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Promoter-independent *In vitro* Assembly of Active Eukaryotic Transcription Elongation Complexes

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Abstract Characterization of the processivity determinants in eukaryotic RNA polymerase Ⅱ is crucial for understanding both the mechanisms of eukaryotic gene expression at the level of promoter escape, pausing, release of the RNA from transcription terminator regions, and the mechanisms of transcription-coupled repair of DNA damage (TCR). The sliding clamp model of transcription processivity suggests the formation of a highly stable elongation complex (EC), in which RNA polymerase is tightly bound to the nascent transcript and template forming the characteristic transcription "bubble". Here, an *in vitro* system for promoter-independent assembly of a functional mammalian RNA polymerase Ⅱ elongation complex using highly purified polymerases and synthetic RNA and DNA oligonucleotides was presented. It was shown that the 9-nucleotide RNA∶DNA template hybrid is necessary and sufficient for the formation of a stable elongation complex with human RNA polymerase Ⅱ, while further inclusion of the non-template DNA strand to form the complete transcription "bubble" actually destabilizes the already formed RNA:DNA:RNA polymerase Ⅱ complex. In addition, by introducing a DNA damage at a specific site in the template DNA, this assay can potentially be used for characterizing the processes of transcription-coupled repair of DNA damages.

Key words RNA polymerase Ⅱ, transcription, elongation complex, TCR

Transcription, the process by which genetic information is transferred from DNA to RNA, constitutes the first step in gene expression. While our knowledge of the molecular details leading to transcription initiation has greatly improved over the past decades, the focus of many recent studies has shifted towards the less well-characterized events taking place after the assembly of the pre-initiation complex, such as promoter clearance, elongation and termination. Since processive elongation by eukaryotic RNA polymerase Ⅱ (RNAP Ⅱ) has been studied using purified RNAP Ⅱ in a promoter- and accessory factor-independent transcription system [1], it is therefore most likely that the basic processivity characteristics are intrinsic to the core RNAP Ⅱ complex. Although the crystal structure of RNAP Ⅱ has clearly shown its multiple contact sites with both RNA and DNA, our knowledge of the mechanisms of transcription elongation is still limited.

It has been proposed that during RNAP Ⅱ transcription, stalling and arrest arising from either intrinsic pausing sites or DNA damage has to be dealt with properly and immediately so that RNAP Ⅱ can continue. In some cases, the permanently arrested RNAP Ⅱ has to be removed so that the gene is unblocked[2]. The idea that the arrested RNAP Ⅱ has to be dealt with promptly is underscored by the fact that DNA lesions in the transcribed strand of active genes are invariably removed much faster than those in the non-transcribed strand, or in the genome overall, *via* a process called transcription-coupled repair (TCR) [2,3]. While accumulating evidence is pointing to the existence of a variety of mechanisms ensuring that the stalled RNAP Ⅱ does not persist long enough to compromise cell viability [2], biochemical details supporting it are still few. A better characterization of these and other related aspects during RNAP Ⅱ transcription at a biochemical level requires a simple, "minimal" *in vitro* system. Recently, a novel approach was developed for obtaining RNAP Ⅱ elongation complexes (ECs) in a promoter-independent manner, which involves direct assembly of the most basic intermediate in the elongation process using purified RNAP Ⅱ from *Saccharomyces cerevisiae* and synthetic RNA and DNA

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oligoncleotides[4]. The resulting complex has structural and functional properties that resemble those of the promoter-initiated ECs. The approach also has an advantage of bypassing the need for other protein factors or for introduction of mismatches in DNA to promote transcription initiation as used in other systems [5,6]. In the present study, we applied the assembly strategy for forming the stable and active ECs *in vitro* involving human RNAP Ⅱ. The effect of the two DNA strands and the RNA transcript on the formation of the ECs was then analyzed. Furthermore, the effect of a DNA lesion introduced into the template DNA to arrest the elongating RNAP Ⅱ was also analyzed.

