A human RNA polymerase II complex associated with SRB and DNA-repair proteins

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We report here the isolation of a human RNA polymerase II complex containing a subset of the basal transcription factors and the human homologues of the yeast SRB (for suppressors of RNA polymerase B) proteins[1–3]. The complex contains transcriptional coactivators and increases the activation of transcription. In addition, some components of the RNA polymerase II complex participate in DNA repair.

A complex containing RNA polymerase II (RNAPII) was purified from HeLa cell nuclear extract using a combination of conventional and affinity chromatography (Fig. 1). Analysis of fractions derived from the last step of purification (gel filtration on a Sepharose CL-4B column) by SDS–PAGE followed by silver staining demonstrated that RNAPII is a component of a large complex with a relative molecular mass of ~2,000K (Fig. 1*a*). To determine the size of the purified RNAPII complex more accurately, we compared the sedimentation of the RNAPII complex to that of 60S and 80S ribosomes in a sucrose gradient. Core RNAPII was used as a control, sedimenting at the top of the gradient (Fig. 1*b*). In contrast, the RNAPII complex sedimented faster, close to the position of the 60S ribosome subunit (Fig. 1*b*). Most of the RNAPII in nuclear extracts sedimented in a broad peak, spanning from the RNAPII complex to heavier forms (Fig. 1*b*).

Quantitative western blot analysis scoring for the presence of the different general transcription factors (GTFs) in the purified RNAPII complex (Fig. 1*a*, and data not shown) demonstrated that transcription factor (TF) IIE (18 fmol μl^{-1}) and TFIIF (14 fmol μl^{-1}) were stoichiometric with subunits of RNAPII (18 fmol μl^{-1}), but that TFIIH (2 fmol μl^{-1}) was limiting (data not shown). TATA-binding protein (TBP), TFIIB and TBP-associated factors were absent from the complex (Fig. 2, and data not shown). The complex also contained stoichiometric amounts of YY1 (13 fmol μl^{-1}) (Fig. 1*a*), a sequence-specific DNA-binding protein implicated in initiator-dependent transcription from TATA-less promoters[4].

We investigated whether the human homologues of yeast SRB proteins are associated with the RNAPII complex. There is significant homology between yeast SRB10/SRB11 and human cyclin C/CDK8 (ref. 5) and a recent report has described the existence of a mammalian RNAPII holoenzyme containing SRB7 (ref. 6). We isolated a complementary DNA clone encoding the human homologue of yeast SRB7 and used the recombinant polypeptide to generate antibodies (data not shown). Western blot analysis of the conventionally (Fig. 1*a*) and affinity-purified (see below) RNAPII complex revealed the presence of human SRB7 and cyclin C/CDK8.

The complex was immunopurified using monoclonal antibodies directed against the large subunit of TFIIF (RAP74). SDS–PAGE analysis of the column eluate revealed the presence of core RNAPII subunits and a number of other polypeptides (Fig. 1*c*). Fractionation of this eluate by gel filtration resulted in a similar elution profile to that of the conventionally purified complex (data not shown), indicating that the affinity-purified eluate is also a large large complex. Western blot analysis of the affinity-purified RNAPII complex confirmed the presence of RNAPII, TFIIE, TFIIH, cyclin C, CDK8 and YY1 (Fig. 1*d*). These proteins were absent from the eluate of the control column (Fig. 1*d*).

The functional analysis supports the above results and demonstrates that the RNAPII complex, but not the core RNAPII, can direct transcription in the absence of TFIIE and TFIIF (Fig. 2*a*). TBP and TFIIB are required for transcription regardless of the form of RNAPII used (Fig. 2*b*). To analyse whether a functional form of TFIIH was present in the complex, reactions were reconstituted in the absence of TFIIH, TFIIE and TFIIF. There was transcription in the absence of added TFIIH which was inhibited by antibodies against the ERCC3 subunit of TFIIH, but not by preimmune sera (Fig. 2*c*). This inhibition was specific as it was neutralized by excess TFIIH (lane 4), but not TBP or TFIIB (lane 3). Addition of TFIIH stimulated transcription, indicating that TFIIH was functionally limiting in the RNAPII complex (lane 6).

