

fragments (~ 50 aa) produced when σ is cut in this region and analyzed with a C-terminal antibody. The comparison of the inferred *Taq* cut sites on σ , β , and β' with the location of fork-junction DNA in the structure⁵⁹—which should show the upstream interactions—is shown in Fig. 12. The binding surface of the upstream DNA on the *Taq* subunits is crudely outlined by the inferred cut sites from the *E. coli* enzyme, which also include cuts caused by downstream DNA that is not included in the structure.

[7] Iodine-125 Radioprobng of *E. coli* RNA Polymerase Transcription Elongation Complexes

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Radioprobng is based on an analysis of DNA or RNA strand breaks produced by decay of Iodine-125 (¹²⁵I) incorporated into one of the strands. ¹²⁵I belongs to a class of radioisotopes called “Auger electron emitters” that decay by electron capture or internal conversion.^{1–3} Both of these processes create a vacancy in an inner atomic shell that results in a cascade of electron transitions accompanied by the emission of a number of orbital electrons, named Auger electrons after Pierre Auger, who first discovered them in the 1920s. Decay of ¹²⁵I produces, on average, 21 such electrons. The energy of the majority of these electrons is below 1 keV, which is considerably less than the energy of nuclear-generated beta particles. Auger electrons produce breaks in DNA or RNA strands either directly or by generation of OH radicals in “bound” water. It is assumed that the strand breaks are due to the energy deposition or radical attack in the sugar-phosphate moiety because damage to the DNA bases generally does not lead to strand scission.⁴ Due to the extremely low energy of Auger electrons, most of the breaks (90%) are located within approximately one helical turn from the ¹²⁵I incorporation site. In addition, the bases located next to that site are affected by the positively charged (+21 on average for ¹²⁵I) daughter nucleus, which, by stripping electrons from the neighboring bonds,

¹ L. E. Feinendegen, *Radiat. Environ. Biophys.* **12**, 85–99 (1975).

² K. S. Sastry, *Med. Phys.* **19**, 1361–70 (1992).

³ K. G. Hofer, *Acta. Oncol.* **35**, 789–96 (1996).

⁴ H. Nikjoo, C. A. Laughton, M. Terrissol, I. G. Panyutin, and D. T. Goodhead, *Int. J. Radiat. Biol.* **76**, 1607–15 (2000).

can ultimately result in breakage of DNA strands. At distances approximately 7 nucleotides from the decay site, the damage produced by diffusible OH radicals generated in “free” water becomes comparable with that of Auger electrons.⁵ The frequency of breaks produced by Auger electrons depends primarily on the distance from ^{125}I to the target atom (or the group of atoms) because nucleic acids and proteins are as “transparent” for the low-energy Auger electrons as water. Therefore, radioprobng, like “low resolution” NMR, allows one to obtain information on the interatomic distances, and, in principle, to reconstruct the 3-dimensional (3-D) structure of nucleic acids in complexes with proteins.

Due to the complex nature of DNA breaks and multiple mechanisms involved, there is no simple analytical relationship between the frequency of breaks at a given base and the distance to the sugar attached to this base. By analyzing breaks in the DNA strands in a DNA-protein complex with known 3-D structure,⁶ we established an empirical relationship between the frequency of breaks and the distance from the site of ^{125}I decay.⁷ Thus, by measuring the intensities of breaks in two strands of the DNA duplex in the RecA protein-mediated synaptic complex, it was possible to estimate the distances from the ^{125}I position in the invading strand to the strands of the duplex.⁸ In addition, the profile of the distribution of breaks along a DNA strand depends on the conformation of this strand. By comparing the distributions of breaks in free DNA and in the complex with cyclic AMP receptor protein (CRP), we were able to detect the kink in DNA induced by the protein.⁶ Therefore, two types of information can be obtained from a radioprobng experiment: the distance from the ^{125}I -labeled strand and the target strand, and the conformation of the target strand. Here we present sample results on the interstrand distances and conformation of the RNA/DNA heteroduplex in *E. coli* RNA polymerase (RNAP) transcription elongation complex.

Experimental Scheme

Transcription elongation complexes (EC) were either assembled on short DNA and RNA oligonucleotides or obtained on a promoter-containing template as reported earlier⁹ and described in detail in Chapter 17 of this

⁵ P. N. Lobachevsky and R. F. Martin, *Radiat. Res.* **153**, 271–8 (2000).

⁶ V. N. Karamychev, V. B. Zhurkin, S. Garges, R. D. Neumann, and I. G. Panyutin, *Nat. Struct. Biol.* **6**, 747–50 (1999).

⁷ V. N. Karamychev, I. G. Panyutin, R. D. Neumann, and V. B. Zhurkin, *J. Biomolec. Struct. and Dynamics* **11**, 156–67 (2000).

