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Imaging and Analysis of Transcription on Large, Surface-Mounted Single Template DNA Molecules

Hua Yu and David C. Schwartz⁺

Laboratory for Molecular and Computational Genomics, Department of Chemistry, Laboratory of Genetics, University of Wisconsin-Madison, UW-Biotechnology Center, 425 Henry Mall, Madison, WI 53706

Abstract

A surface-based approach is presented for the transcriptional analyses of large, single DNA molecule templates and their imaged reaction products by RNA polymerase (RNAP). Results showed surfaces with a charge density supporting stretching of single DNA molecules to 70–80% of their full contour length were ideal for analysis of T7 RNAP transcription complexes on bound single template DNAs. Such DNA molecules were shown to sustain efficient transcription reactions and analysis, which enabled localization of transcription complexes on templates at kilobase resolution. Direct labeling of nascent RNA transcripts by the incorporation of a second fluorochrome into DNA templates promotes more robust and sensitive detection of punctuates. Further characterization by RNase digestions, AFM studies, and fluoro-immuno-labeling revealed a “supercomplex” structure within a punctate where elongation complexes (ECs) aggregate through entanglement of DNA and RNA strands from individual ternary ECs. We have proposed mechanisms that underlie the supercomplex formation process. Whereas supercomplexes develop naturally in free solution, spatial constraints involved in a topologically limited system where template DNA was bound to the surface may facilitate the assembling process by stalling transcriptional elongation.

Keywords

promoter detection; functionalized surfaces; fluorescence microscopy; T7 RNA polymerase; AFM

Introductory Statement

Single molecule studies of transcription have revealed remarkable insights into individual steps, interactions, and associated reactions, and have been recently reviewed in [1;2]. For direct visualization and tracking of RNAP action, a key step involves effective immobilization and manipulation of molecules facing chain dynamics and Brownian fluctuations. Several early approaches have served such purpose by the immobilization of RNAP to surface substrates and monitoring beads that were attached to individual DNA molecules were being transcribed [3], and by use of patterned DNA templates on surfaces enabling tracking of fluorescently labeled polymerase perturbed by fluid flow [4]. Similarly, loose attachment of DNA templates on mica surfaces allowed time lapse AFM imaging of RNAP action [5]. More recently, manipulation of single DNA molecules by optical or magnetic tweezers have provided precise control and resolution for observing transcription [6;7;8] including basepair resolution during “stepping” [9].

⁺To whom correspondence should be addressed: dcschwartz@wisc.edu, 608-265-0546; 608-265-6743 (FAX).

This paper reports the basis for a high-throughput transcription analysis system that utilizes fluorescence imaging and analysis of transcription performed on template DNA molecules stretched and immobilized on surface substrates. Accordingly, to achieve high-throughput, immobilized individual DNA molecules must remain biochemically competent despite their presentation in a stretched linear form. This is important, since precise localization of transcription sites on large DNA templates depends on a usable distribution of stretched analyte molecules. Consequently stretching long DNA molecules from their solution relaxed coil conformation to a linear elongated form has previously been achieved through application of precise mechanical forces in optical and magnetic traps. However, such approaches do not readily foster inherent statistical advantages derived from arrays presenting large numbers of individual analyte molecules. Consequently, arrays of immobilized DNA molecules on surfaces readily support the acquisition of data from statistically meaningful populations of stretched substrates. Such arrays are engendered by means including electrostatic fields [10; 11], fluid flows [12], DNA hybridization [13], and through various chemically patterned surfaces or small scale devices [14]. An important consideration in the development and use of single molecule arrays is preservation of the biochemical competence of deposited analyte molecules. In this regard, a method that used harsh buffer pH conditions produced overstretched DNA molecules to 150% of their contour length, which inhibited transcription activities [15]. In contrast, the Optical Mapping (OM) system creates massive arrays of large DNA molecules that are stretched by fluid flows in microchannels. Immobilized molecules are stretched 50% –90% of their polymer contour length, varied by mounting conditions such as the fluid flow rate, channel dimensions, and surface modifications [11]. We have shown that DNA molecules immobilized on OM surfaces are “bioactive” template molecules that support various biochemical reactions, such as restriction enzyme digestion [16], nick-translation labeling [10], fluorescent nucleotide incorporation [10;17] for sequencing [18].

Recent applications of the OM system [19;20;21;22] enabled comparative analysis for entire genomes [23], and supported imaging of *in vitro* transcription products associated with templates mounted on OM surfaces for visualization, quantitation, mapping locations and statistical analysis [24].

Experiments described here demonstrate that modified OM surfaces support efficient and specific transcriptional initiation from T7 RNAP promoters on surface immobilized template DNA molecules. We show that this approach also enables accurate mapping of transcriptionally active loci for promoter identification on template DNA in ways that portend high-throughput analysis. Properties of transcriptional products formed *in vitro* by our method are examined from the analysis of fluorescence micrographs. Furthermore, the underlying structures that compose observable punctates used to track transcriptional action are thoroughly characterized. A “supercomplex” model for describing the structure of these punctates is proposed here (Figure 1). Briefly, we reasoned that supercomplexes may consist of multiple, co-localized elongation complexes (ECs) that aggregate on DNA templates through entangled networks of DNA/RNA strands wrapped around polymerases. We shall show analysis regarding molecular components, structural conformation and mechanisms that underlie supercomplex formation.