Next we studied the RNAPII complex in activated transcription *in vitro*. Activated levels of transcription using core RNAPII and the acidic activator GAL4-VP16 require cofactors such as PC4 (refs 7, 8) and cofactor A (Cof-A), a new activity (Fig. 3*a*, lanes 1–3, and unpublished results). In contrast to core RNAPII, the RNAPII complex did not require the addition of cofactors to achieve activated levels of transcription, indicating that the complex contains factors capable of mediating transcriptional activation (Fig. 3*a*, lanes 4–6). Western blot analysis of the RNAPII complex indicated the absence of PC4. However, a coactivator activity has been reported to be associated with the C-terminal domain (CTD) of a yeast RNAPII holoenzyme[1,2]. To analyse whether the coactivator activity of the human RNAPII complex is associated (directly or indirectly) with the CTD, the complex was fractionated on an anti-CTD antibody column. Antibodies against the CTD dissociate protein–CTD interactions. Functional analysis combined with western blots revealed that the flow-through fraction did not contain RNAPII, yet could mediate transcriptional activation when added to core RNAPII (Fig. 3*b*). Together, these experiments indicate that the RNAPII complex supports activated transcription and that a coactivator activity is associated with the CTD of RNAPII.

It was previously reported that TFIIH has dual roles in transcription and DNA nucleotide excision repair (NER)[9–11]. Because the RNAPII complex contains TFIIH, we analysed the RNAPII complex in a NER assay. The RNAPII complex only weakly complemented a xeroderma pigmentosum D (XPD) cell-free extract, consistent with limiting amounts of TFIIH in the complex (see above). Surprisingly, the RNAPII complex could efficiently complement XPF-, XPG- and XPC-repair-deficient extracts (Fig. 4*a*). Furthermore, western blot analysis of the RNAPII complex using anti-XPG, -XPC and -ERCC1 (XPF component) antibodies revealed their presence in the RNAPII complex (data not shown). As the yeast homologues of ERCC1/XPF (RAD1/RAD10) have a dual role in nucleotide excision repair and recombinational/double-strand break repair[12–14], we examined the RNAPII complex for other components involved in DNA double-strand break and recombinational repair. The Sepharose CL-4B column (Fig. 4*b*) revealed the coelution of the Ku regulatory subunits of DNA-PK, a protein kinase critical for DNA double-strand break repair and V(D)J recombination[15–19], and human RAD51 (refs 20–22) with the largest subunit of RNAPII (RPB1) and with RNAPII complex transcriptional activity (for transcription, see Fig. 1*a*). Ku interacts with DNA polymerase ε

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(Pol ε)[23], and Pol ε participates in the process of DNA-repair synthesis[24,25]. We therefore examined the RNAPII complex for DNA polymerase activity and immunoreactivity towards different components of DNA-repair synthesis. Western blot analysis of the Sepharose CL-4B fractions revealed the presence of DNA Pol ε, replication protein A (RPA) and replication factor C (RFC). The RNAPII complex was also active in an assay measuring DNA polymerase ε and RFC activity (data not shown). Absent from the complex were ERCC6, DNA pol δ, topoisomerase I. Quantitative western blot analysis revealed that HRAD51, RPA and Ku are

