Translocation and Transcriptional Arrest during Transcript Elongation by RNA Polymerase II*

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RNA polymerase II may stop transcription, or arrest, while transcribing certain DNA sequences. The molecular basis for arrest is not well understood, but a connection has been suggested between arrest and a transient failure of the polymerase to translocate along the template. We have investigated this question by monitoring the movement of RNA polymerase II along a number of templates, using exonuclease III protection as our assay. We found that normal transcription is accompanied by essentially coordinate movement of the active site and both the leading and trailing edges of the polymerase. However, as polymerase approaches an arrest site, translocation of the body of the polymerase stops while transcription continues, leading to an arrested complex in which the 3' end of the transcript is located much closer than normal to the front edge of the polymerase. Surprisingly, mutated arrest sites that no longer block transcription continue to direct the transient failure of polymerase translocation. As transcription proceeds through these sequences, the initially stationary polymerase moves forward 10–15 bases along the template in response to the addition of only 3 bases to the nascent RNA. Mutagenesis studies indicate that the sequences responsible for the transient block to polymerase movement are located downstream of the T-rich motif required for arrest. Our results indicate that blocking translocation is not sufficient to cause arrest.

It is increasingly evident that the elongation phase of transcription by RNA polymerase II is a potential target for regulation (reviewed in Refs. 1–3). Protein factors that influence transcript elongation have been isolated and DNA sequences through which elongation proceeds inefficiently in vitro have been identified (recently reviewed in Ref. 4). A fraction of the RNA polymerases which attempt to transcribe these special sequences cease RNA synthesis, thereby entering a state called arrest. Arrested elongation complexes have not terminated, but they can only resume chain elongation very slowly, even in the presence of high levels of NTPs (5). Rapid recovery from arrest requires the action of the elongation factor SII, or TFIIS (6–8). To resume elongation, an arrested transcription complex must cleave the nascent RNA upstream of the 3' end, thereby generating a new 3' end from which transcription continues (5, 9).

The transcript cleavage reaction thus allows the transcription complex another opportunity to pass the arrest site. Elongation complexes which have paused in transcription simply because of the depletion of NTPs from the reaction mixture, which we refer to as stalled complexes, will resume transcription in an SII-independent manner as soon as NTPs are supplied (10).

The existence of at least two different functional modes during elongation may reflect structural variations among ternary complexes as transcription proceeds. Chamberlin (11) has suggested a model for translocation of the RNA polymerase along the DNA template in which all elongation complexes are not structurally equivalent. It was proposed that, while the active site moves continuously downstream on the coding strand, the body of the RNA polymerase translocates discontinuously. The overall advance of the polymerase was envisioned to occur through an advance of the trailing edge, with the leading edge held stationary, followed by a downstream jump of the leading edge, in the manner of an inchworm (11). This model also suggested a mechanism for arrest. If translocation of the leading edge of the polymerase were somehow blocked, the body of the RNA polymerase could respond with a relaxation in the upstream direction, thereby carrying the active site away from the 3' end of the nascent RNA. In this case the active site might move upstream along the transcript, consistent with the proposal (12) that the transcript cleavage required for relief of arrest occurs at the active site. Several laboratories (13–17) have investigated the translocation behavior of Escherichia coli RNA polymerase by nuclelease protection approaches. Nucler et al. (14) found that the distance from the 3' end of the transcript to the leading edge of the polymerase (the catalytic site-to-front edge, or CF, distance) was the same for most complexes examined. However, at certain template locations the leading edge failed to continue translocation, resulting in a decreasing CF value as RNA synthesis proceeded. This strained configuration of the polymerase was resolved either by a discontinuous forward movement of the front edge of the enzyme or by a failure to continue transcription (see also Refs. 13 and 16). Complexes which were blocked in elongation were much more sensitive to transcript cleavage by the GreB elongation factor (14–16). Gu et al. (18) measured the location of both the leading and trailing edges of several RNA polymerase II transcription complexes. They found that an arrested complex had a much shorter CF distance than a complex which had recovered from arrest via transcript cleavage, suggesting that distortion of the polymerase from transient failure of the leading edge to translocate is involved in the loss of elongation competence for RNA polymerase II as well.

In the studies reported here, we have examined the dimensions of many RNA polymerase II ternary complexes, both

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1 The abbreviations used are: CF, catalytic site-to-front edge; exoIII, exonuclease III; bp, base pair(s).
arrested and stalled. Consistent with earlier work, we find that all complexes which are fully or partially arrested show a shortened CF distance. However, we have also identified a number of compressed complexes which are completely elongation-competent. Thus, the shortening of the catalytic site-front edge distance appears to be necessary but not sufficient for arrest. We also show that sequences which seem to be crucial in blocking translocation of the leading edge of polymerase are located downstream of the T-rich core of the arrest site.

**EXPERIMENTAL PROCEDURES**

Reagents—Ultrapure (FPLC purified) NTPs, dNTPs, and dideoxy-NTPs were obtained from Pharmacia Biotech Inc. and ³²P-labeled NTPs from DuPont NEN. Bio-Gel A1.5m was acquired from Bio-Rad. Exo-nuclease III, plasmonic ribonuclease inhibitor, Taq polymerase, and restriction enzymes were purchased from Life Technologies, Inc. Recombinant human elongation factor SII (rSII), purified as described previously (19), was a generous gift from R. Landick (University of Wisconsin-Madison).

**Plasmids**—All plasmids used in this study were based on pML20, which contains the adenovirus 2 major late promoter cloned into pUC18 (5). Several important modifications were made to generate DNA templates suitable for subsequent enzymatic manipulations. First, linker L1 (see Table I) was inserted between the pML site (±20 relative to transcription start site) and the BamHI site of pML20. The Smal-Apal portion of this intermediate construct was replaced with the 228-bp Smal-Apal fragment from pGR220 (20) (kindly provided by C. Kane), which contains a 135-bp cassette with no T residues on one strand. The 70-bp EcoRI/AflII fragment in the distal part of the promoter (from -181 to -111) was deleted and replaced with linker L2 which contains an SstI site. A 136-bp fragment, extending from a SstI site within the pGR220 insert to the BamHI site, was substituted with linker L3, which contains StuI and XhoI sites. Finally, the StuI-XhoI fragment of DNA was substituted with linker L4 to generate pML20–23.

