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Multisubunit RNA Polymerases Melt Only a Single DNA Base Pair Downstream of the Active Site*

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To extend the nascent transcript, RNA polymerases must melt the DNA duplex downstream from the active site to expose the next acceptor base for substrate binding and incorporation. A number of mechanisms have been proposed to account for the manner in which the correct substrate is selected, and these differ in their predictions as to how far the downstream DNA is melted. Using fluorescence quenching experiments, we provide evidence that cellular RNA polymerases from bacteria and yeast melt only one DNA base pair downstream from the active site. These data argue against a model in which multiple NTPs are lined up downstream of the active site.

Cellular RNA polymerases (RNAPs) are multisubunit enzymes that share high structural and sequence homology from bacteria to eukaryotes. Based upon structural studies, it has been proposed that substrate NTPs might gain access to the active (insertion) site of these enzymes through two alternative routes. The first route is a relatively small opening (10 x 10 Å) called the secondary channel (the "pore" in eukaryotic RNAPs) that leads directly to the active site and is a place where various transcription factors and inhibitors have been shown to bind. An alternative route may direct substrates through the main channel in the RNAP (formed by the two largest subunits), which binds the downstream DNA duplex. The differences in these substrate entry routes and the mechanisms of substrate loading are reflected in three major models. In the first model, the substrate NTP is believed to be loaded through the secondary channel into the insertion site either directly or following initiation binding in the E-site, without preselection for the correct substrate base before its movement into the active site (1, 2). Another model suggests that the incoming substrate forms an incipient base pair with the templating base (n) in a preinsertion site before its movement to the insertion site. This scenario was demonstrated for T7 RNAP and a number of other nucleotide polymerases (3–5) and has also been proposed to operate in bacterial and yeast RNAPs (5, 6). Finally, the third model (the NTP lineup model) suggests that several (up to four) substrate NTPs may bind to the template DNA in the main channel before movement of the template base and its cognate substrate into the active site (7). The latter model would require that the downstream DNA be melted to provide an opportunity for base pairing with the substrate; however, the structural information available so far for the cellular RNAP ECs does not provide an unambiguous answer as to the extent of the downstream melting (2, 6, 8).

In this work, we took advantage of nucleotide analogs whose fluorescence is sensitive to the local DNA environment (9–11) to monitor melting of the downstream DNA. Our data demonstrate that cellular RNAPs melt only one DNA base pair downstream from the active site, a finding that is not consistent with the NTP lineup model, but favors a mechanism of substrate loading through the secondary channel one nucleotide at a time.

EXPERIMENTAL PROCEDURES

Purification of RNAPs and Activity Assays—His-tagged wild type T7 RNAP (pBH161) was purified as described previously (12). Wild type Escherichia coli core polymerase was purified from cell biomass obtained from MRE600 strain as described (13). Yeast RNAP II was prepared as described (14). Polymerase activity was measured by the ability to extend a 32P-labeled RNA primer by one nucleotide in a reaction in which the concentrations of nucleic acid scaffold (R8/TS35/NT35) and RNAP were equimolar (see transcription conditions below).

