RNA Polymerase Switches between Inactivated and Activated States By Translocating Back and Forth along the DNA and the RNA* 

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Important regulatory events in both prokaryotic and eukaryotic transcription are currently explained in terms of an inchworming model of elongation. In this model, RNA extension is carried out by a mobile catalytic center that, at certain DNA sites, advances within stationary RNA polymerase. This idea emerged from the observation that footprints of individual elongation complexes, halted in vitro at consecutive DNA positions, can remain fixed on the template for several contiguous nucleotide additions. Here, we examine in detail the structural transitions that occur immediately after the enzyme stops at sites where discontinuous advancement of RNA polymerase is observed. We demonstrate that halting at such special sites does not “freeze” RNA polymerase at one location but induces it to leave its initial position and to slide backward along the DNA and the RNA without degrading the transcript. The resulting loss of contact between the RNA 3′-hydroxyl and the enzyme’s catalytic center leads to temporary loss of the catalytic activity. This process is equilibrated with enzyme return to the original location, so that RNA polymerase is envisaged as an oscillating object switching between catalytically active and inactive states. The retarded isofrom constitutes a principal intermediate in factor-induced endonucleolytic RNA cleavage. These oscillations of RNA polymerase can explain its apparent discontinuous advancement, which had been interpreted as indicating flexibility within the enzyme.

Ternary elongation complexes consisting of RNA polymerase (RNAP), DNA, and nascent RNA are the essential structures for transcription regulation in both pro- and eukaryotes. Distinct properties of these complexes determine the response of RNAP to pausing and termination signals and to the action of regulatory factors (1–3). The traditional view of the process of transcription has been based on thermodynamic analysis and structural studies of diverse populations of elongation complexes. The entire RNAP molecule was considered to translocate along the template monotonously as each new nucleotide was added to the RNA (4). However, DNA footprinting studies of ternary complexes stopped at one point along the sequence revealed that at certain short sequence intervals, RNAP appeared to remain fixed on the template while the RNA chain increased a few nucleotides in length, leading to a relative contraction of the distance in nucleotides between the 3′ RNA terminus and the front end footprint of RNAP on the DNA (5–11). These special sites where contraction occurs are associated with an increased sensitivity of the RNA near the 3′ end to endonucleolytic cleavage by factor GreB (SII in eukaryotes) and with a predisposition for collapse of the ternary complex into a nonproductive or “arrested” state from which polymerase neither transcribes RNA nor dissociates (7–9). Re-establishment of the catalytic competence of the arrested complexes requires cleavage of the RNA by Gre factors (5, 12, 13). This unusual capability of RNAP to synthesize RNA within the stationary enzyme was recently shown to participate in transcription pausing and termination (9, 10).

To account for the perplexing way elongation and discontinuous movement of RNAP are coupled, an “inchworming” model based on the idea of structural transitions in enzyme consisting of flexibly connected parts was developed (3, 6–9, 14). In this model, at specific DNA sites RNA chain elongation, carried out by a mobile catalytic center advancing within the fixed enzyme, alternates with threading of the transcript through the RNAP in concert with the forward translocation of the enzyme on the DNA. If the translocation goes awry, the catalytic center is supposed to become disengaged from the RNA 3′-OH group and to slide backward within the fixed enzyme, thus causing arrest of elongation. Cleavage of the transcript is thought to return the complex to the active state by producing an appropriate 3′-OH group near the new position of the catalytic center. Thus, the inchworming model was the first insight into detailed structure of elongation complexes, which was shown to depend on a particular sequence which RNAP transcribes.

However, the inchworming model is based solely on data obtained when RNAP is stalled, raising the possibility that such ternary complexes are not necessarily related to the structure of true intermediates in RNA chain extension (6, 7, 15). Indeed, some approaches do reveal differences between stopped and moving RNAP. Transiently halted RNAP is distinguished by bacteriophage T4 factor Alc, since the factor loses its capacity to terminate host transcription in the presence of low concentration of NTPs (15). In addition, kinetic studies of nucleotide misincorporation by Escherichia coli RNAP suggest that a reduced rate of transcription elongation causes a switching between active and inactive conformations at each DNA position (16). Similar conformational changes were proposed for eukaryotic elongation; mathematical modeling of RNA chain growth desynchronization implied that yeast RNAP III
may flip between a “fast-step” state and a “slow-step” state at each nucleotide of the template (17). The most apparent consequence of halting of transcription is the aforementioned arrest of RNAP in sites where unimpeaded elongation would proceed normally (5, 8, 9, 18). Using a combination of DNA and RNA footprinting technique, we recently demonstrated that the loss of catalytic activity during arrest is accompanied by an isomerization of the ternary complex; the RNAP disengages from the 3’ end of the transcript and moves backward along the DNA, while the intact RNA threads through the enzyme leaving its 3’ end free (32). Taken together, these observations suggest that RNAP may adopt multiple conformations, only one of which needs to be capable of forming a phosphodiester bond.