⁸ V. A. Malkov, I. G. Panyutin, R. D. Neumann, V. B. Zhurkin, and R. D. Camerini-Otero, *J. Mol. Biol.* **299**, 629–40 (2000).

volume.¹⁰ In the first case, a 55-nt long transcribed strand DNA oligonucleotide (T-strand) was annealed to a 8-nt long RNA oligonucleotide, followed by addition of RNAP and a 55-nt long nontranscribed strand DNA oligonucleotide (N-strand). EC was attached to Ni²⁺-NTA agarose beads through RNAP histidine tag. Transcription was initiated by the addition of [¹²⁵I]ICTP to the beads followed by an extensive wash with NTP-free transcription buffer. An aliquot of the resulting EC1 (here and below, the index indicates a distance in nucleotides between the [¹²⁵I]IC and the 3' end of the RNA) containing 9-nt long RNA transcript with [¹²⁵I]IC residue in the active center of RNAP was removed at this point for the subsequent breaks analysis. EC3 to EC10 were obtained similarly by adding the appropriate NTPs, washing and removing aliquots as outlined in Fig. 1A. In all

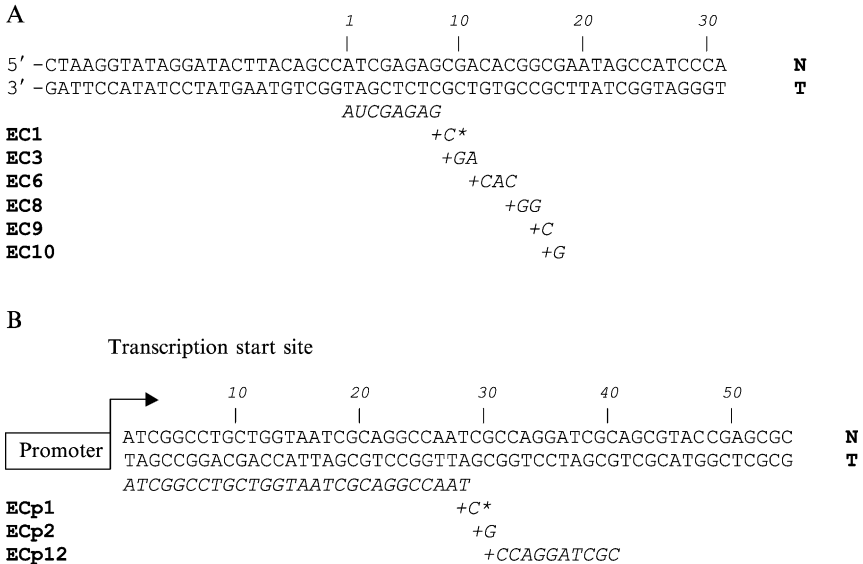


FIG. 1. (A) Sequences of transcribed (T) and nontranscribed (N) strands of DNA duplex and RNA primer (italic). (B) Sequence of the transcribed part of the promoter-containing DNA fragment and the RNA transcript (italic). “+” show the nucleotides added at the consecutive steps of RNAP walking to obtain the corresponding ECs and ECps (listed on the left). C* denote the [¹²⁵I]IC residues.

⁹ I. Sidorenkov, N. Komissarova, and M. Kashlev, *Mol. Cell.* **2**, 55–64 (1998).

¹⁰ N. Komissarova, M. I. Kireeva, J. Becker, I. Sidorenkov, and M. Kashlev, *Methods Enzymol.* **371**, 233–251 (2003).

the complexes, ^{125}I was in the same position relative to the transcribed strand but moved farther away from the active site as the RNAP was “walked” along the template as shown in Figs. 2A, B. DNA strands in the EC were labeled with ^{32}P either at the 5' or 3' end for detection of breaks. The EC samples were stored at -80°C for 14 days for accumulation of DNA breaks (^{125}I half-life is 60 days). The breaks in DNA strands were analyzed in 12% denaturing polyacrylamide gels (see Fig. 3). The intensity of the bands in the gel reflects the frequency of breaks at the corresponding nucleotides. These intensities were measured by fitting the intensity profile of each lane with a series of the Lorentzian peaks representing the individual bands. An example of such a fit for EC1 (see Fig. 3, lane “1”) is shown

