J. Biochem. **133**, 133–138 (2003)
DOI: 10.1093/jb/mvg013

A Rapid Purification Method for Human RNA Polymerase II by Two-Step Affinity Chromatography

Jun Hasegawa[1], Masaki Endou[1], Takashi Narita[1], Tomoko Yamada[1], Yuki Yamaguchi[1,3], Tadashi Wada[1] and Hiroshi Handa[*,1,2]

[1]Graduate School of Bioscience and Biotechnology and [2]Frontier Collaborative Research Center, Tokyo Institute of Technology, 4259 Nagatsuta-cho, Midori-ku, Yokohama 226-8501; and [3]PRESTO, Japan Science and Technology Corporation, Yokohama 226-8503

Received October 7, 2002; accepted November 5, 2002

The molecular dissection of transcription mechanisms is greatly facilitated by constructing and manipulating defined transcription systems *in vitro*. This approach requires highly purified transcription factors. A major enzyme participating in the transcription reaction is RNA polymerase II (RNAPII), which is composed of at least 12 subunits (RPB1–12). Due to its complex structure, it is difficult to prepare highly pure RNAPII by the conventional purification procedure. We transfected HeLa cells with a plasmid expressing RPB3 with a double FLAG-histidine tag on its amino-terminus. A high yielding clone was isolated and its extracts were subjected to immunoaffinity purification and then Co^{2+} affinity chromatography. This resulted in a preparation of RNAPII complexes that consisted of all the core subunits, including the double-tagged RPB3 protein. Transcription reactions with oligo (dC)-tailed templates and transcription assays involving general transcription factors revealed that the double-tagged RNAPII complexes are active and functional in basal and activated transcription. Our method is superior to the conventionally used purification procedure in that the final preparation is markedly more pure (92% *versus* 40%), and the procedures are much less time-consuming. Thus, this two-step affinity purification method is an uncomplicated and effective method by which active and functional RNAPII can be prepared.

Key words: affinity chromatography, RNA-polymerase, transcription, transcription-regulation.

The expression of eukaryotic genes encoding proteins is mediated by RNA polymerase II (RNAPII), which catalyzes the synthesis of mRNA in an α-amanitin-sensitive manner. Eukaryotes have three kinds of RNA polymerases, denoted RNAP I, II, and III, and RNAPII can be separated from the other two enzymes on the basis of its α-amanitin susceptibility. It has been reported that RNAPII consists of at least 12 subunits. Of these, RPB5, RPB6, RPB8, RPB10, and RPB12 are found in all three RNAPs, while RPB1, RPB2, RPB3, RPB4, RPB7, RPB9, and RPB11 are unique to RNAPII (*1*, *2*). In general, the activity of RNAPII is estimated by two methods. One is promoter-independent transcription using oligo (dC)-tailed templates. In this method, RNAPII can transcribe the template without the help of any other factors and starts synthesizing RNA without an initiation reaction. This transcription system thus assesses only the elongation activity of RNAPII itself (*3*, *4*). The other way to measure RNAPII activity is promoter-dependent transcription. Transcription from most eukaryotic promoters requires the presence of general transcription factors (GTFs) such as TFIIB, TBP, TFIIE, TFIIF, and TFIIH (*5*). GTFs support the transcription initiation reaction from promoters. It is believed that RNAPII activity in a transcription system composed of purified GTFs is a measure of its basal level of activity. Although TFIIA is a GTF, it does not participate in basal activity (*6*). Rather, it activates transcription and enhances the response to transcriptional activators when the promoter-dependent transcription system contains TFIID instead of TBP (*7–9*). TFIID is composed of TBP and TBP-associated factors (TAF). The functional difference between TBP and TFIID is also observed when DNA-binding transcriptional activators are used (*10*, *11*). TAF250, one of the human TAFs, has a TAND domain that naturally inhibits TBP-DNA binding activity (*12–14*). It seems that activators interact with TAFs and the interaction is likely to induce a conformational change in TAF250 that eliminates its inhibitory effect on the DNA-binding activity of TBP (*14*, *15*).

Methods to purify RNAPII from eukaryotic cells have been developed by many groups (see Ref. *16* and references therein). These methods employ conventional column chromatography (*17*). Although this allows active RNAPII to be purified, it is time-consuming and requires working in a cold room. In addition, the resulting preparation of RNAPII usually contains some extra polypeptides. Recently, some multiprotein complexes, such as the TIP60 histone acetylase, have been purified from cloned cell lines expressing a tagged protein that belongs to the target complex (*18*). The tag most often used is the FLAG tag. Based on this approach, Kershnar *et al.* have devel-