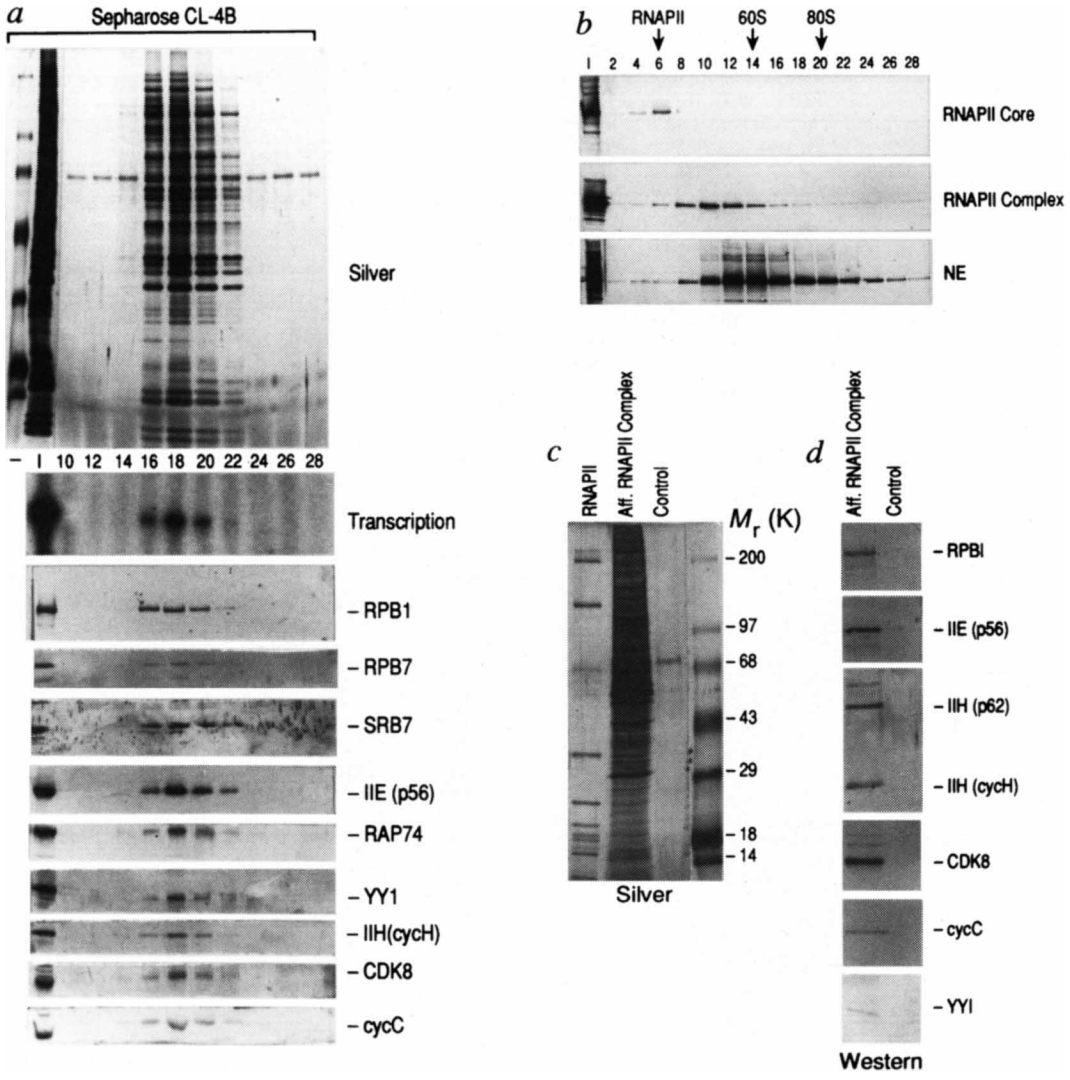
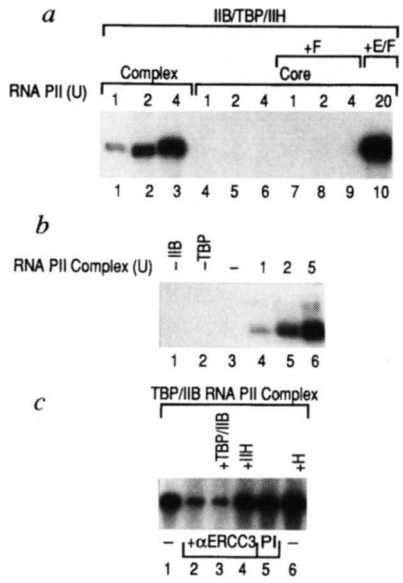