1 Materials and methods

1.1 Cell line and reagents

HeLa cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (Life Technologies, USA), 100 U/ml penicillin and 100 mg/L streptomycin. Protein A Sepharose was purchased from Amersham Biosciences. Streptavidin magnetic beads were from Promega. RNA polymerase Ⅱ antibody 8WG16 was from Promega. N-acetoxy-2-(acetylamino)fluorine (AAF) was obtained from National Cancer Institute, USA. [α-^{32}P]-UTP or [α-^{32}P]-CTP was purchased from Dupont TNT. Sequences of oligos used for the assembly were as follows. Template DNA oligo TDS-1: 5′ CCCTTTCCCCT-ACCTACATACACCACACACCACACCGAG*CCC-AACCACTTACCCCTTCACCTTTACCCTTACCCCTCTCCATACCACACCACCTTACCTACCACCCA-CCTTCCCTTACCCTTCCAx 3′ (x = biotin; asterisk denotes the site of AAF modification); Template DNA oligo TDS-2: 5′ CCCTTTCCGGTACATACACCAC-ACACCACACCGAGTACGCCTCTCCATACCACA CCACCTTACCAx 3′ (x = biotin); RNA oligo: 5'AUGGAGAGG 3′; the non-template DNA oligo NDS-1: 5′ TGGAAGGGTAAGGGAAGGTGGGTG-GTAGGTAAGGTGGTGTGGTATGGAGAGGGGTA AGGGTAAAGGTGAAGGGGTAAGTGGTTGGGC-TCGGTGTGGTGTGTGGTGTATGTAGGTAGGGG-AAAGGG 3′; NDS-2: 5′ TGGTAAGGTGGTGTG-GTATGGAGAGGCGTACTCGGTGTGGTGTGTGG TGTATGTACCGGAAAGGG 3′. The sequence in the TDS-1 and TDS-2 oligo with which the RNA oligo hybridizes is underlined.

1.2 Protein purification

RNA polymerase Ⅱ was purified from HeLa cells using a three-step purification procedure modified from Roeder and co-workers[7]. Briefly, nuclear pellet was made from 1×10^{10} cells by homogenization. The nuclear pellet was resuspended in 2 volume of buffer D (50 mmol/L Tris-HCl, pH 7.9, 1 mmol/L DTT, 20% glycerol, 5 mmol/L EDTA and 5 mmol/L EGTA) plus protease inhibitors. After extensive sonication to shear the chromatin, 30.4 g ammonium sulfate per 100 ml extract was added with constant stirring until all the ammonium sulfate was dissolved within half an hour. The stirring was continued for another half an hour before the extract was centrifuged at 35 000 r/min for 45 mins. The pellet was resuspended in buffer D until conductivity was similar to that of 0.145 mol/L $(NH_4)_2SO_4$. After centrifugation at 40 000 r/min for an hour, the supernatant was loaded onto a 100 ml Heparin-sepharose column and bound proteins were eluted with buffer D containing 400 mmol/L $(NH_4)_2SO_4$. Peak fractions containing RNAP Ⅱ were dialyzed against 8WG16 binding buffer (20 mmol/L Tris-HCl, pH 7.9, 1 mmol/L EDTA, 1 mmol/L DTT, 200 mmol/L $(NH_4)_2SO_4$) and protease inhibitors[8], and insoluble materials were removed by centrifugation at 8 000 r/min in a GSA rotor. The supernatant was incubated in batch with 8WG16-conjugated Sepharose for 3 to 4 hours at 4℃. The collected resin was washed at 4℃, and then at room temperature sequentially with 20 ml, 10 ml, 10 ml of binding buffer plus 500 mmol/L $(NH_4)_2SO_4$. RNAP Ⅱ was eluted at room temperature with the same buffer, but containing 50% (*v*/*v*) ethylene glycol for 20 mins with gentle agitation. Eluted fractions from 8WG16-Sepharose were exchanged for buffer A (20 mmol/L Tris-HCl pH 7.9, 1 mmol/L EDTA, 1 mmol/L 2-mercaptoethanol, 10% glycerol (*v*/*v*), 200 mmol/L KCl and protease inhibitors) using a D10 column (Amersham) prior to being further purified by Mono Q HR5/5 chromatography using an ACTA chromatography system (Amersham) equilibrated with the same buffer. The column was resolved with a salt gradient (0.2～1 mol/L KCl). Highly purified RNAP Ⅱ eluted at 0.6 mol/L KCl.

