Supporting Information

Čabart et al. 10.1073/pnas.1104591108

SI Materials and Methods

Plasmid Constructions and Immobilized Template Preparations. All templates were based on the adenovirus major late promoter construct pML20-40 (1) with variations in the initially transcribed region; most of these constructs (pML-6gW, 8gW, 8g2D, and 11gW) were described previously (2, 3). For this study, two additional variants of 8g2D were created. The pML-8g2D-14a construct was derived from pML-8g2D by changing C and T at +14 and +15 to a double A block on the template strand. The pML-8a2D-75g template was created from pML-8g2D (parental plasmid) and pML-20-49(6g)-2 (4) through the following changes: G and T on the template strand at +8 and +9 were changed to a double A block and a 40-bp DraI-BamHI fragment was replaced by a 54-bp G-less sequence from pML-20-49(6g)-2 with a BamHI site at the downstream end, thereby generating a double G block at positions +75 and +76 on the template strand. The 5′-biotinylated, PCR-amplified working DNA templates (approximately 210 bp) contained a PvuII site 21 bp from the upstream end; transcription initiated 100 bp from the upstream end. Transcription templates were purified using the QIAquick gel extraction kit (Qiagen) and immobilized on Streptavidin-coupled Dynabeads M-280 (Invitrogen) as described by the manufacturer.

Protein Preparations. Recombinant general transcription factors TBP (TATA box-binding protein), TFIIB, TFIIE, and TFIIF were prepared as described (3). TFIIH was purified from HeLa nuclear extracts (3) and polymerase II (pol II) from HeLa nuclear pellets as reported (4, 5). TFIIF was phosphorylated in vitro as follows: 1 μg of His-tagged TFIIF was incubated with 2,000 U of casein kinase 2 (CK2; New England Biolabs) in reaction buffer (20 mM Tris-HCl, pH 7.5, 75 mM KCl, 35 mM NaCl, 10 mM $MgCl_2$, 0.2 mM ATP, 80 μg/mL BSA, and 0.25 mM PMSF) for 1 h at 30 °C in total volume of 400 μL. In parallel, mock-treated TFIIF underwent the same procedure, except that CK2 and ATP were omitted. After 1 h, 1 mL of binding buffer (20 mM Tris-HCl, pH 7.9, 500 mM KCl, 20% glycerol, 0.05% NP40, 10 mM imidazole, 1.5 mM β-mercaptoethanol, and 0.2 mM PMSF) was added and mixed with 20 μL of Ni-NTA agarose beads (Qiagen). The binding reactions were rotated for 2 h at 4 °C. Beads were washed five times with 1 mL of binding buffer. In the last wash, KCl was reduced to 100 mM KCl. Phosphorylated and unphosphorylated (P- and U-) TFIIFs were eluted with 50 μL of elution buffer (20 mM Tris-HCl, pH 7.9, 100 mM KCl, 20% glycerol, 0.05% NP40, 200 mM imidazole, 0.25 mg/mL BSA, 1.5 mM β-mercaptoethanol, and 0.2 mM PMSF) for 30 min on ice.

Assembly of Preinitiation Complexes (PICs). PICs were assembled at 30 °C for 20 min using per 10 μL: 558 fmoles Dynabead-attached template, 95 fmoles TBP, 3.3 fmoles TFIIE, 72 fmoles TFIIB, 46 fmoles unphosphorylated (U) or phosphorylated (P)-TFIIF, 1 μL TFIIH and 6.6 ng pol II in 20 mM Tris-HCl, pH 7.9, 65 mM KCl, 8 mM $MgCl_2$, 1 mM DTT, 0.05 mM EDTA, 5% glycerol, 0.1% NP40, and 1 mg/mL BSA. PICs were washed initially with BC100 buffer (20 mM Tris-HCl, pH 7.9, 100 mM KCl, 20% glycerol, 0.25 mM EDTA, and 1 mM DTT) containing 0.5 mg BSA/mL and 0.1% NP40 and then washed twice with transcription buffer M5 (20 mM Tris-HCl, pH 7.9, 65 mM KCl, 10 mM β-glycerophosphate, 10 mM $MgCl_2$, 5% glycerol, 0.25 mM EDTA, and 1 mM DTT), containing 0.25 mg/mL BSA and 0.1% NP40 before use in transcription or in factor analysis.