Construction of pML20–30 was a two-step process. First, the segment of pML20–23 from the pOl site to the Pol I site in the vector was replaced with a modified DraI-Pol fragment from pADMterm-2 (18) (kindly provided by D. Reines). This 142-bp DNA was generated using the polymerase chain reaction. The upstream primer Pr119 (see Table I) overlapped the DraI site and contained three point mutations to introduce restriction enzyme sites. The primer Pr120 overlapped the Pol I site. The resulting construct was further modified by deleting a 69-bp NsiI-TaqI fragment downstream of the T-rich arrest site within the cloned sequence. The pML20–36 and pML20–38 constructs were assembled by replacing the StuI-HindIII fragment (+157 to +212) DNA of pML20–30 with a modified StuI-HindIII fragment synthesized on the pML20–30 template using primer Pr124 (for pML20–36) or Pr125 (for pML20–38) and the M13/pUC sequencing (-20) 17-mer (Pharmacia) as primers for PCR. The pML20–37 template was assembled by replacing the StuI-HindIII fragment (+147 to +212) fragment of pML20–23 with a DNA fragment synthesized on the pML20–23 template using mismatched primer Pr126 and the M13/pUC 17-mer primer. All of the pML20–XX series templates were sequenced for verification.

**Template Preparation for in Vitro Transcription Reactions**—Plasmid DNA was linearized by digestion with either SfiI for non-template strand labeling or with PolI for labeling of the template strand. Linearized DNA was treated with Photon phosphate with [γ-³²P]ATP to a specific activity of 1.5–3 x 10⁶ cpm/µg using T4 polynucleotide kinase (New England Biolabs). DNA labeled at the SfiI end was subsequently digested with HindIII and EcoRI, which gave three fragments: a uniquely single-end-labeled DNA containing the promoter and the U-free cassette (323 or 325 bp), a small (12 bp) end-labeled EcoRI-SfiI fragment, and the 2.8-kilobase pair unlabeled portion of the pUC18. Similarly, DNA labeled at the 5’ end was digested with HindIII and SfiI. DNA was purified by phenol-chloroform extraction and ethanol precipitation before use as a template for transcription.

**Assembly, Purification, and Analysis of Ternary Transcription Complexes**—Ternary complexes either stalled or arrested at specific positions on the DNA template were generated essentially as described previously with minor modifications (10). Briefly, preinitiation complexes were assembled on end-labeled DNA templates by incubation with HeLa cell nuclear extract; the total DNA concentration in this reaction, which included both the template and the other DNA fragments generated by digestion of the plasmid, was 20–35 µg/ml. Residual NTPs were removed by gel filtration on Bio-Gel A1.5m. Complexes were advanced to +20 (122 complexes) by incubation with 2 mM ApC, 10 µM dATP, 20 µM GTP, 20 µM UTP, and 1 µM [α-³²P]CTP at 30°C for 5 min followed by another 5 min incubation after the addition of CTP to 20 µM. The stalled ternary complexes were further purified by the addition of 1% Sarkosyl and incubation for 5 min at 30°C, followed by another round of Bio-Gel A1.5m gel filtration. To generate ternary elongation complexes stalled at the end of the U-free cassette, Sarkosyl-solubilized U20 complexes were chased by adding 8 mM MgCl₂ and 200 µM ATP, GTP, and CTP for 10 min at 37°C. To create arrested complexes on the pML20–36 template, U20 complexes were chased with all four NTPs at 200 µM. Complexes assembled on the SfiI end-labeled template and paused at the end of the U-free cassette were incubated with restriction enzyme SphI (0.2 unit/µl for 10 min at 37°C). Analogous complexes assembled on templates labeled at the pOl site were treated with both SphI and SmaI (0.2 unit/µl each, 10 min at 37°C). Ribonuclease inhibitor was added to the reaction mixture at this and all subsequent steps to a final concentration 50 units/ml. After the restriction enzyme digestion, complexes were either immediately treated with exonuclease III or gel-filtered a second time and incubated with an appropriate subset of NTPs, as indicated in the text. The exonuclease III concentration and digestion time for each set of experiments is given in the corresponding figure and legend. The reactions were stopped by adding EDTA to 20 mM final concentration. DNA and RNA were purified as described (21). In the indicated cases, half of the sample underwent RNA treatment with RNase A (50 µg/ml, 30 min at 37°C) followed by proteinase K digestion (0.2 mg/ml for 10 min at 37°C), phenol-chloroform extraction and ethanol precipitation. Samples were resolved on denaturing polyacrylamide gels consisting of 6% (19:1) or 10% (19:1) acrylamide: bisacrylamide and visualized either by autoradiography (Kodak X-AR or Kodak Biomax) or with a Phosphorimager (Molecular Dynamics).

**Markers**—The exact length DNA markers were generated by primer extension using the same DNA template employed in the experiment, dNTP mixes with a single dideoxy-NTP, [α-³²P]dCTP, Taq polymerase and synthesized primers. Primers were phosphorylated before addition to the reaction.

**RESULTS**

We used exonuclease III (exoIII) digestion as a probe for structural differences between arrested and stalled RNA polymerase II transcription complexes. Our general strategy was to compare elongation competent and incompetent complexes prepared on templates which were as similar in sequence as possible. The approach is presented schematically in Fig. 1. We constructed a series of templates based on the plasmid pML20 (5), which contains the adenovirus major late promoter. For each template, we added 20 bases of the transcript contain no A residues, and the next 131 or 141 bases of RNA contain no U residues. The template segment encoding the U-free cassette ends at a cleavage site for the restriction enzyme StuI. A variety of sequenced sequences were inserted downstream from the StuI site, allowing RNA polymerase II complexes to be stalled or arrested in this region. We deliberately analyzed complexes which were halted a relatively long distance down-
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Fig. 1. The generation of defined RNA polymerase II elongation complexes for exoIII mapping of the leading (downstream) edge of the transcription complex. The steps needed to move the RNA polymerase forward to the final location for exoIII analysis are shown in the column on the left. Note that most of the template DNAs incubated in HeLa cell nuclear extract are not assembled into a transcription complex. The fate of these DNAs is shown in the column on the right. Note also that the upstream end of the template fragment has a 3’ overhang, so it is a poor substrate for exo-III digestion.