[*]To whom correspondence should be addressed. Phone: +81-45-924-5872, Fax: +81-45-924-5834, E-mail: hhanda@bio.titech.ac.jp

oped a method to purify human RNAPII from a clonal cell line. In this method, the FLAG tag is introduced onto the amino terminus of the RPB9 subunit, and the FLAG-tagged RNAPII is immunopurified from the cytoplasmic S100 fraction which has been subjected to phosphocellulose column chromatography (*19*). The resulting FLAG-tagged RNAPII preparations are capable of mediating basal and activated transcription (*19*). Two other groups have also purified RNAPII from yeast cell extracts using FLAG, histidine, or biotin acceptor peptide tags. Ishihama and his colleague affinity-purified *Shizosaccharomyces pombe* RNAPII by histidine-tagging RPB1 and RPB3. This allowed them to analyze the assembly of the core subunits of RNAPII (*20*). These authors also constructed *S. pombe* expressing FLAG-tagged RPB3 to analyze the cofactors that bind specifically to RNAPII (*21*). The other group, Kireeva *et al.*, constructed a *Saccharomyces cerevisiae* strain that produces histidine-biotin acceptor peptide-tagged RPB3. They succeeded in purifying the active form of RNAPII with avidine resin (*22*).

Here we report an improved method of RNAPII affinity purification using a combined FLAG-histidine tag. We chose the RPB3 subunit to be the target carrying the double tag because purification and characterization studies of yeast RNAPII have revealed that tagged RPB3 does not negatively affect RNAPII function (*20–22*). These studies also showed that FLAG and histidine tags are individually effective in purifying RNAPII from crude cell extracts (*20–22*). Another study has also reported the purification of active FLAG-tagged RNAPII from HeLa cell extracts (*19*), which indicates that the FLAG tag is also effective for purifying human RNAPII. In the method we developed, the FLAG and histidine cluster sequence were placed onto the amino terminus of the human RPB3 subunit and a cell line that efficiently produced the FLAG-histidine tagged RPB3 (FH-RPB3) was established. By using the double-tagged system, we succeeded in purifying a nearly homogeneous preparation of RNAPII complex (FH-RNAPII) containing FH-RPB3. We examined the resulting FLAG-histidine tagged RNAPII (FH-RNAPII) preparation for activity in *in vitro* transcription assays and found that FH-RNAPII is functional in both basal and activated transcription.

MATERIALS AND METHODS

Plasmids—To introduce the tag sequence MHHHHHHDYKDDDDKGH onto the amino terminal region of the RPB3 subunit, the primers FH-5′ CATGGGCGGCCGCACCATGCATCATCATCATCATCACGACTACAAGGATGACGACGACAAGGGACATATGTTGC and FH-3′ TCGAGCAACATATGTCCCTTGTCGTCGTCATCCTTGTAGTCGTGATGATGATGATGATGCATGGTGCGGCCGCC were synthesized. The annealed DNA fragments were cloned between the *Nco*I and *Xho*I sites in pET14b, generating pET14b-FH. To generate pET14b-FH-RPB3, the cDNA of the human RPB3 subunit that had been amplified by PCR with RPB3-5′ ATAACATATGCCGTAGCGCAACCAG and RPB3-3′ AGCAGGATCCTTAATTTATGGTTAGCACA was digested with *Nde*I and *Bam*HI and cloned between the *Nde*I and *Bam*HI sites in pET14b-FH. To generate pcDNA3.1-FH-RPB3, pET14b-FH-RPB3 was digested with *Not*I and *Bam*HI (filled-in) and the DNA fragment was cloned between the *Not*I and *Xba*I sites (filled-in) in pcDNA3.1 (+).

Isolation of Cell Lines Expressing FH-RPB3—pcDNA3.1-FH-RPB3 was introduced into HeLa cells by Effectene reagents (Qiagen) and the cells were cultured in the presence of G418 (800 μg/ml) for two weeks. To isolate the clones that produce FH-RPB3 most efficiently, immunoblot analysis with anti-FLAG antibodies was performed on 200 clones. The cell line FH3 showed the highest expression of FH-RPB3 proteins.

Purification of FH-RNAPII—Approximately 1.4×10^9 FH3 cells were collected and washed with PBS (+), resuspended in buffer A [10 mM HEPES, pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM dithiothreitol (DTT)], and homogenized in a glass Dounce homogenizer using a type B pestle. The cell extracts were then centrifuged at 800 ×*g* for 10 min. After discarding the cytoplasmic fractions, the nuclear pellets were resuspended in buffer B [50 mM Tris HCl, pH 7.9, 25% glycerol, 5 mM $MgCl_2$, 0.5 mM EDTA, 5 mM DTT, 0.5 mM phenylmethylsulfonyl fluoride (PMSF)] in the presence of 0.3 M ammonium sulfate, and then sonicated on ice for 30 min with a pulse mode (TITEC Ultra S. Homogenizer VP-15S). After centrifugation at 22,000 rpm for 10 h (Beckman TYPE 35), the supernatant was recovered and diluted with buffer B to adjust the ammonium sulfate concentration to 0.1 M. The solution was then centrifuged at 32,000 rpm for 1 h (Beckman TYPE 35). The supernatant was transferred to a new tube and powdered ammonium sulfate was added to the supernatant to a concentration of 0.42 g/ml. The sample was rotated for 1 h and centrifuged at 32,000 rpm for 1 h (Beckman TYPE 35). Pellets were resuspended in buffer C (50 mM Tris HCl, pH 7.9, 25% glycerol, 0.5 mM EDTA, 2 mM DTT, 0.2 mM PMSF). After dialysis of the sample against buffer D (20 mM HEPES, pH 7.9, 20% glycerol, 500 mM KCl, 0.2 mM EDTA, 1 mM DTT, 1 mM PMSF, 0.1% Triton X 100), the dialyzed solution was used as whole nuclear extract (WNE).

