Formation and Fate of a Complete, 31-Protein, RNA polymerase II Transcription Initiation Complex

Kenji Murakami¹, Guillermo Calero¹, Chris R. Brown², Xin Liu¹, Ralph E. Davis¹, Hinrich Boeger ² and Roger D. Kornberg¹*

¹ Department of Structural Biology, Stanford University, Stanford, CA 94305, U.S.A.
² Department of Molecular, Cell and Developmental Biology, University of California, Santa Cruz, CA 95064, USA

Running title: Formation of a Complete Transcription Initiation Complex

To whom correspondence should be addressed: Roger D. Kornberg, Department of Structural Biology, Stanford University, Stanford, CA 94305, U.S.A., Tel.: (650)723-6988; Fax: (650)292-2255; E-mail: kornberg@stanford.edu

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Background: Analyses of Pol II transcription are hampered by the difficulty of preparing abundant functional preinitiation complex (PIC).

Result: We reconstituted milligram quantities of complete 31-subunit PIC.

Conclusion: An intermediate comprising TBP, TFIIE, TFIIH, and DNA could be isolated and combined with TFIIB and pol II/TFIID to generate the PIC.

Significance: Enables definitive biochemical and structural studies of the transcription initiation machinery.

SUMMARY

Whereas individual RNA polymerase II (pol II) – general transcription factor (GTF) complexes are unstable, an assembly of pol II with six GTFs and promoter DNA could be isolated in abundant, homogeneous form. The resulting complete pol II transcription initiation complex (PIC) contained equimolar amounts of all 31 protein components. An intermediate in assembly, consisting of four GTFs and promoter DNA, could be isolated and supplemented with the remaining components for formation of the PIC. Nuclease digestion and psoralen cross-linking mapped the PIC between positions -70 and -9, centered on the TATA box. Addition of ATP to the PIC resulted in quantitative conversion to an open complex, which retained all 31 proteins, contrary to expectation from previous studies. Addition of the remaining NTPs resulted in runoff transcription, with an efficiency that was promoter-dependent and was as great as 17.5% with the promoters tested.

INTRODUCTION

The initiation of RNA polymerase II (pol II) transcription is a multistage process, most likely for the purpose of multifactorial control. It extends far beyond the formation of the first phosphodiester bond; a transcript of about 25 nucleotides must be synthesized before a transition occurs from initiation to RNA chain elongation (1,2). The proteins responsible for initiation, a set of general transcription factors (GTFs) and the polymerase, associate
in a so-called pre-initiation complex (PIC) (3,4), and largely dissociate at every round of transcription. Evidence for a PIC was previously obtained with nuclear extract or with partially purified GTFs assembled on immobilized promoter DNA (5); (6);(7);(8);(9). Due to the poor efficiency of the reaction and trace amounts of protein involved, detection was only possible by the synthesis of a radiolabeled transcript and by immunoblotting.

An abundant, homogeneous, soluble PIC is required to elucidate the mechanisms of initiation and regulation of transcription. We sought to assemble a PIC with pure GTFs and pol II from the yeast S. cerevisiae. Four GTFs – TFIIA, TFIIB, the TATA-box binding protein (TBP), and TFIIE – were available in recombinant form, but the remaining GTFs – TFIIF and TFIIH – could only be obtained by isolation from yeast, and presented technical difficulties. Pure TFIIF was largely insoluble, and TFIIH, an 11-subunit complex, invariably dissociated upon isolation (10). The TFIIF problem was solved by identifying a detergent capable of solubilizing the protein without effect on transcriptional activity. Instability of TFIIH was traced to a previously unrecognized subunit, termed Tfb6 (11), which provokes the dissociation of Ssl2, the helicase responsible for conversion of a closed (fully double stranded) promoter to the open state (with about 15 base pairs unwound in the form of a “transcription bubble”). Isolation of TFIIH from a tfb6 deletion strain of yeast resulted in a good yield of the complete 10-subunit protein, lacking only Tfb6 (therefore referred to here as TFIIH*), and active in transcription.

With all the GTFs in suitable form in hand, we investigated the assembly of a PIC, and arrived at an efficient procedure for obtaining a stable, functional complex. We found intermediates consistent with the emerging picture of PIC assembly in vivo. The results were also informative about the fate of the complex upon the initiation of transcription. The way is now open to structure determination of the PIC and to dissection of the initiation process.