FIG. 1 Purification and characterization of the RNAPII complex. *a*, Analysis of the RNAPII complex purified by conventional chromatography from the last gel filtration step (Sepharose CL-4B). The complex was purified after an activity capable of directing specific transcription in an assay reconstituted with recombinant TBP, recombinant TFIIB and native TFIIH. SDS–PAGE followed by silver staining and western blot analysis of the column fractions using the antibodies against factors indicated on the right M_r markers to the far left of the silver staining are the same as in *c*. I, Input to the column (Mono S, 5 μl, 1 μg protein). Numbers, Fractions of the CL-4B column (10 μl). Transcription assays were as described in Fig. 2*a*, *b*, Sucrose gradient sedimentation analysis of the RNAPII complex. The sedimentation of the RNAPII complex and RNAPII in HeLa cell nuclear extracts was compared to that of core RNAPII, rat 80S and 60S ribosome. *c* Silver staining of an SDS–PAGE gel containing the anti-RAP74 affinity-purified RNAPII complex. From left to right are core RNAPII (2 μl, 0.2 μg; Alkyl-superose fraction) purified from HeLa nuclear pellets[28], the eluates from the anti-RAP74 monoclonal antibodies (100 μl eluate, representing ~ 0.5 μg protein) and the anti-β-galactosidase control column. *d*, Western blot analysis of the affinity-purified RNAPII complex (100 μl eluate) using antibodies against factors shown to the right.

METHODS. A full description of the conventional and affinity purification schemes as well as sucrose gradient sedimentation of the RNAPII complex is contained in Supplementary Information. RNAPII complex was purified from HeLa nuclear extract. An aliquot of the Mono-S fraction (0.6 ml, 0.12 mg) was concentrated against 50% glycerol (to 200 μl) in buffer H (20 mM HEPES–KOH, pH 7.9, 0.5 mM EDTA, 5 mM DTT, 10% glycerol, 0.01% NP-40, 0.1 mM PMSF, 1 μg ml^{-1} pepstatin A), containing 0.5 M KCl and was fractionated on a gel filtration Sepharose CL-4B column (12 ml column, 0.7 × 30 cm; Pharmacia). The M_r fractionation of the column ranges between 60K and 20,000K for globular proteins. First, 2 ml was collected in a single fraction, and then fractions of 0.2 ml were collected. The void volume of the column was determined by using polystyrene (M_r 30,000K, Polysciences Inc.) which peaks at fraction 9. The elution of RNAPII complex was compared to that of the M_r markers (blue dextran, M_r 2×10^6; thyroglobulin, M_r 0.7×10^6; alcohol dehydrogenase, M_r 0.15×10^6). The peak of RNAPII complex activity elutes with an apparent M_r of 2,000K (the peak of the blue dextran marker is at fraction 18). For silver staining, 15 μl of each fraction was loaded on a SDS–PAGE. For comparison, 15 μl (3 μg) of the input was loaded on the same gel. Transcription assays were performed with 10 μl of each fraction. For the western blots, 10 μl of the input and 30 μl of each fraction was analysed and the proteins were detected with the indicated rabbit polyclonal antibodies and visualized using alkaline-phosphatase-conjugated secondary antibodies (Promega).