The generation of RNA polymerase II complexes halted at defined sites on the template has been described in detail previously (21) (see also Fig. 1). Briefly, preinitiation complexes were formed by incubation of DNA with HeLa cell nuclear extract and purified by gel filtration. Incubation of these complexes with ApC, GTP, UTP, and [α-32P]CTP resulted in elongation to the end of the A-free cassette. Transcription stalls after incorporation of the U residue at position +20, giving a stable U20 complex. These complexes were purified by the addition of 1% Sarkosyl followed by gel filtration, which removes the detergent, the NTPs and most proteins, including free transcript elongation factors. The Sarkosyl-rinsed U20 complexes were chased with ATP, CTP and GTP to the end of the U-free cassette and gel-filtered again to remove NTPs; the polymerases were then advanced to various stalling sites with subsets of the NTPs. If the template contained an arrest site, the complexes were advanced to three new locations downstream of the StuI site, spanning a total of 9 bases along the template, simply by incubation with subsets of the NTPs. The insert in pML20–23 did not contain any sequences resembling known arrest sites. Complexes were assembled on this template and chased to the end of the U-free cassette, position +151, as described above. The RNAs in these complexes are shown in lane 1 of Fig. 2A. While about half of complexes contained RNA of the predicted size, the other half stalled at position +152. We presume this resulted from trace contamination of chase NTPs with UTP. We have not been able to eliminate this problem, because of the relatively high (200 μM) concentration of ATP, CTP, and GTP needed to obtain efficient elongation to the end of the U-free cassette. After gel filtration, the C151/U152 complexes were advanced with 15 μM UTP (Fig. 2A, lanes 3 and 4), UTp and GTP (lanes 5 and 6), or UTP, GTP, and ATP (lanes 7 and 8); the complexes in lanes 9 and 10 were chased with all four NTPs. To distinguish between the labeled RNA and a background of bands from the labeled DNA, portions of the samples were treated with RNase A (lanes 2, 4, 6, 8, and 10). Note that nearly all of the C151/U152 complexes advanced when NTPs were added. Also, almost all of the U154, G157, and A160 complexes chased when subsequently challenged with all four NTPs (data not shown). Thus, complexes C151, U154, G157, and A160 were genuinely stalled and not arrested. We always observe a small proportion (5–10%) of complexes that fail to restart whenever RNA polymerase II is halted in chain elongation for any length of time. The amount of residual RNA left at positions 151/152 in lanes 3, 5, 7, and 9 is typical for stalled complexes (see also

Stream of +1, instead of using more convenient, promoterproximal stalling sites, because we wanted to guarantee that our complexes had completed any transitions out of the initiating state (14, 22) (but see also Marshall and Price (23)).

The generation of polymerase II complexes halted at defined sites on the template has been described in detail previously (21) (see also Fig. 1). Briefly, preinitiation complexes were formed by incubation of DNA with HeLa cell nuclear extract and purified by gel filtration. Incubation of these complexes with ApC, GTP, UTP, and [α-32P]CTP resulted in elongation to the end of the A-free cassette. Transcription stalls after incorporation of the U residue at position +20, giving a stable U20 complex. These complexes were purified by the addition of 1% Sarkosyl followed by gel filtration, which removes the detergent, the NTPs and most proteins, including free transcript elongation factors. The Sarkosyl-rinsed U20 complexes were chased with ATP, CTP and GTP to the end of the U-free cassette and gel-filtered again to remove NTPs; the polymerases were then advanced to various stalling sites with subsets of the NTPs. If the template contained an arrest site, the complexes were simply advanced to that site directly from U20 by incubation with all four NTPs.

To determine the front edge boundary, elongation complexes were assembled on linear DNA templates labeled with 32P at the 5’-end of the non-template strand. The upstream ends of the templates had 3’ overhangs and were thus resistant to exoIII digestion (see “Experimental Procedures”). Transcription complexes were generated and advanced to the end of the U-free cassette as just described. They were then treated with StuI, which cleaved those DNAs that did not bear a transcription complex. The StuI treatment was necessary because only about 1–2% of DNA templates are transcribed (14, 22) (see also Marshall and Price (23)). The DNA Protection Patterns Generated by RNA Polymerase II Elongation Complexes Stalled at Sequential Pausing Sites along the DNA—We first measured template protection by stalled RNA polymerase II transcription complexes. To generate these complexes at the appropriate location for analysis, we constructed a template, designated pML20–23, with the sequence TTTTGGGAAACCC on the non-template strand immediately downstream of the StuI site at the end of the U-free cassette. This particular sequence allowed us to advance the polymerase to three new locations downstream of the StuI site, spanning a total of 9 bases along the template, simply by incubation with subsets of the NTPs. The insert in pML20–23 did not contain any sequences resembling known arrest sites. Complexes were assembled on this template and chased to the end of the U-free cassette, at position +151, as described above. The RNAs in these complexes are shown in lane 1 of Fig. 2A. While about half of complexes contained RNA of the predicted size, the other half stalled at position +152. We presume this resulted from trace contamination of chase NTPs with UTP. We have not been able to eliminate this problem, because of the relatively high (200 μM) concentration of ATP, CTP, and GTP needed to obtain efficient elongation to the end of the U-free cassette. After gel filtration, the C151/U152 complexes were advanced with 15 μM UTP (Fig. 2A, lanes 3 and 4), UTp and GTP (lanes 5 and 6), or UTP, GTP, and ATP (lanes 7 and 8); the complexes in lanes 9 and 10 were chased with all four NTPs. To distinguish between the labeled RNA and a background of bands from the labeled DNA, portions of the samples were treated with RNase A (lanes 2, 4, 6, 8, and 10). Note that nearly all of the C151/U152 complexes advanced when NTPs were added. Also, almost all of the U154, G157, and A160 complexes chased when subsequently challenged with all four NTPs (data not shown). Thus, complexes C151, U154, G157, and A160 were genuinely stalled and not arrested. We always observe a small proportion (5–10%) of complexes that fail to restart whenever RNA polymerase II is halted in chain elongation for any length of time. The amount of residual RNA left at positions 151/152 in lanes 3, 5, 7, and 9 is typical for stalled complexes (see also