**EXPERIMENTAL PROCEDURES**

**Oligonucleotides**

Short fragments of the HIS4 promoter DNA (HIS4 (-81/+1) and HIS4 (-81/+19)) were obtained by annealing equimolar amounts of complementary oligonucleotides (IDT). Other HIS4 promoter templates were obtained by restriction digestion of a concatemeric form, as described below. HIS4 promoter DNA was amplified by PCR using two primers with EcoRV sites at both ends, and was cloned into pDrive vector (Qiagen). The plasmid construct was digested with EcoRI, and the promoter DNA fragment was purified and concentrated to 240 µg/ml using a Vivaspin 500 (5K MWCO) (Vivascience). The fragment was self-ligated in 20 µl of ligation buffer with 2 units of T4 ligase (NEB) to obtain a concatemer (usually 4-6 copies). The concatemer DNA was purified by agarose gel electrophoresis and was cloned into pUC18. XL10-gold strain (Stratagene) harboring the plasmid was grown in 2-6 L of LB media and the plasmid was isolated using Plasmid Gigaprep (Qiagen). After restriction digestion with EcoRV, the plasmid DNA fragment (~3.8 Kbp) was precipitated by adding PEG6000 and NaCl to final concentrations of 6-8% and 500 mM. The promoter DNA in the supernatant was extracted with phenol:chloroform:isoamyl alcohol (25:24:1) and phenol:chloroform (1:1), and was precipitated with ethanol. The DNA pellet was resuspended with gel filtration buffer (10 mM HEPES (pH7.6), 300mM potassium acetate, and 5 mM DTT) and was fractionated on a Superose 6 column (GE), yielding 1-3 mg of fragment. Thus non-native ATC and GAT were retained at 5' and 3' ends (Figure 1A).

**Run-off transcription**

DNA template (2.5 pmol) was mixed with 3.7 pmol of TFIIB, 3.7 pmol of TFIIA, 2.5 pmol of TBP, 3.7 pmol of TFIE, 1.5 pmol of TFIIH*, and 1 pmol of RNA polymerase II-TFIIF complex in 4 µl of buffer A (50 mM HEPES (pH 7.6), 300 mM potassium acetate, 5 mM DTT, and 5% glycerol). Following addition of 6 µl of buffer B (50 mM HEPES (pH7.6), 5 mM magnesium sulfate, 30 mM potassium acetate, and 5 mM DTT), the mixture was kept for 1 hr at 4°C. Transcription was initiated by adding an equal volume of 2x transcription mixture (1.6 mM ATP, 1.6 mM GTP, 1.6 mM CTP, 40 µM UTP, 0.083 µM of [α-32P] UTP (2.5 µCi), 10
mM magnesium-acetate, and 5 units of RNaseOUT) at 30°C and stopped after 45 min by adding 185 µl of stop buffer (10 mM Tris (pH7.5), 300 mM sodium acetate (pH 5.5), 5 mM EDTA, 0.7% SDS, 0.1 mg/ml glycogen, 0.013 mg/ml of proteinase K). Transcripts were analyzed as described (12).

**PIC reconstitution on a preparative scale**
All reconstitution experiments were performed on ice or at 4°C with proteins purified as before (11,13). Promoter DNA (0.5 nmol) was mixed with 0.75 nmol of TFIIA, 0.75 nmol of TBP, 0.7 nmol of TFIIE, 0.3 nmol of Tb6Δ-TFIIH (TFIH*) in a 40 µl of buffer (500) (20 mM HEPES (pH7.6), 5 mM DTT, 2 mM magnesium acetate, and 5% glycerol, with the mM concentration of potassium acetate in parentheses). The protein mixture was dialyzed in steps of buffer (300), buffer (220), buffer (150) for at least 4 hr at each step, and was then combined with 0.25 nmol of RNA polymerase II-TFIIF complex. The mixture was further dialyzed into buffer (100) and buffer (80), before loading on a 10-40% (v/v) glycerol gradient containing 20 mM HEPES (pH7.6), 5 mM DTT, 2 mM magnesium acetate, 80 mM potassium acetate. After centrifugation at 40,000 rpm in a Beckman SW60 rotor for 9 hr, the gradient was fractionated using a PGF Piston Gradient Fractionator (BioComp). The fractions were kept at -80°C without loss of transcriptional activity. For isolation of the open complex, the PIC was reconstituted in the same way, incubated with 1.6 mM ATP, 0.5 mM GTP, and 0.5 mM CTP for 15 min, and sedimented in a glycerol gradient containing 20 mM HEPES (pH 7.6), 5 mM DTT, 2 mM magnesium acetate, 80 mM potassium acetate, 1.6 mM ATP, 0.5 mM GTP, and 0.5 mM CTP. RNA polymerase II-TFIIF complex was omitted in the reconstitution of an intermediate complex and glycerol gradient centrifugation was for 5 hr at 60,000 rpm in a Beckman SW60 rotor.

