3720–3727 *Nucleic Acids Research, 1999, Vol. 27, No. 18*

Identification of a mammalian RNA polymerase I holoenzyme containing components of the DNA repair/replication system

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Received May 21, 1999; Revised and Accepted July 21, 1999

ABSTRACT

Traditional models for transcription initiation by RNA polymerase I include a stepwise assembly of basic transcription factors/regulatory proteins on the core promoter to form a preinitiation complex. In contrast, we have identified a preassembled RNA polymerase I (RPI) complex that contains all the factors necessary and sufficient to initiate transcription from the rDNA promoter *in vitro*. The purified RPI holoenzyme contains the RPI homolog of TFIID, SL-1 and the rDNA transcription terminator factor (TTF-1), but lacks UBF, an activator of rDNA transcription. Certain components of the DNA repair/replication system, including Ku70/80, DNA topoisomerase I and PCNA, are also associated with the RPI complex. We have found that the holoenzyme supported specific transcription and that specific transcription was stimulated by the RPI transcription activator UBF. These results support the hypothesis that a fraction of the RPI exists as a preassembled, transcriptionally competent complex that is readily recruited to the rDNA promoter, i.e. as a holoenzyme, and provide important new insights into the mechanisms governing initiation by RPI.

INTRODUCTION

RNA polymerase can be purified from *Escherichia coli* in two forms. Only one of these protein complexes, referred to as the holoenzyme, is capable of initiating transcription from gene promoters. Recent studies have demonstrated that eukaryotic RNA polymerase II (RPII) can be isolated in high molecular weight complexes (2000 kDa) associated with various components of the transcription machinery as well as DNA repair enzymes (1–7). Some preparations contain both RPII and components of the general transcription machinery, i.e. TBP (TFIID), required for transcription initiation (3,7); this form of RPII would represent a holoenzyme. On the other hand, some preparations contain components of the general transcription apparatus, but are devoid of TBP and are incapable of specific initiation unless supplemented with TBP (1,5). It has been suggested that the holoenzyme preparations might result from the co-purification of an RPII complex and a general transcription factor complex, because the ligand used in the purification, in this case CDK7 (3), is a component of both complexes (6). RNA polymerase III can also be isolated as a holoenzyme which is capable of specific transcription initiation (37).

Recent reports have demonstrated the existence of various forms of RNA polymerase I (38–40). However, there are some significant differences in the composition of the various preparations which may reflect differences in the mode of isolation or the species from which they were purified. For example, the mammalian polymerase I complex isolated by Seither *et al.* (38) contained the accessory rDNA-specific transcription factor UBF, but was not self-sufficient for promoter-dependent transcription. In contrast, the *Xenopus laevis* RNA polymerase I complex purified by Albert *et al.* (39) did not contain detectable amounts of UBF, but was able to autonomously initiate transcription *in vitro*.

We have previously reported an effective scheme to affinity purify RNA polymerase I from nuclear extracts of an N1S1 cell line that expresses FLAG-tagged A127, the second-largest subunit of rat RPI (8). In the present study, in an attempt to clarify the above discrepancies, we have utilized a combination of both FLAG-affinity purification and gel filtration to examine the possibility that the transcription factors required for specific initiation by RNA polymerase I can be purified as components of a complex that includes the polymerase itself. Both western analysis of the complex and the demonstration that the complex supported specific transcription confirmed that the factors required for initiation had co-purified with RNA polymerase I. In addition, the isolated complex responded to UBF, an activator of rDNA transcription (9; reviewed in 10). As this purification scheme relies upon purification of RPI itself, it is unlikely that we have co-purified complexes that do not contain RPI along with the

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RPI holoenzyme. Thus, in these experiments we establish that RNA polymerase I can be purified as a true holoenzyme.

MATERIALS AND METHODS

Maintenance of cell lines

N1S1C3 cells are a derivative of rat N1S1 cells (rat Novikoff hepatoma cells adapted to tissue culture; ATCC CRL 1604) that express a construct containing the entire coding region of the A127 subunit of rat RPI tagged with the FLAG epitope at the N-terminus (8). N1S1C3 and wild-type N1S1 cells were routinely maintained in spinner flasks containing RPMI 1640 with 5% horse serum and the appropriate antibiotic as described (8).

Preparation of nuclear extracts

Nuclear extracts were prepared from cultured N1S1 and N1S1C3 cells and rat Novikoff hepatoma ascites cells as previously described (11). The extracts were frozen in liquid N_2 and stored at –80°C.