The goal of the present study was to examine in detail the structural transitions that occur immediately after the enzyme stops at sites where discontinuous advancement of RNAP is observed. A combination of approaches, including DNA and RNA footprinting with fast-acting chemical and enzymatic agents and time-resolved correlation of catalytic function and structural parameters, were used. It was revealed that at these sites RNAP repeatedly switches between inactive and active states as it moves back and forth using the DNA and RNA as tracks. The observations give rise to a new interpretation of the discontinuous phase of elongation.

EXPERIMENTAL PROCEDURES

Transcription Template and Transcription Reactions—The standard template for transcription was the purified on PAGE polymerase chain reaction-amplified 386-base pair DNA fragment carrying the T7A1 promoter. Its transcribed sequence (non-template strand) is A1TCGA-GAGGGTAGCCATACC50AATCGACCGG-39CCGGGTGAAT 100TCACT. The permanganate-reactive bubble in EC50, EC56, or EC75 are underlined (see Fig. 1 for details).2 E. coli RNAP bearing a hexahistidine tag genetically fused to the carboxyl terminus of the RNAP β’ subunit was reconstituted from the isolated subunits and immobilized on Ni-NTA-agarose (QIAGEN) as described above in the presence or absence of 200 μM specific oligonucleotide at 16 °C for 10 min. The nonhybridized oligonucleotides were then removed by washing five times with 1 ml of ice-cold TB. 10 μl of each sample were then treated with 1 unit of GreB, or NTPs as described above or specified in figure legends. To melt the oligonucleotides off the RNA, the samples were incubated 5 min at 37 °C, washed once with warm (37 °C) and four times with cold TB and then the assays were performed as described above.

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RESULTS

Halting of Elongation at a Site of Discontinuous Movement Causes Fast Isomerization of RNAP into a Temporarily Inactive Form—In this work a solid-phase transcription system exploiting hexahistidine-tagged RNAP from E. coli immobilized on Ni-NTA-agarose beads was employed. The method allows one to obtain elongation complexes stalled at any site of the DNA by alternating limited RNA extension with washing of the beads (8–10, 19). We focused our study on the complex halted in position 56 of a template containing the T7A1 promoter (EC56). EC56 represents the discontinuous phase of elongation (8). A fraction of EC56 complexes fails to resume transcription after the addition of the missing NTP (in this case ATP), which in our previous study was interpreted as arrested state formation (8). Indeed, the fraction of EC56 not responding to 5 s incubation with 5 μM ATP and UTP retains the RNA after washing of the beads, which rules out the dissociation of the complex (Fig. 1A, lanes 7 and 8; see also Ref. 8). The unimpeaded elongation through position 56 as well as the halting of transcription in the neighboring positions 54, 58, and 60 do not reveal any inactivation of RNAP in the same test (Fig. 1B). Lanes 1–7 of Fig. 1A disclose a distinctive feature of EC56 inactivation; within the first 2 min of the halted state, the complex loses its catalytic competence gradually, but then the inactive fraction maintains a constant value. It is important to notice that the incorporation of ITP instead of GTP in two 3'-terminal positions of the RNA, which weakened the Watson-Crick pairing of the transcript terminus with the template, dramatically increased the rate of catalytic inactivation of EC56 (Fig. 1A, lanes 9 and 10). Unexpectedly, the inactive EC56 turned out to be able to re-enter elongation directly upon incubation with NTPs, bypassing internal cleavage of the transcript. In the experiment shown in Fig. 1C, the fraction of EC56 capable of RNA extension was removed by a 2-min chase with 50 μM each of all four NTPs. After removal of the substrates, the rest of the complex (lane 3) was incubated with 5 μM each of ATP and UTP. As can be seen from lanes 2–4, the remaining EC56 slowly resumes transcription. The rate of NTP addition substantially increased in high salt (lane 5), but it is still far below that in the unimpeaded elongation. However, prolonged incubation with the NTPs ultimately leads to complete reacti-
FIG. 1. Changes of catalytic activity and translocation of transcriptional bubble induced by halting of RNAP in position 56. A, EC56 gradually loses catalytic activity, which depends on the strength of the RNA/DNA pairing near the 3’ end of the transcript. EC54 prepared by walking with RNAP was chased to position 56 with 30 μM GTP (lanes 1–7) or with 300 μM ITP (lanes 9 and 10), for the indicated time periods. The fraction of EC54 unable to continue elongation was then visualized by adding 5 μM ATP and UTP for 5 s. The probe of lane 8 was then washed with cold transcription buffer (indicated by asterisk). All further procedures were performed at 16 °C, unless otherwise indicated. B, the loss of catalytic activity is specific for halted EC56. EC58 (lane 2) and EC54 (lane 3) were obtained from RNA labeled EC54 and EC58, respectively, by incubating with 5 μM subsets of NTPs for 5 s. C, restoration of the catalytic function in EC56. The fraction of RNA labeled EC56 unable to resume elongation after 10 min of halting was purified by chasing with 500 μM each of all four NTPs for 2 min (lane 1). After washing off the NTPs, 5 μM ATP and UTP were added for indicated periods of time in either regular transcription buffer or in transcription buffer containing 300 mM KCl. All procedures were performed at 25 °C. D, potassium permanganate DNA footprinting of EC56 and of the neighboring complexes. Top panel, EC54 obtained on the DNA labeled at 5’ end of the nontemplate strand, was incubated with 100 μM GTP or 300 μM ITP (lanes I) for indicated periods of time before adding potassium permanganate. After 2 s the reaction was stopped with β-mercaptoethanol and the DNA was cleaved with piperidine at 90 °C. EC50, EC54, and EC57 were footprinted after 10 min of halting. In the sequence on the left side of the autoradiogram, the positions of reactive, nonreactive, or partially reactive residues in the nontemplate strand are shown by the filled, empty, or shaded circles, respectively. In EC56 transcriptional bubble moves backward to the position characteristic for EC50 while duration of halting increases.