**Exonuclease footprinting**
For determination of the downstream boundary of the PIC, the 5'-end of an upstream primer (5’-GGATATGACTATGAAACAGTAG-3’) was labeled with [γ-32P] ATP using T4 polynucleotide kinase. HIS4 (-96/+112) was amplified by PCR in 1-2 mL reaction using the 32P-labeled upstream primer and downstream primer (5'-TATTCCATGAGGCCAGATC-3’), and was purified by electrophoresis in a 2% agarose gel. The labeled DNA (1 pmol) was incubated for 1 hr at room temperature with 2 pmol of TFIIA, 1.1 pmol of TBP, 2.4 pmol of TFIIE, 1.5 pmol of TFIIH*, and 1.2 pmol of RNA polymerase II-TFIIF complex in 8 µl of buffer A (50 mM HEPES (pH7.6), 300 mM potassium acetate, 5 mM DTT, and 5% glycerol), then combined with 12 µl of buffer B (50 mM HEPES (pH7.6), 5 mM magnesium sulfate, 30 mM potassium acetate, and 5 mM DTT), and incubated for 1 hr at 4°C. The reconstituted PIC was combined with an equal volume of 2x NTP buffer (1.6 mM NTP(s) or 0.5 mM nonhydrolyzable analog ATPγS, 10 mM magnesium acetate, and 5 units of RNaseOUT) and incubated for 30 min at 30°C. Exonuclease III digestion was performed with 200 U (NEB) for 9 min at 30°C, and was stopped by adding 185 µl of stop buffer (10 mM Tris (pH 7.5), 300 mM sodium acetate (pH 5.5), 5 mM EDTA, 0.7% SDS, 0.1 mg/ml glycogen, 0.013 mg/ml of proteinase K, 0.5mg/ml Salmon Sperm DNA (Invitrogen)). The products were precipitated with 650 µl of 100% ethanol and kept overnight at -20°C. The DNA pellet was recovered by centrifugation at maximum speed for 1hr, dried at 37°C, and resuspended with 10 µl of gel loading buffer (95% formamide, 0.02 % bromophenol blue, 5 mM EDTA, and 0.025 % xylene cyanol). The products were analyzed by denaturing 6% polyacrylamide gel electrophoresis and detected with a PhosphorImager.

**Electron Microscopy**
PICs were sedimented through 10-40% glycerol gradient (containing a gradient of glutaraldehyde from 0 to 0.1%)(Kastner et al., 2008) for 9 hr at 40,000 rpm in a Beckman SW60 rotor. After dilution to 50-200 µg/ml, 2-3 µl were applied to continuous carbon-coated specimen grids (CF300-Cu EMS), washed with 2% uranyl acetate solution, blotted, and dried. Images were collected at a magnification...
Psoralen cross-linking of the PIC
The glutaraldehyde-fixed PIC was combined with psoralen (20 \( \mu \)g/ml) and was irradiated with a 360nm long wavelength ultraviolet fluorescent lamp. DNA was denatured in the presence of glyoxal and spread for electron microscopy as described (15). Grids were scanned at a magnification of 20kx on CCD (4 K \( \times \) 4 K Gatan Ultrascan\textsuperscript{TM} 4000) with a JEOL 1230 electron microscope. The size of denatured bubbles was calculated from the average of the lengths of the two halves of the bubble.