Materials and Methods

DNA preparation

Cosmid DNA (44,675 bp) was isolated from the cosmid LANL-16c_380H5 (“380H5”; accession number AC004233, Los Alamos National Laboratory) that had been transformed into DH10B™ competent cells (GIBCO BRL, Gaithersburg MD). DNA was purified using a QIAGEN-tip 100, linearized with Sal I (New England Biolabs [NEB], Beverly MA), extracted with phenol-chloroform, and ethanol precipitated.

BAC RS218-BAC614 (82.464 kb) DNA was prepared following standard protocols and linearized with Avr II (NEB).

Surface cleaning and derivatization

22×22 mm² glass cover slips (Fisher Scientific) were cleaned in an 8:1 H₂SO₄:30% H₂O₂ solution for 50 min at 70–75° C and then boiled in concentrated hydrochloric acid for 6 hours. Cleaned surfaces are then derivatized with 90–150 µL of N-trimethoxysilylpropyl-N,N,N-trimethylammonium chloride silane (“trimethylsilane”; Gelest Inc., Morrisville PA) in 250 ml distilled water at 50 rpm, 65° C for 17.5 hr and stored in distilled ethanol. Derivatized coverslips were rendered hydrophobic by vapor deposition of 500 µL dimethyldiethoxysilane (DMDES, Gelest Inc.) at 37° C in a sealed 50 mL conical tube until the desired surface contact angle was achieved.

Surface contact angle measurements

Drop shape analysis was performed to measure surface contact angles. A 2 µL droplet of distilled water was pipetted on the coverslip lying flat on a positioning stage and allowed to rest. The stage position was adjusted to obtain a centered video image of the drop through an Intel Play QX3 computer microscope (Intel Corporation, Santa Clara, CA). The diameter (d) and height (h) of the water bead were measured from the captured side image. Surface contact angle (θ) was calculated by the following formula: $\theta = 2 \tan^{-1} 2h / d$.

DNA mounting and *in vitro* transcription reaction

Template DNAs were diluted in TE to a concentration (~0.1 µg/mL) optimal for visualizing under fluorescence microscope. DNA solution was drawn (5 µL) between a coverslip and a glass slide by capillary action, then peeled off on a coverslip and air dried.

Surface-mounted DNA molecules were incubated in 100 µL 1× RNAP Buffer (NEB), 0.5 mM each of ATP, UTP, GTP, and CTP (Roche Applied Science, Indianapolis IN) and 0.02% Triton X-100 (Sigma, St. Louis MO). After initiation by adding 100 units of T7 RNAP (NEB) to the surface, transcription reaction was incubated at 37° C for 15–60 min in a humidified chamber, followed by washing twice with 200 µL water. For incorporating fluorescently labeled UTP, UTP was 0.1 mM and contained 1/20th volume of ChromaTide® Alexa Fluor® 546-14-UTP (Molecular Probes, Inc., Eugene, OR). Other components remained the same as described above. Additionally the surface was washed twice with ATP solution at 37° C for 5 min to reduce fluorescence background.

In vitro transcription reactions (20 µL) for AFM analysis contained the cosmid DNA 380H5 at 10 µg/mL, 5 units T7 RNA polymerase, and 0.5 mM in NTPs (1:5000 3'dNTP:NTP), 1× transcription buffer (40 mM HEPES (N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid), 6 mM MgCl₂, 2 mM spermidine, 10 mM DTT (Dithiothreitol) at pH 8.0). The reactions were incubated at 37° C for 15 min., stopped by putting on ice and crosslinked with 1% formaldehyde (Sigma) at 4° C for 30 min. The mixture was diluted in HEPES buffer (to ~0.1 µg/mL in template DNA) prior to mounting on the surface for AFM analysis.

Digestion after transcription

For RNase digestion, after transcription, 100 µL digestion buffer (1x NEB Buffer 2, 0.02% Triton X-100 [Sigma], with 1 µL RNase H [10 units, Epicentre Biotechnologies, Madison WI] or RNase T1 [1000 units, Epicentre Biotechnologies]) was added to the surface and incubated at 37° C for 15 min. The digestion buffer was then aspirated off and the surface was rinsed with water and TE before staining with YOYO-1.

Restriction enzyme digestion on the surface was done in 100 μ L digestion buffer with enzyme (1x NEB Buffer 2, 0.02% Triton X-100, with 3 units XbaI [NEB]) and incubated at 37° C for 10 to 15 min.

Immuno-labeling of elongation complexes

Following transcription conducted on the surface, surface-mounted molecules were incubated in 100 μ L 10 mM Tris buffer (pH 8.0) with mouse anti-T7 RNA Polymerase Monoclonal Antibody (1:1,000 dilution; Novagen, San Diego CA) and BSA at 0.1 mg/mL (NEB) at 37° C for 60 min. Surfaces were washed twice with 200 μ L high purify water, and incubated in 100 μ L 10 mM Tris buffer (pH 8.0) with Alexa Fluor® 546 goat anti-mouse IgG antibody (H+L; 1:1000 dilution, Molecular Probes) at 37° C for 60 min. Surfaces were then washed with 200 μ L water at room temperature twice for 5 min to remove excess fluorescent molecules.

