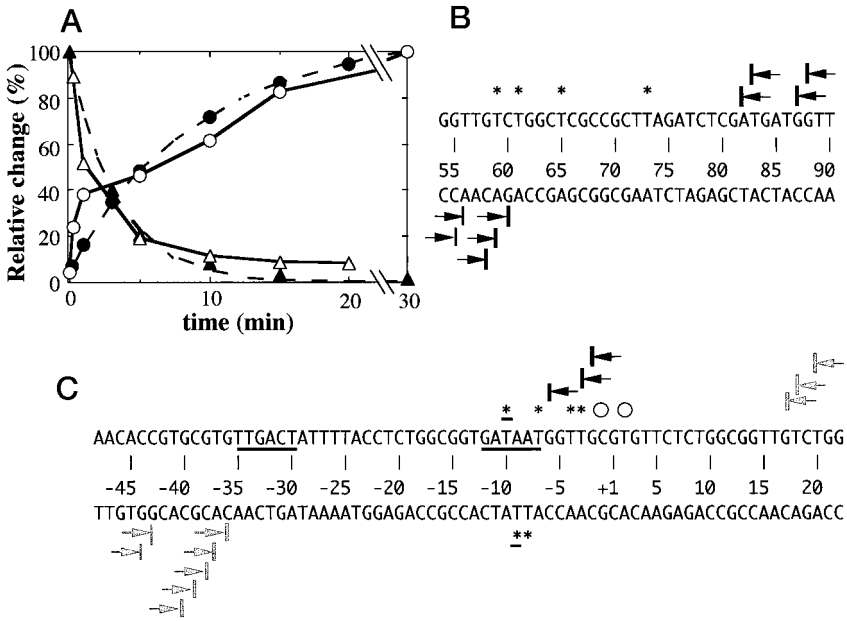

FIG. 3. **Summary of DNA footprinting.** *A*, appearance of exonuclease III footprints at −6 to +12 (*open circles*) and decay of $KMnO_4$ footprints at −10 (*open triangles*). The decay of $KMnO_4$ footprints gave a rate constant of 0.40 min^{-1}. At the indicated time after initiation with unlabeled nucleotides, transcripts were pulse-labeled with [γ-^{32}P]GTP for 5 min (5), and the decay of the labeled 9-mer band is plotted (*closed triangles*). The decay of 9-mer pulse-labeled band denotes binary complex (more exactly, moribund binary complex), and the best fit curve gave a rate constant of 0.30 min^{-1} (the *broken decay curve*). These decays are presented in a scale of 100% at $t = 0$. The time course of 9-mer band synthesized (*closed circles*) was previously obtained (3), and the best fit single exponential curve of the amount of 9-mer (the *broken saturating curve*) gave a rate constant of 0.11 min^{-1}. This is the conversion rate of moribund complex into the dead-end complex under this condition. The time course of the exonuclease III footprints at −6 to +12 is presented in a scale that gives 100% at 30 min. The time course can be well approximated with the same single-exponential except the early small burst, which is approximated with the rapid decay of 0.3–0.4 min^{-1}. *B*, summary of footprints of elongation complex stalled at +73 by eliminating ATP. The *asterisks* denote $KMnO_4$ footprints, and strong bands are *underlined*. The *bars* with *arrows* denote exonuclease III footprints. *C*, summary of footprints of complex formed near the promoter. *Symbols* are as described for *B*. The *asterisks* represent $KMnO_4$ footprints that decay after the addition of substrates. The *circles* indicate $KMnO_4$ footprints that transiently appear after the addition of substrate and then disappear. The *gray bars* with *arrows* represent exonuclease III footprints of binary complex that rapidly decays.

have been shown to affect the arrest (5). The complexes arrested at the promoter were prepared using the same immobilized template, and hydroxyl radical cleavage of σ^{70} was carried out under a single-cut condition as described previously (12). When compared with the binary complexes, no changes were detected in the N-terminal half of σ^{70}. The most prominent change was observed in region 3 (the domain *a* in Fig. 4). Region 3 is exposed in free form but becomes protected in holoenzyme and binary complex (Ref. 12; Fig. 4). The promoter-arrested complexes showed a protection intermediate between those of free σ^{70} and binary complexes. In addition, the −35 interacting domain (domain *b*) was reproducibly shown to be less protected in these promoter-arrested complexes than in binary complexes. One of the possible interpretations is that these complexes have weaker interaction with −35 region of the promoter than the binary complexes, although other interpretations are equally possible.