Sephacryl HR500 gel filtration chromatography

Nuclear extracts of rat Novikoff hepatoma ascites cells (0.5 ml, 11.5 mg/ml) or N1S1C3 cells (0.5 ml, 14 mg/ml) were fractionated by chromatography over a Sephacryl HR500 gel filtration column (1.6 × 22 cm) equilibrated in solution D (20 mM HEPES, 0.1 M KCl, 0.2 mM EDTA, 20% glycerol, 0.5 mM PMSF, 0.5 mM DTT) containing 2.5 μM distamycin. Aliquots of each 0.5 ml fraction were assayed for either RPI activity or used in western blots as described below. In some cases fractions were assayed for specific transcription or immunoprecipitated with anti-FLAG affinity resin and western blotted as described below.

Ligand and immunoaffinity purification of RNA polymerase I

RPI complexes containing FLAG-tagged A127 were immunopurified using 0.2 ml of packed anti-FLAG resin (2.5 mg/ml; ICI/Kodak) equilibrated in C20 buffer (11) containing 200 mM NaCl (C20/200) and 0.1% NP-40. The resin was tumbled for 2 h with 5 ml of nuclear extracts, packed into columns, drained and washed with 100 column vol of C-20/200 containing 0.1% NP-40. Bound proteins were then eluted with C-20/200 containing 0.5 mg/ml FLAG peptide. The eluted fractions as well as the flow-through were analyzed by immunoblot analysis as described below. In some cases the bound proteins were subjected to washes in the presence of increasing amounts of NaCl (i.e. 200, 400, 600 and 800 mM) prior to elution with the FLAG peptide. To control for proteins that might non-specifically bind to the FLAG-affinity resin, nuclear extracts from non-transfected control N1S1 cells were subjected to affinity purification and western analysis in parallel with extracts containing tagged proteins.

RPI complexes containing FLAG-tagged A127 were immunopurified from HR500 gel filtration column fractions using 0.2 ml of packed anti-FLAG resin. The resin was equilibrated in C-20/200 containing 0.1% NP-40 and then tumbled for 2 h with pooled HR500 gel filtration column fractions containing either Holo-RPI (fractions 33–36, see Fig. 3A) or the Core-RPI (fractions 58–63, see Fig. 3A), after which they were packed into columns and washed with 10 column vol of C-20/200. Bound proteins were then eluted with FLAG peptide and aliquots of the eluted fractions were analyzed by immunoblot analysis as described.

Immunoblotting

Protein determinations used the Bio-Rad Bradford assay kit with bovine serum albumin (BSA) as the standard. Western blots were carried out as described previously (8). Proteins of interest were detected by incubating filters with the appropriate primary antibodies at dilutions between 1:100 and 1:5000, followed by horseradish peroxidase-conjugated anti-rabbit or anti-mouse antibodies (Amersham). Immunoreactive proteins were visualized by the enhanced chemiluminescence method (Amersham). The molecular size of the immunodetected proteins was verified by comparison to the migration of prestained protein markers (Bio-Rad) electrophoresed in parallel.

In vitro transcription

Specific transcription by RPI was carried out essentially as described (11) using nuclear extracts from N1S1 cells, rat Novikoff hepatoma ascites cells and FLAG-affinity purified RPI fractions. Specific transcription was carried out in the presence of 200 μg/ml α-amanatin with 10 μl of the extracts/fractions and 0.1 μg of *Eco*RI-linearized template DNA, pU5.1E/X (11), which contains the rat 45S rDNA promoter (–286 to + 630). The *in vitro* synthesized RNA was purified and analyzed by urea–PAGE and autoradiography. In some cases partially purified UBF (HS600), prepared as described (11), was included in the transcription reactions. TFIC was assayed as described (28).

The ability of nuclear extracts, immunopurified FLAG-tagged RPI or high molecular weight RPI complexes fractionated over Sephacryl HR500 to carry out non-specific RNA synthesis was assayed in the presence of 200 μg/ml α-amanatin as previously described (8). Non-specific transcription reactions contained 25 μl of the starting extracts or anti-FLAG affinity column eluate and nicked calf thymus DNA as template as described previously (8). The radioactivity incorporated was determined by liquid scintillation spectrophotometry.