Template, based on Ad 2 ML promoter:

1. Form preinitiation complex; gel filter

2. A-less transcription; add Sarkosyl and gel filter

3. U-less transcription; cleave with StuI; gel filter

4. Digest with exo III
Fig. 2. RNA polymerase II translocates monotonously during transcription of the pM20–23 template, a DNA sequence which does not cause arrest. Panel A, elongation complexes with label in both DNA and RNA were generated as described under “Experimental Procedures.” 5’ labeled nascent RNA transcripts were chased to the end of the U-free cassette (lanes 1 and 2), gel-filtered, and supplied with the indicated set of NTPs to form other complexes (lanes 3–8) or run off (lanes 9 and 10). RNAs were resolved on a 6% gel. Lengths of the transcripts from the stalled elongation complexes are shown to the left of the autoradiogram; the locations of the 3’ ends of these RNAs within the overall transcript sequence are shown at the bottom of the figure. Panel B, front edge boundaries of stalled elongation complexes were determined with DNA labeled at the 5’ end of the non-template strand. Complexes stalled at C151/U152 were treated with StuI, gel-filtered, and incubated with the indicated NTPs, followed by exoIII digestion for 2 min at 37°C as indicated. DNAs were resolved on a 6% gel along with exact markers (lanes 9–12) generated by primer extension using the same DNA template. A segment of non-template DNA strand sequence downstream of the StuI site is shown; the numbers indicate distance from the labeled 5’ end. Dots mark the position of major complex boundaries. Panel C, rear edge boundaries of stalled elongation complexes were obtained as in B except that the DNA was labeled at the 3’ end of the template strand and RNA was not labeled. Samples were resolved on a 10% gel. The portion of DNA template strand sequence upstream of the StuI site is shown at the bottom of the figure, with numbers indicating length from the labeled end; note that the DNA is presented in 3’ to 5’ orientation. The band corresponding to total StuI-digested DNA (at 49 nucleotides) is much fainter, relative to the boundary bands, than in B. This occurred because the 49-mer is too small to be excluded from the gel filtration column that was run between restriction digestion and exoIII treatment.
The downstream, or front edge, boundaries of template protection for the C151/U152, U154, G157, and A160 complexes were determined using exoIII digestion after StuI cleavage, as described above. The results are shown in Fig. 2B. As expected, most of the DNA was cleaved by StuI (lane 1); the ratio of the intensities of the full-length and StuI-cut bands was about 1:50. Note that, in Figs. 2–6, all RNAs and DNAs are labeled according to their length. In the case of DNA, this indicates distance from the 5' end label, not distance from the start of transcription. The locations of the ends of DNA fragments produced by exoIII digestion relative to the 3' ends of the RNA in each complexes are summarized in Fig. 6. When the StuI-cleaved C151/U152 complexes were treated with exoIII, the 323-nucleotide full-length DNA was truncated to a set of bands, the most prominent at 284 nucleotides (Fig. 2B, dotted band, lanes 3 and 4). This places the front edge of the polymerase about 19 nucleotides downstream of the position of the catalytic site. For ease of description, we will refer to the last base transcribed as the location of the catalytic site. It is possible, particularly in arrested complexes, that the active site is no longer located near the position where the last bond was formed, a point to which we will return under “Discussion.” For each of the other complexes, a similar protection edge with a single prominent band was obtained after exoIII digestion. Significantly, the major boundary (indicated by the dots) was displaced downstream in rough synchrony with transcription, although the front edge moved exactly the same number of bases as the active site in only one case (U154 to G157).

A number of controls gave us confidence that the bands indicated in Fig. 2B actually represent the boundaries of the various transcription complexes. First, the putative boundary bands were absent when the transcription complexes were chased to run-off before exoIII treatment (lanes 23 and 24). Second, if the transcription reactions were treated with α-amanitin at the U720 complex stage and then carried through the transcript elongation-exoIII digestion procedure, none of the boundary bands appeared (data not shown). The boundary locations did not depend strongly on exoIII concentration, not only for the 2-fold range shown in Fig. 2 but also for a factor of two on either side of this range (data not shown). This was usually but not always true of the exoIII boundaries that we measured in this study; the boundaries in Figs. 3, B and C, for example, were more sensitive to digestion levels.

To map the rear edge boundaries of the stalled complexes, we used a fragment of pML20–23 labeled at the 5' end of the template strand. The experiment was identical to that described above except that C151 complexes were incubated with both StuI and SmaI. The SmaI treatment was necessary to generate a blunt end, at which exoIII will efficiently begin digestion, relatively close to the transcription complex (123 bp upstream of the StuI site). The rear edge boundary reproducibly consisted of several equally prominent bands, rather than a single intense band, for several of the complexes (Fig. 2C). However, it is clear that rear edge of the polymerase, like the front edge, moves forward as transcription proceeds. As was the case for the front edge, the bands corresponding to the boundaries were absent when the complexes were chased to run-off before exoIII digestion (lanes 19 and 20).

The experimental results from Fig. 2 are summarized in Fig.

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Fig. 3. Exonuclease III footprints of an arrested transcription complex on the pML20–30 template. Panel A, transcripts produced by chase (200 μM NTPs; 5 min at 37 °C) of 5'-labeled RNAs without SII (lanes 1 and 2) or with SII (1.5 μg/ml; lanes 3 and 4) were resolved on a 6% gel. Labels in the left margin indicate the major arrest site (Ia), a cryptic arrest site (arrow), and run-off (RO). Panel B, front edge boundaries were determined with complexes chased in the absence (lanes 1–4) or presence (lanes 9–12) of SII, followed by treatment with StuI and exoIII digestion (2 min at 37 °C) as indicated. The resulting DNAs and exact length size markers (lanes 5–8) were resolved on a 6% gel. The sequence of the non-template strand near the StuI site is shown at the bottom of the figure; the numbers indicate distance from the 5' end label. Panel C, rear edge boundaries were obtained as in B, except that the DNA was labeled at the 3' end of the template strand and exoIII digestion was for 8 min. DNAs were resolved on a 10% gel. DNA template strand sequence upstream of the StuI site is shown at the bottom of the figure; the numbers indicate distance from the 5' end label. Note that the DNA is presented in 3' to 5' orientation. Dots represent the rear edge footprint of the arrested complex.
TABLE II  Summary of exoIII protection experiments