RNA and DNA Oligonucleotides—The following synthetic oligonucleotides were used (all sequences are 5’ to 3’). RNA oligomers (Dharmacon) are as follows: R8, GCGGCGAU; R9, GC GGCGCAUA; R9m, CGGGCGAU; R9 N2, CGCGGCCGAU; R9N3, GCCGGCGCGA. DNA oligomers (Integrated DNA Technologies) are as follows: TS35, CCTGCTGAAATGATATCCGG; NT42U2, GATAGTCAGACAGG; NT1C, AGATA GTCAAGACC; NT2C, AAGATAGTCAGACAGG; NT3C, TAAGATAGTCAGACAGG; NT491, TGGCTATTGACAGG; NT38, ATCAGATTCAGACAGG; NTFl, AGTGATTC AGACAGG; NTF2, CTTATGTCAAGACAGG; NT35, TCGAT TCAGACAGG. The following DNA oligomers containing fluorescent analogs were custom synthesized by Integrated DNA Technologies: TS42PC, CCTGCTGACTATCPTATGCGCGG; TSC1, CCTGCTGACTATCPTATGCGCGGCTC-
Assembly of ECs and Transcription Conditions—Nucleic acid scaffolds were assembled by annealing equimolar concentrations of complementary RNA and DNA oligomers as described previously (15). To test the efficiency of complex formation and NTP incorporation, RNA oligomers were labeled at their 5′ ends using [γ-32P]ATP and T4 polynucleotide kinase (New England Biolabs) prior to assembly. To assemble elongation complexes, RNAP (0.2–1 μM) was incubated with an equimolar concentration of scaffold for 5 min at room temperature in 10 μl of transcription buffer containing: 20 mM Tris-HCl (pH 7.9 at 25 °C), 100 mM NaCl, 10 mM MgCl2, and 5 mM 2-mercaptoethanol (for E. coli RNAP); 20 mM Tris-HCl (pH 7.9 at 25 °C), 40 mM KCl, 5 mM MgCl2, 1 mM 2-mercaptoethanol (for yeast RNAP II); 20 mM Tris-HCl (pH 7.9 at 25 °C), 10 mM MgCl2, 5 mM 2-mercaptoethanol (for T7 RNAP). Primer extension was achieved by incubation of complexes with substrate NTPs (500 μM) for 2 min at 37 °C (T7 and e. coli RNAP) or 30 °C (yeast RNAP II). Reactions were terminated by the addition of 90% formamide, 50 mM EDTA solution, and products were resolved in 20% PAGE in the presence of 6 M urea and visualized by PhosphorImagerTM (GE Health).

Fluorescence Spectroscopy—Fluorescence measurements were carried out using a Fluoromax-3 fluorometer (HORIBA Jobin Yvon) equipped with a F-3004 thermostatted sample holder and a LFI-3751 temperature controller (Wavelength Electronics). Quartz cells (30 μl) with 1.5 × 1.5-mm optical length paths (Hellma) were used. Samples containing pyrrolo-cytosine were excited at 350 nm, and fluorescence emission was monitored at 450 nm with both excitation and emission slits set at 5 nm. The nucleic acid scaffolds carrying a fluorescent probe were prepared as described above by annealing together RNA and DNA oligonucleotides. The ECs were prepared by preincubating 200 nm scaffold with 300 nm yeast RNAP II, T7, or E. coli RNAP in a corresponding transcription buffer (see above) for 10 min at 23 °C. When needed, the resulting fluorescent complexes were incubated with or without cognate NTP (0.5 mM) for 5 min at 23 °C, and emission was averaged over 10 min with a 1-s interval. All measurements were performed at 23 °C and repeated at least three times for each EC in independent experiments. The effect of protein and NTP absorption on the emission of the pyrrolo-cytosine was determined to be negligible.

RESULTS

Fluorescence emission of NTP analogs such as 2-aminopurine and pyrrolo-cytosine (pC) is sensitive to the local environment of the nucleic acid polymer in which they are incorporated (9, 10, 16). It is thought that quenching of fluorescence is due to base stacking interactions with the adjacent bases, which is increased in the case of double-stranded DNA (9). Thus, changes in fluorescence emission can be used to monitor the transition from double-stranded (ds) DNA to single-stranded (ss) DNA, for example, during promoter melting (17). In this work, we took advantage of pC probes incorporated at specific positions into the template (T) strand of the nucleic acid scaffold used to assemble transcription elongation complexes. Two types of scaffolds were used: scaffolds with a ds-DNA duplex (ds-scaffolds) having both T and non-template (NT) strands in the downstream region or scaffolds that contained only RNA and T DNA strand (ss-scaffolds).

To find out whether and how far bases in the downstream DNA region of EC are melted, we placed the fluorescent probe in the T strand at different positions in immediate proximity to the active site (i.e., at positions n, n+1, n+2, and n+3). The principle of this experiment is illustrated in Fig. 1A. In a scaffold prepared by annealing together only T DNA strand and an RNA primer, relatively high emission of the pC probe is detected (supplemental Fig. S1). As expected (9), the addition of an NT strand results in quenching of fluorescence (supplemental Fig. S1). Incubation of RNAP with ss- or ds-scaffolds results in the formation of an EC (18). In the case of an ss-scaffold EC, the fluorescence emission of the probe is expected to remain high since a complementary DNA region is not present. When an EC is formed using a ds-scaffold, two possibilities exist. In one scenario, RNAP melts open the G:pC base pair, and an increase in emission up to the level of the ss-scaffold EC would be expected (Fig. 1A). Alternatively, if the GpC pair remains intact (is not melted), no changes in fluorescence emission should be registered.