Staining, fluorescence image acquisition and processing

For YOYO-1 staining, surfaces were mounted onto a glass slide with 6 μ L 0.2 μ M oxazole yellow homodimer(YOYO-1, Molecular Probes) in 20% (v/v) β -mercaptoethanol in TE, and sealed with nail polish.

Surfaces were imaged by fluorescence microscopy with a 100x objective (Zeiss) and a CCD camera (Princeton Instruments). YOYO-1 stained DNA (green) images were collected with XF115-2 filter set (Omega Optical), while red images of AlexaFluor-546-UTP were collected with XF108-2 filter set (Omega Optical). Image collection was performed by a fully automated image acquisition system developed by our laboratory (Channelcollect) [11]. Image analysis was done similar to the process described previously [24]. Transcription products were manually marked and analyzed with the image editing software, Omari, which assigns contour length in pixels for each DNA fragment and EC, as well as integrated fluorescence intensity for each fragment and EC.

Atomic Force Microscopy (AFM)

Freshly cleaved mica surfaces (20 mm $\times$ 20 mm; Ted Pella, Redding CA) were incubated with 20 μ L 0.01% aminopropyltriethoxysilane (APTES; Gelest Inc.) water solution at room temperature for 10 min, which was then aspirated off. A drop of 5 μ L of cosmid DNA 380H5 (0.1 μ g/mL) bearing elongation complexes (ECs) was spread on a mica surface by covering with a clean glass cover slip. The cover slip was peeled off after 5 min, and the mica surface air dried before imaging. Tapping mode scans on mica in air with a Metrology Probetm Tap 300 (Ted Pella, Inc., Redding CA) tips were used on a BioScope with NanoScope IIIa controller (Digital Instruments, Santa Barbara CA).

Results

Transcription on hydrophobic surfaces

Our previous results have shown that *in vitro* transcription conducted in “test tubes” produce ECs at positions of transcript synthesis that are readily identified by fluorescence microscopy imaging after staining with YOYO-1 [24]. However, transcription by T7 RNAP performed on pre-mounted template DNA on standard OM surfaces was inefficient – few template molecules (<10%) were observed bearing punctates corresponding to transcription products (Figure 2a).

We found that transcription efficiencies of DNA templates immobilized on surfaces were significantly improved by increasing the hydrophobicity of the supporting surface. Standard OM surface modification procedures use trimethylaminosilane for patterning positive charges on glass surfaces for the electrostatic binding of DNA templates. OM surfaces were then rendered hydrophobic by treating with a hydrophobic silane, dimethyldiethoxysilane

(DMDES), prior to DNA mounting. We tested cosmid 380H5 DNA that bears a T7 RNAP promoter sequence as the template for T7 RNAP transcription. DNA molecules and associated transcription products were then imaged following staining with YOYO-1. As illustrated on Figure 2b, a majority of the template DNA molecules were found to have associated ECs appearing as bright punctates, indicating significantly improved transcription efficiency on template bound to DMDES treated OM surfaces.

Transcription efficiency increases as surfaces are rendered more hydrophobic (higher contact angle). The optimal surface contact angle (hydrophobicity) was determined to be between 80° and 90°. For example, 46.9% of template DNA molecules were found to have associated punctates in an experiment conducted using surfaces derivatized by 150 μ L trimethylsilane and a contact angle of 85°. Encouragingly, this yield was comparable to previous *in vitro* transcription reactions conducted in test tubes. However, aggressive derivatization rendered surfaces with contact angles above 90°, suppressing the spreading of reaction mixtures.

DNA elongation on hydrophobic charged surfaces

Surfaces that were derivatized with various amounts of trimethylsilane (90 to 150 μ L yielding “90” to “150” surfaces) had similar profiles of contact angle alteration during the course of hydrophobic treatment by DMDES (Supplementary Figure 1), with surface contact angles optimized at 80° to 90° after 2 to 3 days of vapor treatment. DNA stretching varied due to differences of charge densities distributed on the surface substrate, so that DNA molecules were stretched to 70–80% of their full contour length on 150 trimethylsilane surfaces (Supplementary Figure 2), but only to 50–60% on 90 trimethylsilane surfaces (data not shown), even though surface hydrophobicity was held constant as measured by surface contact angle (~85°).

Because the imaged length of DNA fragments was used for calculating their sizes, optimally stretched DNA templates offered greater spatial resolution and precision in determining EC positions as well as minimizing the occurrence of poorly stretched “blobs” of DNA that would obscure EC recognition. It was noted, however, that transcription efficiency dropped when too much charged silane was conjugated to the surface since higher charge densities promoted DNA overstretching, which has been previously shown to inhibit transcription activity [15]. We determined that stretching template molecules to 70% to 80% of their full contour length was optimal for identifying ECs with confidence while keeping sufficient transcription efficiency, which was the case on 150 trimethylsilane surfaces that we determined to use in subsequent experiments.