| Construct no. | Stalled (S) or arrested (A)? | Front to rear edge | Active site to front edge | Active site to rear edge |
|---------------|-----------------------------|--------------------|--------------------------|-------------------------|
| pML20–23      |                             |                    |                          | bpsi                    |
| a             | S                           | 32–35              | 19                       | 12–16                   |
| b             | S                           | 33                 | 18                       | 15                      |
| c             | S                           | 33                 | 18                       | 15                      |
| d             | S                           | 31–34              | 17                       | 14–17                   |
| pML20–30      |                             |                    |                          | bpsi                    |
| a             | S                           | 29–33              | 15–18                    | 13–16                   |
| b             | A                           | 28–32              | 7–9                      | 20–24                   |
| pML20–36      |                             |                    |                          | bpsi                    |
| a             | S                           | 33–36              | 18–20                    | 13–16                   |
| b             | S                           | 28–32              | 12–13                    | 16–18                   |
| c             | S                           | 30–34              | 9–10                     | 21–24                   |
| d             | S                           | 29–33              | 6–7                      | 23–26                   |
| e             | S                           | 34                 | 19                       | 15                      |
| pML20–37      |                             |                    |                          | bpsi                    |
| a             | S                           | 32–35              | 18–20                    | 14–16                   |
| b             | S                           | 33                 | 18                       | 15                      |
| c             | S                           | 29–34              | 15–17                    | 14–17                   |
| d             | S                           | 33                 | 17                       | 16                      |
| e             | S                           | 33                 | 18                       | 15                      |
| pML20–38      |                             |                    |                          | bpsi                    |
| a             | S                           | 32–36              | 17–20                    | 14–17                   |
| b             | S                           | 29–32              | 10                       | 19–22                   |
| c             | S                           | 25–32              | 7                        | 18–25                   |
| d             | S                           | 34–37              | 18–19                    | 16–18                   |
| e             | ?                           | 29–32              | 4                       | 25–28                   |

6 (pML20–23, a–d) and in numerical form in Table II. The elongation complexes are represented by boxes and the positions of the catalytic site are designated by dots. For the sake of simplicity, only the non-template strand of DNA is shown, even though the rear edges were mapped on the template strand. We found that the overall structure of the complex, as judged both by the length of template protected and by the location of the catalytic site within the protected region, did not change substantially among the four stalled complexes. We also generated a second set of stalled complexes by walking the polymerase forward on another template similar to pML20–23, but with a more random DNA sequence placed downstream of the StuI site. Preliminary measurements of boundary positions and active site locations (data not shown) agreed with those given in Fig. 6 for the pML20–23 complexes. Our results are also consistent with those obtained by Reines and colleagues for the exoIII boundaries of elongation-competent complexes (18). Thus, for a small set of stalled RNA polymerase II elongation complexes, synthesis of the RNA transcript and translocation of the elongation complex are closely coupled.

The DNA Protection Patterns Generated by Arrested RNA Polymerase II Elongation Complexes—To generate an arrested elongation complex we constructed a new template, pML20–30, with a strong arrest site sequence downstream of the U-rich cassette. The arrest site sequence (see Fig. 6) was transferred from the pADTerm-2 plasmid (18) (kindly provided by D. Reines). This sequence, originally designated Ia, is the strongest in a set of arrest sites derived from the histone H3.3 gene (24). The core of the arrest site contains the sequence TTTTTTTTC-CTTTTTTT on the non-template strand; arrest is expected to occur near the end of the first T run (25). In the assembly of pML20–30, we changed three base pairs upstream of the first T run to create a StuI site, but the sequences downstream of the T runs were retained from pADTerm-2 (see Fig. 6). Note that these sequences differ from those downstream of the stalled sites in pML20–23.

Arrested complexes were generated on pML20–30 by chasing Sarkosyl-rinsed U20 complexes with all four NTPs at 200 μM. About half of the transcription complexes arrested under these conditions (Fig. 3A, lanes 1 and 2), as expected (18). High resolution mapping revealed two major stops, after the incorporation of the fifth and sixth U residues encoded in the first part of the arrest site (see Fig. 6; data not shown). The majority of the complexes arrested on pML20–30 chased to the end of the template (Fig. 3A, RO, lanes 3 and 4) after incubation with the elongation factor II and NTPs. Some of the complexes arrested at a cryptic arrest site downstream (Fig. 3A, arrow). The front and rear edge boundaries of complexes arrested at the Ia site were determined essentially as described above for the stalled complexes; the results are shown in Fig. 3, B and C. The front boundary (Fig. 3B), which consisted of two bands (lanes 3 and 4), and the roughly four-band rear edge boundary (Fig. 3C, lanes 3–5) were both greatly reduced, as expected, in SII control reactions (Fig. 3, B, lanes 11 and 12, and C, lanes 8–10). We also measured the exoIII boundaries of complexes stalled on the pML20–30 template at the beginning of the first T run of the arrest site (data not shown). These complexes were genuinely stalled, because they efficiently chased up to the arrest site when UTP was added (data not shown).

The boundaries of the arrested and stalled complexes on the pML20–30 template are summarized in Fig. 6 and Table II. It is remarkable that the RNA polymerase appears to move in the opposite direction to transcription as the complex approaches arrest. The stalled pML20–30 complex has essentially the same boundaries and edge-to-active site dimensions as the stalled pML20–23 complexes. The arrested and stalled pML20–30 complexes both protected the same length of template, but the catalytic site is much closer to the front edge in the arrested complex. This latter result was obtained by Reines and colleagues in an earlier study of the boundaries of RNA polymerase II complexes at the histone H3.3 Ia site (18). We also studied the exoIII boundaries of complexes arrested on another template, pML20–21, in which the arrest site was derived from the pML5–4NR plasmid which we had originally used to study arrest (5). The efficiency of arrest at the pML5–4NR site after recloning into pML20–21 was rather low, which made it difficult to map the complex boundaries. Nevertheless, we were able to show that on pML20–21, as on pML20–30, the catalytic site was located much closer to the front edge in the arrested complex than in the preceding stalled complex. However, in the pML20–21 case there was no apparent retrograde movement of the complex between stalling and arrest; both the arrested and stalled complexes had essentially identical protection boundaries (data not shown). Thus, at least two different arrested complexes show a striking structural difference when compared to the average stalled complex.