ANTI-FLAG M2-Agarose Affinity gel (240 μl, Sigma) was mixed with 4 ml of WNE and the mixture was rotated for 3 h. The gel was collected and washed five times with 2 ml of wash-buffer A (20 mM HEPES, pH 7.9, 20% glycerol, 400 mM KCl, 0.2 mM EDTA, 1 mM PMSF, 0.3% NP-40) and then twice with 2 ml of wash-buffer B (50 mM sodium phosphate, pH 7.0, 500 mM NaCl, 5% glycerol, 0.1% NP-40). Bound proteins were eluted by adding 500 μl of wash-buffer B containing 0.2 mg/ml FLAG peptides (Sigma). The elution step was repeated four times. The elution fractions (total 2 ml) were then applied to 60 μl of TALON Metal Affinity Resin (CLONTECH). After 60 min of rotation, the resins were collected and washed three times with 1 ml of wash-buffer B. Bound proteins were eluted by adding 90 μl of wash-buffer B containing 100 mM imidazole. The elution step was repeated and the elution fractions were pooled. After dialysis against 20 mM HEPES, pH 7.9, 20% glycerol, 100 mM KCl, 0.2 mM EDTA, 1 mM PMSF, 0.1% NP40 for 6 h, the dialyzed solution was used as the FH-RNAPII preparation. Protein concentrations were measured by the BioRad Protein Assay.

Purification of C-RNAPII—Purification of C-RNAPII was performed as described (*17*).

Preparations of Proteins—Recombinant TFIIA was prepared as described (*23*). Gal4-VP16 was prepared as described (*24*). Partially purified TFIIH was prepared from HeLa cell nuclear extracts (*25*). The extracts were diluted with an equal volume of HGE (20 mM HEPES, pH 7.9, 20% glycerol, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF) and loaded onto a P11 phosphocellulose column (Whatman) equilibrated with HGE containing 0.05 M KCl. The proteins were eluted by a step gradient of 0.1, 0.3, and 1.0 M KCl in HGE. The 1.0 M KCl fractions contained TFIIH activity. The fractions were dialyzed against HGE containing 0.15 M KCl and loaded onto a DEAE-Sepharose FF column (Amersham Biosciences) equilibrated with HGE containing 0.15 M KCl. The proteins were eluted by a step gradient of 0.15, 0.3, 1.0 M KCl in HGE. The DEAE 0.3 M KCl TFIIH-containing fractions were dialyzed against HGE containing 1.0 M $(NH_4)_2SO_4$ and loaded onto a Phenyl-Superose column equilibrated with HGE containing 1.0 M $(NH_4)_2SO_4$. TFIIH was eluted with a linear gradient from 1.0 M to 0 M $(NH_4)_2SO_4$. The TFIIH-containing fractions were pooled and dialyzed against HGE containing 0.1 M KCl. The dialyzed Phenyl fractions were used as partially purified TFIIH. TFIIH was monitored and examined by immunoblot with antibodies against CDK7 and *in vitro* transcription assays with linearized DNA templates (*26*).

In Vitro Transcription Assays—Transcription reactions with oligo (dC)-tailed templates were performed essentially according to the method described previously (*4*). Reactions (23 μl) containing the oligo (dC)-tailed templates dC3.8 (100 ng) and RNAPII in 10 mM HEPES, pH 7.9, 10% glycerol, 5 mM $MgCl_2$, 50 mM KCl, 0.5 mM DTT, 0.5 mM EDTA, 0.2 μg/μl bovine serum albumin were incubated for 30 min. To start elongation, 2 μl of an NTP mix (final concentrations 60 μM ATP, 600 μM CTP, 120 μM GTP, and 5 μM UTP containing 5 μCi [α^{32}P]UTP) was added to the reaction. The reaction mixture was incubated for 4 or 16 min.

A kinetically synchronized transcription reaction containing 30–40 ng of supercoiled DNA templates was performed as described previously (*26*). Briefly, we incubated a 23 μl reaction mixture that contained recombinant TBP (Proteinone), TFIIB (Proteinone), TFIIE (Proteinone), TFIIF (Proteinone), partially purified TFIIH, and DNA templates in TRX buffer (25 mM Tris HCl, pH 7.9, 10% glycerol, 4% polyethyleneglycol 8000, 5 mM $MgCl_2$, 50 mM KCl, 0.5 mM DTT, and 0.5 mM EDTA) for 45 min at 30°C. Nucleotides in TRX buffer (final concentrations 60 μM ATP, 600 μM CTP, and 5 μM UTP containing 5 μCi [α^{32}P]UTP) and 50 units of RNase T1 (Ambion) were then added, and the mixture was incubated for an additional 45 min (Fig. 3A) or 60 min (Fig. 3B).

