Affinity Purification of a Human RNA Polymerase II Complex Using Monoclonal Antibodies against Transcription Factor IIF*

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Five different monoclonal antibodies that immunoreact with RAP74, the large subunit of general transcription factor (TF) IIF, were produced and characterized. Using one of these antibodies, an affinity purification procedure was devised to isolate a human RNA polymerase II complex. This procedure is fast, simple, and reproducible and does not require extensive purification. The RNA polymerase II complex isolated using this procedure contains SRB (suppressor of RNA polymerase B) polypeptides, transcription factors IIE and IIF, limiting amounts of TFIIF, and the TATA-binding protein, but was devoid of TFIIB.

Accurate initiation of transcription by RNA polymerase II (RNAPII) is a complex process that requires six general transcription factors (GTF; known as TFIIA, TFIIB, TFIID, TFIIE, TFIIF, and TFIIH) in addition to RNAPII and regulatory factors (reviewed in Refs. 1 and 2). The formation of a transcription complex begins with the recognition of the TATA motif by the TATA-binding protein (TBP) subunit of TFIIID. The resulting protein-DNA complex provides a recognition site for the other factors that can enter either sequentially or as components of a pre-assembled complex, called the “RNAPII holoenzyme” (reviewed in Refs. 2–4).

A combination of genetic and biochemical experiments with the yeast *Saccharomyces cerevisiae* uncovered the existence of an RNAPII complex and demonstrated its biological significance (5, 6). The level of conservation between the transcription systems of yeast and higher eukaryotes suggested that a similar RNAPII complex exists in mammals. The first indication that such a complex exists in higher eukaryotes came from studies using rat liver extracts (7). It was found that antibodies directed against the CTK7/MO15 subunit of TFIIH immunoprecipitated RNAPII and the entire set of GTFs, except TFIID. The immunoprecipitates could support RNAPII transcription *in vitro*. This was taken as evidence for a large RNAPII holoenzyme in mammals; however, it was not determined whether the immunoprecipitated material represented a single “holoenzyme” complex or many TFIIH-GTF complexes, as previous studies have shown that TFIIH can independently interact with many of the GTFs (8); nor was it demonstrated that the complex contained mammalian homologues of yeast SRB (for suppressor of RNA polymerase B; Refs. 9–13) proteins. The SRB genes were identified as suppressors of the cold-sensitive phenotype associated with the partial truncation of the carboxyl-terminal domain (CTD) of RNAPII in *S. cerevisiae* (9, 14, 15). The CTD is an unusual motif present at the carboxyl terminus of the largest subunit of RNAPII, composed of a heptapeptide repeated 26 times in yeast and 52 times in mammals. The SRB proteins are hallmarks of the yeast RNAPII holoenzyme and distinguish the mammalian complexes as true RNAPII complexes.

Confirmation of the existence of mammalian RNAPII complexes came from studies using calf thymus and HeLa cells. The calf thymus complex contains substoichiometric amounts of the GTFs TFIIE and TFIIF and cannot respond to activators (12), while the HeLa cell complex contains stoichiometric amounts of TFIIE and TFIIF and a substoichiometric amount of TFIIH and can respond to activators (13). More significantly, both complexes contain homologues of yeast SRB proteins: hSRB7 (12, 13) and cyclin C/CDK8 (hsRBB11/hsRBB10; Ref. 16).

From the results described thus far, it is clear that the RNAPII complexes isolated contain different subsets of the GTFs. Adding complexity to the RNAPII complexes are the findings demonstrating that the mammalian RNAPII complexes contain a large number of other polypeptides, some of which may play important roles in nucleotide excision repair, DNA double strand break repair and/or cell cycle checkpoint control (13), and chromatin remodeling (16); however, the majority remain elusive.

A known polypeptide that exists in stoichiometric amount with respect to the largest subunit of RNAPII in both the yeast (6, 15) and the human RNAPII complex (13) is transcription factor IIF. The subunits of TFIIF, RAP30 and RAP74, were first identified through their ability to interact with immobilized RNAPII (17). Soon after, TFIIF was independently purified as an essential RNAPII initiation factor (18–20). In addition to its role in initiation, TFIIF performs additional functions that increase the specificity and efficiency of RNAPII transcription. By stably associating with RNAPII, TFIIF can increase the rate of transcription elongation (19, 21–25). Additionally, TFIIF prevents spurious initiation by inhibiting, and reversing, the binding of RNAPII to non-promoter sites on DNA, drawing comparisons with the bacterial σ factors (26, 27).