Transcription product detection by fluorescence microscopy

Our previous results showed that imaged punctates were ECs being paused or arrested at the DNA backbone during the transcription process under certain experimental conditions [24]. An EC may consist a long strand of nascent RNA produced from a single round of transcription [24], while the majority of ECs we observed consist of a “supercomplex” which resulted from multiple rounds of transcription from the same promoter being packed together. The molecular component and structure for “supercomplex” will be discussed in greater detail in later sections of this paper. Because OM generates ordered restriction patterns of DNA templates, ECs can be mapped to specific locations of their synthesis based on the alignment of template restriction patterns to a reference map produced from the DNA sequence [11;19;25].

To locate ECs, template cosmid 380H5 was subsequently digested by XbaI and mapped by OM. Gaps produced at XbaI cleavage sites on DNA backbones marked DNA digestion patterns, revealing a given molecule’s orientation [26] and potentially, its identity, when dealing with random genomic DNA template molecules [25]. Examples of fluorescence images

of XbaI digested template molecules bearing ECs are shown in Figure 2c. Through visual examination of restriction fragments, one quickly determines the molecular orientation and thus the EC location. The DNA molecules shown here were not fully digested into 4 fragments—the cut near the promoter was missing in all 4 of the DNA molecules (Figure 2c). However, the resulting pattern was still sufficient for correctly assigning DNA orientation. In fact, in this case only one cut would be sufficient for establishing template orientation. A total of 227 DNA molecules that showed associated ECs and a correct XbaI digestion pattern were analyzed. Peaks in the fluorescence intensity profiles above the baseline intensity of the DNA backbone indicate the presence of ECs. Sizes of DNA fragments (in kb) bearing ECs were assigned by OM alignment to the reference map (Figure 2c). Subsequently, EC positions were mapped by measuring integrated fluorescence intensity of intervening YOYO-1 signals on the DNA template, which scales with mass [27].

The resulting distribution of EC positions is shown in Figure 3a. Although there was not a termination sequence associated to the cosmid DNA, ECs produced in this way generally did not move more than a few kilobases downstream of the promoter (15.961 kb). The histogram of EC in Figure 3a was fit by a Gaussian where the peak position was determined at 16.5 ± 0.1 kb, which is 0.5 kb downstream from the known promoter.

The apparent spread of the distribution is partly due to the resolution and errors associated with our DNA fragment sizing method. Such errors may also explain possible outliers appearing as transcription products upstream from the promoter, in Figure 3a, left to the 15 kb bar, where ECs are not expected. Additionally, non-specific transcription could also contribute to the presence of punctates at unexpected positions [24].

One-way blind test for localizing a T7 RNAP promoter on an unknown template

Since ECs produced on surfaces can be trapped and detected as a distribution encompassing a known promoter site, a “blind test” was then performed in which a linearized 82.464 kb bacterial artificial chromosome (BAC) was used as the transcription template, the identity of which was not known at the time of promoter prediction. While most of the template DNA molecules had only one associated punctate that corresponded to the presence of EC, occasionally few DNA template molecules were detected bearing two or more punctates. Generally, such punctates were closely adjacent to each other, suggesting a spacing characteristic of multi-round transcriptional events originating from the same promoter. Thus, these results strongly suggested that there was only one promoter on the unknown DNA template. A histogram plot of the EC distribution on 562 analyzed DNA molecules is shown in Figure 3b. Gaussian fit to the distribution determined the EC peak position to be at 16.8 ± 0.1 kb along the DNA backbone.

During the course of transcription, the ability of a T7 RNAP to elongate downstream while dragging the nascent transcript along on a surface substrate was considered to be mainly restricted by local attachments of the transcription complex to the surface. Accordingly, the electrostatic and hydrophobic interactions presented by derivatized surfaces most likely governed the tabulated locations of ECs near promoters. These interactions affected DNA template stretching and non-specific absorption that competed with an EC's processive binding to templates.

To predict the promoter location, a 0.5 kb offset was included that accounted for the distance of an EC to transcribe downstream from the promoter along the DNA template; this offset was obtained from the experiment described earlier using identically prepared surfaces. Thus, the promoter was located at 16.3 kb, which was deliberately offset 0.5 kb upstream from the experimentally determined position of EC (16.8 ± 0.1 kb) from analysis of the histogram shown in Figure 3b. The actual location of the T7 RNAP promoter on this BAC template DNA was

later revealed to be at 15.487 kb from the sequence. Thus, this promoter prediction, based on the detection and mapping of transcription products showed kilobase accuracy.