1.3 Assembly of the RNAP Ⅱ ternary complex

RNAP Ⅱ ternary complexes were assembled from oligonucleotides, essentially as described[4]. Briefly, a biotinylated "coding strand", TDS oligonucleotide, was annealed to a complementary 9 nucleotide RNA oligonucleotide prior to mixing with RNAP Ⅱ. The non-transcribed strand, NDS oligonucleotide, was then

added in an excess of TDS (typically 15∶1 in these experiments, though less has also later been found to suffice), and the mixture was incubated further at 37℃ for 30 min prior to isolating the RNA:DNA:RNAP Ⅱ complexes *via* biotin-mediated binding to magnetic Streptavidin beads equilibrated in transcription buffer (20 mmol/L Tris-HCl, pH 7.9, 40 mmol/L KCl, 5 mmol/L $MgCl_2$, 1 mmol/L 2-mercaptomethanol). Upon magnetic separation of the beads, they were washed three times with 1 ml ice-cold transcription buffer. Both the beads and the corresponding supernatant were boiled in SDS sample buffer before being resolved on 4% ～12% Tris-glycine polyacrylamide gels. Western blot was performed by probing with anti-CTD antibody 8WG16 and subsequent detection using the ECL reagents (Amersham Pharmacia Biotech). In some cases, the bead-bound complexes were incubated further in transcription buffer with 1 mol/L KCl at 30℃ for 5～10 min followed by being washed twice with 1ml transcription buffer. For transcription assays, the pelleted beads were adjusted in an appropriate volume (10～15 μl) of transcription buffer and the transcription was initiated by adding 50 μmol/L of the appropriate NTPs. The transcripts were labeled by incorporation of 48×10^4 Bq of [α-^{32}P] NTP (11.1×10^{13} Bq/mol; NEN Life Science Products) at 25℃. Analysis of the RNA transcripts was performed by denaturing electophoresis in a 20% polyacrylamide sequencing gel.

1.4 AAF adduct introduction into template oligonucleotide TDS-1

A bulky adduct at a specific guanine site was introduced by treatment of TDS-1 oligonucleotides with N-acetoxy-2-(acetylamino) fluorine (AAF)[9]. The adduct-containing oligonucleotides were purified by denaturing Urea-PAGE. The final concentration of the adducted oligonucleotides was determined by absorbance at a wavelength of 260 nm, and they were then used exactly like the untreated oligonucleotides in the assembly of transcription elongation complexes and transcription reactions.

2 Results

2.1 A DNA∶RNA hybrid is necessary and sufficient for the assembly of the RNAP Ⅱ elongation complex

To obtain human RNAP Ⅱ elongation complexes in the absence of promoter sequence and other necessary transcription factors, an *in vitro* system was set up, in which a single-stranded synthetic DNA oligonucleotide was used as a template DNA strand (TDS) for EC assembly. A 9-nucleotide synthetic RNA oligonucleotide complementary to a region of the TDS was then added to provide the scaffold for the assembly of the EC. Purified RNA polymerase Ⅱ was added to the prehybridized DNA/RNA oligos allowing the formation of a RNAP Ⅱ-RNA-DNA complex. The complementary non-template DNA strand (NDS) was finally incubated with the preformed complexes to allow for the formation of the complete elongation complex. The formed ECs were purified making use of the biotin group attached at the 3′-end of the TDS, by specifically binding to the streptavidin beads (Figure 1). When purified RNA polymerases were added to the prehybridized RNA∶DNA oligos at molar ratio of 1∶10, up to half the polymerases added were incorporated into the RNAP Ⅱ-RNA-DNA complexes. A large increase in the ratio of oligonucleotides to RNAP Ⅱ did not significantly improve the incorporation of RNAP Ⅱ (Figure 2b). However, if RNA polymerases were added directly to the template that had not been prehybridized with RNA oligonucleotide, hardly any RNAP Ⅱ was retained by the TDSs. Interestingly, addition of the non-template strand seemed to destabilize the already formed RNAP Ⅱ-RNA-DNA complexes, as less RNAP Ⅱ was detected when the initial RNAP Ⅱ-RNA-DNA complex was incubated with the NDS oligos (Figure 2c, lane *3* and *5*). Because the structure