viation of the complex. The omission of GTP from the added NTPs rules out participation of internal RNA cleavage in the activation, since the 3’-terminal nucleotide in EC56 is guanine. Here and in all further experiments involving halted EC56 and all other ternary complexes, the 3’-terminal labeling of the transcripts was used to exclude the possibility that shortening of the RNA was responsible for all observed effects of halting (data not shown). Below, the inactivated form of EC56 is defined as temporarily arrested on account of the remarkable reversibility of the inactivation, to distinguish it from irreversible arrest described elsewhere (20, 32). Thus, the fact that after 2 min of halting no further net change in the system occurs reflects an equilibrium between the active and inactive forms. After removal of the active fraction by incubating with NTPs, the equilibrium soon restores at the level typical for halted EC56 (compare lane 7 in Fig. 1A with lane 2 in Fig. 1C).

Appearance of Temporarily Arrested RNAP Is Associated with the Backward Translocation of Transcriptional Bubble— Because of the extremely brief appearance of the fully active form of EC56 (Fig. 1A), a fast acting footprinting agent was employed to examine the structural difference between the active and the temporarily arrested isoforms. GTP was added to EC54 to synthesize EC56 which completed in 2 s. After different periods of time following the addition of GTP (ranging from 2 s to 5 min), the complex was exposed for 2 s to potassium permanganate, which allowed visualization of the transcriptional bubble by modifying single-stranded pyrimidine residues of the nontemplate DNA strand. Lanes 3 and 4 of Fig. 1D show that within the first 10 s after the halting, the leading edge of the bubble in EC56 occupies a position 1–3 nucleotides downstream of the 3’ tip of the RNA, corresponding to the normal location for the bubble in the majority of the complexes tested so far (11). However, while the halted state is maintained, the bubble steadily shifts backward to a position similar to that in EC50 (lanes 1 and 5–9). In the control experiment, RNA in EC56 was labeled at the 3’ end by incorporating [α-32P]GTP to positions 55 and 56 of the transcript. This labeled fraction maintained a constant value during 10 min of incubation of the complex at room temperature, which excluded the possibility that backward shift of the bubble was caused by shortening of the RNA in the complex (data not shown). Remarkably, the translocation of the bubble in EC56 coincides in time with the RNAP inactivation both ceasing at about 2 min after the halting. The substitution of two 3’-terminal guanosines with inosines in the RNA increased the rate of bubble isomerization (lane 10) in accord with the much higher rate of inactivation of inosine-substituted EC56 (Fig. 1A, lane 9). The transcriptional bubble in EC56 and EC57, which we took as representative of the monotonous mode of transcription, have the normal locations relative to the 3’ ends of their transcripts (lanes 1 and 11).