To test our experimental setup, we employed T7 RNAP elongation complexes assembled on scaffolds containing a pC probe at different positions downstream from the active site. Scaffolds ECs of T7 RNAP having a fully complementary downstream region starting from base n–1 or n were previously crystallized, and the crystal structures showed that the DNA is melted only one base pair downstream from the active site in these complexes (5, 19). When we compared the fluorescence emission of pC probes positioned at n, n+1, n+2, or n+3 in ss- and ds-scaffold ECs formed with this enzyme, we found that the quantum yield was reduced 1.6–1.8-fold in all positions except for base n (Fig. 1, B and C). This indicates that, in agreement with the structural data and prior fluorescence data obtained with the promoter-originated ECs having 2-amino purine (11), T7 RNAP melts open only one base pair ahead of the active site (Fig. 1C). Also consistent with the structural data and prior observations (20), the absolute value of fluorescence intensity of the probe placed at position n in the ss-scaffold EC was significantly higher as compared with the unbound scaffold, reflecting a 90° kink in the DNA in this region (Fig. 1, B and D).

We next used the same approach to examine melting of the downstream region in bacterial RNAP ECs. Previously, we demonstrated that depending upon the length of the RNA-DNA hybrid in the scaffolds used for their assembly, bacterial ECs may exist in either the pretranslocated or the post-translocated states (18). In this study, we utilized scaffolds having a 9-bp RNA-DNA hybrid, which results in the formation of E. coli RNAP EC that is in the post-translocated state and therefore should have a similar location of the 3′ end of the RNA relative to the insertion site as in T7 RNAP.
We found that, as in the case with T7, the pC probe placed at base \( n \) had the highest fluorescence emission in both ss-scaffold and ds-scaffold ECs (Fig. 2A), suggesting a similar kink in the DNA strand in this complex, as has been observed for other RNAPs (19, 21). Also, as in the case with T7 RNAP, we observed 1.8–2-fold reduction of the fluorescence emission in ds-scaffold ECs as compared with ss-scaffold ECs at positions \( n+1 \), \( n+2 \), and \( n+3 \) (Fig. 2B) but not at position \( n \). The same data were obtained when 2-aminopurine was used at position \( n \), \( n+1 \), and \( n+2 \) in the template strand of bacterial EC (supplemental Fig. S2), indicating that the quenching was sequence-independent.

Prior to fluorescence measurements with yeast RNAP II, we analyzed the conformation of ECs formed on scaffolds having different lengths of RNA:DNA hybrid in a pyrophosphate sensitivity assay (supplemental Fig. S3). We found that RNAP II ECs having a 9-bp RNA:DNA hybrid were resistant to PP\(_i\), and were thus in the post-translocated state (18). Analysis of the fluorescence emission of the pC probe in RNAP II ECs revealed the same pattern of downstream DNA melting as in bacterial RNAPs, i.e. melting of the \( n \) base pair only (supplemental Fig. S4). To confirm that multisubunit RNAPs melt only a single base pair downstream of the active site, we performed “walking” experiments in which the RNAP II active site was shifted one base pair at a time downstream by providing the ds-scaffold ECs with the next incoming NTP (Fig. 2C). As expected, incorporation of GMP into the RNA in a ds-RNAP II EC having pC at position \( n \) resulted in a quench of fluorescence emission due to inclusion of the probe into the RNA:DNA hybrid (\( n-1 \) position). In a scaffold EC where the pC probe was at position \( n+1 \), the fluorescence emission is low. Upon primer extension, following the addition of ATP, the probe moves to position \( n \), where it becomes unpaired, causing an increase in the fluorescence quantum yield. At the same time, no changes in fluorescence emission were observed when ECs containing the pC probe at position \( n+2 \) and \( n+3 \) were incubated with the cognate substrates, confirming that no DNA melting occurs at this region.

In a separate experiment, we examined whether the addition of several cognate NTPs results in stabilization of a possible transient melting of the downstream DNA in RNAP EC. For this, we incubated the ds-scaffold *E. coli* EC (R9/TSC1/NTC1) having pC at the \( n+1 \) base with AMPCP (\( n \) and \( n+2 \) NTP) and GTP (\( n+1 \) NTP). No changes in fluorescence emission were
detected, indicating that the presence of the substrate NTPs does not affect melting of the downstream DNA (data not shown).