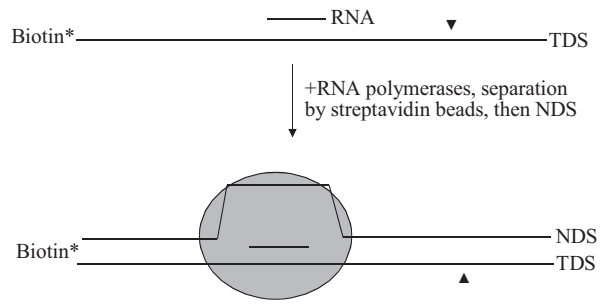

Fig. 1 Schematic presentation of the assembly of active transcription elongation complexes from DNA and RNA oligonucleotides in a promoter-independent manner

The template DNA oligonucleotide(TDS) was first hybridized with the complementary RNA oligonucleotide in an equal molar ratio. Purified RNA polymerase was then added into the hybridization reaction at the indicated molar ratios to oligonucleotides. Finally, the non-template strand oligonucleotide(NDS) was included and the reaction incubated for half an hour at 37℃. The formed elongation complexes were separated from non-incorporated RNAP Ⅱ by taking advantage of the biotin attached at the 3′ end of the TDS. *: Biotin; ▼:AAF.

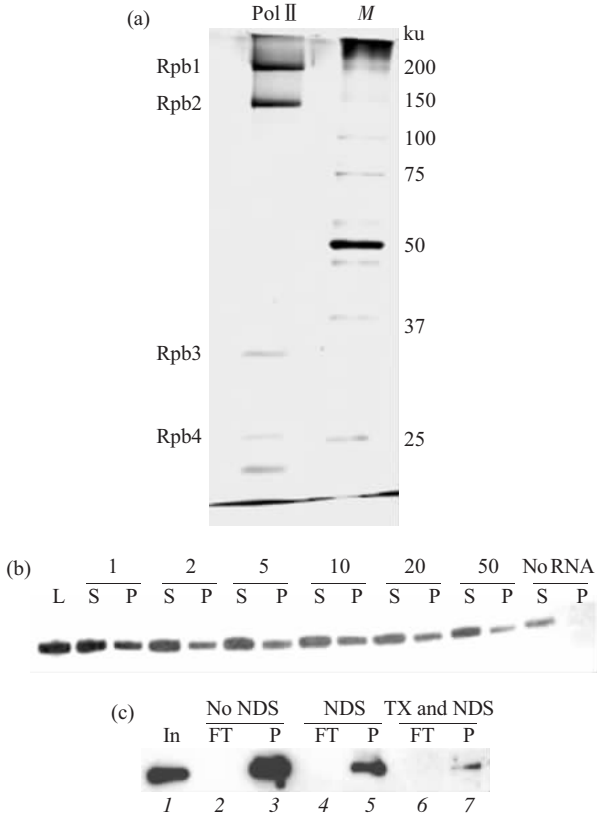

Fig. 2 Assembly of the DNA-RNA-RNAP Ⅱ ternary complex
(a) Silver staining of human RNA polymerase Ⅱ, purified from the nuclear pellet of HeLa cells. (b) A comparison of assembly efficiency when different molar ratios of RNAP Ⅱ was assembled with RNA：DNA oligonucleotides or TDS alone, as described in Figure 1. RNA Pol Ⅱ was detected by using anti-Rpb1 antibody 8WG16 in Western blots. One-tenth of the RNAP Ⅱ used in the assembly reaction was loaded as input control. (c) Purified RNA polymerase was incorporated into the RNA：DNA hybrids. The resultant RNP Ⅱ-nucleotide complex was allowed to walk for two or three additional nucleotides before introducing NDS-2 (TX and NDS). RNA Pol Ⅱ in the beads pellet and in the supernatant was detected by using anti-Rpb1 antibody 8WG16 in Western blots. S: Supernatant; FT: Non-incorporated fractions; P: Streptavidin bead-associated; TX: Transcription in the presence of GTP, CTP and UTP.