RESULTS AND DISCUSSION

Affinity purification of RPI using FLAG-affinity chromatography

The goal of these experiments was to determine whether transcription factors required for specific initiation by RPI can be purified as components of a complex that includes the polymerase itself. RPI and putative RPI-associated complexes were affinity purified from nuclear extracts of a rat N1S1 cell line (N1S1C3) that expresses a construct containing the entire coding region of the A127 subunit of RPI tagged with the FLAG epitope at the N-terminus (8). Nuclear extracts derived from either N1S1C3 cells or control, untransfected N1S1 cells were applied to pre-equilibrated anti-FLAG affinity columns and after extensive washing in buffer containing 200 mM NaCl and 0.1% NP-40 the columns were eluted with FLAG peptide (Fig. 1A and Materials and Methods). The eluted fractions prepared in this manner were subsequently analyzed for RPI activity and by western blot analysis. To purify ‘core’ RPI

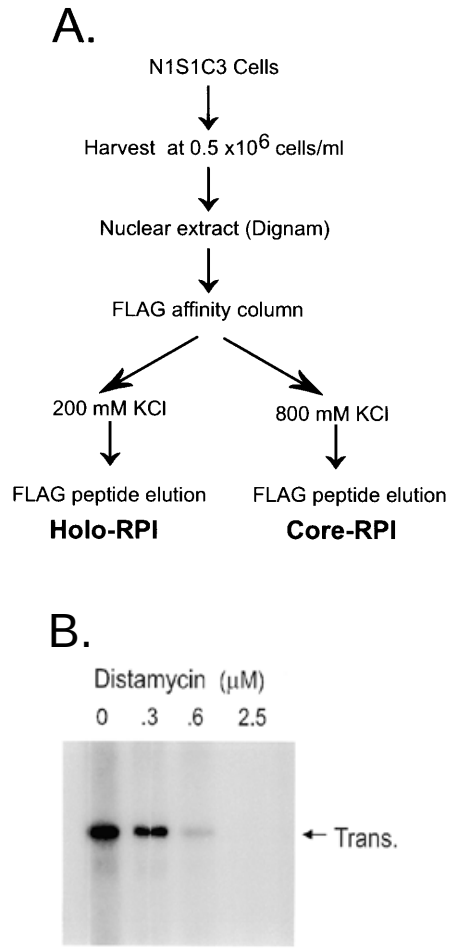

Figure 1. (**A**) Schematic of the procedure used to affinity purify RPI (Core-RPI) and RPI-associated complexes (Holo-RPI) from nuclear extracts of NISIC3 cells. (**B**) Distamycin inhibits transcription by RNA polymerase I. *In vitro* transcription, using the rat rDNA promoter (pU5.1E/X), was carried out as described (11) in the presence of the indicated amounts of distamycin.

devoid of associated complexes, the affinity columns were washed with buffer containing 800 mM NaCl prior to FLAG elution (Fig. 1A and discussed below).

Prior to carrying out those experiments, it was necessary to eliminate the possibility that such a complex might arise from the co-association of the components with DNA fragments generated during preparation of the extracts. To address this question we fractionated the extracts in the presence of distamycin. Distamycin binds in the minor groove of DNA, inhibits DNA binding and transcription by RNA polymerase II (3) and at 2.5 μM distamycin inhibits transcription by RPI (Fig. 1B). Subsequently, all affinity purifications were performed in the presence of 2.5 μM distamycin.

As shown in Figure 2A, eluates from the N1S1C3 column fractionated in the presence of distamycin contained significant RPI activity. Western blot analysis demonstrated that the RPI activity co-purified in the same fractions as did the FLAG-tagged A127 subunit and the untagged core subunits of RPI, A194 (8) and AC40 (12) (Fig. 2A). As expected, the anti-FLAG immunopurified RPI complex also contained significant amounts of PAF53 (Fig. 2B), a polymerase-associated factor thought to be involved in the regulation of RPI transcription initiation (8,12,13). In contrast, neither RPI activity nor RPI subunits were detected in eluates from control N1S1 cells.

The affinity-purified RPI complex contains both transcription initiation and transcription termination factors

We next examined what other components of the RPI transcription apparatus might co-purify with the affinity-purified RPI. In addition to RPI, a *trans*-acting factor, selectivity factor-1 (SL-1), is absolutely required for initiation of transcription from the rDNA promoter (14,15). SL-1 consists of TBP and three TATA-associated factors (TAFs) specific for rDNA transcription (16) and is the RPI homolog of TFIID. Western blot analysis of the anti-FLAG immunopurified RPI complex, isolated in the presence of 2.5 μM distamycin, demonstrated the presence of TBP, TAF_I110 and TAF_I48, three subunits of SL-1 (16), indicating that this factor had co-purified with the RPI complex (Fig. 2B).