**DISCUSSION**

The mechanism by which substrate NTPs are selected and loaded into the RNAP active site is of fundamental importance to our understanding of the transcription process (and indeed to all template-directed nucleotide polymerization reactions) (22). In view of the different models that have been proposed for this step, it is important to establish the extent of melting of the downstream DNA duplex and the availability of the template strand bases for substrate binding and formation of incipient base pairs. Structural analysis for multisubunit RNAP ECs may eventually provide an answer to this question, as they did in the case of T7 RNAP (19, 23). Biochemical approaches to probe melting of the DNA duplex have included endonuclease and permanganate footprinting; however, these assays are not usually able to provide single-base resolution (24). As fluorescence quenching assays have successfully been applied to questions involving the topology of nucleic acids in a number of protein complexes (4, 11, 17, 20, 25), we utilized this approach in this work.

Our choice of pC as a fluorescent analog was determined by the emission spectra of the probe, which is far (450 nm) from the protein emission, which thus brings little if any interference into the experimental data. This probe is sensitive to the local DNA environment and was successfully used in promoter melting experiments with T7 RNAP (17). With regard to RNAP studies, the structural picture of substrate binding is the most complete for T7 RNAP as high resolution structures are available for both initiating and elongation complexes. Importantly, in all post-translocated T7 RNAP ECs, the downstream DNA region was formed with fully complementary DNA oligonucleotides, and melting of that region observed in the crystal structure is therefore the result of RNAP binding. Our experimental data with T7 RNAP EC are in agreement with the structural data and indicate that melting of only one base pair in the downstream DNA region is necessary for elongation. Prior to the analysis of the downstream DNA melting in structurally unrelated multisubunit RNAPs from bacteria and yeast, it was important to establish whether the assembled complexes are in the pre-translocated (the 3′ end of the RNA occupies the insertion site) or post-translocated (the 3′ end of the RNA is in the product site) conformation as this may affect availability of unpaired DNA in the downstream region. Thus, in the crystal structure of the pretranslocated T7 RNAP EC, in contrast to the post-translocated EC, the base closest to the RNA-DNA hybrid is paired (26). We therefore used only post-translocated complexes in measurements of fluorescence emission (18).

Importantly, the level of fluorescence emission of pC at positions n+1, n+2, and n+3 for each RNAP (Figs. 1 and 2 and supplemental Fig. S4) is similar and well below the level of fluorescence observed at position n, indicating that there are no significant changes in the topology of the downstream DNA (such as a kink at base n). Therefore, the observed difference in fluorescence emission between ss- and ds-scaffold ECs clearly represents melting of the DNA duplex. The results of the fluorescence quenching assay with both bacterial and yeast RNAP indicate that these RNAPs, similar to T7 RNAP, melt only the base pair closest to the active site, whereas the rest of the downstream duplex remains intact. It thus appears that not only the length of the RNA-DNA hybrid but also the size of the transcription bubble may be conserved between single and multisubunit RNAPs.

The data obtained in our fluorescence experiments apparently contradict the NTP lineup model, which requires the availability of several unpaired template bases downstream from the active site (7). Although diffusion of substrate NTP
into the RNAP active site through the main channel remains possible, loading through the secondary channel should be direct and sufficient for the delivery of the cognate substrate to the place of its base paring with an acceptor DNA base. The extent of melting of the downstream DNA duplex has implications with regard to transcription fidelity. We have recently demonstrated that a number of misincorporation errors in cellular RNAPs can be generated by an NTP-stabilized template strand misalignment mechanism (25). Importantly, the rate of misincorporation by misalignment is significantly increased when the $n+1$ base pair is melted (by means of an abasic or non-complementary site in the non-template strand) (25, 27), suggesting that melting of no more than a single base at a time downstream of the active site is essential for the accurate transcription.

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On page 17849 in Fig. 2B, the Western blot for HMGB1 release in the +DHIQ parp-1−/− panel was inadvertently duplicated in the +DHIQ WT panel during preparation of the final figure. The duplicated panel has been replaced with the correct photomicrograph, and the corrected figure is shown below. The correction does not affect the conclusions of the work.