Immunoblot Analysis—Immunoblot assays were performed as described previously (*27*, *28*). The blot filter was developed with the ECL system and reprobing strategies were used according to the manufacturer's protocol (Amersham Biosciences). Anti-FLAG antibodies were purchased from Sigma and anti-CTD antibodies were purchased from BAbCO.

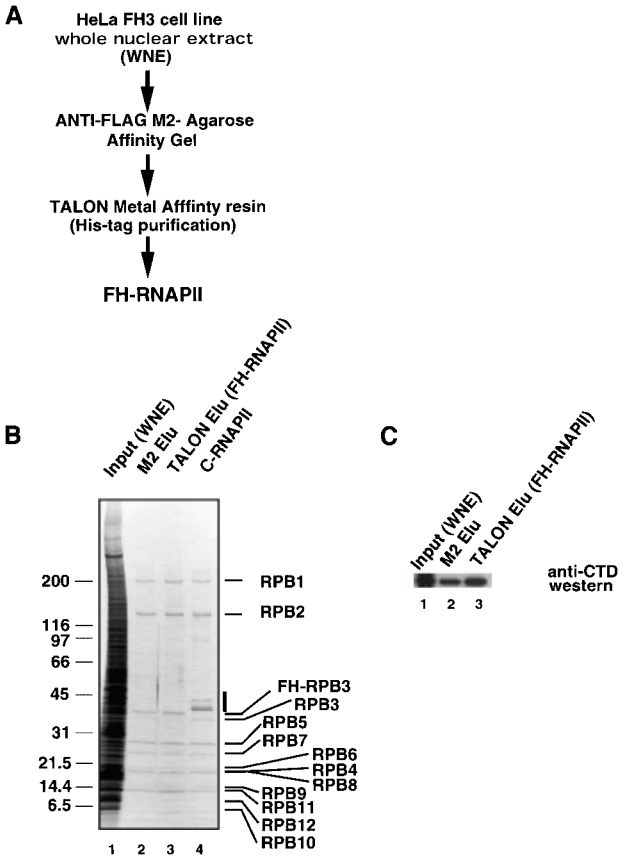

Fig. 1. **Purification of FH-RNAPII.** (A) The scheme for the purification of FH-RNAPII is illustrated. (B) Aliquots of the purified fractions were analyzed in a 5–20% SDS–polyacrylamide gel and the proteins were visualized by silver staining. Numbers to the left of the gel indicate the protein molecular size standards. Each subunit of RNAPII is identified on the right according to the published molecular weights (*2*, *29*). Input, WNE (8.8 μg, lane 1); M2 Elu, eluate of ANTI-FLAG M2 Agarose Affinity Gel (110 ng, lane 2); TALON Elu, eluate of TALON Metal Affinity Resin (110 ng, lane 3); C-RNAPII, RNAPII purified by a conventional column method (110 ng, lane 4). (C) Immunoblotting with anti-CTD antibodies. WNE (35 μg, lane 1), M2 Elu (110 ng, lane 2), and TALON Elu (110 ng, lane 3) were analyzed by immunoblotting with the anti-CTD antibody.

RESULTS AND DISCUSSION

Isolation of HeLa Cell Lines Expressing FLAG- and Histidine-Tagged RPB3 (FH-RPB3)—To purify RNAPII core subunits, we introduced the 17 amino acid tag MHHHHHH**DYKDDDDK**GH onto the amino terminus of human RPB3. The underlined amino acids indicate the histidine tag and the bold-faced amino acids indicate the FLAG tag. We transfected HeLa cells with a plasmid expressing FH-RPB3 and cultured them in the presence of G418. Immunoblot analysis with anti-FLAG antibodies was used to identify the clone FH3 that expressed high levels of FH-RPB3. Whole nuclear extracts (WNE) were prepared from 1.4×10^9 FH3 cells and first subjected to immunoaffinity purification with ANTI-FLAG M2-Agarose Affinity Gel. To remove the IgG proteins derived from the anti-FLAG antibodies and the FLAG peptides in the resulting eluate, we further performed Co^{2+} affinity purification employing TALON Metal Affinity Resin. The

Table 1. **Purification of FH-RNAPII and C-RNAPII.**

Enzyme	Fraction	Protein (mg)[a]	RNAPII (μg)[b]	Purity (%)[c]	Yield (%)[d]
FH-RNAPII	Input (WNE)	35 (1.4×10^9 cells)	1.6×10^2	0.46	100
	M2 Elu	0.084	5.7×10^1	68	36
	TALON Elu	0.012	1.1×10^1	92	6.9
C-RNAPII	Input	3.7×10^2 (8.0×10^{10} cells)	1.6×10^3	0.43	100
	P-11	0.52	2.1×10^2	40	13