In Vitro Transcription and Abortive Initiation. Transcription was initiated with 1 mM CpA (Dharmacon), which pairs at $-1/+1$ with the template strand, or with 1 mM ApC (Sigma), which pairs at $+1/+2$, along with 25 μM dATP as the energy source; alternatively 0.5 mM ATP alone was used, which directs initiation at +1 (see the figure legends). Reactions to produce labeled RNA 6 nt or longer also contained 2 μM [α-^{32}P] CTP (PerkinElmer), 0.5 mM UTP, and RNasin (0.4 U/μL). When RNA was not labeled, 0.5 mM CTP was used; for the experiments in Figs. 1*A* and 5, the chain terminator 3′-deoxy-GTP (TriLink Biotechnologies) was added at 0.1 mM. Initial transcription reactions were performed at 30 °C for 5 min. If longer RNAs were to be made in a two-step procedure (Figs. 3*C*, 4, and 6 and Fig. S3 *A* and *B*), the initial elongation complexes (ECs) were washed twice with M5 plus 0.25 mg/mL BSA and 0.1% NP40 (M5/BSA/NP40) and then chased with 0.5 mM of the appropriate NTPs for 1–4 min (Fig. 4 and Fig. S3) or 2 min (Figs. 3*C* and 6) at 30 °C. Abortive initiation assays (Fig. 3*A* and Fig. S1*B*) were run for 5 min with 1 mM CpA, 25 μM dATP, and 1 μM [α-^{32}P] CTP. Denaturing polyacrylamide gels of 20% (or 28% when trinucleotides were produced) were used to resolve labeled RNAs, which were quantified using a Storm Imager and ImageQuant software.

Analysis of Proteins Retained on and Released from the Templates. To generate complexes for immunoblotting assays, PIC assembly was scaled up three times from the procedure described above and nonlabeled NTPs were used. At the end of all reactions, supernatants were separated from beads, complexes washed once in 5 μL of M5/BSA/NP40, and the washes combined with supernatants for subsequent analysis. To obtain the template-bound fraction, complexes after the M5/BSA/NP40 wash were resuspended in 15 μL of M5/BSA/NP40 and treated with PvuII (New England Biolabs; 3.3 U/μL) for 10 min at 30 °C followed by addition of herring sperm DNA (1 ng/3 ng of template) for 1 min at room temperature. The supernates from the PvuII digest were collected, the beads were washed with 5 μL M5/BSA/NP40, and the combined supernatants used for analysis. Proteins were resolved on SDS-12% polyacrylamide gels, which were transblotted onto polyvinylidene fluoride membranes (Millipore). Immunodetection was performed by enhanced chemiluminescence (Millipore) as recommended by the manufacturer. Antibodies against TFIIB (sc-274), TFIIF-RAP74 (sc-234), TFIIF-RAP30 (sc-134081), and TFIIH-p62 (sc-292) were purchased from Santa Cruz Biotechnology. TBP was detected with anti-FLAG antibody and the Rbp7 antibody was a gift from Erica Golemis (Fox Chase Cancer Center, Philadelphia, PA). Membranes were exposed to film to generate signals in the linear range. We generally had two intensity standards loaded and exposed the films so that the ratio of signals reflected the ratio of the standard masses—that is, detection between the two standards would be linear. Scanning was done with a Bio-Rad Molecular Imager and results quantified with ImageJ software.

Determination of RNA Synthesis Levels Supported by PICs. The amount of TFIIB in an aliquot of PIC was measured as described above. The amount of RNA that could be made by an identical volume of PIC was measured by determining the intensity of a ^{32}P-labeled 20-mer transcript using a Storm Imager and comparing that to the intensity obtained with known amounts of ^{32}P-CTP spotted on filter paper and exposed along with the gel in which the 20-mer was resolved. The template was 8g2D and initiation was with ApC as in most TFIIB release/retention assays, which

gives a 20-mer transcript in the absence of ATP. In order to match transcription conditions with the factor release assays, the 20-mer was synthesized with 1 mM ApC, 25 μM dATP, 0.5 mM UTP and GTP, 2 μM [α-^{32}P]-CTP, and 498 μM CTP for 5 min at 30 °C. We found that 1.8 fmole of TFIIB in transcription complex corresponded to the synthesis of 0.66 fmole of RNA in the 5-min reaction.