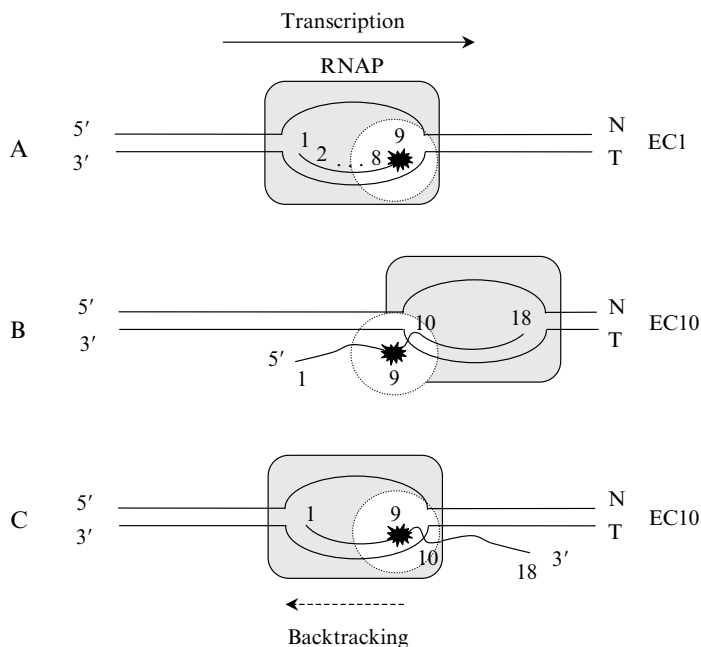


FIG. 2. Experimental scheme. RNAP EC was assembled with two complementary DNA strands and 8-mer RNA primer. (A) At the first step, the ^{125}I ICTP (shown as an “explosion”) was added and incorporated into the active center of RNAP, resulting in EC1. The ribonucleotides are numbered in the 5'-3' direction. (B) Walking of RNAP along the DNA template results in elongation of the RNA transcript and movement of the ^{125}I IC residue relative to the EC. (C) Backtracking of the EC leads to exposure of the 3'-end of the RNA transcript and reverse movement of the ^{125}I IC residue relative to the EC. The circles around the ^{125}I IC residues show the approximate range of ^{125}I decay-induced breaks (i.e., the range of measurable RNA-DNA distances).

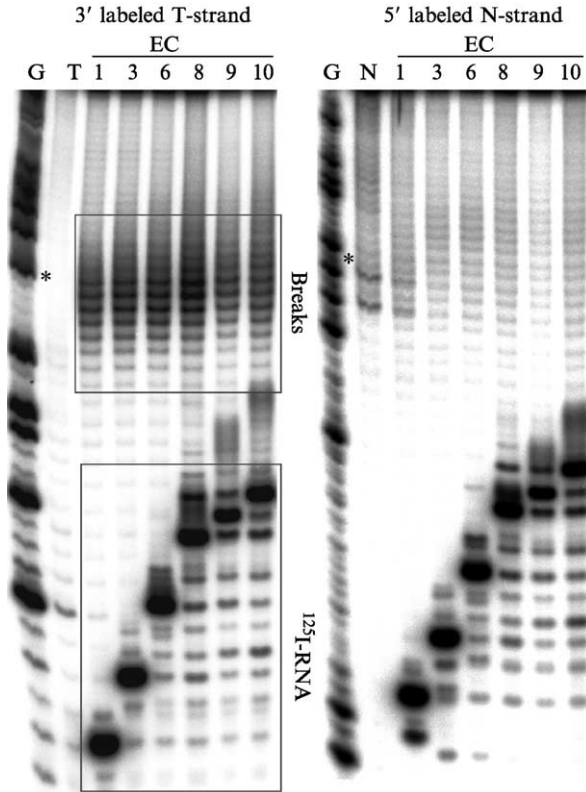


FIG. 3. Analysis of DNA strand breaks in the transcribed and nontranscribed strands in 12% denaturing PAGE. The numbers on the top of the lanes correspond to the analyzed EC. “G”—Maxam-Gilbert G sequencing ladder. “T” and “N” are the respective strands from the assembled ECs before addition of [^{125}I]ICTP. Bands corresponding to ^{125}I -produced breaks and ^{125}I -labeled RNA transcripts are outlined with boxes on the panel of the transcribed strand. Stars mark the positions of the guanine in the T-strand complementary to [^{125}I]IC, and the corresponding cytosine in the N-strand. The T-strand was ^{32}P -labeled at the 3' end and the N-strand was labeled with ^{32}P at the 5' end.

in Fig. 4. The resulting values were normalized and shown as graphs of break distributions for EC1 to EC10 (see Figs. 5 and 6). Based on these distributions, we analyzed DNA and RNA fold in the RNAP EC.