Preparations of Input (WNE), M2 Elu, and TALON Elu from the FH3 cell line are described under "MATERIALS AND METHODS." Preparations of Input (chromosomal fraction) and P-11 (phosphocellulose fraction) from HeLa cells have been described (*17*). [a]Total protein was measured by the Bio Rad Protein Assay. [b]The amounts of RNAPII were estimated by Western blotting with anti-CTD (8WG16) or silver staining. [c]Percentage of purity is calculated as follows: Purity (%) = RNAPII (μg)/Protein (mg) $\times 10^{-1}$. [d]Percentage of yield is calculated as follows: Yield (%) = RNAPII (μg)/RNAPII (μg) of Input $\times 10^2$.

purification scheme is summarized in Fig. 1A. As a control, we purified RNAPII by conventional column chromatography (*17*). The native human RNAPII purified by this latter method is referred to as C-RNAPII (control RNAPII). C-RNAPII and the RNAPII preparations after the first and second affinity chromatographic steps were examined by SDS polyacrylamide gel electrophoresis and silver staining (Fig. 1B). The RNAPII subunits were identified according to their published molecular weights (*2*, *29*). M2 Elu, the eluted preparation after FLAG-immunoaffinity purification, contained all of the core subunits of human RNAPII. The final preparation after the second affinity purification step (TALON Elu) also contained all of the core subunits. Additional, non-RNAPII elements in this preparation were negligible. However, the C-RNAPII preparation still contained some proteins that were not RNAPII subunits. These were not characterized further and are indicated by a bar (Fig. 1B). Immunoblotting with an antibody (*8*WG16) specific for the carboxyterminal domain (CTD) of the largest subunit of RNAPII showed that this subunit is present in both the M2 Elu and TALON Elu preparations (Fig. 1C). Thus, this two-step affinity chromatographic method was successful in purifying the core subunits of human RNAPII. We refer to the purified RNAPII complexes in TALON Elu as FH-RNAPII. The recovery rate and purity of RNAPII in TALON Elu were 6.9 and 92%, respectively (Table 1). In contrast, the recovery and purity of C-RNAPII were 13 and 40%, respectively (Table 1). Although in both methods, the rate of recovery was almost the same, the purity in our method was greatly superior to that of the conventional column chromatographic method. Thus, C-RNAPII still contained other proteins. Furthermore, while the yield of FH-RNAPII was approximately 1.1×10^1 μg from 1.4×10^9 cells, we obtained approximately 2.1×10^2 μg of C-RNAPII from 8.0×10^{10} cells (Table 1). Thus, to obtain 100 μg of purified RNAPII by either method, 1.3×10^{10} cells would be needed for our two-step affinity chromatographic method while 3.8×10^{10} cells would be needed for the conventional column procedure. Thus, the yield of our method is about 3-fold higher than that of the conventional procedure. We also compared the time spent on purification. The time was measured after the preparation of cell extracts. Our method took 13 h, including the dialysis step, while the conventional column chromatographic method took about one week (144 h). Although the purification scale of C-RNAPII was larger than that of FH-RNAPII, we believe that our procedure considerably reduces the time needed to purify RNAPII.

Characterization of FH-RNAPII—We performed transcription assays with oligo (dC)-tailed templates and found that FH-RNAPII was active in the synthesis of RNA (Fig. 2). At a low concentration of α-amanitin, the production of RNA was specifically inhibited (Fig. 2, lanes 5, 6, 11, and 12), which indicates that there is no detectable contamination by RNA polymerases I and III in the purified FH-RNAPII preparation. We assessed the efficiency of the transcription elongation by comparing the lengths of the RNA transcripts produced after 4- and 16-min of incubation (Fig. 2). Notably, the length of the transcripts produced by FH-RNAPII and C-RNAPII did not differ, regardless of the elongation time. Thus, the rates of RNA synthesis by FH-RNAPII and C-RNAPII are identical (Fig. 2). This supports the notion that the double tag on the RPB3 subunit does not block or hinder the transcription elongation activity of RNAPII.

We further assessed the ability of FH-RNAPII to perform *in vitro* transcription reconstituted with purified GTFs including TFIIB, TBP, TFIIE, TFIIF, and TFIIH. We tested basal transcription from supercoiled $pG5C_2AT$ templates that have five Gal4-binding sites, an adenovirus major late promoter (MLP), and a 390 base pair G-less cassette. Efficient transcription was observed when increasing amounts of FH-RNAPII were supplied (Fig. 3A), indicating that FH-RNAPII is functional in basal transcription. C-RNAPII was also active in the reconstituted transcription system. C-RNAPII produced more RNA transcripts than equivalent amounts of FH-RNAPII, which suggests that the specific activity of C-RNAPII is higher than that of FH-RNAPII. This difference may be due to the as yet uncharacterized proteins found in the C-RNAPII preparation (Fig. 1B).