Incorporating fluorescently labeled nucleotides during transcription on surface-bound template DNA

Direct labeling of nascent RNA transcripts by the incorporation of a second fluorochrome into DNA templates may promote more robust and sensitive detection of punctuates. Accordingly, AlexaFluor-546-UTP (red) was chosen for transcriptional incorporation since its spectrum is distinguishable from YOYO-1 stained DNA (green) using two-color imaging techniques. Figure 4a–c shows a set of representative images of T7 RNAP incorporated AlexaFluor-546-UTPs on the 82.464 kb BAC, which is the same BAC DNA template used in the previous blind test experiment. The correlation between the labeled transcripts (red) and DNA backbone (green) features (shown in Figure 4c) indicates the presence of ECs. To remove excessive AlexaFluor-UTPs after labeling, the surface was incubated and rinsed with ATP solutions. Fluorescent background from the AlexaFluors is greatly eliminated this way, although residual AlexaFluors attach to the glass surface, contributing to the background that is not associated with DNA templates. Nevertheless, intense fluorescent signals from incorporated multiple AlexaFluors within individual ECs are easily distinguished from background noise that colocalizes with the DNA backbone.

Detected ECs were divided into two groups: (i) ECs that were captured in both green and red channels, and (ii) ECs that were captured only in the red channel while not in the green channel due to weak morphological signatures possibly buried in DNA backbone signal. All ECs that were detected in the green channel, utilizing punctate morphology, also appeared in the red channel (group (i), see molecule #1 in Figure 4 for an example). In contrast, a subset of labeled transcripts, although readily detected in the label (red) channel, did appear sufficiently morphologically distinct from DNA backbones to be detected in the green channel (group (ii), see molecules #2, 3 in Figure 4). This experiment demonstrated that while one-color YOYO-1 staining and imaging approach, efficiently detects ECs (group (i)), it is not as sensitive as the two-color approach, based on spectral discrimination, at detecting morphologically indistinct punctuates (group (ii)). As such, a larger population of ECs (group (i) and (ii) combined) can be confidently detected in the two-color approach than in the one-color YOYO-1 staining. These results showed that fluorochrome-labeled RNA transcripts and two-color imaging augmented the detection sensitivity of ECs while reducing the requirements for optimally stretched analytes.

To examine the positions of ECs detected by labeling (including groups (i) and (ii)), the distribution of ECs on BAC DNA were plotted in Figure 4d. Aside from the primary peak at position similar to ECs produced without the labeling, an additional weaker peak that is further downstream is present. The distribution was fit by several Gaussians and the positions for the primary and secondary peaks were determined to be at 17.2 ± 0.2 kb and 22.0 ± 0.2 kb, respectively.

The primary EC peak is slightly downstream from the position determined by previous experiments on the same BAC DNA template (16.8 kb). This difference may be explained by less extended DNA template observed in labeling experiments which apparently allows the EC to transcribe further downstream. This possibility is confirmed by a relative wider spread of ECs downstream from the promoter on the template.

The secondary peak showing at 22.0 kb may originate from aggregates of incorporated labels in the nascent transcripts which accumulate to the extent that prohibits the EC from elongating further. The peak is 6.5 kb from the promoter, which allows approximately 80 labeled UTPs to be incorporated assuming equal incorporation capability between labeled and unlabeled

UTP. The number of labels within an EC of this size may pose a considerable obstacle that arrests ECs, possibly increasing the presence of ECs at this arrested site.

Distribution in (e) (representing EC group (i), with a primary peak located at the 17 kb and 18 kb bin) shifts slightly downstream from the promoter (located at 15.487 kb) as compared to distribution in (f) (representing EC group (ii), with a primary peak located at the 16 kb bin) since only signals in the green channel are considered. This is expected, as ECs would need to transcribe a longer distance downstream for accruing fluorescence signals sufficiently distinct from that of the DNA backbone in the background. Importantly, these results show that detection of incorporated AlexaFluor-546-UTP signals (red channel) reveals additional ECs, which may not be confidently identified in the DNA (green) channel.

RNase digestion

We wanted to examine molecular components of T7 RNA polymerase (RNAP) transcription products that hug surface-bound template DNA molecules after nuclease digestion (Figure 5). T7 RNAP transcribed surface mounted cosmid DNA (“380H5”) depositing punctates along their backbones that were then treated with RNase T1, which cleaves single-stranded RNA. This step eliminated ECs, indicating that transcript RNA is an essential component for EC maintenance. However, RNase H digestion, which degrades RNA/DNA hybrids, did not annihilate punctates residing on DNA templates, suggesting punctate stabilization involves an entangled network of RNA and ECs rather than solely DNA/RNA hybrids.

Supercomplex structural analysis by AFM

Since atomic force microscopy (AFM) discerns structural information at the nanometer scale, we used it for revealing the morphology, composition and functionality of surface bound EC/template complexes. However, published EC structures are commonly formed on very short DNA templates (<1 kb) [28] because relaxed large DNA coils obscure imaging of small complexes. To solve this problem, we have developed a technique termed “fluid fixation” to elongate lambda bacteriophage DNA molecules by convective fluid flow within an evaporating droplet of DNA solution on mica surface [29]. Here we prepare elongated DNA molecules for AFM imaging by adaptation of a method used in preparation of aminopropyltriethoxysilane (APTES) modified OM glass surfaces that elongates DNA molecules in high-throughput [16]. Accordingly, freshly peeled mica was briefly treated by APTES in water. The cosmid 380H5 DNA bearing ECs was then immobilized onto APTES-mica surface by sandwiching DNA solution between a coverslip and a modified mica surface. This approach, confirmed by fluorescence microscopy, produced uniformly elongated large single DNA molecules so that associating ECs could be discerned by AFM without interference from relaxed DNA strands. Transcription reactions were conducted in a nucleotide mixture containing 3'-dNTPs which produced ECs at approximately 5 kb from the promoter.