We have taken advantage of the physical association between TFIIF and the human RNAPII complex to immunofluorescence purify a human RNAPII complex using monoclonal antibo-
ies (mAbs) that recognize the large subunit of TFIIF. We also report the characterization of five different anti-RAP74 mAbs.

MATERIALS AND METHODS

Buffers—Buffer C contained 20 mM Tris-HCl, pH 7.8, 0.2 mM EDTA, pH 8.0, 1 mM dithiothreitol, 20% (v/v) glycerol (otherwise indicated), and 1 mM phenylmethylsulfonyl fluoride. TTBS (×10) solution contained 100 mM Tris-HCl, pH 7.5, 2 mM NaCl, and 0.5% (v/v) Tween 20. Laemmli buffer (×1) contained 2% (w/v) SDS, 100 mM dithiothreitol, 50 mM Tris-HCl, pH 6.8, 0.001% (w/v) bromophenol blue, and 10% (v/v) glycerol.

Proteins—Escherichia coli BL21 (DE3) transformants containing RAP74 deletion constructs (ZB317, ZB304, ZB325, ZB275, ZB329, and ZB268) were a gift from Dr. Z. Wilm (28). HelA cell extracts were fractionated on a phosphocellulose (Sigma) column as described previously (29). Proteins eluting in the 0.3–0.5 M KCl wash were dialyzed against 1 ml of buffer C containing 0.1 M KCl and loaded onto a DEAE-cellulose (Whatman, DE52) column as described previously for the purification of TFIIH (19). Bound proteins were eluted with buffer C containing 0.5 M KCl and dialyzed to 0.15 M KCl prior to immunoaffinity purification. Protein factors used in the in vitro transcription reactions were purified as follows. Recombinant human TBP (30), TFIIH (31), TFII-p56 (32), TFII-p34 (32), TFII-FAP30 (17), and TFII-FAP74 (28, 33, 34) were isolated from bacteria using previously published procedures (35). TFII and TFII-FAP activities were reconstituted by mixing the isolated recombinant subunits as described (35). Human RNA-polymerase II (RNAPII) was affinity-purified from bacterial cell extracts using a monoclonal antibody isotyping kit from Boehringer Mannheim.

Western Blot Analysis—For epitope mapping, extracts from E. coli BL21 (DE3) strains expressing RAP74 deletion proteins (28) were prepared and separated by electrophoresis on a 15% SDS-polyacrylamide gel. Proteins were transferred to nitrocellulose membranes, which were subsequently blocked for 30 min in 3% nonfat dry milk in 1× TTBS, then washed extensively with 1× TTBS and incubated with ascitic fluid derived from each of the anti-RAP74 mAbs at a dilution of 1:500 in 0.1% bovine serum albumin and 1× TTBS. To prevent the mAbs from nonspecifically cross-immunoreacting with bacterial proteins, E. coli BL21 (DE3) extracts (50 μg/ml) prepared from a strain lacking RAP74 were mixed with the mAbs during the incubation. Alkaline phosphatase-conjugated anti-mouse IgG antibodies (Promega) were used as secondary antibodies. After incubation with the secondary antibodies, the membranes were washed with 1× TTBS and developed with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Bio-Rad) according to the manufacturer’s instructions.

For the characterization of the anti-RAP74 affinity-purified RNAPII complex, an aliquot of each fraction (30 μl) was loaded onto a 5–15% SDS-polyacrylamide gradient gel. Polypeptides were transferred to polyvinylidene difluoride membrane (Millipore) and probed with various antibodies as indicated. Western analysis was performed as above without the addition of E. coli extract.