Termination of mammalian rDNA transcription is effected by the binding of a nucleolar protein, TTF-1 (130 kDa), to a repetitive sequence motif (the Sal box) within the intergenic spacer (17). Sal box-mediated termination results from a specific interaction between TTF-1 and RP1 (18). As the two proteins can interact, we examined the possibility that TTF-1 might be associated with the affinity-purified RPI. As shown in Figure 2C, western blot analysis of the anti-FLAG immunopurified RPI complex isolated in the presence of 2.5 μM distamycin demonstrated the presence of TTF-1, indicating that this factor was a component of the affinity-purified RPI complex.

A second *trans*-acting factor, upstream binding factor (UBF), is required for efficient transcription from the 45S rDNA promoter (10,14,15). Current evidence suggests that UBF functions as an activator of rDNA transcription (9). *In vitro* assays for protein–protein interactions have demonstrated that UBF has the potential to interact with RPI (13,19) and TAF_I48 (20) and UBF has been found in one RPI holoenzyme preparation (37). Co-immunoprecipitation experiments using anti-UBF antisera have confirmed the interaction between UBF and SL-1 (21), but have failed to detect an interaction between UBF and RPI (8,21). In agreement with the latter observation, western blots demonstrated that UBF did not co-purify with the RPI complex (Fig. 2C). Although the titers of the anti-UBF and anti-A127 antisera used in these experiments were approximately equal, the anti-UBF antiserum failed to detect UBF in the RPI complexes that clearly contained A127 (even after prolonged exposures). These results confirm our previous studies (8,21) and suggest that either UBF–RPI and/or UBF–SL-1-RPI complexes do not form in the absence of DNA or they do not survive the relatively gentle immunopurification procedures used in these experiments. In addition, it is interesting to note that the RPI complexes did not contain either TFIIF (RAP 30/74) or SRB11 (CDK8), components of the RPII holoenzyme (1–6; Fig. 2C). These results demonstrate differences between the RPI and RPII holoenzymes, as well as the specificity of the immunopurification procedure, i.e. components of the RPII holoenzyme do not co-purify with the RPI complex.