Alternatively, ECs were formed on a 124-bp long DNA fragment containing A1 promoter of bacteriophage T7. Transcription was initiated from the promoter and 28-nt long RNA transcript was synthesized by RNAP walking as described above. At this point, [^{125}I]ICTP was incorporated into

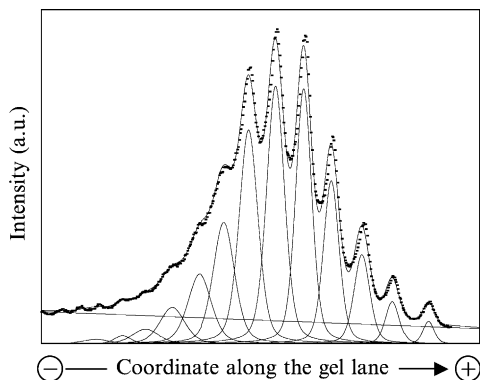


FIG. 4. Deconvolution of the experimental (dots) intensity profile (lane EC1 for the T-strand, Fig. 3) with a series of Lorentz-type peaks corresponding to individual bands. The resulting best fit of the experimental data (the sum of all peaks) is also shown. X axis—distance along the gel; Y axis—intensity in arbitrary units (a.u.).

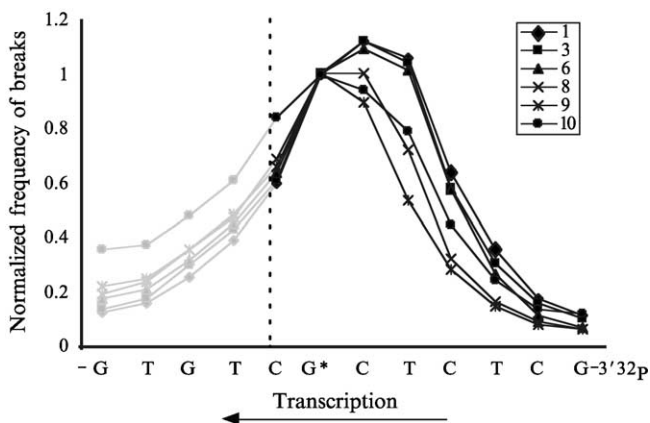


FIG. 5. Relative frequencies of breaks at the individual bases in the T-strand labeled at the 3' end normalized on the intensity at the G* opposite [125 I]IC. Symbols are assigned as follows: “diamond”—EC1, “square”—EC3, “rectangle”—EC6, “cross”—EC8, “star”—EC9, “circle”—EC10. Data points on the left are grayed out (see text for details). Note that the direction of transcription in this orientation of the T-strand is from right to left.

the transcript and two aliquots of ECp1 (“p” stands for promoter-initiated, to distinguish from the previously described EC1) were removed for the subsequent RNA length and DNA breaks analysis. The remaining ECp was walked farther along DNA template. DNA breaks in the T-strand of ECp1, ECp2, and ECp12 were analyzed in denaturing PAGE.

Materials and Equipment

Oligonucleotides were synthesized on an ABI-394 DNA synthesizer (Applied Biosystems, Foster City, California) and purified by polyacrylamide gel electrophoresis (PAGE). T4 polynucleotide kinase and bovine serum albumine were purchased from New England Biolabs (Beverly, MA). Hexahistidin-tagged *E. coli* RNAP was purified and immobilized on Ni²⁺-nitrilotriacetate (NTA)-agarose as described.¹¹ The concentration of the protein was determined by BSA* Protein Assay (Pierce, Rockford, IL). Terminal deoxynucleotidyl transferase (TdT) was purchased from Fermentas (Hanover, MD). RNase A was from Sigma (St. Louis, MO). All chemicals were at least of analytical grade and were purchased from Sigma. Radiochemicals were purchased from PerkinElmer Life Sciences (Boston, MA), except where indicated otherwise. HPLC was performed on model 1050 chromatography system (Hewlett Packard, Palo Alto, CA) equipped with UV and γ -RAM (IN/US Systems, Fairfield, NJ) detectors using PRP-1 (150 \times 4.1 mm, 3 μ m) C18 column (Hamilton, Reno, NV).

Synthesis of [¹²⁵I]ICTP

Solution of Ti(NO₃)₃ in HNO₃ was prepared by addition of 20 mg Ti₂O₃ to 500 μ l of concentrated HNO₃ in a quartz tube. The mixture was heated on the top of a magnetic stirrer until Ti(NO₃)₃ was completely dissolved. The solution is ready to use after cooling down to room temperature and can be stored at room temperature for two weeks. One microliter of Ti(NO₃)₃ in HNO₃ was mixed with 30 μ l of 0.5 M acetate buffer (pH 4.5), 1 μ l (10 nmol) of 10 mM CTP, and 3 μ l of Na¹²⁵I (0.5 mCi) in 0.1 M NaOH (0.2 nmol, 2200 Ci/mmol). After 15 min at 75 °C, the reaction was quenched with 10 μ l of sodium bisulfite (10 mg/ml). The [¹²⁵I]ICTP was purified from unreacted CTP and other components of the reaction by reverse phase HPLC. Separation was performed on a 150 \times 4.6 mm C18 column using 2% to 10% gradient of acetonitrile in 50 mM triethylammonium

¹¹ M. Kashlev, E. Nudler, K. Severinov, S. Borukhov, N. Komissarova, and A. Goldfarb, *Methods Enzymol.* **274**, 326–34 (1996).