AFM imaging (Figure 6) revealed an assembly of globular elements localized as punctates binding DNA backbones, consistent with our “supercomplex” model (Figure 1), which predicts co-localized masses comprising multiple ECs associated to templates. Each of these globular elements was approximately 4 nm in height, in agreement with dimensions of T7 RNAP [30], suggesting a ternary EC configuration. Meanwhile, upstream from a relatively large punctate stalled at the expected position, a few additional punctates of smaller sizes existed. Such smaller complexes would likely be too small to be discerned from DNA backbones based merely on fluorescence imaging without specific labeling. It was estimated that there were generally 10 or more ECs in a fluorescently detectable supercomplex.

Fluoro-immuno-labeling of RNAP for estimation of number of ECs

Immunological fluorescent labeling was used for estimating the number of ECs composing a supercomplex. RNAPs were assayed using a mouse T7 RNAP monoclonal antibody targeting RNAPs after transcription. A secondary goat anti-mouse IgG (H+L) conjugated by AlexaFluor-546 dyes was then applied to form a sandwich complex to the RNAP. Aside from labeled ECs that associated with DNA templates, significant fluorescence signals distributed randomly on surfaces were also detected. This was not observed in control experiments where the primary antibody was spotted on surface and later incubated with the secondary antibody, suggesting immuno-labeled RNAP that randomly stick to the surface as the origin of these signals. Given the low concentration of RNAP and wash conditions that were used, we assumed no substantial aggregation of RNAPs that randomly absorbed to surfaces. Because both the binding of antibodies to their target sites and the number of fluorescent dyes that have been attached to the secondary antibody were quantitative, individual labeled RNAP can be fluorescently imaged and the number of RNAPs in supercomplexes quantitated. Therefore the fluorescence intensities of these signals (plotted in Figure 7, peaks at $(6.29 \pm 0.16) \times 10^4$ a.u.) could serve as the reference that represents the fluorescence signal of an individual immuno-labeled RNAP allowing rough estimation of composition of a given EC.

Fluorescence images were compared after immuno-labeling and YOYO-1 counter-staining. Fluorescence signals in the labeling channel were categorized as: (1) “YOYO-1 non-detectable supercomplex” – small complexes that could not be recognized in the YOYO-1 channel, while the signal in the labeling channel could be assigned to DNA backbone at expected position close to promoter; (2) “YOYO-1 detectable supercomplex” – large complex that could be recognized in both YOYO-1 channel and labeling channel; (3) “reference” – immuno-labeled RNAP that randomly distributed on the surface. Distributions of group (1) and group (2) were plotted in Figure 8. Peak intensities of immuno-stained supercomplexes were divided by the intensity of the reference peak (Figure 7), resulting the estimated number of RNAPs/ECs in a typical YOYO-1 detectable supercomplex (group 2) to be 19, whereas in a typical YOYO-1 non-detectable supercomplex (group 1) the estimated number of RNAPs/ECs is 10.

Discussion

By carefully controlling the surface charge density as well as hydrophobicity, optimal conditions for transcription of surface-bound template DNA molecules were established. Since hydrophobic surfaces lacking any charge were poor surfaces for retaining DNA molecules efficiently (data not shown), charges present on modified OM surfaces may provide DNA attachment sites irregardless of whether the surface is hydrophilic or hydrophobic. The approach presented here for arraying long DNA templates supported transcription on surfaces and enabled several detection schemes. Consequently, such experimental conditions fostered DNA stretching which allowed transcription reactions with efficiencies comparable to *in vitro* transcription reactions conducted in test tubes, as well as restriction endonuclease action, sufficiently robust for OM analysis. Meanwhile, DNA molecules were sufficiently elongated for enhancing the spatial resolution of transcription complexes for kb discernment of promoter locations.

There are several plausible reasons explaining why rendering surfaces hydrophobic markedly improves their ability to efficiently support transcription. One possibility is that the synthesized RNA is retained locally on the surface by hydrophobic interactions, contributing to the formation and stabilization of the ternary DNA-RNAP-RNA complex during transcription. Another consideration concerns RNAP enzyme activity on surfaces. Interactions between surface charges and the RNAP binding sites may inhibit the promoter recognition function of the RNAP, as well as prevent the RNAP from elongating along templates. Hydrophobic features on surfaces may help to work against these inhibiting interactions imposed by charged

surfaces and therefore free up RNAP molecules for optimal transcriptional action. We also could not rule out the hypothesis that DNA elongates in a different manner on hydrophobic surfaces favoring the transcription process, although the apparent extent of DNA stretch may be the same as on hydrophilic surfaces.