Immunoprecipitations—Asctic fluid, containing anti-RAP74 mAbs (1C11/1G7, 4F9/8G11, 6H10/F10, 7B3/E10, and 7E7/G11, 20 μl) were each bound to 10 μl of protein G-agarose beads (Boehringer Mannheim) for 1.5 h at 4°C. The beads were subsequently washed with 1% acetic acid-buffered saline, followed by 1 ml of buffer C containing 0.1 M KCl, 0.1% (v/v) Nonidet P-40, and 0.05% (v/v) Triton X-100. The beads were then incubated for 2 h at 4°C with the DEAE-cellulose column-bound fraction (150 μg, see “Proteins”) that had been dialyzed in buffer C containing 0.1 M KCl, 0.1% (v/v) Nonidet P-40, and 0.05% (v/v) Triton X-100 (v/v). Each immune complex was washed three times with 1 ml of buffer C containing 0.15 M KCl, 0.1% Nonidet P-40 (v/v), and 0.05% Triton X-100 (v/v), and 1 μl of buffer C containing 0.05 M KCl. The immune complexes were eluted by boiling in 2 × Laemmli buffer and analyzed by Western blot using either affinity-purified rabbit polyclonal antibodies against the CTD of RNAPII or RAP74. For peptide inhibition experiments, 5 μg of peptide were preincubated with mAbs bound to protein G-agarose beads in buffer C containing 0.1 M KCl for 30 min at 20°C, then washed extensively with buffer C containing 1 M KCl and buffer C containing 0.05 M KCl prior to incubation with the DEAE-cellulose protein fraction. Immune complexes were analyzed by Western blot for RAP74. The following polypeptides containing sequences of RAP74 were synthesized by HMM/UCSF Protein Structure Laboratory (a, b, c, d, e).

Electrophoretic Mobility Shift Assay—The TAB (promoter DNA complex containing TBP, TFIIA, and TFIIIB) and TBPoFEH (promoter DNA complex containing TBP, TFIIIB, RNAPII, TFIIIE, TFIIIF, and TFIIH) complexes were formed on a 32P-labeled DNA fragment containing sequences of the adenovirus major late promoter (Ad-MLP) as described previously (39). Protein G-purified anti-RAP74 and anti-FLAG (Eastman Kodak Co.) mAbs were added (8 ng/ml) to the TAB and TBPoFEH complexes and incubated for an additional 30 min at 28°C. The complexes were separated by electrophoresis on a 3.5% native polyacrylamide gel and visualized by autoradiography.

Affinity-Purification of a Human RNA Polymerase II Complex—Monoclonal antibody 73E10 or anti-β-galactosidase (Promega) was covalently cross-linked to protein G-agarose beads (1 ml) using 20 mM dimethylpimelidate as described (40). Prior to incubation with the protein sample, the resin was equilibrated with buffer C containing 0.15 M KCl, 0.1% Nonidet P-40 (v/v), and 0.05% (v/v) Triton X-100. Proteins derived from the DEAE-cellulose column 0.5 M wash were dialyzed against buffer C containing 0.15 M KCl, 50 mM MgCl2, 0.1% (v/v) Nonidet P-40, and 0.05% (v/v) Triton X-100. An aliquot of the dialyzed fraction (5 mg) was subsequently incubated with 0.1 volume of the protein fraction with protein G-agarose beads for 30 min at 4°C, which was then removed by centrifugation at 500 × g for 5 min. This precleared fraction was incubated with the resin (1 ml) for 3 h at 4°C. The resin was washed with 30 column volumes of buffer C containing 0.2 M KCl, followed by 10 column volumes of buffer C containing 0.05 M KCl (Fig. 4A, (W)). The resin was then incubated (for 15 min at 4°C) with 1 column volume of buffer C containing 0.05 M KCl and control peptide α (2.5 mg/ml; Fig. 4A, (C)). The RNAPII complex was then eluted by incubating the resin with 1 column volume of peptide c containing the epitope sequences (2.5 mg/ml in buffer C containing 0.05 M KCl) for 15 min at 4°C (fraction 1). Three further fractions were collected by eluting each with 1 column volume of buffer C containing 0.05 M KCl (fractions 2–4). For transcription analysis, fractions 1 and 2 (2 ml) were pooled and concentrated to 0.5 ml on a concentrator (Millipore) and dialyzed against buffer C containing 0.1 M KCl. The gel filtration analysis was performed with fractions 1 and 2 (2 ml), which were concentrated to 1 ml and dialyzed against buffer C containing 0.5 or 2% (v/v) glycerol (0.05% (v/v) Nonidet P-40, and 0.05% (v/v) Triton X-100). Anti-RAP74 affinity-purified complexes (100 μl) were loaded onto a 4-m (0.5 cm × 20 cm) Sepharose CL-4B (Pharmacia) column. Fractions of 120 μl were collected at a flow rate of 30 μl/min in buffer C containing 0.5 M KCl, 20% (v/v) glycerol, 0.01% (v/v) Nonidet P-40, and 0.05% (v/v) Triton X-100. For Western blot analysis, every other fraction was precipitated with trichloroacetic acid, washed with 80% ethanol, and loaded onto a 5–15% SDS-polyacrylamide gradient gel. The gel filtration step was performed also with a crude fraction derived from the DEAE-cellulose column (the input used for the affinity chromatography). The protein pool (144 mg/170 ml) was concentrated on a 40-ml S-Sepharose ion exchange column (98 mg/70 ml) before chromatography on the Sepharose CL-4B gel filtration column. The concentrated sample was dialyzed against buffer C containing 0.5 M KCl, 50% (v/v) glycerol, 0.01% (v/v) Nonidet P-40, and 0.05% (v/v) Triton X-100. Proteins (10.5 mg) were loaded onto the Sepharose CL-4B column (350 ml, 2.5 cm × 75 cm), and the column was developed with buffer C containing 0.5 M KCl, 20% (v/v) glycerol, 0.01% (v/v) Nonidet P-40, and 0.05% (v/v) Triton X-100. Fractions (1.2 ml) were collected at 2 ml/min. Every other third fraction was analyzed by Western blot analysis using 4% or for 1 h at 4°C with 3% TBP, TFIIA, and TFIIIB. Both Sepharose CL-4B columns were calibrated with thyroglobulin and blue dextran (Sigma).