Next, the ability of FH-RNAPII to participate in activated transcription was examined using TFIIA and Gal4-VP16 and two kinds of DNA templates, namely, $pG5C_2AT$ or $pdMLC_2AT$, which carry an MLP and a 270 base pair G-less cassette. In addition, a purified TFIID fraction purchased from Proteinone was employed in this assay instead of recombinant TBP because activated transcription is observed when the reconstituted transcription system contains TFIID (*10*). The addition of Gal4-VP16 activated transcription only from the $pG5C_2AT$ template since it bears Gal4 binding sites while $pdMLC_2AT$ does not (Fig. 3B, lanes 3 and 8). In contrast, the addition of TFIIA induced transcription from both templates (Fig.

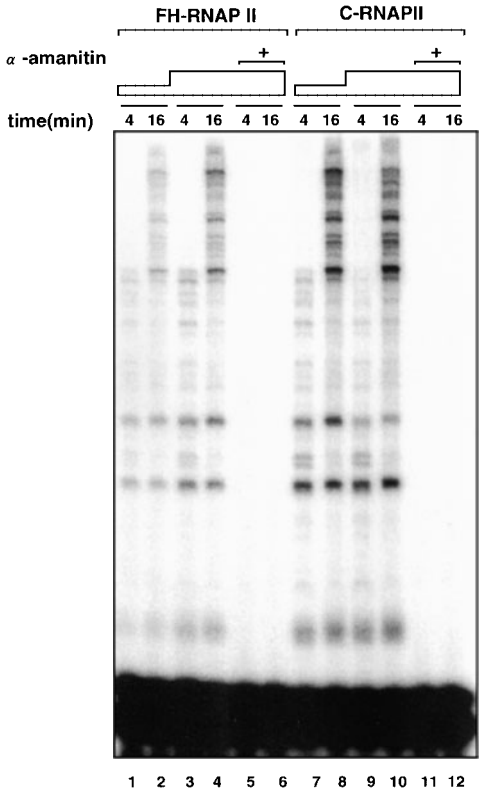

Fig. 2. **FH-RNAPII mediates the synthesis of RNA in an α-aminitin–sensitive manner.** Transcription assays were performed with oligo (dC)-tailed templates. FH-RNAPII (lanes 1 and 2, 50 ng; lanes 3 to 6, 150 ng) or C-RNAPII (lanes 7 and 8, 50 ng; lanes 9 to 12, 150 ng) were used in the reactions. Numbers above the gel indicate the elongation time after the nucleotides were added. α-Aminitin was added to lanes 5, 6, 11, and 12 at a final concentration of 1 ng/μl 5 min before adding the nucleotides.

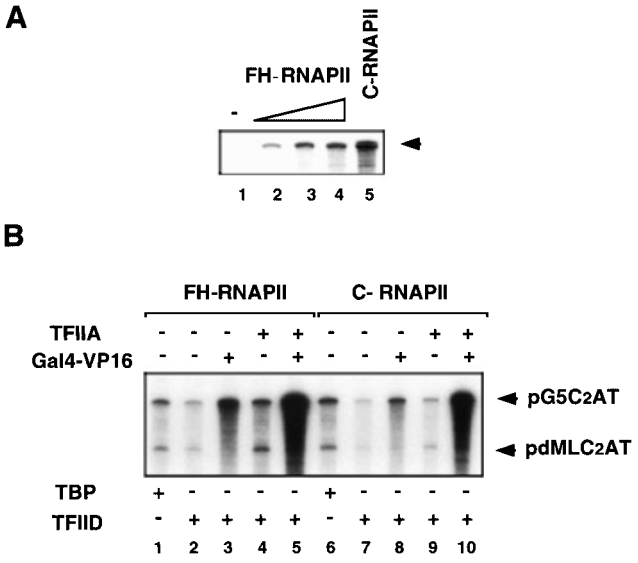

Fig. 3. **FH-RNAPII is functional in *in vitro* transcription.** (A) FH-RNAPII (lane 2, 6.25 ng; lane 3, 18.8 ng; lane 4, 56.5 ng) and C-RNAPII (lane 5, 6.25 ng) were analyzed in *in vitro* transcription assays with GTFs and pG5C_2AT (30 ng). Reaction products are shown by an arrowhead. (B) FH-RNAPII (lanes 1 to 5, 25 ng) and C-RNAPII (lanes 6 to 10, 25 ng) were analyzed in *in vitro* transcription assays as described in (A) except TFIID was used (lanes 2 to 5 and lanes 7 to 10) instead of TBP (lanes 1 and 6) and pdMLC$_2$AT (10 ng) was used as well as pG5C_2AT (20 ng). Gal4-VP16 (lanes 3, 5, 8, and 10, 45 ng) and TFIIA (lanes 4, 5, 9, and 10, 75 ng) were supplied. The positions of the 390 nucleotide RNA transcripts from pG5C_2AT (upper arrowhead) and the 270 nucleotide RNA transcripts from pdMLC$_2$AT (lower arrowhead) are indicated on the right.