sequencing ladder. The transcribed sequence of the promoter template is shown on the right of the gel. The position of the guanine in the T-strand complementary to [¹²⁵I]ICMP, and the corresponding cytosine in the N-strand are boxed and starred in the sequence. The 3' ends of the RNA in ECp1, ECp2, and ECp12 are also indicated on the sequence. The [¹²⁵I]-labeled RNA is not visible on the gel because the samples were treated with RNase A before the electrophoresis. The RNA was analyzed on a separate gel (right panel).

bicarbonate (pH 7.5). The peak corresponding to the product (retention time 12 min) was collected and lyophilized in a vacuum concentrator. The samples were dissolved in 3 μ l H₂O and kept at -80°C . Radio-HPLC and TLC analysis showed 96% purity. The stock solution can be stored for 2 days; after that, degradation of [¹²⁵I]ICTP reduces the yield of incorporation of [¹²⁵I]ICTP into RNA. Note that the presence of unreacted CTP in the [¹²⁵I]ICTP preparation also reduces the incorporation of [¹²⁵I]ICTP into RNA, leading to low frequency of breaks in DNA targets. The complete removal of CTP (retention time 4 min) from [¹²⁵I]ICTP (retention time 12 min) was achieved by shallow gradient of acetonitrile (from 2% to 10% for 20 min).

Labeling of DNA Oligonucleotides

The template strand (see Fig. 1B), was labeled at the 3' end with [3'- α -³²P]dATP and TdT or at the 5' end with [5'- γ -³²P]ATP and T4 polynucleotide kinase. The nontemplate strand was labeled at the 5' end with [5'- γ -³²P]ATP and T4 polynucleotide kinase. The maximum specific activity of labeled oligonucleotides was achieved by using 2 to 3 times molar excess of fresh [5'- γ -³²P]ATP and [3'- α -³²P]dATP relative to oligonucleotide. The ³²P-labeled oligonucleotides were purified on a MicroSpin G-50 column (Amersham Biosciences, Piscataway, NJ), evaporated to desired concentration, and equilibrated with transcription buffer (TB; 20 mM Tris-HCl (pH 7.9), 40 mM KCl, 5 mM MgCl₂, 1 mM $\beta\beta$ -mercaptoethanol). The incorporation of ³²P into oligonucleotides was measured in a liquid scintillation counter (Packard model 2200CA).

Assembly of Transcription Elongation Complex and RNAP "Walking"

The T-strand (30 pmol), ³²P-labeled at the 5' or 3' end, was incubated with RNA-oligonucleotide (70 pmol) in 100 μ l reaction mixture in 1 \times TB buffer for 5 min at 45 $^{\circ}\text{C}$. After the samples were cooled down to room temperature, 6 μ l (26 pmol) of RNAP were added, and the samples were mixed and kept for 10 min at room temperature, followed by the addition of 3 μ l (120 pmol) of N-strand and incubation at 37 $^{\circ}\text{C}$ for 10 min. Similarly, ECs were assembled with ³²P-labeled N-strand. In this case the T-strand was not ³²P-labeled. The concentration of all reagents in the experiments with ³²P-labeled N-strand was the same as in the experiments with ³²P-labeled T-strand. The assembled ECs were transferred to a 1.5 ml plastic tube containing 50 μ l Ni²⁺-NTA agarose prewashed with TB, mixed immediately, and agitated for 5 min to ensure uniform binding of EC to the solid phase. The Ni²⁺-NTA agarose pellet was then washed 4

times with 1 ml of ice-cold TB, and the final reaction volume was adjusted to 30 μl with TB. The elongation was started by adding 0.4 mCi [^{125}I]ICTP in 3 μl H_2O . After 5 min, the agarose pellet was washed 4 times with 1 ml of ice-cold TB and two 1 μl aliquots were removed. One of the aliquots was diluted with 2.5 μl of RNA loading dye (9.8 M urea, 50 mM EDTA, 0.1% xylencyanol) and analyzed by 20% denaturing PAGE for ^{125}I incorporation. Another 1 μl aliquot was immediately frozen at -80°C . The remaining assembled complex was “walked” in the presence of 5 μM of appropriate NTP subsets for 5 min at room temperature, then washed with TB 4 times, and 2 1- μl aliquots were analyzed/frozen as in the previous step. The procedure was repeated to obtain the RNA transcripts of various lengths. The sequence of the addition of NTPs is shown in Fig. 1A. The analysis of viability of the EC complexes after storage at -80°C for 14 days showed that 80–90% of the complexes were able to produce runoff transcript after thawing and addition of all 4 NTPs (data not shown).