Temporarily Arrested and Active Forms of RNAP Have Different Front End Positions—The irreversible arrest of E. coli RNAP is associated with a similar retreat of the transcriptional bubble, which involves backward sliding of the whole enzyme along the DNA (32). We suggested that an analogous retreat of RNAP accompanies the appearance of the temporarily arrested
form. But, in our previous report (8), the mapping of protection of the nontemplate DNA strand using 3’ to 5’ degradation with ExoIII did not discriminate between the active and inactive isoforms of EC56 and detected only a single position of the ExoIII did not discriminate between the active and inactive form. Our own preliminary results also showed that prolonged incubation of some ECs with ExoIII led to the push RNAP upstream may have eliminated the footprint of the nontemplate DNA strand, where the RNAP front end coincides with that in EC 50. Nevertheless, our previous report (8) that the transition is accompanied by the forward translocation of RNAP along the DNA by 6 nucleotides.

To visualize the rearrangement of the DNA/protein contacts associated with RNAP reactivation, we chased EC56 with all four NTPs and then quickly removed them by washes with the ico-cold transcription buffer. Although footprinting performed immediately after NTP removal ceases to detect the regular boundary in EC56, ExoIII added after several minutes of incubation without NTPs reveals that the remaining retreated boundary reproduces the regular one at the preexisting level (lanes 9, 10, and 3). The reorganization of the front end footprints correlates with the restoration of the limited catalytic activity; the fraction of EC56 that is catalytically inactive right after removal of the NTPs regains the elongation competence characteristic for the complex within 5 min (lanes 22 and 23 of the bottom panel). It indicates that EC56 comes out of the arrested state slower than it enters. Thus, the reactivated form of EC56 originates from the retreated inactive intermediate, and this transition is accompanied by the forward translocation of RNAP along the DNA by 6 nucleotides.

Active-to-arrested Transition Involves Backward Sliding of RNAP along the DNA—The reverse process, backward translocation of RNAP during the development of the arrested fraction, cannot be detected with exonuclease III directly after halting of EC56 because the equilibrium between the temporarily arrested and active isoforms is established faster than the relatively slow-acting nuclease reaches the RNAP location by processive digestion of the linear template from the 3’ end. In a separate experiment, we demonstrated that in the applied conditions it took 5–7 min for ExoIII to reach location of EC56 by progressive degradation of nontemplate DNA strand from the 3’ end and this time cannot be decreased by adding more ExoIII to the reaction. It suggests that acquisition of the arrested state is faster than ExoIII can probe it. To visualize the process of reverse translocation, we employed an alternative approach utilizing short deoxyoligonucleotides complementary to the 56-nt transcript immediately behind RNAP (Fig. 3A). The analogous oligonucleotides were previously shown to suppress the irreversible arrest of transcription by blocking enzyme retreat (32). In the experiment of Fig. 3B,
EC56 was synthesized from EC54 in the presence of the oligonucleotides, and the arrested fraction was then evaluated by adding ATP and UTP. As expected, oligonucleotides 12–14, complementary to the RNA at a distance of about 14 nucleotides from the 3' end (which corresponds to the end of the zone of tight contact between the enzyme and the transcript, see below), preserve the catalytic activity of the complex (lanes 4–6), whereas the oligonucleotides complementary to other parts of the RNA have no effect (lanes 3, 7, and 8). Although ExoIII footprinting of EC56 performed at room temperature reveals only the retarded position of the RNAP front edge (compare lane 3 of Fig. 3C and lane 3 of Fig. 2; see also Ref. 8), the arrest-suppressing oligonucleotides completely shift this boundary forward to the regular position characteristic for the active form of the complex (lanes 5, 7, and 8). The control experiments performed with the other oligonucleotides and with EC50 and EC57 establish the specificity of this effect (lanes 1, 2, 4, and 9–12). Oligonucleotide 12 protects the corresponding region of the transcript in EC56 and EC57 from degradation with single-strand specific ribonucleases, indicating that it forms a hybrid with the RNA (data not shown). Oligonucleotides 12–14 even added to already formed EC56 can switch it to the active state and shift the footprint forward on the DNA (data not shown).