Reconstituted Transcription Assays—Transcription reactions (40 μl) were reconstituted on a linear DNA containing the Ad-MLP fused to a 392-nucleotide G-less cassette (41). Transcription factors were purified as described above and were added as indicated. Factors used were...
human TBP (5 ng), TFIIH (5 ng), TFIIE (15 ng), TFIIIF (23 ng), and TFIIH (500 ng), and RNAPII (50 ng). α-Amanitin was added to the reaction mixture to 2 μg/ml prior to the addition of nucleotides as indicated. Products of the reactions were separated on a 7% urea, 6% polyacrylamide gel and quantitated using a phosphorimager (Bio-Rad).

RESULTS AND DISCUSSION

Epitope Mapping of Anti-RAP74 Monoclonal Antibodies—A series of mAbs directed against the large subunit of TFIIF (RAP74) were generated. Immunization was performed with bacterially expressed, hexahistidine-tagged RAP74 that was purified by metal affinity chromatography. The particular epitopes recognized by the various antibodies were defined by two methods: immunoreactivity of RAP74 truncated polypeptides and immunoprecipitation studies.

Five different RAP74 mAbs (1C11/G7, 4F8/G11, 6H10/F10, 7B3/E10, and 7E7/G11) were selected and analyzed for their ability to recognize different truncated RAP74 polypeptides using Western blots (Fig. 1). This analysis allows us to approximate the domain of RAP74 recognized by various mAbs. For example, mAb 1C11/G7 (1C11) specifically recognizes polypeptide ZB275 (amino acids 407–517), while it does not recognize polypeptide ZB370 (amino acids 363–452). Therefore, the epitope recognized by mAb 1C11 resides in the boundary of amino acids 453–517. The same approach was applied to the other mAbs. Monoclonal antibodies 4F8/G11 (4F8) and 6H10/F10 (6H10) recognize an epitope located between amino acids 207 and 258, whereas mAbs 7B3/E10 (7B3) and 7E7/G11 (7E7) recognize an epitope within residues 407–452 in RAP74.

We next analyzed whether the different RAP74 mAbs could immunoprecipitate TFIIIF and TFIIH-associated factors such as RNAPII. Western blot analysis using polyclonal antibodies against the large subunit of RNAPII and RAP74 showed that four of the antibodies (4F8, 6H10, 7B3, and 7E7) did co-immunoprecipitate RNAPII (Fig. 2A). Monoclonal antibody 1C11 failed to immunoprecipitate TFIIH. Co-immunoprecipitation of RNAPII was not a result of cross-reactivity with RNAPII, since none of the antibodies directly immunoprecipitate RNAPII when a TFIIH-free RNAPII fraction was used (data not shown).