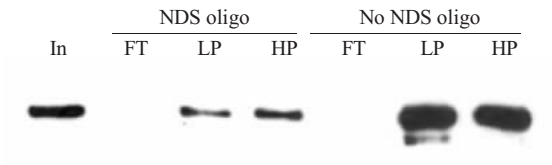

Fig. 3 Assembled complexes are stable in high salt
Effect of high salt incubation on the stability of formed ternary complexes or complexes without incorporation of NDS. Separated ternary complexes assembled with incorporation of NDS-2 (labeled NDS oligo), or complexes assembled without the incorporation of NDS-2 (labeled no NDS oligo), were incubated with 1 mol/L KCl, and RNAP Ⅱ in the bead-associated elongation complexes was detected by Western blot. LP: streptavidin bead-associated fractions after low-salt incubation; HP: Streptavidin bead-associated fractions after high-salt incubation.

of the natural ECs formed during promoter-initiated transcription is very stable and resistant to disassembly by high concentrations of salt, the stability of the formed ECs using this approach was also tested by being incubated in 1 mol/L KCl (Figure 3). Both the initial RNAP Ⅱ-RNA-DNA complexes and the final ECs were stable against such incubations, indicating that stable elongation complexes had indeed been formed.

2.2 The assembled ternary complex is active in transcription elongation

To test whether the assembled EC was functional and competent in transcription elongation in the presence of substrate NTPs, ECs formed with TDS-2 and NDS-2 were incubated with [α-^{32}P]-CTP and found to produce a 10 nt labeled RNA product, as expected, while further including the un-labeled GTP alone, or both GTP and UTP gave rise to 11 nt and 12 nt products, respectively (Figure 4, lane *1*, *2* and *3*). Transcript elongation was template specific, because addition of the inappropriate [α-^{32}P]-UTP alone, instead of [α-^{32}P]-CTP, did not allow the extension of the RNA oligonucleotide (Figure 4, lane *4* and *5*). Further addition of all four nucleotide substrates produced "run-off" transcripts, and part of the run-off products appeared in the transcription reaction supernatant as a result of the RNAP Ⅱ running off the template, thus disassembling the EC (Figure 4, lane *6*

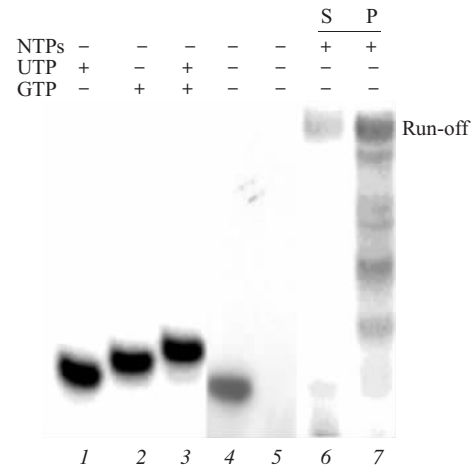

Fig. 4 The assembled ternary complexes are transcription-competent
Ternary complexes formed with TDS-2/NDS-2 were incubated with [α-^{32}P]-CTP (lanes *1*, *2*, *3*, *4*, *6*, *7*) or [α-^{32}P]-UTP (lane *5*) for 5 min first, and then non-radio-labeled UTP (lane *1*), GTP (lane *2*), GTP plus UTP (lane *3*) or all NTPs (lanes *6* and *7*) were added to allow further transcription. The final transcription products were detected by autoradiography after 20% UREA-PAGE. S: Supernatant; P: Streptavidin bead-associated.

and *7*). Thus, the assembled elongation complex with mammalian RNAP Ⅱ is active, with the 3′-end of the hybridized 9 nt RNA oligonuleotide properly located in the active center of the enzyme and in the correct register with the template DNA strand. While the RNA∶DNA hybrid is necessary and sufficient for the assembly of the RNP Ⅱ-nucleotide complex, the length of the RNA oligonucleotide was found to be crucial for the formation and thus the stability of the ECs. When the initially formed RNP Ⅱ-nucleotide complexes were allowed to walk for two or three additional nucleotides before introducing the NDS, the amount of RNP Ⅱ in the final ECs dropped dramatically compared with the initial ECs formed with 9 nt oligonucleotides (Figure 2c, lane *5* and *7*). This result is consistent with previous results indicating that the RNA oligonucleotides of more than 9nt would decrease the stability of EC formed by yeast RNP Ⅱ [4].