CTD phosphorylation
Pol II (0.14 \( \mu \)M) was treated with 0.4 \( \mu \)M TFIIK (16) in 20 mM HEPES (pH 7.6), 7.5 mM magnesium acetate, 100 mM potassium acetate, 10 mM DTT, 5% glycerol, 0.1% 3-(decyldimethylammonio) propanesulfonate (Sigma), and 1 mM ATP for 1hr at room temperature. Reactions were stopped by adding EDTA and analyzed by SDS-PAGE.

RESULTS
Assembly and isolation of yeast Pol II PIC
To identify a suitable DNA for assembly of a PIC, we performed transcription with a series of fragments of the \( HIS4 \) promoter and a mixture of pure transcription proteins (TFIIA, TFIIIB, TBP, TFIIE, TFIIF*, and pol II-TFIIF complex). All fragments extending from -84 to +74 with respect to the first transcription start site at +1 (Figure 1A) yielded runoff transcripts of the expected lengths (Figure 1B). A fragment truncated at +50 failed to support transcription, consistent with a previous study, which identified a requirement for promoter DNA extending at least 50 bp downstream from transcription start site (17). All proteins except TFIIA were required for transcription (Figure 1C), consistent with previous studies of transcription in vitro (12,18). The amount of pol II-TFIIF complex was limiting, with all other proteins and DNA added in molar excess and saturating for activity; the yield of the reaction was 0.076 transcripts per pol II-TFIIF complex. Run-off transcription was ~95% complete in 15 min and was limited to a single round (Figures 1D-E). Similar results were obtained with other promoters, except for variation in the level of transcription (Table 1).

It was not possible to perform the reaction on a preparative scale by increasing the concentration of components, as a 10-fold increase resulted in precipitation, and no transcription was obtained (not shown). A concentrated mixture was, however, soluble at elevated ionic strength. TFIIH* could be combined with excess TFIIA, TFIIB, TBP, TFIIE, and \( HIS4 \) promoter DNA (-96/+112) in 0.5 M potassium acetate and dialyzed to 0.15 M without precipitation. The resulting GTF-DNA complex was combined with pol II-TFIIF complex, dialyzed to 0.1 M potassium acetate, and sedimented in a 10-40 \% glycerol gradient. A single peak in the center of the gradient (lanes 7 and 8 in Figure 2A) contained equimolar amounts of all transcription proteins (Figure 2E), as shown by SDS-PAGE and densitometry, which resolved the 31 proteins in the PIC. The same results were obtained with a minimal DNA fragment (-81/+1) (lane 2 in Figure 2E).

Electron microscopy provided support for the assembly of a complete PIC. Peak glycerol gradient fractions, embedded in stain, disclosed fields of particles, virtually identical to one another except for differences in direction of view (Figure 3A). The particles often appeared bipartite, with each part comparable in size to a pol II – TFIIB – TBP – DNA complex. No uniform particles were obtained when the experiment was repeated with the omission of any one of the GTFs.

The location of the PIC on the \( HIS4 \) promoter DNA fragment was mapped by reaction with psoralen, which cross-links the two DNA strands (Figure 3B). A region of DNA bound by protein is protected from cross-linking and appears as a single stranded bubble following denaturation. Analysis of the PIC in this way gave rise to a bubble at one end of the promoter fragment (Figure 3B), in keeping with the location of the TATA box towards one end of the fragment. The contour length of the bubble corresponded to 67.6 \( \pm \) 2.6 bp, (average of 57 molecules)(Table 2), and increased by 10.9 bases (to 78.5 \( \pm \) 5.0 bp)
upon addition of ATP, GTP, and CTP (average of 85 molecules) although the percentage of molecules with a bubble was much lower in this case (Table 2).

The fully assembled PIC isolated by gradient sedimentation utilized the same start site as PICs obtained by simple mixing (Figure 2B). The level of transcription, 0.106 (± 0.012) transcripts per PIC, was similar to levels obtained by simple mixing of pol II with all factors added in excess. There was no increase in transcription upon supplementation with additional TFIIB, TBP, TFIIE, or TFIIH*, further attesting to the completeness of the isolated PIC (Figure 2C).