The epitopes recognized by the different antibodies were further defined by designing peptides overlapping the residues mapped above. The peptides were analyzed for their ability to specifically compete with the immunoprecipitation of RAP74. The regions of RAP74 that were recognized by the different antibodies, defined in Fig. 1, were analyzed by the ANTIGENIC program of the Genetics Computer Group (GCG Package). This algorithm generates a prediction of the antigenicity of a particular peptide sequence. Two overlapping peptides, each containing predicted antigenic sequences, were designed and synthesized for regions of RAP74 mapped by Western blot. Monoclonal antibody 1C11 failed to immunoprecipitate RAP74, and therefore was not used in this analysis. Peptides a and b were derived from amino acids 207–258 (recognized by mAbs 4F8 and 6H10), and peptides c and d were from amino acids 407–452 (recognized by mAbs 7B3 and 7E7).

The reactivities of mAbs 4F8 and 6H10, which recognize the same set of RAP74 deletion mutants (Fig. 1), were distinguished by this method. Immunoprecipitation of RAP74 by antibody 4F8 was competed by peptide a, but not peptide b (Fig. 2B, lanes 3 and 4), whereas immunoprecipitation by antibody 6H10 was competed by peptide b, but not peptide a (Fig. 2B, lanes 6 and 7). This demonstrates that mAbs 4F8 and 6H10 recognize unique epitopes located between amino acids 231–258 (peptide a) and 202–226 (peptide b), respectively. In a similar manner, mAbs 7B3 and 7E7 were further characterized. Immunoprecipitations of RAP74 by mAbs 7B3 and 7E7 were specifically competed by peptide c (Fig. 2B, lanes 9 and 13), but not by peptides d or a or b (Fig. 2B, lanes 10, 11, 14, and 15). This inhibition by peptide c was specific to mAbs 7B3 and 7E7, because this peptide has no effect on mAb 4F8 (Fig. 2B, lane 17). Therefore, we concluded that the epitope(s) recognized by mAbs 7B3 and 7E7 is (are) located between amino acids 411 and 435 (peptide c).

Monoclonal Antibodies Recognize TFIIH within a Transcription Preinitiation Complex—Our immediate goal was to isolate antibodies that allow affinity purification of TFIIH-containing protein complexes, i.e. RNAPII complexes. We reasoned that mAbs recognizing RAP74 within a transcription pre-initiation complex, could, in principle, recognize RAP74 present in RNAPII complexes. Toward this goal, we analyzed whether the different mAbs affected the migration of the TBPolFEH complex formed on the Ad-MLP (Fig. 2C, lane 1, top). As a control, the preinitiation complex intermediate TAB (Fig. 2C, lane 1, bottom), which lacks TFIIH but contains TBP, TFIIA, and TFIIIB, was also formed on the Ad-MLP.

Monoclonal antibodies 6H10 and 7B3 were found capable of supershifting the complex containing TFIIH, but not the TAB complex (Fig. 2C, lanes 5 and 6). In contrast mAbs 1C11, 4F8, and 7E7 have no effect on the complexes (Fig. 2C, lanes 2, 4, and 7). These observations allowed us to distinguish mAbs 7B3 and 7E7. The epitope recognized by mAbs 7B3 and 7E7 is contained between amino acids 411 and 435 in RAP74 (Fig. 2B), yet mAb 7B3, but not mAb 7E7, supershifted the TB-
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Immunoadfinity Purification of RNA Polymerase II Complex—Our previous studies have demonstrated that the conventionally purified human RNAPII complex contains stoichiometric amounts of TFIIF, with respect to the largest subunit of RNAPII (13). Therefore, the mAb 7B3 was used to affinity purify an RNAPII complex from a HeLa cell-derived crude protein fraction.

Anti-TFIIF and anti-β-galactosidase mAbs were covalently linked to agarose beads coated with protein G and used in the purification as described in Fig. 4A and “Materials and Methods.” Protein fractions from the second step of the conventional chromatographic RNAPII complex purification procedure (13) was used as input material for the affinity purification. Briefly, HeLa cell nuclear extracts were fractionated on a phosphocellulose column as described previously (29). The 0.3–0.5 M KCl wash was applied onto a DEAE-cellulose column, and the bound proteins were eluted as described previously (19). This fraction was dialyzed to 0.15 M KCl before loaded onto the antibody affinity column (Fig. 4A; DEAE 52 0.5 M). Proteins that bound nonspecifically to the column were washed with buffer C containing 0.2 M KCl, 0.1% (v/v) Nonidet P-40, and 0.05% (v/v) Triton X-100, followed by a second wash with the same buffer containing 0.05 M KCl (Fig. 4A, W). Bound proteins were further washed with a buffer containing 0.05 M KCl and the irrelevant peptide a (see “Materials and Methods” (Fig. 4A, C)). Proteins that remain bound were eluted from the column with peptide e, which contains the epitope recognized by the 7B3 mAbs.