2.3 Introduction of AAF adduct at a specific guanine site block the transcription elongation

During RNAP Ⅱ transcription elongation, frequent pausing and stalling of the polymerase occur and an important role of the many transcription accessory factors may be to minimize the negative effect of such events on transcription[10]. In the extreme case, DNA lesions, such as thymine dimers generated by UV irradiation, in the transcribed strand will result in the irreversible stalling or arrest of RNAP Ⅱ. It has been shown that a process called transcription-coupled nucleotide excision repair (TC-NER) is responsible for the faster remove of the damage, which would otherwise allow for transcription to continue. However, the precise biochemical mechanisms underlying TC-NER are still elusive. To better understand the fate of irreversible stalled RNAP Ⅱ in the presence of DNA damage at the basic biochemical level, an *in vitro* assay reconstituting the whole reaction seems pertinent. With the above system being active in transcription elongation, an AAF abduct was introduced at a specific site in the transcribed strand TDS-1 as described in **Materials and methods**. Introduction of the AAF abduct had no effect on the assembly of the ternary complex. Importantly, the assembled complexes were active in transcription elongation (Figure 5a and 5b, lane *2* and *4*). However, when the assembled complexes were allowed to transcribe in the presence of all substrate NTPs, the transcribing polymerase stalled prematurely compared with the complexes assembled on the "un-damaged" coding strand (Figure 5b, lane *3* and *4*). This indicated that the introduced DNA "lesion" blocked transcription elongation and thus caused the stalling/arrest of RNAP Ⅱ. Such a stalled ternary complex would be an ideal substrate for further characterizing most of the ensuing reactions involved in rescuing the stalled RNAP Ⅱ.

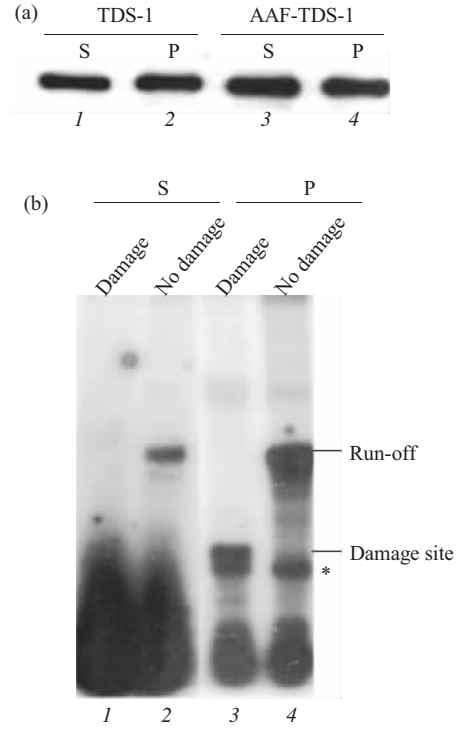

Fig. 5 Introduction of an AAF abduct in the TDS blocks transcription at a specific site

(a) AAF-damaged TDS-1 has no effect on the assembly of the ternary complex. (b) Ternary complexes involving AAF-damaged TDS-1 are transcription competent, but are blocked by AAF at a specific site during transcription elongation. Asterisk denotes intrinsic pause-site. Arrow shows the arrest site due to DNA damage.

3 Discussion

One of the most difficult problems in conducting *in vitro* analyses of transcription elongation in eukaryotes is the formation and subsequent isolation of ECs in the absence of multiple other transcription factors. A novel strategy for EC assembly independent of promoter and many other transcription factors has been described for RNA polymerase from *Escherichia coli* and *Saacharomyces cerevisiae*, which exploits the ability of RNA polymerase to bind the 3′-end of a short RNA oligonucleotide pre-hybridized to a single-strand DNA oligonuelotide serving as a transcription template [4,11]. It has also been shown that the structure of the assembled EC closely resembles