Since octanucleotides can be easily melted off the RNA by increasing the temperature, one can launch RNAP inactivation by washing the arrest-suppressing oligonucleotide off the immobilized complex at 37 °C (lane 9 of Fig. 3B). Lanes 5 and 6 of Fig. 3C demonstrate the crucial result of the present experiment; the loss of the catalytic activity upon oligonucleotide departure coincides with the restoration of the retarded position of the enzyme front end. This observation shows that RNAP shifts backward by 6 nucleotides in the course of catalytic inactivation, which reveals the missing element in the cycle of the RNAP active-to-arrested interconversion.

The Rear Edge of Temporarily Arrested RNAP Moves along the DNA in Synchrony with Its Front Edge—We used the arrest-suppressing oligonucleotides to confirm that the position of the rear edge of the temporarily arrested RNAP is also shifted backward compared with its position for the active enzyme. ExoIII footprinting performed in the standard conditions reveals that the rear edge of the protein in EC56 occupies the same position as in EC50, whereas the rear edge in EC57 is located 7 nucleotides downstream (Fig. 3C, lanes 13, 14, and 16). At the same time, EC56, maintained in the active state by oligonucleotide 12, has the rear edge shifted forward at the distance of 6 nucleotides (lane 15). Thus, in halted EC56, the whole RNAP mainframe seems to move back and forth along the DNA.

Reverse Threading of the Transcript in the Temporarily Arrested Ternary Complex—Using ribonuclease RNA footprinting, we previously showed that the irreversible arrest of RNAP involved the reverse threading of the transcript through the enzyme (32). Fig. 4A represents the results of RNA footprinting.
in a series of halted EC52–58, labeled near the 3′ end, with ribonuclease T1 which is specific to single-stranded G residues. In the majority of the complexes tested so far, RNAP protected about 14 nt of the 3′-proximal RNA from degradation with different ribonucleases.4 Arrows indicate the major cuts introduced by RNase T1, after the G residues shown in bold, and the cylinders represent the segments protected by RNAP from the cleavage. B, EC56 labeled both in the 12C and 54U positions was incubated with 30 μM GTP for indicated periods of time before adding 200 ng of GreB for 5 s. The 3′ and the 5′ products of the cleavage are indicated by the arrows on the left side of the autoradiogram. C, EC56 and EC57 labeled in the 12C position were obtained from EC54 either in the presence or in the absence of the oligonucleotides and were treated with 2 (+) or 200 (+ +) ng of GreB for 5 min. In the probes of lanes 11 and 12, oligonucleotide 12 was washed off at 37 °C before adding GreB. The arrows on the left side of the autoradiogram indicate the full-size 56-nt transcript and the 5′ products of the cleavage.

4 M. Kashlev, unpublished data.
RNAP Halted at Other Sites of Discontinuous Elongation Translocates Back and Forth along the DNA—

The data of Fig. 5 argue that the ternary complexes stalled at other known sites of discontinuous movement (6, 8, 9) undergo the same isomerization as EC56 does. EC80, halted at the oligo(T) sequence of a phage λ TR2 terminator derivative from which the hairpin has been deleted, demonstrated the dominance of the retarded position of the enzyme’s front end on the DNA (compare lanes 2–4 and 8–10 of Fig. 5A), high sensitivity to GreB (lanes 11 and 12) and partial loss of catalytic activity upon halting (see Ref. 9). Inactivation of EC80 turned out to be reversible (data not shown), and the complex, obtained in the presence of the oligonucleotide complementary to the RNA behind RNAP, has the downstream boundary shifted forward, decreased sensitivity to GreB (Fig. 5A, lanes 11 and 12 and lanes 12 and 13, respectively), and normal catalytic activity during the whole period of halting (data not shown). All these effects can be completely reversed by the removal of the oligonucleotide from its target in the RNA (lanes 6 and 14). The analogous oligonucleotide acts similarly on RNAP front end footprint in EC27 (Fig. 5B), where the phenomenon of discontinuous elongation was originally described (6, 8). The control oligonucleotides do not affect the parameters of the complexes (lane 7 in Fig. 5A and lane 3 in Fig. 5B).