In general terms, the promoter-arrested complexes are a mixture of moribund and the dead-end complexes. We have more likely footprinted the dead-end rather than moribund complexes, because more than half of moribund complexes are converted into the dead-end complexes during our preparation longer than 10 min. Since moribund complexes might be dissociated during the preparation process, there could be a contribution of signals from the resultant free holoenzyme. Nevertheless, this does not vitiate our overall conclusion, because an altered conformation of the arrested complexes was detected as increased sensitivity in regions where free holoenzyme and binary complexes show a similar or higher degree of protection.

The role of region 3 is not fully understood, but it is located within a short distance from the initiating nucleotide and RNA 3′-end (13), suggesting its involvement in the initiation process. A mutation in this region decreases the level of accumulation of moribund complexes (5). Presumably, the increased sensitivity of this region to hydroxide radical attack in the arrested complexes arises from the displacement of this region from the active center, which in turn could be a reason for the inactivation.

DISCUSSION

Here we have presented biochemical evidence for the existence of a branched pathway involving inactivation during transcription initiation. The results obtained agree well with those of previous kinetic studies. Previously, the existence of moribund complexes has been demonstrated kinetically by a persistent production of abortive transcripts after completion of full-length synthesis (3). This persistence also indicates the existence of binary complex that is destined to abort its incoming transcript, because abortive synthesis turns over in multiple rounds without dissociation of holoenzyme from a promoter (3, 11). A possible interpretation is that both binary and ternary complexes in the moribund state share a common feature in their structures that is maintained throughout the abortive cycle.

In this study, the complexes arrested at the promoter were isolated and were shown to produce only abortive transcripts. By using immobilized template in a batch process (3) or in a minute column (4), it has been shown that the dead-end complexes (inactivated ternary complexes) retaining short transcripts are formed slowly at a rate of 0.1 min^{-1} (Fig. 3*A*). Although the structural difference between moribund and dead-end complexes is less clear because of their transient nature, we have shown here distinctive characteristics of these complexes arrested at the promoter: the exonuclease III foot-

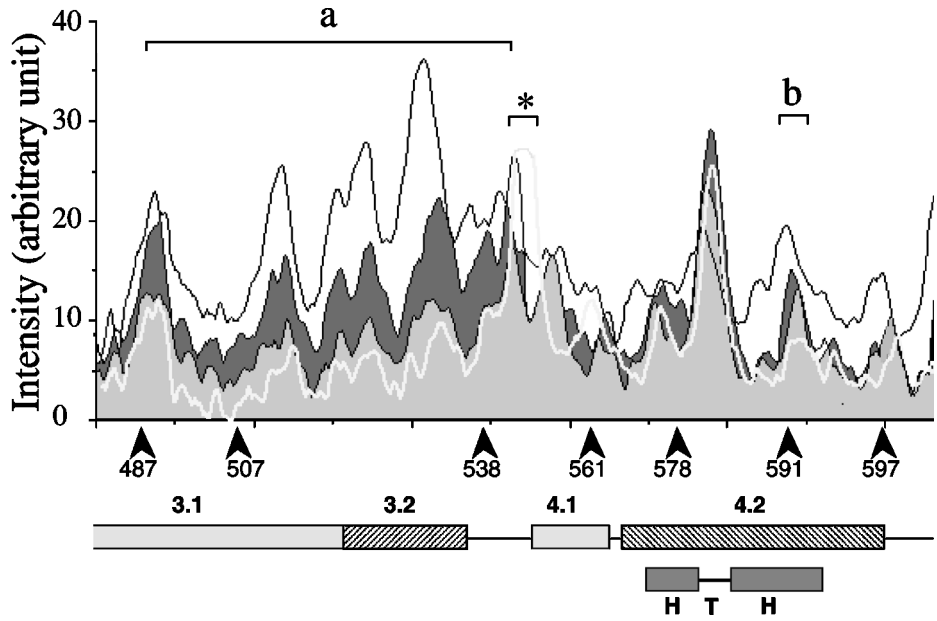

FIG. 4. **Intensity profiles of the protein footprints of C-terminal domain of σ^{70} in free form (*black line*), in holoenzyme (*light gray shading*), in promoter-arrested complexes (*dark gray shading*), and in binary complexes (*white line*).** The positions of conserved regions and a predicted helix-turn-helix in region 4.2 are shown *below* the profiles. The *arrowheads* denote the residue numbers that were determined from the mobility standards obtained from σ^{70}. The footprinting exploits a tag peptide for labeling with ^{32}P, which had been fused to the C terminus of σ^{70} (14). The major conformational changes observed in the promoter-arrested complexes are indicated by *a* and *b*. An apparent hypersensitivity of the segment marked with the *asterisks* was due to a stain on the gel and not a true signal.