The DNA Protection Patterns Generated by RNA Polymerase II Elongation Complexes Stalled at Mutated Arrest Sites—Our studies with pML20–30 and pML20–21 suggested that the abnormally close approach of the active site to the leading edge is an important component of transcriptional arrest. However, on the pML20–30 template, we could only compare arrested complexes with complexes stalled 4 or 5 bases upstream of the arrest site. At what point does the transcription complex begin to undergo the transition into the arrested structure? We could not stall the polymerase at defined locations as it traverses the initial T run of the arrest site. It has been shown that continuous T runs on the non-template strand are strongly correlated with efficient arrest (24). We reasoned that slight alterations of the T-rich regions of pML20–30 should eliminate the ability to cause arrest, thus providing a template on which elongation-competent complexes could be walked to the same position at which arrest occurs in pML20–30. If unusual interactions of the front edge of the polymerase with the template, such as failure of translocation, were still observed on such templates,
Fig. 4. RNA polymerase II translocates discontinuously during the transcription of the mutated Ia arrest site sequence in pML20–36. Panel A, RNAs from complexes stalled at sequential positions were resolved on a 6% gel. Complexes were chased to the end of the U-free cassette (lane 1), gel-filtered, and incubated with the indicated NTPs. In lanes 4 and 5, a second gel filtration step was performed (designated by **). The lengths of the transcripts are shown on the left of the figure. RNA sequence complementary to the DNA template strand and the positions of the transcript 3′ ends in the stalled elongation complexes are shown at the bottom of the figure. Dots adjacent to lane 5 indicate elongation competent (open dot) and incompetent (solid dot) complexes. Panel B, front edge boundaries for stalled complexes were obtained with DNAs labeled at the 5′ end of the non-template strand. Complexes stalled at the end of the U-free cassette were treated with StuI, gel filtered, advanced with NTPs, and digested with exonIII (2 min at 37°C) as indicated. The reaction in lane 2 was RNase A-treated. DNAs were resolved on a 6% gel. The sequence of the non-template strand near the StuI site is shown at the bottom of the figure, with distances from the 5′ end label indicated. Dots represent the positions of the major boundaries; the open dots indicate the boundary after chase with U, G, and A.

Fig. 6, compare complexes a–d on pML20–36 to TTGGTATCCCGAGTTT, creating template pML20–36. Complexes on this template showed no arrest at downstream locations. Complex U168 was subjected to gel filtration (Fig. 4A, asterisks, lane 4) to remove NTPs and then supplied with 200 μM CTP to form C171 complex (lane 5). After gel filtration, the C161/U162 complexes advanced to U163, U166, or U168 when incubated with appropriate subsets of the NTPs. Note that in this case 200 μM NTPs were added, instead of 15 μM, to walk the complexes forward. The higher NTP concentration was used to minimize any possibility of arrest, but it did result in higher levels of leak-through to more downstream locations. Complex U168 was subjected to gel filtration (Fig. 4A, asterisks, lane 4) to remove NTPs and then supplied with 200 μM CTP to form C171 complex (lane 5). Complexes C161, U163, and U166 were stalled and not arrested; when these complexes were chased with excess NTPs they advanced very efficiently (data not shown). However, only half of the U168 complexes left the pausing site and advanced three bases forward (lane 5, open dot) when supplied with CTP. The complexes which remained at position 168 (solid dot) were still associated with DNA (see below). This failure to elongate by some of the U168 complexes was not simply the result of the extra round of gel filtration, because performing an additional gel filtration on the U166 complexes did not reduce their ability to continue RNA synthesis (data not shown).

We mapped the front and rear boundaries of the complexes on the pML20–36 template as described above. The front edge boundaries are shown in Fig. 4B; data for the back edges are not shown. Results of both the front and back edge mapping experiments are summarized in Fig. 6 (pML20–36, a–d) and Table II. The initial stalled complex, C161/U162, showed essentially the same template protection length and location of the catalytic site as the stalled complexes we investigated earlier. We expected that the other pML20–36 stalled complexes, except perhaps for C171, would have the same dimensions as C161/U162 but simply be displaced downstream, as we observed for the stalled complexes on pML20–23. We were surprised to find a very different result. As transcription proceeded on pML20–36, the front and back protection boundaries shifted upstream, as we observed with transcription on pML20–30. Thus, as transcription continued the catalytic site grew progressively closer to the front edge compared with C161. In particular, the conformations of the elongation-competent U166 and U168 complexes, which stopped transcription at locations analogous to the site of arrest on pML20–30, are nearly identical to the conformation of the arrested pML20–30 complex (in Fig. 6, compare complexes c and d on pML20–36 to complex b on pML20–30). Therefore, the close approach of the catalytic site to the front edge of the RNA polymerase is not, by itself, sufficient to force arrest.

A dramatic transformation occurred when complex U168 advanced to complex C171 on the pML20–36 template. Although the catalytic site shifted only three bases forward, the entire complex translocated 14–15 bp downstream, thereby resuming the usual conformation characteristic of a stalled complex. As
noted above, only about half of the U168 complexes advanced to C171, while the other half remained at U168. This partitioning of complexes at a functional level was reflected by a partitioning on structural level. We observed two edge boundaries (Fig. 4B, lanes 15–16) in the C171 complexes. We assume that the downstream boundary corresponds to the complexes which actually advanced to position +171, while the upstream boundary, which is identical to that of the U168 complex, represents complexes which failed to resume transcription. The U168 complex is the most strained of the stalled complexes in the set we produced on the pML20–36 template, in that the active site is displaced forward to the greatest extent in this complex. It is interesting that the U168 complex partitions between transcriptional activity and what appears to be arrest. Thus, although all strained complexes are not necessarily arrested, highly strained complexes may decay into arrest.