FIG. 2 Transcription factors IIE, IIF and IIH are present in the RNAPII complex. *a*, Transcription assays were reconstituted on the Ad-MLP promoter with recombinant human TFIIB (20 ng), recombinant human TBP (10 ng) and purified TFIIH (500 ng, phenyl-Superose fraction)[10]. Equal western blot units (1, 2, 4) of RNAPII complex (lanes 1–3) and core RNAPII (lanes 4–6) were analysed. Purified TFIIF (60 ng, phenyl-superose fraction)[29] was added to the core RNAPII (lanes 7–9). A partially purified fraction (500 ng, Superdex 200) of TFIIE/TFIIF was added to the core RNAPII in lane 10. Western blot units of RNAPII were determined using anti-CTD antibodies. One unit was arbitrarily defined as the amount of RNAPII complex which gives a signal equivalent to 0.04 pmol of the largest subunit of purified core RNAPII (hRPB1). *b*, Transcription by the RNAPII complex requires TFIIB and TBP. Transcription assays were reconstituted with 5 units (5U) of the RNAPII complex or as indicated. TFIIB (lane 1), TBP (lane 2) and RNAPII complex (lane 3) were omitted. The indicated units of RNAPII complex were added in the presence of TFIIB, TBP and TFIIH in lanes 4–6. *c*, Transcription directed by the RNAPII complex is sensitive to anti-ERCC3 antibodies. Reactions are described above. The ERCC3 polypeptide was not detected by western blot in the RNAPII complex preparations, most probably because of its low abundance. Transcription reactions were reconstituted as indicated. In lanes 2–4, the RNAPII complex (Mono-S fraction, 1.2 μg) was incubated for 30 min at 4 °C with 0.6 μg affinity-purified anti-ERCC3 polyclonal antibodies. Lane 5, the complex was treated with preimmune IgG (1 μg) as in lane 2. In lane 3 the amount of TBP/TFIIB was increased (40 ng each, this amount does not inhibit transcription). Lanes 4 and 6 were supplemented with TFIIH (500 ng, phenyl-superose fraction).



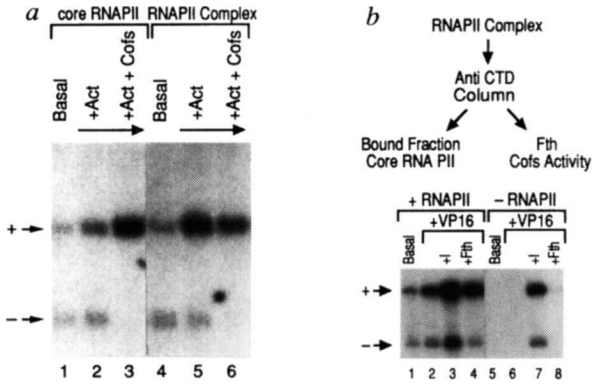


FIG. 3 The RNAPII complex contains activities involved in transcriptional activation. *a*, The RNAPII complex does not require additional cofactors (other than TAFs) to respond to the activator GAL4-VP16 (Act). Transcription reactions were reconstituted using two different templates containing the Ad-MLP (20 ng of each). Plus, Transcript generated by pG5MLT, a construct based on the adenovirus MLP with 5 GAL4 sites upstream of the TATA box. Minus, Transcript generated by p210, a similar construct lacking GAL4 sites driving a shorter G-less cassette. Transcription reactions were reconstituted with: affinity-purified eTFIID (50 ng), TFIIF/H (500 ng, phenyl-superose fraction), core RNAPII (DEAE-5PW fraction, 150 ng)[28], recombinant TFIIA (40 ng), recombinant TFIIB (20 ng), and recombinant TFIIE (20 ng). Reactions also contained recombinant PC4 (10 ng)[7,8] and Cof-A (1 μl of HQ50 fraction), collectively referred to as Cofs, and GAL4-VP16 (10 ng) as indicated. In lanes 4–6, RNAPII complex (Mono-S fraction, 600 ng) was used. Additions and omissions are as shown. *b*, The RNAPII complex contains a CTD-associated coactivator activity. Reactions were reconstituted as described in *a*. The input (I, RNAPII complex Mono-S fraction, 600 ng) and flow-through (Fth, 2 μl; 400 ng) of an anti CTD column were assayed for their activity as cofactors when core RNAPII was used (lanes 1–4).

METHODS. The coactivator fraction was separated from the RNAPII complex by anti-CTD affinity chromatography. The RNAPII complex from the MonoS fraction (600 μl, 120 μg) was dialysed against buffer H containing 0.5 M KCl and incubated for 45 min at 4 °C with anti-CTD antibodies (8WG16, Promega) bound to protein A-agarose (150 μl, 150 μg antibodies). The flowthrough from the affinity column was recovered, and the column was washed with the above buffer. The flow-through and the wash were pooled and concentrated to 500 μl by dialysing against buffer H containing 0.1 M KCl and 50% glycerol.

present in stoichiometric amounts (20 fmol μl^{-1} for each protein) with the largest subunit of RNAPII (Fig. 4*c*).