Further evidence that the PIC represents a stable entity, rather than a mixture capable of transcription with additional factors, came from a template challenge experiment. A PIC formed on HIS4 (-96/+112) was transcribed in the presence of the shorter promoter fragment HIS4 (-96/+74). Only the pair of runoff transcripts from the longer fragment was observed (Figure 2D, lanes 1-3), and not the transcripts expected from the shorter fragment (Figure 2D, lane 4).

**Isolation of GTF-DNA intermediate**

We investigated the intermediate formed in the first step of PIC assembly by sedimentation in a glycerol gradient. Two complexes were resolved, one containing equimolar amounts of four GTFs - TFIIC, TBP, TFIIE, and TFIIH* - as well as promoter DNA (Figure 4A), and a second, slower sedimentating complex, containing TFIID, TBP, TFIIE, and promoter DNA. The four-GTF complex, when supplemented with TFIIB and pol II-TFIIF, supported transcription (Figure 4B), whereas little transcription was obtained when supplemented with pol II-TFIIF complex alone, confirming the absence of TFIIB from the gradient-purified preparation. The four-GTF complex therefore provides a platform onto which pol II-TFIIF and TFIIB assemble to form the PIC.

**Isolation of open complex**

The PIC assembled by simple mixing of all components at low concentration exhibited downstream and upstream barriers to digestion by exonuclease III at about positions -9 and -70 respectively with respect to the first transcription start site (Figure 5A). Upon addition of ATP (but not the nonhydrolyzable analog ATPγS), the downstream barrier disappeared, and pauses or stops in digestion downstream, between +7 and +46, were intensified, indicative of essentially complete conversion from closed to open complexes. Open complex formation was dependent on the inclusion of TFIIE and TFIIH* in the PIC (Figure 5). The shift in the downstream boundary of the PIC is consistent with the idea of “promoter scanning” proposed to explain the location of transcription start sites 40-120 bp downstream of the TATA box in yeast(19). There was no further change in the pattern of exonuclease III digestion upon the addition of GTP, CTP, and UTP, consistent with the small percentage of open complexes that give rise to runoff transcripts (about 7.6%, as noted above).

The PIC in the presence of ATP, and also GTP and CTP appeared in faster sedimenting fractions (Figures 6A and D). Following sedimentation in the glycerol gradient, the PIC retained its entire complement of 31 polypeptides (Figure 6B). The largest subunit of pol II, Rpb1 was hyper-phosphorylated (Figure 6C), producing a quantitative mobility shift from the starting (“IIa”) position to that characteristic of a hyperphosphorylated state (“IIo”). Electron microscopy revealed a bipartite structure of the same size, regardless of presence or absence of nucleotides (Figure 6E).

**DISCUSSION**

The notable finding from this work is the isolation of an abundant, homogeneous RNA polymerase II transcription preinitiation complex. It was uncertain, even doubtful, that such a complex could be formed, and that it would resist the rigors of isolation. In our experience, complexes of pol II with TFIIB, with TFIIF, and with DNA, dissociate during handling; we hoped, however, that a complex with all the factors would be more stable than those with individual ones, and that
The procedure we developed for the assembly of the complete complex may resemble the pathway for PIC formation *in vivo*. An intermediate comprising TBP, TFIIE, TFIIH, and DNA could be isolated and then combined with TFIIB and pol II–TFIIF to generate the PIC. Support for the significance of this intermediate comes from studies of transcription reinitiation *in vitro* and *in vivo*. Following the addition of NTPs to a PIC formed with nuclear extract on immobilized promoter DNA, four GTFs – TFIIA, TFIID, TFIIE, and TFIIH – as well as Mediator, were retained on the DNA, as revealed by immunoblot analysis. Addition of TFIIB, TFIIF and pol II to the immobilized complex enabled transcription. The complex retained on the DNA was termed a reinitiation “scaffold” (7). A similar complex may be responsible for the identification by ChIP-ChIP analysis of genes enriched for TFIIH and Mediator (20). The assembly intermediate we have isolated may be regarded as a scaffold in the full sense of the word: it supports promoter DNA positioned with respect to pol II, following a path around pol II with little, if any, direct contact to the pol II surface.