Since pML20–36 differed from pML20–30 within the arrest site but was identical in the downstream region (see Fig. 6), this suggested that at least part of the signal for the translocation blockade was encoded in the sequences downstream of the arrest site. Therefore, we replaced the sequences downstream of the mutated arrest site in pML20–36 with the corresponding segment from pML20–23, where no translocation block was observed, creating pML20–37. Five successive stalled complexes were generated on this template and the exoIII boundaries were determined as described previously. The results of these experiments are summarized in Fig. 6 (primary data not shown). We found that the polymerase advanced on the pML20–37 template exactly as it had on the pML20–23; that is, translocation was coordinated with the growth of the transcript. No translocation blockade or reverse movement of the elongation complex was observed. Thus, the block to translocation must be at least partially determined by the sequence downstream of the T runs.

To reinforce this idea, we constructed another template in which the mutated T runs from pML20–36 were replaced by the TTTGGAACCT sequence from pML20–23, generating construct pML20–38. C161, U164, G167, and A170 complexes were assembled on the pML20–38 template (Fig. 5A). As expected, all of these complexes were functionally stalled since they chased very effectively when supplied with NTPs (data not shown). Note that part of the complexes released from position +161 with all four NTPs became arrested at a cryptic arrest site further downstream (Fig. 5A, arrow, lane 6). The front and rear edge exoIII boundaries of the stalled pML20–38 complexes are shown in Fig. 5, B and C, and are summarized in Fig. 6 (pML20–38, a–d) and Table II. Note that the complexes stopped at the cryptic site were also detected (Fig. 5, B, open dot, and C, solid dots). We found that the elongation complex on the pML20–38 template translocated backward while the catalytic site moved forward from position +161 to the position +164. Further advance of the catalytic site from +164 to +167 was not accompanied by movement of the complex itself. Finally, the transition from C167 to A170 was marked by an 11–15 bp downstream translocation of the entire elongation complex. Thus, the general pattern of translocation observed on the pML20–38 template reproduced with only minor variations the pattern seen on the pML20–36 template (Fig. 6, compare a–d, pML20–36, with a–d, pML20–38). However, there was one crucial difference in the results. The transition from U168 to C171 on the pML20–36 template resulted in an apparent partial arrest. Some of the U168 complexes could not extend their nascent RNAs, and these complexes remained in their original location on the template as judged by exoIII protection. In contrast, while all of the U168 complexes on the pML20–38 template advanced to A170 complexes, the footprint revealed two populations with different edge boundaries: complexes which translocated further downstream (Fig. 5B, asterisk, lanes 12 and 13) and complexes which did not move (Fig. 5B, solid dot, lanes 12 and 13). This partitioning was more prominent in case of rear edge boundaries (Fig. 5C, note particularly lanes 13 and 14). The A170 complexes are represented in Fig. 6 (complex d of pML20–38) by solid and dashed boxes, respectively. This is the only example we observed of a complex with two distinct, stable conformations.

**DISCUSSION**

The mechanism by which RNA polymerase II translocates along the DNA has recently been the subject of considerable research (11, 13–18, 20). A particularly interesting point is the relationship of the translocation process and transcriptional arrest. As noted in the Introduction, recent models have predicted that a transient failure of the leading edge to translocate is directly coupled to arrest (11, 14–16). Through the use of exonuclease III footprinting, we have investigated the movement of RNA polymerase II during transcription on a number of templates, including two with well-characterized arrest sites. For all complexes, both stalled and arrested, the length of the protected template was roughly the same. Our results agree with the prediction that a strained configuration of the transcription complex, resulting from the inappropriately close approach of the active site to the leading edge of the polymerase, is a necessary feature of arrest. This was not only observed at the strong histone H3.3 (Fig. 3B) arrest site, confirming the earlier report of Gu et al. (18), but also at the weaker pML5–4NR arrest site (data not shown), and interestingly with the U168 complex on template pML20–38 (Fig. 4B, lanes 12–16). In this latter case, the mutated T-rich region could no longer cause arrest, but failure of the leading edge to translocate still occurred. About half of the most highly strained complexes generated by walking the polymerase forward on this template, those halted at +168, were unable to resume transcription after a 5-min chase.

It is important to emphasize that we have also shown that strain within the transcription complex is not sufficient to cause arrest. The most dramatic example of this was seen with the complexes stalled at +170 on the pML20–38 template. In a subset of these complexes (Fig. 6, pML20–38, d), the active site had advanced to within four bases of the leading edge, and yet all of these complexes were elongation competent (Fig. 5A, lanes 5 and 6). We did observe tight coupling of translocation and RNA synthesis using the pML20–23 and pML20–37 templates; all complexes assembled on these templates remained fully elongation competent. The structural data for these latter complexes are very similar to each other (Table II) and are quite different from the values of the arrested complexes.

While nuclease protection measurements are informative in studying transcription complexes, it is important to acknowledge some fundamental limitations of this approach. We must assume that the conformation of stalled transcription complexes as revealed by the exoIII method is representative of polymerases during the normal elongation phase. Our translocation and footprinting experiments typically take several minutes, which is two orders of magnitude longer than the time required for bond formation under optimal conditions. Thus, we can study only stable and metastable configurations of elongation complexes. Because of the progressive nature of exonuclease III digestion, the boundaries we detect may represent either the most stable or the most compressed intermediates of the elongation complex in question. We have referred to the position of the catalytic site as the last base transcribed. However, we have previously suggested (10) that the active site may actually be in equilibrium between the 3′ end of the RNA and other
FIG. 5. RNA polymerase II also elongates discontinuously on the pML20–38 template. Panel A, RNAs from stalled complexes were resolved on a 6% gel. Complexes were chased to the end of the U-free cassette (lane 1), gel-filtered, and incubated with the indicated NTPs. The arrow indicates RNA from complexes arrested at a cryptic arrest site. The lengths of the transcripts are shown on the left of the figure. The RNA sequence complementary to the DNA template strand and the positions of the transcript 3' ends in the stalled elongation complexes are shown at the bottom of the figure. Panel B, front edge boundaries for stalled complexes were obtained with DNA labeled at the 5' end of the template strand. Complexes stalled at the end of the U-free cassette were treated with StuI, gel-filtered, advanced with NTPs, and digested with exoIII (2 min at 37 °C) as indicated; the sample in lane 2 was also treated with RNase. DNAs were resolved on a 6% gel. The sequence of the non-template strand near the StuI site is shown at the bottom of the figure, with distances from the 5' end label indicated. Dots and asterisks represent the positions of the major boundaries; the open dot indicates complexes arrested at the downstream cryptic site. Panel C, rear edge boundaries were obtained as in B, except that the DNA was labeled at the 3' end of the template strand and exoIII digestion was carried out for 8 min. All samples except lanes 2, 6, 9, 12, and 15 were treated with RNase A. DNAs were resolved on a 10% gel. Dots correspond to the rear edge boundary of the complex arrested at the cryptic arrest site. DNA template strand sequence upstream of the StuI site is shown at the bottom of the figure in 3' to 5' orientation, with distance from the labeled end indicated.
locations within the ternary complex. This is particularly likely in complexes in which the active site has approached abnormally close to the front edge of the polymerase.