Transcription products were defined in these experiments by their substantially higher fluorescence intensity above DNA backbones. Evidence for the need for DNA wrapping around polymerase during transcription have been reported for T7 RNAP [31] and *E. coli* RNAP [32;33], which may contributed to the elevated fluorescence intensity measured at the EC site. Nevertheless, the combination of synthesized RNA strands and compressed DNA template within an EC may not be sufficient to give rise to significant jumps in local fluorescence intensity. Accordingly, we have proposed a “supercomplex” model that considered multiple ECs originating from the same promoter as becoming entangled with each other forming bright aggregates that were readily flagged by just YOYO-1 staining.

Labeling ECs through the incorporation of fluorescently labeled nucleotides increases the sensitivity of detection of transcriptional events by revealing morphologically undetectable ECs through spectral discrimination. Out of a total of 291 template molecules bearing labeled ECs, 150 molecules had ECs that were morphologically indistinct in the YOYO-1 detection channel, but were revealed in the Red, AlexaFluor channel. Since multiple fluorescently labeled nucleotides can be incorporated within a single round of RNA elongation, shorter transcription length and fewer ECs within a supercomplex would be needed to be recognized as an EC by fluorescence detection. Since the enhanced detection sensitivity enables the detection of smaller complexes, the system may be used to detect less efficient transcription as well as weak promoters. In the case where a better control of positioning ECs at locations closer to promoter is required, possible experiments may involve using more stringent surface or reaction conditions, such as higher charge density, or a high content of 3'-dNTP in the reaction mixture. ECs prepared in this way would contain multiple fluorescent labels at position at close proximity to the promoter (e.g. tens to a few hundreds of bp away), which might greatly increase the precision in the promoter identification. The results presented here suggest that although such labels could be incorporated nearby promoters, their detection and localization to a template will be affected by several factors that include EC structure, fluorochrome brightness, and the variance of DNA stretching. Depending on the number of fluorescent dyes incorporated, the imaging of the ECs can be achieved either by 2-color imaging (as shown in the experiment and result), or by fluorescence resonance energy transfer (FRET) between YOYO-1 stained DNA and the labeled RNA transcript [34].

Transcription elongation by T7 RNAP is highly robust *in vivo*, with little tendency to pause, arrest and backtrack. Nevertheless elongation is often impeded *in vitro* by potential intrinsic and extrinsic elements, resulting in transient or permanent stalling of transcription complexes. Topological constraints in transcription exist in both topologically open systems and fixed boundaries. Long nascent transcripts in a free solution may accumulate enough frictional drag that would eventually inhibit RNAP translocation and rotation [7;35]. Meanwhile transient torque develops as RNAP pushes, pulls and twists DNA even in linear unanchored templates [36;37]. Both mechanisms produce punctates we have observed in free solution [24]. On the other hand, for transcription reactions performed on surfaces, RNAP alone has to overcome spatial and rotationally hindrance imposed by the surface as well as intrinsic transcription stress. Electrostatic interactions between RNA and positive charges on surface impose additional drag against EC translocation. In addition, since only segments between DNA anchoring points can be actively transcribed, transcription pausing/arresting may occur if specific anchoring was too strong due to charge aggregations on the surface. Finally, accumulating supercoils as topologically limited DNA template is treaded through the RNAP [38;39] pose growing barriers against transcription elongation [40]. All these topological

limitations involved in the system explain the observation that ECs would transcribe only a limited distance, usually within a kb, in transcription supported by surface-immobilized DNA template.

Once a leading EC is stalled, it may act as a “roadblock” that induces the formation of a supercomplex from trailing ECs initiated from the same promoter, as observed in our AFM images. ECs may behave as “partners” to rescue the leading EC by traversing intrinsic and extrinsic obstacles so that the transient supercomplex, which as demonstrated in the immunolabeling experiments has approximately 10 ECs, would dissociate and transcribe further [41;42]. In the case of large supercomplexes, DNA/RNA wrapping and entangling from neighboring RNA chains [43] may cause attempts to push the leading EC forward to fail. ECs then accumulate further and form stable supercomplex that typically consists of 19 ECs and can be detected by fluorescence imaging.

In summary, we have revealed the molecular organization of supercomplexes comprising ECs produced by *in vitro* transcription which provided insights into how these complexes form. This understanding will aid development of the Transchip system for large-scale, transcription analysis, by improving optical detection of RNAP products, localization of punctates at defined positions, and controlling the lengths of transcripts.

The single molecule modalities approach presented demonstrate great stability, efficiency and specificity for high-throughput transcription analysis on large, surface-bound DNA template. Identification and characterization of promoters or transcription starting sites (TSSs) based on this method was shown to be within kilobase accuracy, making this approach a viable system for rapid genome-wide identification of TSSs. This approach also has the potential for systematic investigation of gene expression and regulation on a whole-genome scale, given *in vitro* systems or *in vivo* crosslinking of transcripts to templates.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. “Supercomplex” structure

This supercomplex model describes transcription complexes (punctates) produced by *in vitro* transcription using the methods described in this paper. As proposed, a supercomplex comprises multiple ECs that form an entangled network of DNA/RNA strands wrapped around polymerases.