3B, lanes 4 and 9). Moreover, TFIIA strongly enhanced Gal4-VP16-mediated activation (Fig. 3B, lanes 5 and 10). Similar activities were observed with FH-RNAPII and C-RNAPII (Fig. 3B). Although the level of TBP-mediated basal transcription by FH-RNAPII was lower than that of C-RNAPII (Fig. 3B, compare lane 1 with lane 5), much stronger transcriptional activation was observed with FH-RNAPII compared to the activation of C-RNAPII transcription (Fig. 3B, compare lanes 3 to 5 with lanes 8 to 10). This suggests that the uncharacterized proteins in C-RNAPII may affect the transcription products.

Thus, FH-RNAPII purified by our two-step affinity chromatographic method is active and functional in basal and activated transcription. Our method can be performed at room temperature with an ICE box, which makes it a considerably less arduous procedure than the conventional column chromatographic method. In addition, our procedure is markedly less time-consuming and more efficient than the conventional column method.

This work was supported by a Grant-in-Aid for Scientific Research on Priority Areas from the Ministry of Education, Science, Sports and Culture of Japan to T.W. and H.H., a research grant from the CREST of JST Corporation, and a grant from NEDO to H.H. T.N. is a JSPS research fellow. We are indebted to Drs. Tetsuro Kokubo, Kohji Hisatake, and Yoshiaki Ohkuma for many helpful discussions. We also thank Dr. Sachiko Okabe for technical support and members of the Handa Lab for helpful suggestions.

REFERENCES

1. Shpakovski, G.V., Acker, J., Wintzerith, M., Lacroix, J.F., Thuriaux, P., and Vigneron, M. (1995) Four subunits that are shared by the three classes of RNA polymerase are functionally interchangeable between *Homo sapiens* and *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **15**, 4702–4710
2. Khazak, V., Estojak, J., Cho, H., Majors, J., Sonoda, G., Testa, J.R., and Golemis, E.A. (1998) Analysis of the interaction of the novel RNA polymerase II (pol II) subunit hsRPB4 with its partner hsRPB7 and with pol II. *Mol. Cell. Biol.* **18**, 1935–1945
3. Kitajima, S., Chibazakura, T., Yonaha, M., and Yasukochi, Y. (1994) Regulation of the human general transcription initiation factor TFIIF by phosphorylation. *J. Biol. Chem.* **269**, 29970–29977
4. Yamaguchi, Y., Takagi, T., Wada, T., Yano, K., Furuya, A., Sugimoto, S., Hasegawa, J., and Handa, H. (1999) NELF, a multisubunit complex containing RD cooperates with DSIF to repress RNA polymerase II elongation. *Cell* **97**, 41–51
5. Orphanides, G., Lagrange, T., and Reinberg, D. (1996) The general transcription factors of RNA polymerase II. *Genes Dev.* **10**, 2657–2683
6. Ma, D., Watanabe, H., Mermelstein, F., Admon, A., Oguri, K., Sun, X., Wada, T., Imai, T., Shiroya, T., Reinberg, D., and Handa, H. (1993) Isolation of a cDNA encoding the largest subunit of TFIIA reveals functions important for activated transcription. *Genes Dev.* **7**, 2246–2257