The association of DNA-repair factors with the RNAPII complex was further analysed by immunoprecipitation and immunoaffinity purification. Western blot analysis of the affinity-purified RNAPII complex revealed the presence of DNA-PKcs (catalytic subunit), HRAD51, DNA polymerase ε, RPA70K subunit, and RFC37K subunit (Fig. 4*e*). These proteins were not detected in the control β-galactosidase column eluate (Fig. 4*e*). Furthermore, antibodies against RNAPII or TFIIF, but not control antibodies, immunoprecipitated RFC (Fig. 4*d*) and other polypeptides of the RNAPII complex (data not shown). The integrity of the RNAPII complex was not affected by DNase I treatment (Fig. 4*d*; or micrococcal nuclease, or if the complex was fractionated on a gel-filtration column containing ethidium bromide; data not shown), thereby ruling out the possibility that the complex is held together by nucleic acids. The immunoprecipitated complexes were also analysed functionally in a transcription assay devoid of RNAPII, TFIIE and TFIIF. Antibodies to RFC, RPA, TFIIF (RAP74) and TFIIH (ERCC3) immunoprecipitated a transcriptionally active RNAPII complex, whereas immunoprecipitates with control antibodies had no RNAPII activity (Fig. 4*f*). Antibodies against CDK7, a subunit of TFIIH, have previously been used to isolate a putative RNAPII holoenzyme containing all the GTFs[26]. TFIIH is known to interact independently with RNAPII, TFIID, TFIIB, TFIIE, TFIIF and other factors[27], therefore immunoprecipitation of all the GTFs and RNAPII by anti-CDK7 antibodies does not unequivocally