Promoter-pol II interaction takes place upon the addition of ATP and open complex formation. We show that virtually all PICs undergo the transition to the open complex (Figure 5), which can be reisolated, without loss of any of the 31 protein components (Figure 6). In this respect, our findings differ from the unstable open complex obtained by others with immobilized templates: in the human system, PICs lose activity 1 min after addition of ATP (21,22) and the activity can be partially restored by additional TFIE (8); in the yeast nuclear extract system, PICs incubated with ATP rapidly undergo dissociation of pol II, TFIIB, and TFIIF (7,23). The loss of pol II is unexpected, in light of studies demonstrating the high affinity of pol II for open promoter DNA. Indeed if pol II is truly lost from the complex formed on immobilized DNA, it is unlikely to represent an intermediate on the pathway to transcription. We cannot explain this previous result of others, but the very stable open complex we have isolated, retaining all protein components, is demonstrably relevant, as shown by its capacity for transcription.

We have mapped the location and extent of the PIC on promoter DNA in both closed and open complexes by nuclease digestion and psoralen cross-linking. The barriers to exonuclease III digestion of the PIC around -70 to -9 are in good agreement with the observed size and location of the 68bp psoralen bubble at the end of the DNA fragment, and consistent with previous protein-DNA cross-linking studies performed with yeast nuclear extract (24) and with a reconstituted human system (25). The TATA sequence, between -56 and -63, would be located off center within the PIC, with an additional 40 bp lying within the PIC on the downstream side.

There was no loss of transcriptional activity of the PIC upon isolation. The level of transcription with the purified PIC was approximately same as that obtained by direct mixing of pol II with excess GTFs, but was much higher than that obtained by direct mixing of pol II and equimolar amounts of GTFs (data not shown), indicating that one or more of the GTFs was only partially active.

The level of transcription was promoter-dependent, ranging from 0.04 to 0.175 transcripts per PIC with the three promoters tested. In view of the homogeneity of the isolated PIC, and the virtually complete transformation from closed to open complexes, it seems likely that the failure to achieve 100% transcription efficiency reflects some limitation(s) of the initiation process itself, in formation of the first phosphodiester bond, or in events leading to a transcript length of about 25 nucleotides, or in the subsequent transition from initiation to elongation. It is noteworthy that the observed initiation rate *in vitro* (on the order of 0.1 per min, Figures 1D, 1E) is much slower than the rate of initiation *in vivo*, for
example about 0.15 per sec at an enhanced HIS3 promoter (26), and 0.25 per sec at the hsp70 promoter (27). The fraction of productive PICs and the initiation rate are doubtless influenced by other factors, such as activators and coactivators (28). There is now the possibility of separating inactive complexes from those engaged in transcription and determining the basis for the difference between them.

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FIGURE LEGENDS

Figure 1. Run-off transcription
(A) The template strand sequence of the promoter HIS4 (-96/+112) is shown. Non-native ATC and GAT were retained at 5' and 3' ends, respectively. The in vitro transcription start sites are indicated by black arrows. (B) The templates indicated over each lane were prepared as described in Experimental procedures. HIS4 promoter DNA (2.5 pmol) was mixed with 3.7 pmol of TFIIB, 3.7 pmol of TFIIA, 2.5 pmol of TBP, 3.7 pmol of TFIIE, 1.5 pmol of tfb6Δ-TFIH (TFIIH*), and 1 pmol of RNA polymerase II-TFIIF complex. Transcription was initiated by adding an equal volume of 2x transcription mixture containing 1.6 mM ATP, 1.6 mM GTP, 1.6 mM CTP, 40 µM UTP and 0.083 µM [α-32P] UTP. (C) Requirement for each factor in run-off transcription assay. The complete reaction was performed as in (B) with HIS4 (-96/+112) template DNA. Other reactions contained all the factors except the component indicated over the lane. (D) Time course of run-off transcription. After PIC formation, transcription was initiated by the addition of all four NTPs and [α-32P] UTP, and reactions were terminated at 5, 15, 30, 45, or 60 min by the addition of stop buffer. (E) Same as (D) for 60 min without [α-32P] UTP. After 60 min, [α-32P] UTP was added and reactions were terminated at 65, 75, 90, 105, or 120 min by the addition of stop buffer.