print is backtracked, the −10 region is less open, the interaction with the template strand of DNA is weak or absent, and the conformation of region 3 of σ^{70} in these complexes is more like that of the free σ^{70}. The overall results are consistent with the view that the loss of elongation activity of these promoter-arrested complexes is due to inappropriate alignment of the catalytic site, RNA, and DNA.

During elongation, the loss of activity is known as elongation arrest (14, 15) and is caused by backtracking of the enzyme and extrusion of the 3′-end of the RNA from the active site (16, 17). This movement is concerted with a loss of 8–9-nucleotide base pairing between the 3′-end of a transcript and the template strand (18), and GreB relieves the arrest by restoring the 3′-end of transcript at the catalytic center (17–20). The backtracking of dead-end complexes during initiation, discovered here, may share these characteristics of a complex arrested during elongation. We speculate that the inactivation during initiation could similarly result from an intrinsically weak hybridization of short RNA transcripts to the DNA. Furthermore, Gre factors reduce abortive synthesis at promoters including λP_R (21) and enhance productive transcription from weak promoters (22, 23). Indeed, Gre factors also inhibit the promoter arrest at the λP_RAL promoter,[2] suggesting that Gre factors may also rearrange transcripts at the catalytic center during initiation. A more striking similarity is found in the generation of branched pathways due to misincorporation. In elongation, misincorporation leads to a branched pathway, forming a kinetic trap that can be relieved by GreA (20). The biological significance of this branching pathway during elongation is believed to be its ability to maintain high fidelity in transcription by preventing further elongation of misincorporated transcripts (18, 20). In initiation, moribund complexes also commit misincorporation due to slippage at the λP_RAL promoter (2), which is also inhibited by the Gre factors.[2] Furthermore, the factors introduce reversibility between otherwise irreversible branched pathways, and a similar introduction of reversibility has been found as an effect of a mutation in region 3 of σ^{70} (5).

The fact that moribund complex can elongate short transcript means that the spatial arrangement among the catalytic center, template DNA, and 3′-OH of nascent transcript is similar to the one in productive complex, at least during a limited time period. The changes as fast as several minutes in footprints near the promoter (Fig. 2*A*), however, indicate that interaction between DNA and holoenzyme is altered in the moribund complex. Since a back and forth movement of RNA polymerase occurs at an arrested site in elongation (20), an interesting model for moribund complexes would be a group of complexes alternating between active and inactive configurations, in which a correct arrangement for elongation is occasionally attained. This hypothesis could explain the slow elongation rate, weaker affinity for substrate nucleotides, and frequent release of transcripts, which are all characteristics of moribund complexes. An alternative model for moribund complex is that a large bend of DNA brings both the +1-position and upstream DNA into the same complex. In the both models, dead-end complex may be a stably backtracked complex with an improper positioning of the 3′-OH of its transcript, which is harmonized with the observed high stability of the complex (3).

The biological significance of the branching in initiation is not yet clear, but a contribution to regulation has been suggested. *In vivo*, blocking of many promoters by dead-end complexes would be lethal, and probably transcription factors including the Gre proteins prevent their accumulation. In fact, transcription of more than 200 genes is reduced in a double-disrupted strain of *greA* and *greB*, and the reduction seems to occur in initiation in the majority of cases, raising the possibility that the present mechanism is working at some endogenous genes.[3] The mechanism preventing initiation complexes from being arrested at the λP_RAL promoter is common for a mutant σ^{70} (5) and Gre factors,[2] and for CRP at the *malT* promoter (24, 25), increasing the rate of interconversion of binary complexes

[2] R. Sen, H. Nagai, and N. Shimamoto, unpublished results.

[3] T. Kubori, M. Susa, and N. Shimamoto, unpublished results.

between the productive and nonproductive pathways. This generality suggests that the existence of these two pathways is biologically significant as a component of the overall regulatory mechanism of transcription initiation. This hypothesis has to be tested for other regulatory systems in future.

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