**DISCUSSION**

In the studies of elongation, pausing, and termination utilizing the method of halted transcription, the retention of catalytic activity and stability were generally accepted criteria for considering halted complexes as true representatives of actively transcribing RNAP (6–11). The results of the present research demonstrate that the apparent ability to catalyze RNA chain extension does not necessarily signify that the halted enzyme remains permanently in the original elongation prone conformation.

Here we have studied the effect of halting using complexes of E. coli RNAP stopped at three DNA regions where discontinuous advancement of the enzyme was previously detected (6, 8, 9). Stopping of the enzyme in position 56 of the template, which was selected for the most comprehensive analysis, does not affect the ternary complex stability but causes its fast arrest-like inactivation. The fraction of inactive EC56 remains constant after 2 min of halting. Since the inactivated fraction turned out to be able to re-enter normal elongation directly, bypassing internal transcript cleavage, we defined it as temporarily arrested. The inactivation of the enzyme proceeds in good synchrony with backward translocation of the transcriptional bubble as revealed by the footprinting with potassium permanganate. ExoIII maps the front end boundary of the active form of EC56 as 6 nucleotides downstream from the front edge of its temporarily arrested counterpart. Remarkably, the footprint of the active form is situated at the same regular distance from the 3′ end of the RNA as in the majority of elongation complexes that advance monotonously along the template. We demonstrate that this footprint shifts backward in the process of EC56 inactivation, and that reactivation is accompanied by its return to the original location. The rear end footprint in the active form of EC56 translocates in concert with its front end. In that view, results of ribonuclease T1 RNA footprinting, showing that the protected segment of the transcript is more extended to the upstream direction in EC56 than in the closely

**FIG. 5.** The effect of oligonucleotides on the position of the front edge of RNAP on the template and on GreB-induced cleavage in EC80 and EC27. The sequences at the top represent the 3′-proximal part of the transcripts in EC27 and EC80 and the oligonucleotides complementary to different parts of these transcripts used in the experiment. A, the format of the experiment was the same as in Figs. 3C (top panel) and 4C (bottom panel), except that the dose of GreB was 60 ng/probe. The RNA in EC80 used for the cleavage with GreB was labeled at position 12. B, the autoradiogram shows the positions of the RNAP front end determined by ExoIII footprinting of EC27 performed as described in the experiment shown in Fig. 3C.
located complexes representing the monotonous mode of elongation, can be also explained by the reverse translocation of RNAP along the RNA chain. The sensitivity of the complex to transcript cleavage induced by factor GreB substantially increases in line with enzyme inactivation. Only the temporarily arrested, retreated form of RNAP is susceptible to the cleavage, while its catalytically competent form is resistant. All other complexes of the discontinuous phase of elongation studied in this work display similar halting-dependent rearrangements, whereas the complexes in the monotonous state do not.

In combination with our data that RNAP disengages from the 3' end of the transcript and moves backward along the DNA and the RNA in the process of irreversible arrest (32), the most reasonable interpretation of the present results is that RNAP, halted at sites of discontinuous elongation, moves back and forth along the DNA and RNA, repeatedly switching between inactivated and activated states. These "oscillations" proceed without breaking down the RNA and do not alter the correct alignment of RNA-DNA base pairing. Thus, such halted complexes are represented by at least two convertible isoforms co-existing in a state of dynamic equilibrium (see Fig. 6A).

The fact that incorporation of IMP instead of GMP into the 3' terminus of the transcript drastically facilitates backward translocation (Fig. 1, A and D) supports the idea that nucleic acid rather than protein-nucleic acid interactions primarily determine the direction of lateral motion. Clearly, the shift of the bubble and the reverse threading of the transcript are associated with spreading apart of the DNA and RNA chains.