Fractions from the control and 7B3 columns were analyzed for the presence of different polypeptides using Western blot analysis and silver staining. The results from the Western blot analysis demonstrate that the column containing the mAb 7B3, but not the control column containing the β-galactosidase mAb, retained the large subunit of RNAPII (IIa), and CDK8, the human homologue of Srb10 (10, 11), and a hallmark of the yeast RNAPII holoenzyme (9) among other factors (Fig. 4B and data not shown; see below). These polypeptides were specifically retained by the mAb 7B3 column, and specifically eluted with peptide e (Fig. 4B, lanes 4–7), since these polypeptides were absent in the eluate obtained with the unrelated peptide a (Fig. 4B, lane 3). Moreover, silver staining of an SDS-polyacylamide gel, containing the protein pool derived from the control and 7B3 mAb columns, reveals that a large number of polypeptides are present in the peptide-eluante fraction derived from the 7B3 mAb column (Fig. 4C, lane 2). No polypeptides were detected in the peptide-eluante fraction derived from the control column (Fig. 4C, lane 1).

Analysis of the polypeptides present in the silver-stained gel reveals the presence of the different subunits of RNAPII (Fig. 4C, compare lanes 2 and 3). The two most predominant polypeptides, however, correspond to the two subunits of TFIIF. This is not an unexpected result, as the fraction used in the affinity purification was crude and contains “free” TFIIF as well as TFIIF within the RNAPII complex. We have observed that fractionation of the input sample (DEAE-cellulose) on a DEAE-5PW column prior to affinity chromatography results in the separation of free TFIIF from the RNAPII complex (data not shown). Consistent with our previous report (13), a large number of unidentified polypeptides were also present in the affinity-purified sample.

To analyze whether the affinity-purified sample represents one complex or interactions of TFIIF with multiple complexes, the sample was analyzed by gel filtration on a Sepharose CL-4B column. Sepharose CL-4B resolves polypeptides/complexes between 6 × 104 and 2 × 107 daltons and was previously

PolIFEH complex (Fig. 2C, lanes 6 and 7). None of the mAbs affected transcription, under conditions where rabbit polyclonal-anti-RAP74 antibodies inhibited transcription (data not shown).

From the studies described above, we concluded that mAbs 7B3 and 6H10 are likely to be effective in isolating TFIIF-containing RNAPII complexes, as both antibodies co-immunoprecipitated RNAPII and were capable of recognizing RAP74 epitopes within the transcription pre-initiation complex. All of the mAbs were classified as IgG1 and κ isotype. The properties of the different monoclonal antibodies are summarized in Fig. 3.

**Fig. 2.** Characterization of the specificity of the different mAbs by immunoprecipitations and electrophoretic mobility shift assays. A, monoclonal antibodies co-immunoprecipitate RNAPII. Different monoclonal antibodies were used to immunoprecipitate TFIIF and analyzed for their ability to co-immunoprecipitate RNAPII. The protein fraction used was the DEAE-cellulose, which contained TFIIF and RNAPII. Immunoprecipitation and Western blots were performed as described under “Materials and Methods.” The antibodies used are indicated on the top of the lanes. lla denotes the largest subunit of RNAPII, which was detected using polyclonal antibodies against the CTD. The lower panel indicates the immunoprecipitation of RAP74, which was detected using polyclonal antibodies. B, epitope mapping of the RAP74 mAbs using synthetic peptides to inhibit immunoprecipitation of RAP74. Synthetic peptides were preincubated with each mAb prior to incubation with the DEAE-cellulose protein fractions. Immunoprecipitations followed by Western blot analysis were performed as described under “Materials and Methods.” Lanes 2, 5, 8, 12, and 16 show immune complexes formed without peptide preincubation. Lanes 3, 6, 11, 15, and 19 show immune complexes formed after preincubation with peptide a, lanes 4 and 7 show immune complexes after preincubation with peptide b. Lanes 9, 13, and 17 show immune complexes after preincubation with peptide c, lanes 10, 14, and 18 show immune complexes formed after preincubation with peptide d. The mAbs used in the immunoprecipitation are indicated above the Western blot. Lane 1 is 10% of the input sample that was directly loaded onto the gel. C, monoclonal antibodies 6H10 and 7B3 change the electrophoretic mobility of the TBPolFEH pre initiation complex, but not of the TAB preinitiation complex. All of the different monoclonal antibodies are summarized in Fig. 3.
used successfully to characterize the RNAPII complex (13). Fractionation of the affinity-purified sample on this column resulted in the resolution of two peaks containing RNAPII, as detected by Western blot using antibodies against the largest subunit of RNAPII (Fig. 5A). The smaller peak (fraction 21–25) eluted with an apparent mass of approximately 1.3 to 1.5 MDa, whereas the second peak (fraction 15–17) of RNAPII eluted with an apparent mass larger than 2 MDa (blue dextran).