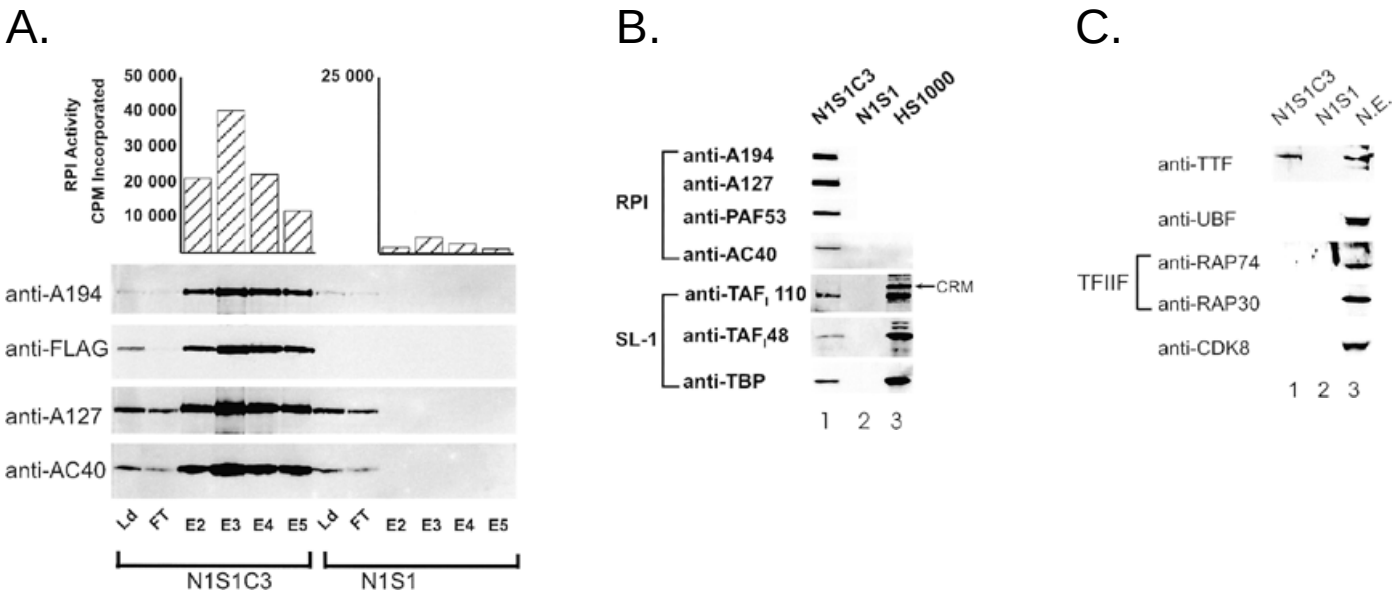

Figure 2. Analysis of the proteins that assemble with the FLAG-tagged A127 subunit of RPI. RPI complexes were affinity purified from nuclear extracts of a rat N1S1 cell line (N1S1C3) that constitutively expresses a construct containing the A127 subunit of RPI tagged with the FLAG epitope at the N-terminus (8). (**A**) Nuclear extracts derived from either N1S1C3 cells or control N1S1 cells were applied to anti-FLAG affinity columns (Ld, column load, FT, flow-through material). The columns were eluted with FLAG peptide (0.5 mg/ml) in C20/200 buffer (20 mM HEPES, 5 mM $MgCl_2$, 0.2 mM EDTA, 5 mM DTT, 100 mM PMSF, 20% glycerol, containing 200 mM KCl) and the eluted fractions (E2–E5) analyzed for both RPI activity (8) and for the presence of different polypeptides by SDS–PAGE and western blot analysis. (**B**) Nuclear extracts of N1S1C3 (lane 1) and N1S1 (lane 2) cells were fractionated by affinity chromatography over immobilized anti-FLAG antibody in the presence of 2.5 μM distamycin as described above and analyzed by western blotting for the subunits of RPI (A194, A127, PAF53 and AC40) or the subunits of SL-1 (TAF_I110, TAF_I48 and TBP). A sample (5 μl) of partially purified (~300-fold) SL-1 (HS1000) (11) was included in lane 3 as a positive control for SL-1 (note that this fraction does not contain detectable RPI activity). CRM, material unrelated to TAF_I110 that cross-reacts with the anti-TAF_I110 antibodies. (**C**) Nuclear extracts of N1S1C3 (lane 1) and N1S1 (lane 2) cells were fractionated by affinity chromatography over immobilized anti-FLAG antibody in the presence of 2.5 μM distamycin as described above and analyzed by western blotting for the presence of TTF, UBF, TFIIF (RAP 30/74) and SRB11 (CDK8). A sample (20 μg) of a N1S1 cell nuclear extract (N.E) was included in lane 3 as a positive control.

A high molecular weight RPI complex can be identified by molecular sieve chromatography

We then determined if this RPI complex could be identified by molecular sieve column chromatography. Nuclear extracts were fractionated over a Sephacryl 500-HR gel filtration column in the presence of 2.5 μM distamycin. The majority of the RNA polymerase I activity eluted with an equivalent molecular mass of ~500–600 kDa, the size predicted for core RPI (Fig. 3A). However, ~5–10% of the RPI activity chromatographed with an apparent mass of 2000 kDa. Western blots of both forms of RPI demonstrated the presence of the 194 kDa subunit (Fig. 3A, anti-A194, lower panel; note that the left and right sides of the panel are exposed for different lengths of time) and other core subunits of RPI (results not shown), confirming that these fractions contained RPI. In a complementary series of experiments, nuclear extracts of NISIC3 cells expressing FLAG-tagged A127 were fractionated over a Sephacryl 500-HR gel filtration column in the presence of 2.5 μM distamycin. Immunoaffinity purification of the column fractions using anti-FLAG resin demonstrated that SL-1 was associated with the 'high' molecular weight RPI complex (Holo-RPI) but did not co-purify with the 'low' molecular weight complex (Core-RPI) (Fig. 3B). Considered with the affinity purification experiments presented in Figure 2, these results demonstrate that a subset of RPI (<10%) can be isolated in the form of a high molecular weight complex pre-assembled with some of the basal components of the RPI transcription machinery.