Analysis of Breaks in DNA

After 14 days of storage at -80°C the samples were thawed, immediately mixed with 1 μl of formamide-based Stop solution (USB, Cleveland, OH), and analyzed by 12% denaturing PAGE for fragmentation of the ^{32}P -labeled DNA strands. Maxam-Gilbert sequencing reactions were performed as described.¹² The gel was fixed in 10% acetic acid, dried on Whatman 3-mm Chr paper with DrygelSr (Hofer Scientific, San Francisco, CA) and digitized with a BAS 1500 BioImaging Analyzer (FUJI Medical Systems, Stanford, CT). To measure the intensity of the individual bands, the intensity profile of each lane was generated from the digitized gel image using Fuji Lab Image Gauge software (FUJI Medical Systems, Stanford, CT). The profile was deconvoluted on a series of the Lorentz-type peaks corresponding to individual bands as described in detail in.¹³ The best-fit curves were produced with the PeakFit software package for PC (SPSS Inc., Richmond, CA). In the ECs assembled on 5'- ^{32}P -labeled T-strand, ^{125}I -labeled RNA transcripts comigrated in the gel with the DNA fragments produced by ^{125}I (data not shown). To degrade the RNA and to clean up the gel for a more accurate measurement of break distribution, we treated these samples with RNase A before loading on the gel.

¹² A. M. Maxam and W. Gilbert, *Methods Enzymol.* **65**, 499–560 (1980).

¹³ S. E. Shadle, D. F. Allen, H. Guo, W. K. Pogozelski, J. S. Bashkin, and T. D. Tullius, *Nucleic. Acids Res.* **25**, 850–60 (1997).

Radioprobng of Promoter-Initiated Elongation Complexes

A stable EC containing 14-nt long RNA was obtained by preincubating 25 pmoles of the template containing T7 A1 promoter (124-nt PCR-generated, agarose-purified fragment; see Fig. 1B for the sequence of the transcribed region) with 10 pmoles of *E. coli* RNAP in 120 μ l TB for 5 min at 37 °C and subsequently adding 100 μ M of trinucleotide RNA primer ApUpC and 10 μ M of UTP, CTP, and GTP for 5 min at 37 °C. The EC was next immobilized on 45 μ l of Ni²⁺-NTA agarose beads suspension (50% v/v) prewashed with TB. After a 5-min incubation at 25 °C, the NTPs were washed off with TB, the reaction volume was brought to 50 μ l, and the DNA was labeled by a 10-min incubation of the immobilized complex with 50 units of T4 polynucleotide kinase (New England Biolabs) and 1 mCi of [γ -³²P] ATP (7000 Ci/mmol; ICN Biomedicals, Inc.) at 25 °C. The complex was washed with TB, incubated for 5 min with TB containing 1M KCl to remove nonspecifically bound DNA, and washed again with regular TB. The complex was walked in the presence of 5 μ M of appropriate NTP subsets until a 28-nt long RNA was synthesized. Then [¹²⁵I]CTP was incorporated as described above. The resulting complex, ECp1, was walked to form ECp2 and, subsequently, ECp12. The DNA breaks were analyzed by PAGE as described previously.

Breaks in the T-strand and N-strand of DNA

Analysis of DNA strand breaks produced by decay of ¹²⁵I incorporated in RNA strand of RNAP ECs is shown in Fig. 3. The left panel represents the experiments with the 3'-end-labeled T-strand and the right panel with the 5'-end-labeled N-strand. Bands corresponding to the ¹²⁵I-labeled RNA transcripts were located in the lower part of the gels (¹²⁵I-RNA box). The positions of the G complementary to the [¹²⁵I]IC in the T-strand and the corresponding C in the N-strand are marked with asterisks (G* and C*). Bands corresponding to the ¹²⁵I-induced breaks in the T-strand are outlined with a box (left panel). The bands appear as ladders with a sharp bell-shaped distribution of intensities. For the N-strand, the intensity of the bands corresponding to the breaks is considerably lower and they are spread along the lanes without defined maxima.

We observed a similar pattern of breaks previously in the displaced identical strand of the RecA synaptic complex.⁸ Therefore, radioprobng data indicate that in the RNAP EC, the newly transcribed RNA is in a close contact with the T-strand for at least 10 nt upstream from the RNA 3' end, while the corresponding fragment of the N-strand is apparently displaced and does not interact with RNA.

Distribution of Breaks in the T-strand

The frequencies of breaks at individual nucleotides in EC1 through EC10 (calculated as outlined in Fig. 4) are presented in Fig. 5. For comparison of the break distribution in different ECs, the intensities of individual Lorentzian peaks were normalized, such that the intensity for G*, the nucleotide complementary to [¹²⁵I]IC, equals 1 for all the profiles (Fig. 5). Note that the intensity of a band does not directly correspond to the frequency of breaks at a given base because one decay can produce multiple breaks in the same DNA strand. Strictly speaking, the intensity of a band corresponds to the frequency of breaks at that base under the condition that there are no other breaks between that base and the labeled 3' end. Therefore, we consider the data points close to the 5' end unreliable, and, thus, they are grayed out in Fig. 5.