Figure 2. Isolation and characterization of 31-subunit PIC
(A) PIC assembled with HIS4 (-96/+112), sedimented in a glycerol gradient, and fractions analyzed by SDS-PAGE. (B) Run-off transcription. The peak fractions of the PIC from (A) were combined with an equal volume of 2x transcription mixture containing 1.6 mM ATP, 1.6 mM GTP, 1.6 mM CTP, 40 µM UTP and 0.083 µM [α-32P] UTP, and the resulting transcripts were analyzed by gel electrophoresis. Arrows indicate the two major promoter-specific transcripts. (C) Run-off transcription using 1.5 pmol of the isolated PIC (lane 1) supplemented with 0.65 pmol of TFIIH* (lane 2), 1.3 pmol of TFIIE (lane 3), 1.0 pmol of TBP (lane 4), or 1.3 pmol of TFIIB (lane 5). (D) Run-off transcription of the isolated PIC (3 pmol) supplemented with 2.2 pmol (lane 2) or 4.5 pmol (lane 3) of pol II-TFIIF was challenged with shorter DNA fragment HIS4 (-96/+74)(2.2 pmol). For lane 4, transcription was initiated by simple mixing of each component as in Figure 1B with 2.2 pmol of HIS4 (-96/+112) and 2.2 pmol of HIS4 (-96/+74). Black and red arrows indicate two major promoter-specific transcripts from HIS4 (-96/+112) and HIS4 (-96/+74), respectively. (E) SDS-PAGE of peak glycerol gradient fractions (left panel), scanned and plotted (right panel). Lane 1: PIC assembled with HIS4 (-96/+112); lane 2: PIC assembled with HIS4 (-81/+1); lane 3: an internal standard, in which equimolar amount of TFIIA, -IIB, TBP, -IIE, -IIF, -IIH, and pol II were combined. lane 4: mixture of TFIIA, TFIIB, TBP; lane 5: mixture of TFIIH, TFIIE.

Figure 3. Electron microscopy analysis of the PIC
(A) Representative images of negatively-stained particles, complete 31-polypeptide PIC with HIS4 (-81/+19)(upper left), pol II-TFIIB-TBP-DNA complex (upper right), PIC assembled without TBP (lower left), PIC assembled without TFIIB (lower right). Scale bar, 100 nm. (B) Analysis of PIC on HIS4 promoter by psoralen crosslinking of DNA. The PIC with the HIS4 (-96/+112) was exposed to an interstrand DNA crosslinking agent. The DNA was then deproteinized and spread under fully denaturing conditions. The micrograph reveals ~68bp bubble on one end, where proteins blocked access of the crosslinker to the DNA.
Figure 4. Isolation of intermediate complex
(A) HIS4 (-92/+8) (0.9 nmol) was mixed with TFIIB (0.6 nmol), TFIIA (0.6 nmol), TBP (0.6 nmol), TFIIE (0.6 nmol), and TFIIH (0.3 nmol) and sedimented in a glycerol gradient. SDS-PAGE of the 31-polypeptide PIC (left) and the intermediate complex containing TFIIA, TFIIE, TFIIH, and TBP (right) are shown. (B) The intermediate complex was reconstituted with HIS4 (-96/+112) and sedimented in a glycerol gradient. NTPs were added to initiate transcription along with the components indicated above the lanes.

Figure 5. Exonuclease III Footprints of the PIC
(A) The 5'-end of the template strand of HIS4 (-93/+109) was labeled with 32P to determine the downstream boundary. The labeled DNA (1.0 pmol) was incubated with TFIIB (2.0 pmol), TFIIA (1.9 pmol), TBP (1.0 pmol), TFIIE (2.4 pmol), TFIIH (1.0 pmol) with or without nucleotides as indicated over the lanes, followed by treatment with exonuclease III and gel electrophoresis. Positions of protected fragments are indicated on the right and positions of molecular markers are indicated on the left. The assay was performed in the presence (left panel) or absence of TFIIE and TFIIH* (right panel). (B) To determine the upstream boundary, the 5'-end of the nontemplate strand of HIS4 (-132/+109) was labeled with 32P, incubated with the pol II and GTFs, and was treated with 3'-exonuclease. The arrow indicates the upstream boundary of the DNA of the closed complex. The assay was performed in the presence (left panel) or absence of TFIIE and TFIIH* (right panel).