The purified RPI complexes support specific transcription from the rDNA promoter and are responsive to activators

The presence of the transcription initiation factor SL-1 in the immunopurified RPI complex and high molecular weight RPI complex obtained by molecular sieve column chromatography led us to examine if the complexes would be capable of accurately transcribing rDNA. Cell-free transcription assays demonstrated that the high molecular weight complex isolated by molecular sieve column chromatography could support specific transcription from the rat rDNA promoter in the absence of exogenously added SL-1 (Fig. 4A, fractions 34 and 35). In contrast, the low molecular weight complex (Core-RPI) isolated by molecular sieve column chromatography did not support specific transcription (results not shown). Holoenzyme purified by FLAG immunoaffinity chromatography from extracts of N1S1C3 cells also supported specific transcription (Fig. 4B, lane 1). It is important to note that the holoenzyme retains its ability to respond to UBF, an activator of rDNA transcription (Fig. 4B, lanes 2 and 3; 9).

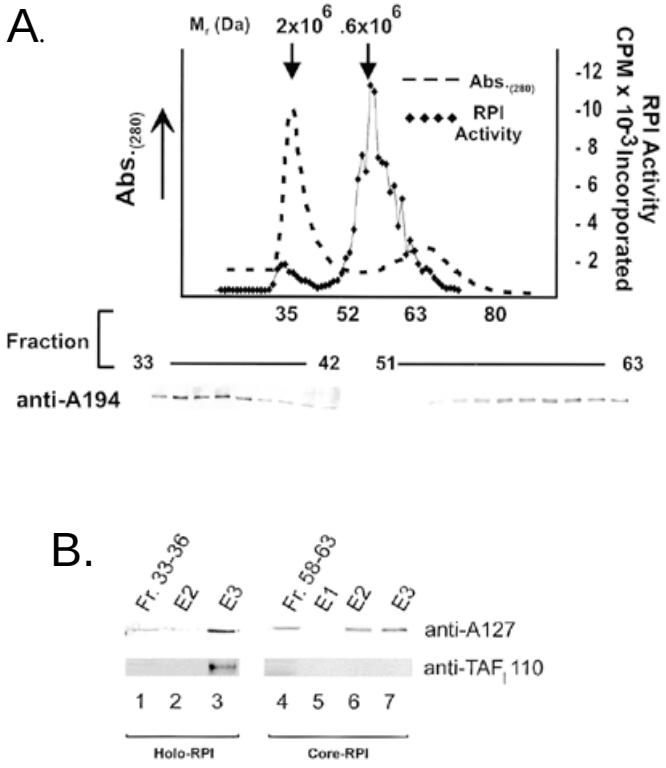

Figure 3. A fraction of RPI exists as a complex of >2000 kDa. (**A**) (Top) Nuclear extracts were fractionated by chromatography over a Sephacryl HR500 gel filtration column (in the presence of 2.5 µM distamycin) and 25 µl of each fraction (0.5 ml/fraction) were assayed for RPI activity (8). (Bottom) Aliquots of the fractions containing the high (fractions 33–42) and low (fractions 51–63) molecular weight forms of RPI were analyzed by western blotting for the presence of A194. The blots containing the high and low molecular weight forms of RPI were exposed for 20 min and 30 s, respectively. (**B**) Nuclear extracts from NISIC3 cells were fractionated by chromatography over a Sephacryl HR500 gel filtration column. The pooled fractions containing the Holo-RPI (fractions 33–36) and the Core-RPI (fractions 58–63) were immunopurified with immobilized anti-FLAG antibodies. The bound proteins were eluted with FLAG peptide (E2 and E3) and analyzed by western blotting for RPI (A127) and SL-1 (TAF_I110). The anti-A127 blots containing the high and low molecular weight forms of RPI were exposed for 20 min and 30 s, respectively. The anti-TAF_{110} blot containing the high and low molecular weight forms of RPI were exposed for equal amounts of time (30 min).

A polymerase-associated factor, referred to as TFIC, has been shown to be required for transcription. Thus, by inference the holoenzyme contains TFIC. However, this can be more directly demonstrated. Thompson's laboratory (28) has demonstrated that TFIC is stable at 45°C (whereas RPI is heat labile), but it has a short half-life. Thus, when cells are treated with cyclohexamide, TFIC is depleted and extracts from these cells provide the basis for a quantitative TFIC assay. As shown in Figure 4C, the RPI holoenzyme is inactivated by heating to 45°C for 15 min (lanes 2 and 3). However, the heat-treated holoenzyme can complement an S100 fraction from cyclohexamide-treated cells (lane 4), demonstrating the presence of TFIC.