that obtained by initiation from a DNA end[12]. In this work, the approach was essentially applied to the quantitative analysis of the functional assembly of human RNA polymerase Ⅱ elongation complex. While RNA oligonucleotide is absolutely necessary for the stability of the formed RNAP Ⅱ/DNA/RNA complex, the complementary un-transcribed DNA strand proved to not be required for the assembly of a transcription-competent complex, a result similar to that reported for yeast RNAP Ⅱ-ECs[4], though it should be pointed out that the processivity of this artificial RNA：DNA：RNAP Ⅱ complex has not been tested further to see how many nucleotides it can incorporate in the absence of non-template strand before it disassemble. Rather, the addition of the non-template DNA strand seems to destabilize the preformed RNAP Ⅱ/DNA/RNA complex, displacing almost half the already incorporated RNAP Ⅱ from the final ECs, suggesting that some RNA：DNA hybrid bound RNA P Ⅱ might not positioned properly in this preformed RNAP Ⅱ/DNA/RNA complex, and introduction of the non-template DNA just serves to eliminate them while only keeping those whose active sites appropriately harbors the 3′ end of RNA oligo. Moreover, if the original RNA oligonucleotide was allowed to be extended for another two to three nucleotides by the pre-assembled RNAP Ⅱ/DNA/RNA complex, addition of the non-template DNA strand further destabilized the preformed RNAP Ⅱ/DNA/RNA complex, supporting the idea that the 9 nt RNA：DNA hybrid was critical for the stabilization and thus processive capabilities of ECs not only *in vivo*, but also *in vitro*. Nevertheless, the existence of non-template DNA strand in the ECs may function to regulate the ECs stability during elongation by modulating the hybrid length between the newly transcribed RNA and template DNA through its interaction with the upstream part of the RNA：DNA hybrid[13]. Thus, in the actual elongation complexes, the non-template DNA strand may be important to prevent the formation of an abnormally long RNA：DNA hybrid by displacing the nascent RNA in the ECs, which, as shown elsewhere, has a dramatic destabilizing effect on the RNAP Ⅱ EC.

It is evident that DNA damage stalled RNAP Ⅱ during transcription elongation can either be allowed to continue after the damage has been repaired or be removed from the lesion site[2]. Recent evidences have shown that RNAP Ⅱ undergoes ubiquitylation and proteosome-mediated degradation in response to UV-generated DNA damage[14,15], and the nucleotide depleting elongation inhibitor 6-azauracil[16], thus ensuring that the arrested RNA Pol Ⅱ does not persist for long enough to compromise cell function. However, the biochemical studies to elucidate the specific mechanisms underlying RNAP Ⅱ ubiquitylation and degradation, and how this relates to RNAP Ⅱ removal are not yet conclusive. To better mimic the actual situation where RNA Pol Ⅱ is stalled by DNA lesions, we introduced an AAF chemical abduct at a specific site in the transcribed DNA strand. When the assembled ECs were allowed to elongate, RNAP Ⅱ was found to stop at the adducted site, as expected. By applying the above reported system using yeast RNAP Ⅱ, it has recently been shown that RNAP Ⅱ in an elongation complex is a much better substrate for ubiquitylation than free, or DNA-associated RNAP Ⅱ. Moreover, the rate of ubiquitylation is further increased when transcription arrest is caused by DNA damage[16]. With the simplicity of the described *in vitro* system, and with future incorporation of nucleosomes onto the DNA template, it is anticipated that a reconstitution reaction could be set up using pure, physically relevant factors, which will make it possible to dissect the roles played by various factors involved in processes such as transcription-coupled repair and transcription through chromatin structure.

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真核基因启动子非依赖的功能转录延伸复合物的体外组装

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摘要 真核生物 RNA 聚合酶Ⅱ的持续合成能力对基因转录过程中每一个阶段，包括启动子脱离、转录暂停、转录终止以及转录偶联 DNA 损伤修复过程的调节至关重要. 在 RNA 聚合酶Ⅱ介导的转录延伸过程中，其和模板 DNA 及转录产物 RNA 紧密结合，形成一个非常稳定的延伸三维复合物 (elongation complex，EC). 此特征性“泡”状结构的形成是 RNA 聚合酶Ⅱ持续合成能力所必需的. 在不依赖启动子及众多转录起始因子的条件下，利用人工合成的 RNA 与 DNA 寡核苷酸，在体外组装形成具有功能转录活性的延伸复合物. 结果表明，长度为 9 个核苷酸的 RNA 与模板 DNA 形成的杂合分子对转录延伸复合物的形成是必需的，而非转录模板 DNA 链的加入导致最终活性转录“泡”状复合物的形成，并可转录形成与模板相关的转录产物，进一步通过在模板 DNA 的特定位置引入一个乙酰氧乙酰氨基芴修饰基团，可特异性地阻断转录延伸过程，从而显示该系统在研究真核基因转录及转录偶联 DNA 损伤修复机制中的潜在应用价值.

关键词 RNA 聚合酶，转录，延伸复合物，TCR
学科分类号 Q78

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