Figure 6. Isolation of PIC in the presence of nucleotides
(A) The reconstituted PIC was subjected to glycerol gradient sedimentation in the presence of ATP, GTP, and CTP, and was analyzed by SDS-PAGE. (B) SDS-PAGE of the PIC in the presence (right) or absence (left) of nucleotides. Peak glycerol gradient fraction was analyzed by 4%–12% NuPAGE, followed by staining with Coomassie blue. (C) The PIC was analyzed as in (B) in a 5% polyacrylamide gel (lanes 1 and 2). As a control, 0.14 µM of pol II alone was treated with or without 0.4 µM TFIIK and analyzed by SDS-PAGE (lanes 3 and 4). The positions of the hyperphosphorylated Rpb1 subunit (Ilo) and the unphosphorylated subunit (IIa) are indicated on the right. (D) Fractionation of the PIC in the absence or presence of nucleotides. (E) Representative images of the negatively stained particles of the PIC with HIS4 (-96/+112) in the presence of ATP, GTP, and CTP. Scale bar, 100 nm.
|         | Transcription activity * |
|---------|-------------------------|
|         | Mean ± S.E.              |
| HIS4    | 0.076 ± 0.012 (n=7)     |
| SNR20   | 0.175 ± 0.03 (n=3)      |
| SNR14   | 0.041 ± 0.02 (n=2)      |

* The activity is defined as the mean number of transcripts per pol II
| No NTP (with fixation*) | 57 | 25 (43.8%) | 67.6±2.62 |
|------------------------|----|------------|-----------|
| +A,G,C (with fixation) | 85 | 10 (11.8%) | 78.5±5.0  |
| DNA alone              | 100| 11 (11.0%) | 65.5±5.34 |

* The PIC was fixed with Glutaraldehyde.
Figure 1

A

\[\text{TATA} \quad \text{atCGA\textsuperscript{TATGACTATGAACAGTAGTATACTGTG}TATA\textsuperscript{TATA}}\]

\[\text{GGACGTTATATTCACTCCTACCTGATGTTGCTATACAT} \quad \text{+10} \quad \text{+20} \quad \text{+30}\]

\[\text{AAAAATATCTAGAGCAACATGCTGTTGTACATACAT} \quad \text{+40} \quad \text{+50} \quad \text{+60} \quad \text{+70}\]

\[\text{CATGTTTACAAATTTTTTTCTGAAATATGTTTTG} \quad \text{+80} \quad \text{+90} \quad \text{+100} \quad \text{+110}\]

\[\text{CGATCTAACCTTTAATTAAATCTTGGCFCATGOAATA} \quad \text{at}

B

| TBP | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|-----|---|---|---|---|---|---|---|---|---|----|----|----|
|     |   |   |   |   |   |   |   |   |   |    |    |    |

C

D

stop reaction

\[\text{factors + NTPs} \quad \text{+ [\text{32P}] UTP}\]

\[5 \quad 15 \quad 30 \quad 45 \quad 60 \text{ min}\]

E

stop reaction

\[\text{factors + NTPs} \quad \text{+ [\text{32P}] UTP}\]

\[65 \quad 75 \quad 90 \quad 105 \quad 120 \text{ min}\]
Figure 3

A

PIC

PolII-TFIIb-TBP-DNA

PIC without TBP

PIC without TFIIb

B

68.6 bp

65.6 bp

100nm

5' -96  +112  3'

5' downstream

~68bp
Figure 4

A

B

pol II-TFIIA

four-GTF complex (TBP-GA-GD-GDm)

pol II-TFIIB

four-GTF complex (TBP-IA-IE-IEm)
Figure 5

A

PIC

without IIH and IIE

DNA alone

ATP

GTP

CTP

UTP

1

2

3

4

5

6

+46

+7

-12

B

PIC

without IIH and IIE

DNA alone

ATP

GTP

CTP

UTP

1

2

3

4

5

6

-132

-99

-79

-60

-63

-63

-93

-9

+109

+109

-109

-109

-70

-63

-60

-63

-63

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Figure 6
Formation and Fate of a Complete, 31-Protein, RNA polymerase II Transcription Initiation Complex
Kenji Murakami, Guillermo Calero, Chris R. Brown, Xin Liu, Ralph E. Davis, Hinrich Boeger and Roger D. Kornberg

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