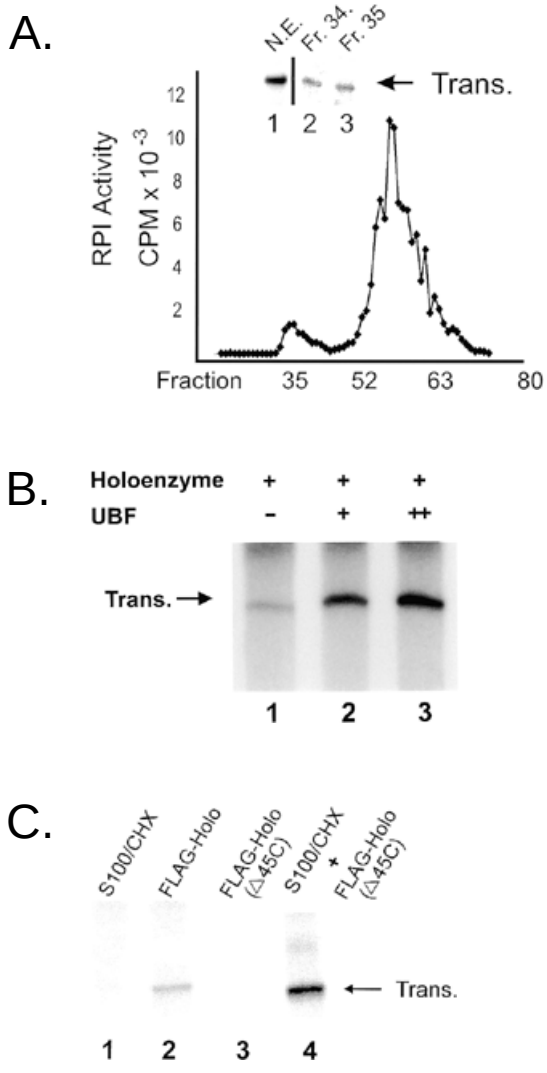

Figure 4. The affinity-purified and high molecular weight RPI contains all the factors sufficient to initiate specific transcription from the rDNA promoter. (**A**) Nuclear extracts from N1S1 cells (1 µl, N.E., lane 1) or Sephacryl HR500 column fractions containing Holo-RPI (100 µl, Fr. 34–35, lanes 2–3) were added to *in vitro* transcription reactions (final volume 200 µl) containing the rat rDNA promoter (0.1 µg) (11). The *de novo* synthesized transcript was isolated, fractionated by urea–PAGE and detected by autoradiography as described (11). (**B**) RPI holoenzyme (10 µl) purified by anti-FLAG affinity chromatography from nuclear extracts of N1S1C3 cells was added to a cell-free transcription reaction (final volume 50 µl, 0.01 µg/ml template) containing increasing amounts of partially purified rat UBF (1 and 2.5 µl, lanes 2 and 3, respectively) (11). (**C**) Whole cell extracts of cyclohexamide-treated N1S1 cells (10 µl, S100/CHX), RPI holoenzyme (FLAG-Holo) (10 µl) purified by anti-FLAG affinity chromatography from nuclear extracts of N1S1C3 cells, heat-treated RPI holoenzyme (heated at 45°C for 15 min, Δ45C) and the indicated combinations were added to a cell-free transcription reaction (final volume 50 µl, 0.1 µg/ml template). The *de novo* synthesized transcript was isolated, fractionated by urea–PAGE and detected by autoradiography as described (11). The mobility of the transcript which resulted from correct initiation (638 nt) is indicated (Trans.).

The transcription experiments complement and extend the western blots, which showed the presence of SL-1, suggesting

that the complex might be transcriptionally active. The transcription assays establish that the Holo-RPI complex is both transcriptionally active and responsive to activators.

Components of the DNA repair/replication system are associated with the RPI holoenzyme

Topoisomerase I (TopoI) is thought to have a functional role in the relaxation of DNA superhelices during DNA replication and transcription (22). In addition, TopoI co-localizes with RPI in immunohistochemical assays and co-purifies with RPI during biochemical purification of the enzyme (23,24). These observations suggest a specific role for TopoI in rDNA transcription. Western blot analysis of the eluates from the N1S1C3 column demonstrated that TopoI is a component of the FLAG-affinity purified RPI holoenzyme (Fig. 5A). Interestingly, TopoI was not present in affinity-purified RPII complexes (5), indicating that this enzyme is not a general component of RNA polymerase holoenzymes.