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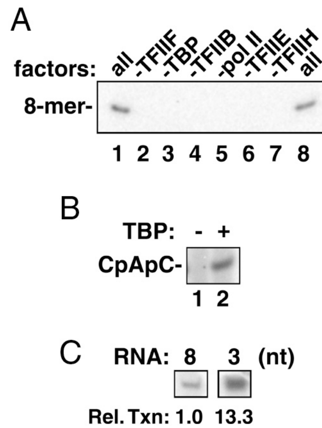

Fig. S1. (*A*) Formation of initiation-competent PICs is specific to addition of each individual factor. PIC assembly reactions were performed on 8g2D in the presence of all factors (lanes 1 and 8) or in the absence of TFIIF, TBP, TFIIB, pol II, TFIIE, or TFIIH (lanes 2-7, respectively). To generate 8-mer RNAs, reactions contained 1 mM CpA, 25 μM dATP, 2 μM [α-^{32}P] CTP, and 0.5 mM UTP. Reactions were run for 5 min at 30 °C. (*B*) Abortive initiation is dependent on TBP—TATA box interaction. PIC assembly reactions were performed as in *A* with or without TBP (lanes 2 and 1, respectively). Washed PICs were incubated for 5 min with 1 mM CpA, 25 μM dATP and 1 μM [α-^{32}P] CTP to generate CpApC. (*C*) Efficiency of multiple round transcription. PICs assembled on 8g2D were supplemented with 1 mM CpA, 25 μM dATP, and 1 μM [α-^{32}P] CTP to monitor abortive initiation (3-nt RNA) or with 0.5 mM UTP added for productive initiation (8-nt RNA). RNA levels were quantified for the 3-mer and 8-mer transcripts; relative transcription levels were corrected for the CTP content of the 8-mer, with the single round value set to 1.0.

Fig. S2. (*A*) PICs assembled with P-TFIIF fail to retain both subunits of TFIIF. Assembly reactions on 8g2D contained U-TFIIF (lane 2), P-TFIIF (lane 3), or no TFIIF (lane 1). Retention of RAP74 and RAP30 in the PICs was analyzed by Western blotting as described in *Materials and Methods*. The two left-hand lanes (Std) contained 2 ng of U- or P-TFIIF. (*B*) Neither unphosphorylated nor phosphorylated TFIIF forms a stable complex with promoter DNA in the absence of other factors. U-TFIIF (lane 3) and P-TFIIF (lane 4) were incubated with 8g2D under the same conditions (including concentrations of TFIIF and template) described for PIC assembly in *Materials and Methods*. Binding reactions were washed and the template-bound fraction was analyzed by immunoblotting with antibody specific to RAP74. Lanes 1, 2 and 5, 6 contained 2 ng and 1 ng of U-TFIIF or P-TFIIF, respectively. (*C*) TBP governs specific recruitment of factors in PIC assembly. PIC assembly reactions on 8g2D were performed as described in *Materials and Methods* in the presence (lane 2) or in the absence of TBP (lane 1). The retention of TFIIB, Rpb7, TFIIH (p62), or RAP74 within the PICs was determined by immunoblotting. The left-hand lane (Std) in each panel represents 0.75 ng TFIIB, 0.5 ng Rpb7, 1 μl TFIIH, or 2 ng TFIIF.

Fig. S3. TFIIB becomes destabilized within the EC when the transcript is extended from 7 to 13 nt. PICs were assembled with unmodified TFIIF on 8g2D (for eventual synthesis of 8- or 10-mers), 8g2D-14a (for 13-mers), or 8a2D-75g (for 74-mers and 107-nt runoff RNA). On all templates, pol II was advanced to +7 with ApC, CTP, and UTP (plus dATP) and the ECs were washed. (*A*) 7-mer ECs were chased with GTP, UTP, and CTP (+7 → 13), ATP, UTP, and CTP (+7 → 74), or all four NTPs (+7 → RO) for the indicated times. For control reactions, stalled 7-mer ECs were incubated with ATP (8g templates) or GTP (8a templates) only. Proteins retained on the templates or released from the templates were analyzed for TFIIB and pol II (Rpb 7) by Western blot as described in *Materials and Methods*. The Std lane contained TFIIB (1 ng) or Rpb7 (0.5 ng) in each panel. (*B*) As in *A*, except that 7-mer ECs were advanced with GTP (+7 → 8) or GTP and UTP (+7 → 10); only the chased reactions are shown.