With these caveats in mind, we can consider the implications of the results reported here for our understanding of the signals which cause arrest. It is well known that long T-rich segments of the non-template strand can provoke arrest by RNA polymerase II (7, 24). Arrest typically occurs in the initial part of this T-rich region, indicating that sequences downstream of this location must act at the DNA level. Sequences flanking the T-rich region have been reported to affect the efficiency of arrest (24), but the mechanism by which this occurs is not known. In the assembly of the pML20–30 construct, we completely replaced the original sequence upstream of the first T run of the histone H3.3 arrest site with unrelated DNA. Somewhat surprisingly, neither the arrest efficiency nor stability of the arrested complex changed significantly (data not shown). Thus, in our hands sequences upstream of the T-rich region did not have a strong impact on the efficiency of arrest at one strong arrest site. This is similar to results obtained with a bacterial terminator, in which mutation of upstream sequences reduced the half-life of the paused complex but not the efficiency of pausing (16).

In creating the pML20–36 template, we successfully eliminated the arrest site of pML20–30 by mutating both T runs. However, as noted, translocation was as effectively blocked as

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**Fig. 6. Schematic summary of ExoIII protection experiments.** Only the non-template strand of DNA is shown. Note that the numbers on the DNA sequence indicate the distance from the 5' end of the template fragment, not the distance downstream of transcription start. The elongation complexes are represented by the boxes. The positions of transcript 3' ends are designated by dots. The dashed box in pML20–38, d, represents complexes which did not translocate (see text).
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it was on pML20–30. The mutated T-run of pML20–36 did not block translocation in the context of a different downstream sequence (the pML20–37 template); therefore, we may conclude that the sequences downstream of the mutated T-rich region in the pML20–36 template are able, by themselves, to block translocation by the RNA polymerase. Since we have examined the effect of only two downstream sequences (the segment in pML20–23/37 versus the segment in pML20–30/36/38) we cannot identify the length or the particular sequence features of the downstream region which hinder translocation. It may seem surprising that sequence elements which are largely unprotected by RNA polymerase II, as judged by the exoIII assay, are nevertheless important in blocking translocation. In this context it is useful to recall that polymerase II shows more extensive upstream and downstream interactions with the template when DNase I is used as a probe. For example, RNA polymerase II stalled at +35 downstream of the adenovirus major late promoter protects more than 40 bp of template from attack by DNase I (22). Finally, we cannot exclude a contribution from the T-rich regions to the translocation blockade, since we did not, for example, assay the T-rich region of pML20–30 in the context of downstream sequences, such as those from pML20–23, which clearly cannot cause blockades on their own. It is interesting to note that Rice et al. (26) observed an apparent upstream retreat in the leading edge of the DNase I footprint of an RNA polymerase II transcription complex after the addition of three U residues to the nascent RNA.

As expected from earlier work with the histone H3.3 site (24), mutations in the first T run of pML20–30 abolished arrest completely in the presence of high UTP concentrations. However, at low (20 μM) levels of UTP, some polymerases still arrested. In this case the position of arrest was shifted downstream, to the middle of the remaining T run (data not shown). Thus, the downstream sequence context in the mutated template substituted, rather inefficiently, for the second T run and downstream sequences in the normal arrest site. This suggests that blocking the progress of the polymerase’s leading edge is only one of several roles which sequences downstream of the first T run must perform in an efficient arrest site.

At this point we can say very little with certainty concerning the mechanism by which downstream sequences might block translocation. We can speculate that some DNA sequences have less affinity than others for the leading edge of the RNA polymerase. The polymerase would have difficulty binding to such sequences as transcript elongation proceeds, thereby at least temporarily stopping translocation while the active site continues to advance. This model would not predict the retreat of the leading edge which we observed, for example, during transcription of the pML20–30 template. A trivial explanation for this effect would be that exoIII simply pushes the polymerase backward along the DNA during the determination of the front edge of protection. However, if this had happened, we would not have also observed an upstream retreat by the rear edge of the polymerase, since for rear edge measurements exoIII would be pushing the polymerase downstream. The transient upstream movement which we observed during transcription of the pML20–30, -36, and -38 templates might be explained by recalling that the transcription bubble must also translocate as chain elongation proceeds. The approach of the unwound region toward the leading edge could place torsional strain on the point of interaction of the leading edge with the template and thereby actively dissociate downstream sequences from the polymerase. If maintaining contact between the polymerase and DNA is energetically favorable, then the entire body of the polymerase would shift upstream in order to reacquire the protein-DNA contacts which were lost.

In summary, the results we report here emphasize that arrest by RNA polymerase II occurs in response to a very complex set of signals. Blocking translocation is almost certainly a requirement for arrest to occur under optimum transcription conditions, but we showed that the translocation block alone does not necessarily cause arrest. Strain within the transcription complex due to transient failure of translocation may couple with an unusually weak interaction between the active site and U-rich regions of the transcript to facilitate loss of contact between the active site and the 3′ end of the nascent RNA at strong arrests sites. This model is attractive based on our results and those of many other workers in this field. However, it does not suggest a role for the downstream portion of the T-rich region, which is required for efficient arrest and does it explain the tendency of certain stalled complexes with U-rich 3′ ends to decay into arrest (10). Further structural studies on the transcription complex, and a more extensive collection of well characterized arrest sites, will be needed to refine the existing model.

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