**Fig. 6.** A model of EC rearrangements induced by RNAP halting and effect of RNAP oscillations on apparent protection of the DNA from footprinting agents. A, the halting of RNA extension (the boxed diagram) provides a "window of opportunity" in which RNAP can initiate reverse translocation (process 1). It causes the disengagement of the catalytic center from the RNA 3'-end and inactivation of the complex. From the upstream site where further retreat becomes inhibited, RNAP is able to return to its original location (process 2), thus regaining catalytic competence. Processes 1 and 2 maintain the equilibrium between the two isoforms of the EC. The addition of nucleotides draws into elongation the RNAP molecules currently in contact with the 3' end of the transcript (process 4). The oligonucleotides hybridized to the 5' site of the transcript affect the equilibrium by preventing its reverse threading necessary for the formation of the retreated complex. If the return of the retreated enzyme is unfavorable, the complex becomes arrested irreversibly (process 3). Transcript cleavage in the retreated EC clips the extruded RNA and abolishes the RNAP return to the original position (process 5). B, factors explaining the high variability in footprint parameters between different ECs. Downward arrow, in the case of high frequency motion, slow-acting footprinting agents may never be able to damage the DNA in the sites temporarily covered by RNAP, which leads to the apparent expansion of the protected region. Upward arrow, if a footprinting agent acts quickly enough, the equilibrium concentrations of the isoforms would dictate the apparent size and location of the footprint. When these concentrations are close to 50%, the protected zone would appear shorter, producing the effect of footprint contraction. Only when either the active or retreated form of the enzyme substantially dominates, the size of the footprint can correspond to the actual dimension of RNAP.
ahead of the catalytic center and with restoring the DNA duplex at the leading edge of the bubble. In all catalytically active ECs, the 3’ end of the transcript is always positioned right next to the downstream DNA branching point, suggesting that the elementary step in backward sliding may involve a direct competition between DNA/DNA and DNA/RNA pairing at the leading edge of the bubble. Since the relative stability of each DNA-DNA versus DNA/RNA base pair varies significantly, there must be template positions where stronger DNA/DNA hybrid can easily displace the 3’ end of the RNA from the template, thus dragging RNAP backward and causing its inactivation. Although in this work we describe two extreme states of RNAP, the “zipping” model of lateral sliding suggests that the oscillating enzyme has the option to move back or forth at each nucleotide of the DNA and RNA it passes by in the course of isomerization.

It is necessary to discuss our unexpected findings in the relation to the inchworming model of elongation. That novel view was initially proposed based on the pioneering Krummel and Chamberlin (6), who analyzed deoxyribonuclease I footprints in a series of successive complexes of E. coli RNAP. In those complexes, the front end of the enzyme appeared to advance in a leap of several nucleotides, whereas the rear end moved forward steadily with the growth of the RNA (see Ref. 7). Subsequent studies of pro- and eukaryotic RNAPs exploiting ExoIII and hydroxyl radial as footprinting agents demonstrated that the size of the footprints and their positions relative to the 3’ end of RNA substantially varied, depending on the particular complex and on probe applied (10, 11, 22, 23). Extensive studies with ExoIII of a great number of halted complexes detected variations in footprint size only in short template regions, some of which were involved in transcription termination and pausing, whereas in the majority of complexes the RNAP progressed monotonously (8, 9, 24). From all these results emerged the principal idea of the inchworming model that a mobile catalytic center is capable of synthesizing RNA within the stationary enzyme (3, 7). Our new findings introduce an alternative interpretation of the footprinting data without the need to assume unusual flexibility of RNAP, since the footprint of the complexes halted in the sites of discontinuous synthesis is determined by at least two convertible isoforms. In such a dynamic system, the size and location of the DNA segment protected by the protein and the momentary location of the active center relative to the 3’ end of the transcript should depend primarily on such parameters as the amplitude and the frequency of oscillations and the equilibrium concentrations of the isoforms as well as on the nature of footprinting agent (see Fig. 6B). In this view, the characteristic leaplike forward translocation of the footprints in some complexes, earlier interpreted as actual “jumps” (see also Introduction), may signify RNAP passage to a site where it does not oscillate.

The idea of RNAP internal plasticity is supported by cross-linking experiments where linking of the priming substrate to the 5’ “face” of RNAP active center in the promoter complex did not interfere with the synthesis of the RNA up to 9 nucleotides long (25). However, the authors themselves agreed that the extension of the cross-linked nucleotide might proceed through the DNA looping between the 5’ and 3’ faces of a nonflexible catalytic center. The extremely slow rate of RNA synthesis observed in those experiments (it required 1 h of incubation with 1 mM NTPs to synthesize 9 nt of RNA chain), suggests that cross-linking of the primer might induce temporary inactivation of RNAP via shifting backward.