The break distributions in the T-strand for EC1, EC3, and EC6 are almost identical and resemble the break distributions we observed earlier for the DNA/RNA hybrid in the T7 RNAP elongation complex.⁷ Such a distribution of breaks, where the maximum is shifted from the G* position towards the 3' end, is characteristic for A-DNA.^{7,14} This result is consistent with formation of a relatively stable and uniform DNA/RNA duplex with a conformation close to the canonical A-form. The break distributions in EC8, EC9, and EC10 deviate from this pattern; in particular, the cleavage intensities in the 3'-end shoulder gradually go down, thereby indicating increase in the distance between RNA and the DNA T-strand⁷ (Fig. 5). In EC8 and EC9, the [¹²⁵I]IC is located at the edge of the 8-9-bp long DNA/RNA duplex,^{15,16} therefore, the observed effect (increase in the RNA-DNA distance) can be explained by separation of the 2 strands.

The cleavage pattern for EC10 deserves special discussion, however. In EC10, the [¹²⁵I]IC should be located beyond the 8-9-bp long DNA/RNA duplex (see “explosion” in Fig. 2B). Nevertheless, the total amount of breaks in the T-strand of EC10 is still comparable to that of EC1 (Fig. 3). This apparent contradiction can be explained by RNAP backtracking along the template with simultaneous translocation of the transcription bubble and the RNA/DNA hybrid.¹⁷ The ECs assembled on short templates are known to be particularly susceptible to backtracking.¹⁸ For example, in

¹⁴ E. K. Gaidamakova, R. D. Neumann, and I. G. Panyutin, *Nucl. Acids Res.* **30**, 4960–65 (2002).

¹⁵ A. L. Ghatt, P. Cramer, J. Fu, D. A. Bushnell, and R. D. Kornberg, *Science* **292**, 1876–82 (2001).

¹⁶ N. Korzheva and A. Mustaev, *Curr. Opin. Microbiol.* **4**, 119–25 (2001).

¹⁷ N. Komissarova and M. Kashlev, *Pros. Natl. Acad. Sci. USA.* **94**, 1755 (1997).

¹⁸ M. L. Kireeva, N. Komissarova, D. S. Waugh, and M. Kashlev, *J. Biol. Chem.* **275**, 6530 (2000).

EC10, the backtracking would relocate the [^{125}I]IC to the downstream edge of the hybrid, where the “explosion” is positioned in Fig. 2C. As a result, after backtracking the [^{125}I]IC-DNA distance would be as in the duplex. On average, the intensity of breaks in the EC10 would be intermediate between those for the positions in Figs. 2B, and 2C and thus close to the intensity for EC1.

Notice that backtracking would not affect the breaks distribution in EC1, EC3, and EC6 because, in all possible positions of RNAP, the [^{125}I]IC always stays within the DNA/RNA hybrid. In the “forward” position the [^{125}I]IC is located between the ribonucleotides 10 and 18 (Fig. 2B), and in the “backward” position the [^{125}I]IC is located as shown in Fig. 2C.

Breaks in the T-strand of the Promoter-Initiated EC

To prevent backtracking, we obtained ECs of the *E. coli* RNAP on a 124-nt long double-stranded linear template. Transcription was initiated not by assembly but rather in a conventional way, from the strong A1 promoter of bacteriophage T7 inserted in the template. First, the 28-nt long RNA capable of forming a secondary structure was synthesized by walking, then [^{125}I]IC was incorporated into the 3' end. The ^{125}I decay induced breaks in the complementary T-strand of the DNA strand (see ECp1 in Fig. 6). Importantly, no breaks were detected when ECp1 was walked to form ECp12, which did not backtrack because a strong secondary structure formed in the nascent RNA behind RNAP. Therefore, in the absence of backtracking, the [^{125}I]IC located 12 nt from the RNA 3' end was no longer involved in the complementary interactions with the template DNA strand (Fig. 6, ECp12).

DNA Conformation in RNAP Active Center

The breaks distribution in ECp1, where [^{125}I]IC is located in the RNAP active center, is different from that of ECp2 (Fig. 6). Indeed, the frequency of breaks at the nucleotides located to the 5' end from the G^* (C and G) seems lower in ECp1 than in ECp2, implying that these nucleotides were at greater distance from the [^{125}I]IC.