When the input sample used in the affinity purification step was directly loaded onto a similar gel filtration column, two distinct RNAPII complexes were also resolved (Fig. 5B). The resolution of two RNAPII-containing peaks is not unexpected. The human RNAPII previously isolated, using multiple chromatographic steps, was estimated to have a mass of approximately 1.5 MDa, and found to sediment close to the 60 S ribosomal subunit (13). However, the RNAPII in HeLa cell nuclear extracts sedimented on sucrose gradients in a broad peak, between the 60 S and 80 S ribosome subunits (13). These previous results were interpreted to suggest that factors present in a large RNAPII complex were removed during extensive chromatography. Since the purification procedure described in the present studies involves only three steps, it is possible that the integrity of a larger RNAPII-containing complex is maintained. We suspect that the smaller complex isolated in the present studies corresponds to the RNAPII complex isolated previously using conventional chromatography.

In agreement with this assessment, we found that the smaller RNAPII complex copurifies with TFIIF, TFIIE, and CDK8 (human SRB10; Refs. 9–11), the catalytic subunit of the DNA-dependent protein kinase (DNAPKcs; Ref. 42), and other previously identified factors such as cyclin C (human SRB11; Refs. 9–11) and human SRB7 (Fig. 5 and data not shown). This complex was transcriptionally active in an assay reconstituted only with TBP, TFIIH, and TFIIF (Fig. 5B and see below). The larger RNAPII-containing complex copurifies with DNAPKcs, but surprisingly, this complex was devoid of the GTFs, as exemplified by the
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Our previously characterized RNAPII complex, purified by conventional chromatography, was found to contain stoichiometric amounts of TFII E, TFII H, and limiting amounts of TFII H, and was devoid of TFIIB, TBP, and TBP-associated factors (13). We next functionally analyzed which GTFs were present in the complex purified by affinity chromatography. Transcription reactions were reconstituted with the GTFs and core RNAPII in each preparation was estimated using quantitative Western blots and antibodies against three subunits of RNA-P II: RPB1, RPB2, and RPB7 (data not shown). Similar to our previously characterized RNAPII complex in vivo, and suggest that these complexes were generated during chromatography from a larger RNAPII-containing complex.

**Functional Characterization of the Affinity-purified RNAPII Complex**—Our previously characterized RNAPII complex, purified by conventional chromatography, was found to contain stoichiometric amounts of TFII E, TFII H, and limiting amounts of TFII H, and was devoid of TFIIB, TBP, and TBP-associated factors (13). We next functionally analyzed which GTFs were present in the complex purified by affinity chromatography. Transcription reactions were reconstituted with the GTFs and core RNAPII that was affinity-purified over an anti-CTD mAb column (Fig. 4C, lane 3), or the RNAPII complex purified on an anti-RAP74 (mAb 7B3) column (Fig. 4C, lane 2). The results presented in Fig. 6 demonstrate that the affinity-purified complex contains a transcriptionally active form of RNAPII (lane 10). Transcription was sensitive to low concentrations of α-amanitin (Fig. 6A, lane 14). Transcription directed by the core RNAPII was, as expected, dependent on each of the GTFs (Fig. 6A, lanes 2–7); however, transcription directed by the RNAPII complex was only dependent upon TFIIB (Fig. 6A, lane 9). The omission of TFII E or TFII F from the reconstituted system was without an effect on transcription directed by the RNAPII complex (Fig. 6A, lanes 11 and 12). Consistent with our previous findings, we observed that the RNAPII complex contains limiting amounts of TFII H (Fig. 6A, lane 13); however, contrary to our previous observations, we also found limiting amounts of TBP (Fig. 6A, lane 8). The presence of TBP within the affinity-purified RNAPII complex, but not in the conventionally purified RNAPII complex (13), is perhaps not surprising since our previously characterized RNAPII complex included a larger number of chromatographic steps that, most likely, removed TBP from the complex. The important finding, however, is that both conventionally and affinity-purified RNA-P II complexes contain TFII E and TFII F, are devoid of TFIIB, and contain limiting amounts of TFII H.