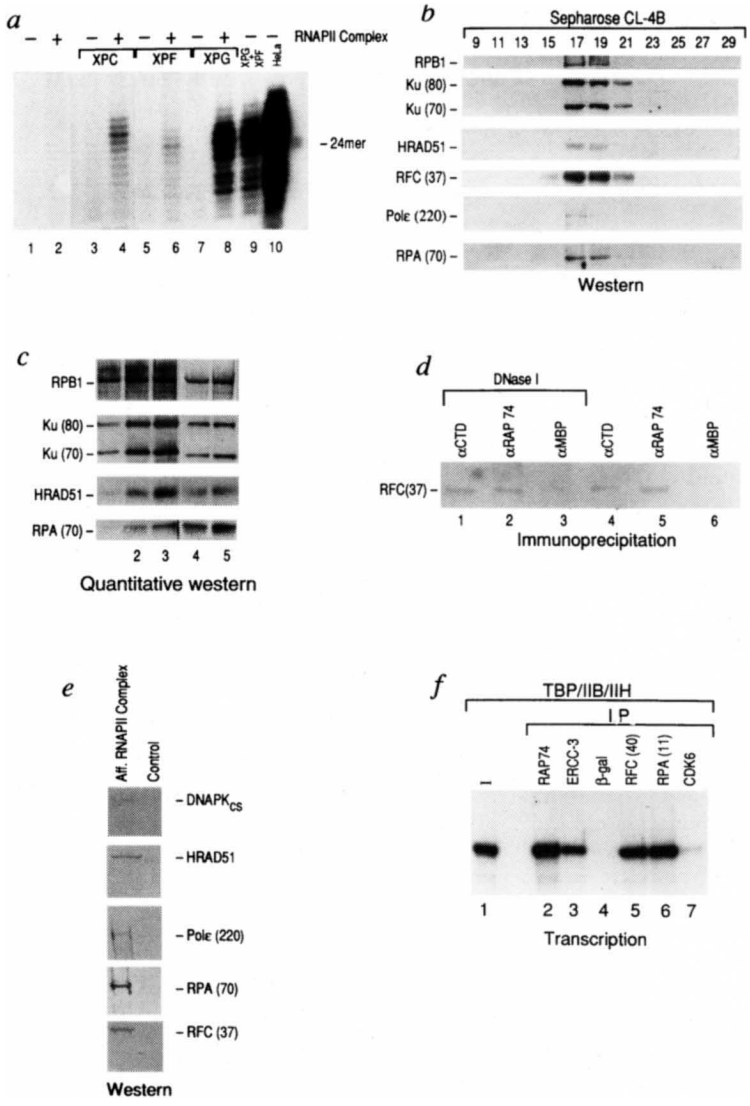


FIG. 4 RNAPII complex contains components required for DNA repair. *a*, Nucleotide excision assay scoring for complementation activity of RNAPII complex (3–4 μl; 0.6–0.8 μg, MonoS) using XPC- (lanes 3 and 4), XPF- (lanes 5 and 6), and XPG- (lanes 7 and 8) deficient extracts. Lanes 1 and 2 are in the absence of any extract, lane 9 is a mixture of XPG and XPF extracts and lane 10 is the control HeLa extract. Excision assays were described previously[30]. *b*, Western blot analysis of Sepharose CL-4B gel filtration column fractions (50 μl, see Fig. 1*a*) using antibodies to proteins delineated on the left. *c*, Quantitative western blot analysis of RNAPII complex (lanes 4, 5, 10 μl (2 μg) and 20 μl (4 μg) of MonoS pool, respectively). Lanes 1, 2 and 3 are: RNAPII, 0.25, 0.5, 0.75 pmol; Ku, 0.14, 0.7, 1.4 pmol; HRAD 51, 0.06, 0.3, 0.6 pmol; RPA, 0.05, 0.25, 0.5 pmol, respectively. *d*, Immunoprecipitation using RNAPII (lanes 1 and 4) and RAP 74 (lanes 2 and 5) antibodies revealed the presence of the 37K subunit of RFC. Lanes 3 and 6 are the immunoprecipitates of the control MBP antibody. Lanes 1–3 denote immunoprecipitation in the presence of 100 units of DNase I as described[31]. The AcA22 (8 μg) fraction was used as an input for the immunoprecipitation experiments as described[10]. Quantification of western blots is described in Supplementary Information. *e*, Analysis of components of DNA repair in the affinity-purified RNAPII complex purified by monoclonal anti-RAP74 antibodies. The same quantity of protein as in Fig. 1*c* was used. *f*, Antibodies against components of the RNAPII complex can immunoprecipitate a complex which requires the addition of TBP, IIB and IIH for specific basal transcription. Lane 1 represents transcription of 15% of the input fraction. Lanes 2–7 contain transcription from the immunoprecipitates of the corresponding antibodies (monoclonal antibodies against RAP74, ERCC-3 and β-galactosidase (Promega), and polyclonal antibodies against RFC (40), RPA (11) and CDK6 (Santa Cruz Biotechnology) as indicated. To immunoprecipitate the RNAPII complex, 3 μg of different antibodies (as indicated on the top) were bound to 15 μl of protein G-agarose beads for 90 min, then extensively washed with buffer H containing 0.1% NP40, 1 mM DTT, 100 mM KCl and 20% glycerol. AcA22-purified RNAPII complex fraction (0.2 ml, 40 μg) was incubated with each antibody at 4 °C for 2 h and extensively washed with buffer H as described. Transcription from the AdMLP driving a G-less cassette was reconstituted by the addition of recombinant TBP (10 ng), recombinant IIB (20 ng), TFIIH and 15 μl of each immunoprecipitate. Anti-β-galactosidase and anti-CDK6 immunoprecipitates were assayed as negative controls.

demonstrate the presence of a large RNAPII complex and may represent individual subcomplexes to transcription factors and TFIIH. The results presented here clearly demonstrate the existence of such an RNAPII complex which contains both transcriptional coactivators and components with roles in DNA repair. □

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SUPPLEMENTARY INFORMATION. Requests should be addressed to Mary Sheehan at the London editorial office of *Nature*. Supplementary Information is also available at the *Nature* Web site http://WWW.nature.com.

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CORRESPONDENCE and requests for materials should be addressed to D.R. (e-mail: Reinberg@mbcl.rutgers.edu). The cDNA sequence of the human homologue of SRB7 is lodged with EMBL, accession number U52960.