The view of RNAP as a flexible protein appeared independently as the explanation of internal transcript cleavage in ternary complexes induced by the protein factors GreB or SII (8, 14, 26–28). According to the inchworming model, the point of the cleavage demarcates two operationally defined product binding sites, different in their affinity for RNA: a loose site from which the 3’-terminal fragment falls out and a tight site where the 5’ fragment is held. In the series of successive complexes having the invariant position on the DNA, GreB removes the 3’ RNA increments of increasing length without any translocation of the enzyme. Leaping along the DNA, which re-establishes the monotonous mode, coincides with a drop in sensitivity to cleavage, which thereafter results only in removal of mono- and dinucleotides from the 3’ end. This much less efficient RNA cleavage was shown to causes the retreat of the enzyme along the template (8, 23, 29). The inchworming model considers internal cleavage as indication of a two-stroke process of RNA extension in the discontinuous phase of elongation, where filling of the loose site within the stationary RNAP alternates with threading of the recently synthesized RNA increment into the tight site when the enzyme leaps ahead (7, 8, 14). The resistance of monotonous complexes to cleavage was attributed to difficulty in pushing the enzyme backward (8).

Most recent observations imply that the cleavage function belongs to the catalytic center of RNAP (30, 31). This could be incorporated into the inchworming model by assuming that a mobile catalytic center temporarily draws back within the enzyme to execute cleavage. However, at the moment of cleavage in the irreversibly arrested complexes, the active center is located at an internal RNA position due to backward translocation of the whole enzyme along the DNA and RNA (32). Additionally, the data of the present paper show that for the cleavage to occur in the complexes of discontinuous phase, RNAP must be temporarily inactivated through the same kind of retreat. The shifting backward prior to the cleavage explains why transcript truncation does not affect the position of the enzyme halted in the sites of discontinuous movement (see Fig. 6A). Since such a retreated complex has the 3’ end of the transcript extruded out of the RNAP, it is not necessary to propose a special 3’-proximal loose product binding site to explain why the terminal fragment dissociates after cleavage. The above model can be extended to explain cleavage in the monotonous phase, if we assume that these complexes still can temporarily retreat by 1–2 nucleotides from their original positions even though they display neither unusual footprints nor loss of catalytic activity. Cleavage alone makes this otherwise hidden retreat visible by fixing the shifted conformation and prohibiting enzyme from returning to the original position. Thus, GreB sensitivity, the pattern of the cleavage, and its effect on RNAP footprint must be determined by specific parameters of the oscillations in various complexes: the amplitude of the translocations and the ratios of the active and inactive forms. Since the same parameters will determine both the catalytic activity of the complex and the DNA footprinting, the elongation complexes will be misclassified as persisting in monotonous (regular) or discontinuous (strained) phase in all their characteristics.

The reversible switching between the alternative states may have an important implication to a not yet understood phenomenon of transcriptional pausing. It is easy to imagine that during transcription of template positions where the time interval required for RNAP to start moving backward is less than that required for the next NTP addition, the ternary complex can momentarily fall into the temporarily arrested conformation. The retreated RNAP has much less chance to escape quickly, even when supplied with substrates, which may ex-
plain general pausing of transcription by the kind of feedback mechanism. Favoring that idea, strong intrinsic pausing at some DNA positions is completely suppressed by the oligonucleotide complementary to the transcript immediately behind RNAP.3

The dynamic rearrangements discovered in the halted transcription can also be relevant to the proofreading function of RNAP. Data obtained from an in vitro study of misincorporation kinetics of E. coli RNAP suggest that elongation complexes may exist in equilibrium between active and inactive states at each template position (15). The retreat of RNAP can comprise the central component in this mechanism. In accord with this view, replacement of 3'-terminal guanines with inosines, which weakens DNA/RNA pairing at the 3' end of the transcript, may be considered as a prototype for the misincorporation of an NMP into the 3' end of the RNA. As observed with inosines, such a mismatch may trigger fast temporary isomerization, facilitating the displacement of the transcript 3' terminus from the template by invasion of nontemplate DNA strand. Suspension of transcript extension may provide a chance for RNAP to remove the mismatch by pyrophosphorolysis or endonucleolytic cleavage.

Lateral oscillations at the run of thymidines taken from the normal bacterial terminator (Fig. 5A, Tρ terminator of bacteriophage λ) suggest that sliding could be a step in the normal termination process. The backward sliding of RNAP at a full terminator sequence, which always contains a region of dual symmetry immediately preceding the oligo(T) tract, may proceed amiss due to formation of the hairpin-like structure in the RNA near the upstream edge of the transcriptional bubble. Since such structure may compete with DNA/RNA rehybridization necessary for the backward translocation, it may initiate simultaneous closing of the bubble from both sides, leading to collapse of the bubble, and to release of RNAP.

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