To further compare the transcription activity directed by core RNAPII and the RNAPII complex, the exact amount of RNAPII in each preparation was estimated using quantitative Western blots and antibodies against three subunits of RNA-P II: RPB1, RPB2, and RPB7 (data not shown). Similar amounts of core RNAPII and affinity-purified RNAPII complex were added to reconstituted transcription systems containing all of the GTFs (Fig. 6B), or only TBP, TFII B, and TFII H (Fig. 6C, lanes 2–7). We found that the core RNAPII was approximately 1.5-fold more active than the RNAPII complex in a
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The "RNA polymerase holoenzyme" term was initially applied to the bacterial RNA polymerase, which contains the core polymerase and σ subunits (44). An important property of the bacterial RNA polymerase holoenzyme is its ability to recognize promoters and initiate transcription. In higher eukaryotic systems, such a complex has yet to be defined. As discussed above, none of the RNA polymerase II complexes thus far isolated do have a complete set of GTFs. These complexes are not, therefore, capable of specifically initiating transcription. Additional factors (GTFs) are required. We therefore believe that using the term holoenzyme, when referring to the eukaryotic RNA polymerase II complexes isolated thus far, is misleading. We suggest that they be termed RNAPII complexes. We do not imply, however, that there are no RNA polymerase II holoenzymes complexes in vivo. A purification scheme with minimal manipulations is more likely to result in the purification of intact and physiological RNA polymerase II complex. In this respect, the procedure described here should provide the basis to isolate true RNA polymerase II holoenzyme complexes from eukaryotic cells.

CONCLUSION

In these studies we described an affinity purification procedure, using monoclonal antibodies against the large subunit of TFIIH, that permits the isolation of the human RNAPII complex. The procedure was based on previous observations, demonstrating that the yeast (5, 6) and human (13) RNAPII complexes contain stoichiometric amounts of TFIIH. The procedure is fast, simple, and reproducible and yields a transcriptionally active form of the human RNAPII complex. In addition, the present studies also describe five different monoclonal antibodies, which recognize different regions of the large subunit of TFIIH. These reagents will be valuable tools in attempts to understand the roles of TFIIH during the transcription cycle.

The purification procedure described here for the human RNAPII complex resulted in the isolation of a multiprotein complex. Transcription assays and Western blot analyses indicate that the complex contains stoichiometric amounts of TFIIH and TFIIIC and limiting amounts of TFIIA, and is devoid of TFIIA. Identical results were obtained with the human RNAPII complex isolated using an elaborate conventional chromatographic procedure (13).

Different RNAPII complexes have been isolated (6, 12, 13, 15). Each of these complexes contains a different subset of the GTFs. For example, the yeast complex isolated by Kornberg and colleagues contains only TFIIA (6), which is present in stoichiometric amounts, whereas the yeast complex isolated by Young and colleagues contains stoichiometric amounts of TFIIA, TFIIIC, and TFIIH but is devoid of TFIIA and TBP (5, 15). As for the mammalian complexes, the complex isolated from calf thymus contains substoichiometric amounts of TFIIA and TFIIH and is devoid of TBP, TFIIA, and TFIIH (12), while the conventionally purified human RNAPII complex contains TFIIA and TFIIH in stoichiometric amounts, contains limiting amounts of TFIIH, and is devoid of TFIIA and TBP (13). Similar results were obtained with the affinity-purified human RNAPII complex. The only difference was the presence of limiting amounts of TBP in the affinity-purified complex. We suspect that TBP, TFIIA, and TFIIH are not integral components of the RNAPII complex, and their association may simply reflect the ability of these factors to interact with multiple components in the RNAPII complexes. Interestingly, each of the factors we found absent in the RNAPII complex have been suggested to participate in steps of the transcription cycle that are subject to regulation by activators (43).

The purification scheme described here should provide the basis to isolate true RNA polymerase II holoenzyme complexes from eukaryotic cells.
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