Figure 2. EC detection and analysis

(a) Typical fluorescence images on a standard OM surface after transcription on cosmid 380H5 DNA. Few DNAs were seen to have associated ECs. (b) Fluorescence images on a DMDES treated OM surface after transcription. Surfaces were derivatized using 150 μ L trimethylsilane, and rendered hydrophobic (contact angle = 85°). Punctates associated to DNA templates correspond to transcription complexes, or ECs, generated on surface. The bottom section is a gallery of representative individual template DNA molecules bearing punctates (indicated by arrows). (c) Locating transcription products (ECs) on restriction mapped molecules. Top section is an XbaI restriction map of the linearized cosmid DNA. Arrow indicates the T7 RNAP transcription start site and its direction. At the bottom is a gallery of fluorescence images of

cosmid DNA molecules that were digested by XbaI. Digestion sites (gaps between DNA fragments) are indicated by unfilled arrowheads. ECs are marked by arrows. Size bars: 5 μ m.

Figure 3. Transcription product distribution on DNA templates. (a) EC distribution on cosmid 380H5 template

The peak location of the EC distribution is 16.5 ± 0.1 kb based on a Gaussian fit to the histogram. Compared to the known promoter location at 15.961 kb, ECs are detected on average at +0.5 kb downstream of promoter. This means that in this specific experiment, the T7 RNAP transcribed an average of 0.5 kb downstream from the promoter. **(b) EC distribution on BAC DNA template ("unknown").** The peak of the EC distribution is at 16.8 ± 0.1 kb position on the template. A prediction to the promoter location of 16.3 kb was made based on the estimation that the transcribed length was the same 0.5 kb on this BAC DNA as on the previously analyzed cosmid DNA (a) immobilized on surfaces treated the same way.

Figure 4. Identification and localization of fluorescently labeled RNA transcripts on BAC DNA (promoter at 15.487 kb). (a–c) A set of representative images Scale bar: 20 μ m. (a) YOYO-1 stained DNA (green channel); (b) AlexaFluor-546 (red channel); (c) superimposition of (a) and (b). Labeling signals associated with DNA backbone images (judging from the superimposed image) are identified as transcription products. Labeling is used to confirm the presence of transcription products identified in the YOYO-1 channel (marked by arrows, molecule #1). Meanwhile, labeling improved the detection sensitivity by identifying transcription products that could not be identified solely by YOYO-1 staining (marked by circles, molecules #2, #3). (d) **Histogram distributions of ECs detected by labeling.** This distribution, which was identified by labeling (red), includes (e) and (f). The distribution was fit by two Gaussians, with the primary peak at 17.2 ± 0.2 kb, and a weaker secondary peak at 22.0 ± 0.2 kb. (e) Distribution of ECs detected in both YOYO-1 (green) channel and labeling (red) channel; *i.e.* molecule #1 in (a–c). (f) Distribution of ECs not detected in the green, but captured in red channel, such as molecules #2, #3 in (a–c). By comparing distributions in (e) and (f), we show that in average, ECs that are detected only in

labeling (f, primary peak at 16 kb bin) are distributed at positions slightly closer to the promoter than ECs that are detected in both labeling and DNA staining (e, primary peak at 17 and 18 kb bins).

Figure 5. Fluorescence micrographs after RNase digestion

Bottom panels (scale bar: 5 μ m) are closeup images that correspond to representative molecules (numbered) from the top panels (scale bar: 10 μ m). RNase T1 (left) stripped virtually all punctates from DNA templates. In contrast, after RNase H digestion (right), most punctates remained attached to templates. Punctates are indicated by arrowheads.

Figure 6. AFM images of supercomplexes

Left: elongated DNA strands bearing punctates (ECs). Right: a closeup scan of representative complexes from the left panel. Straight lines which span across the images are portions of elongated DNA. Only complexes that were above certain size were believed to be detectable by fluorescence microscopy, e.g., the two larger complexes on the left that consist of tens to hundreds of ECs.

Figure 7. Histogram of immuno-labeled reference RNAP based on integrated fluorescence intensity measurements

Gaussian fit to the histogram shows a peak fluorescence intensity value of $(6.29 \pm 0.16) \times 10^4$ a.u.. This value is used as the “reference” in the RNAP/EC estimation. The breadth of this distribution is mediated by errors stemming the quantitation of fluorescence intensity measurements performed on images and from a slight variation of the number of dyes conjugated to the secondary antibody.

Figure 8. Immuno-labeled supercomplexes distribution by fluorescence intensity and RNAP/EC counting

Representative fluorescence micrographs show molecules (highlighted by white circles) imaged in the YOYO-1 channel (left) and label channel (right). Bars: 5 μm . **(a) YOYO-1 non-detectable supercomplexes.** Peak integrated fluorescence intensity is $(6.48 \pm 0.32) \times 10^5$ using a Gaussian fit; **(b) YOYO-1 detectable supercomplexes.** Peak integrated intensities is $(1.20 \pm 0.03) \times 10^6$ from Gaussian fit. Typical number of RNAPs/ECs in a supercomplex can be estimated by comparing peak intensities of supercomplexes to the reference (Figure 7) (see results).