To further investigate this effect, we obtained the break distributions for assembled ECs with the T-strand labeled at the 5' end (Fig. 7). These data complement the results for the 3'-end-labeled T-strand (Fig. 5) by revealing the break distributions between G^* and the 5' end of the T-strand. The break distribution for EC1 is radically different from the other ECs; in particular, the frequency of breaks in the nucleotides shifted to the 5'-end from the RNAP active center is abruptly reduced. This reduction in the

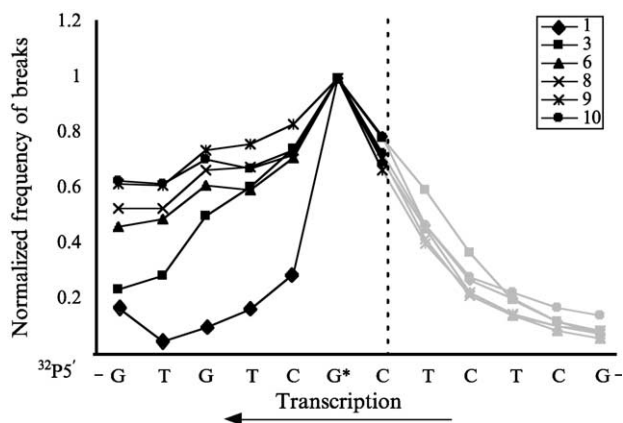


Fig. 7. Relative frequencies of breaks at the individual bases in the T-strand labeled at the 5' end normalized on the intensity at the G* opposite [125 I]IC. Symbols are assigned as in Fig. 5. Data points on the right are grayed out.

DNA cleavage is consistent with the sharp turn in the T-strand of DNA similar to that observed in the yeast RNA polymerase II (Pol II) elongation complex¹⁵ and proposed for the bacterial RNAPs as well.¹⁶

Concluding Remarks

Radioprobings of the *E. coli* RNAP elongation complex demonstrate that approximately 9 bp upstream from the active site of RNAP, the DNA/RNA hybrid undergoes a drastic conformational change, and the two strands become separated. This result is in agreement with the crystal structure of the Pol II elongation complex,¹⁵ where the heteroduplex is 9 bp long, and the crystal/crosslinking-based model for the bacterial elongation complex,¹⁶ where the length of the heteroduplex is assumed to be 8-9 bp. In addition, our data allow direct comparison between two RNA polymerases, the single subunit T7 RNAP, and the multi-subunit *E. coli* RNAP. Indeed, the breaks distribution presented here for the EC9 and EC10 (Fig. 5) is similar to that observed by the same method for the EC7 in the case of T7 RNAP.⁷ This non-A-like distribution of the breaks reflects the local separation of the RNA and the DNA T-strand.⁷ Therefore, we conclude that the DNA/RNA hybrid formed inside the *E. coli* RNAP is 1-2 bp longer than the hybrid inside the T7 RNAP. Again, this is consistent with the crystal structure of the T7 elongation complex,¹⁹ where the

¹⁹ Y. W. Yin and T. A. Steitz, *Science* **298**, 1387 (2002).

DNA/RNA hybrid is shown to be 7 bp (that is, less than in the multisubunit Pol II elongation complex¹⁵).

In summary, the ¹²⁵I radioprobing is a unique approach useful for revealing the DNA and RNA trajectories in nucleoprotein complexes, which are too large and flexible for the conventional high-resolution methods such as x-ray crystallography and NMR in solution. The advantage of radioprobing over other footprinting and crosslinking methods is the ability of the DNA/RNA breaking agent—Auger electron—to freely penetrate proteins and nucleic acids.

Acknowledgments

We are grateful to Jodi Becker for her expert technical support.

[8] Formation of Long DNA Templates Containing Site-Specific Alkane–Disulfide DNA Interstrand Cross-Links for Use in Transcription Reactions

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The ability to “walk” transcription complexes to discrete positions by performing repetitive rounds of nucleotide starvation has proven extremely useful.¹ However, generating a uniform population of elongation complexes is complicated on some natural templates, because some sequences require far too many rounds of walking to produce the desired complex. Other templates have positions that undergo significant backtracking or arrest, complicating the simple walking procedure. We present a technique to generate a uniform population of elongation complexes at any template position on any template in a single step by employing interstrand DNA cross-links.

Advances in oligonucleotide synthesis allow incorporation of a wide range of chemically modified bases. Verdine and colleagues introduced the use of “convertible nucleosides” during standard oligonucleotide synthesis at internal positions.^{2–4} Conversion of these modified bases allows production of a wide range of alkylamine derivatized nucleotides;

¹ B. Krummel and M. J. Chamberlin, *J. Mol. Biol.* **225**, 221 (1992).

² A. E. Ferentz and G. L. Verdine, *J. Am. Chem. Soc.* **113**, 4000 (1991).

³ A. M. MacMillan and G. L. Verdine, *J. Org. Chem.* **55**, 5931 (1990).

⁴ A. M. MacMillan and G. L. Verdine, *Tetrahedron* **47**, 2603 (1991).