7. Yokomori, K., Zeidler, M.P., Chen, J.L., Verrijzer, C.P., Mlodzik, M., and Tjian, R. (1994) Drosophila TFIIA directs cooperative DNA binding with TBP and mediates transcriptional activation. *Genes Dev.* **8**, 2313–2323
8. Ozer, J., Moore, P.A., Bolden, A.H., Lee, A., Rosen, C.A., and Lieberman, P.M. (1994) Molecular cloning of the small (gamma) subunit of human TFIIA reveals functions critical for activated transcription. *Genes Dev.* **8**, 2324–2335
9. Sun, X., Ma, D., Sheldon, M., Yeung, K., and Reinberg, D. (1994) Reconstitution of human TFIIA activity from recombinant polypeptides: a role in TFIID-mediated transcription. *Genes Dev.* **8**, 2336–2348
10. Goodrich, J.A., Hoey, T., Thut, C.J., Admon, A., and Tjian, R. (1993) Drosophila TAFII40 interacts with both a VP16 activation domain and the basal transcription factor TFIIB. *Cell* **75**, 519–530
11. Kokubo, T., Takod, R., Gong, D.W., Roeder, R.G., Horikoshi, M., and Nakatani, Y. (1993) Identification of TFIID components required for transcriptional activation by upstream stimulatory factor. *J. Biol. Chem.* **268**, 17554–17558
12. Burley, S.K. and Roeder, R.G. (1998) TATA box mimicry by TFIID: autoinhibition of pol II transcription. *Cell* **94**, 551–553
13. Kotani, T., Miyake, T., Tsukihashi, Y., Hinnebusch, A.G., Nakatani, Y., Kawaichi, M., and Kokubo, T. (1998) Identification of highly conserved amino-terminal segments of $dTAF_{II}230$ and $yTAF_{II}145$ that are functionally interchangeable for inhibiting TBP-DNA interactions *in vitro* and in promoting yeast cell growth *in vivo*. *J. Biol. Chem.* **273**, 32254–32264
14. Liu, D., Ishima, R., Tong, K.I., Bagby, S., Kokubo, T., Muhandiram, D.R., Kay, L.E., Nakatani, Y., and Ikura, M. (1998) Solution structure of a TBP-TAF(II)230 complex: protein mimicry of the minor groove surface of the TATA box unwound by TBP. *Cell* **94**, 573–583
15. Kotani, T., Banno, K., Ikura, M., Hinnebusch, A.G., Nakatani, Y., Kawaichi, M., and Kokubo, T. (2000) A role of transcriptional activators as antirepressors for the autoinhibitory activity of TATA box binding of transcription factor IID. *Proc. Natl Acad. Sci. USA* **97**, 7178–7183
16. Dignam, J.D., Martin, P.L., Shastry, B.S., and Reoder, R.G. (1983) Eukaryotic gene transcription with purified components. *Methods Enzymol.* **101**, 582–598
17. Reinberg, D. and Roeder, R.G. (1987) Factors involved in specific transcription by mammalian RNA polymerase II. Purification and functional analysis of initiation factors IIB and IIE. *J. Biol. Chem.* **262**, 3310–3321
18. Ikura, T., Ogryzko, V.V., Grigoriev, M., Groisman, R., Wang, J., Horikoshi, M., Scully, R., Qin, J., and Nakatani, Y. (2000) Involvement of the TIP60 histone acetylase complex in DNA repair and apoptosis. *Cell* **102**, 463–473
19. Kershnar, E., Wu, S.Y., and Chiang, C.M. (1998) Immunoaffinity purification and functional characterization of human transcription factor IIH and RNA polymerase II from clonal cell lines that conditionally express epitope-tagged subunits of the multiprotein complexes. *J. Biol. Chem.* **273**, 34444–34453
20. Kimura, M., Ishiguro, A., and Ishihama, A. (1997) RNA polymerase II subunits 2, 3, and 11 form a core subassembly with DNA binding activity. *J. Biol. Chem.* **272**, 25851–25855
21. Kimura, M., Suzuki, H., and Ishihama, A. (2002) Formation of a carboxy-terminal domain phosphatase (Fcp1)/TFIIF/RNA polymerase II (pol II) complex in *Schizosaccharomyces pombe* involves direct interaction between Fcp1 and the Rpb4 subunit of pol II. *Mol. Cell. Biol.* **22**, 1577–1588
22. Kireeva, M.L., Komissarova, N., Waugh, D.S., and Kashlev, M. (2000) The 8-nucleotide-long RNA:DNA hybrid is a primary stability determinant of the RNA polymerase II elongation complex. *J. Biol. Chem.* **275**, 6530–6536
23. Li, F.Q., Takemaru, K., Goto, M., Ueda, H., Handa, H., and Hirose, S. (1997) Transcriptional activation through interaction of MBF2 with TFIIA. *Genes Cells* **2**, 143–153
24. Orphanides, G., LeRoy, G., Chang, C.-H., Luse, D.S., and Reinberg, D. (1998) FACT, a factor that facilitates transcript elongation through nucleosomes. *Cell* **92**, 105–116
25. Dignam, J.D., Lebovitz, R.M., and Roeder, R.G. (1983) Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. *Nucleic Acids Res.* **11**, 1475–1489
26. Wada, T., Orphanides, G., Hasegawa, J., Kim, D.K., Shima, D., Yamaguchi, Y., Fukuda, A., Hisatake, K., Oh, S., Reinberg, D., and Handa, H. (2000) FACT relieves DSIF/NELF-mediated inhibition of transcriptional elongation and reveals functional differences between P-TEFb and TFIIH. *Mol. Cell* **5**, 1067–1072
27. Wada, T., Watanabe, H., Usuda, Y., and Handa, H. (1991) Different biological activities of the hetero- and homodimers formed by the 47- and 43-kilodalton proteins of transcription factor ATF/E4TF3. *J. Virol.* **65**, 557–564
28. Wada, T., Takagi, T., Yamaguchi, Y., Kawase, H., Hiramoto, M., Ferdous, A., Takayama, M., Lee, K.A., Hurst, H.C., and Handa, H. (1996) Copurification of casein kinase II with transcription factor ATF/E4TF3. *Nucleic Acids Res.* **24**, 876–884
29. Acker, J., de Graaff, M., Cheynel, I., Khazak, V., Kedinger, C., and Vigneron, M. (1997) Interactions between the human RNA polymerase II subunits. *J. Biol. Chem.* **272**, 16815–16821