The observation that TopoI was associated with the RPI complex led us to examine if other DNA modifying enzymes, e.g. components of the DNA replication/repair systems, might also be associated with the affinity-purified RPI complex. Western blots (Fig. 5A) of affinity-purified RPI demonstrated the presence of Ku70 and Ku80, the regulatory subunits of DNA-PK, proteins essential for DNA double-strand break repair and V(D)J recombination (25–28). In contrast, other components of the DNA repair/replication process reported to be present in the RPII holoenzyme (5), such as TFIIH (p62 and p44), replication protein A (RPA) (Fig. 5B) and replication factor C (RFC) (results not shown) were absent from the RPI complex. These findings accentuate the similarities and the differences between the RPI and RPII complexes. It is intriguing to note that lack of the TFIIH–NER complex appears to correlate with observations that, in comparison to genes transcribed by RPII, rDNA is a poor substrate for nuclear excision/repair (29,30).

The presence of Ku70/80 in the RPI complex is of particular interest. Ku70/80, also identified as E_1BF, has been shown to contribute to the regulation of rDNA transcription *in vitro* (31,32). In addition, Ku70/80 has been reported to demonstrate a PCNA-like localization to the nucleolus (33), consistent with our proposal for a role for Ku in rDNA transcription.

The relative strengths of the associations between the components of the FLAG-affinity purified RPI holoenzyme were examined. Affinity purifications were performed in which the FLAG-affinity columns containing bound holoenzyme were washed (100 column vol) with buffers containing increasing concentrations of NaCl. As shown in Figure 6A, the majority of the SL-1 was dissociated from the complex above 400 mM NaCl. Consistent with this observation, RPI affinity purified under salt conditions above 400 mM NaCl no longer retained the ability to support specific transcription from the rDNA promoter in the absence of exogenously added SL-1 or UBF (results not shown). Interestingly, Ku70/80 dissociated from the RPI complex at approximately the same concentration of salt. In contrast, at least 50% of the core subunits of RPI and the RPI-associated enzyme CK2 remained associated with each other and with the FLAG beads in the presence of 800 mM NaCl (8).

The difference between peptide compositions of the RPI complexes affinity purified under low and high salt conditions was further examined by SDS–PAGE and silver staining. After

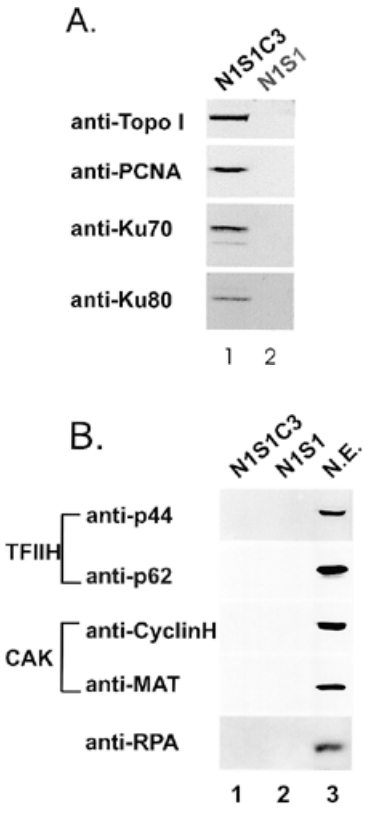

Figure 5. DNA-modifying enzymes are associated with the affinity-purified RPI. (**A**) Nuclear extracts of N1S1C3 and N1S1 cells were fractionated by FLAG-affinity chromatography in the presence of 2.5 μM distamycin and analyzed by western blotting for the presence of TopoI, proliferating cell nuclear antigen (PCNA) and Ku70 and Ku80. (**B**) Nuclear extracts of N1S1C3 (lane 1) and N1S1 (lane 2) cells were fractionated by affinity chromatography over immobilized anti-FLAG antibody as described above and analysed by western blotting for TFIIH (p44 and p62), CAK (cyclin H and MAT1) and RPA. A sample (20 μg) of an N1S1 cell nuclear extract (N.E.) was included in lane 3 as a positive control.

washes with 800 mM NaCl the subunit composition of the eluted FLAG-affinity purified RPI included the core subunits of RPI enzyme, including A194 and A127, AC40 and A27, A20, A19, A18, A16, A14 and A12 and a cluster of polypeptides between 54 and 49 kDa which correspond to the polymerase-associated factors (24) PAF53, PAF51 and PAF49 (Core-RPI, Fig. 6B and C, lanes 3). This subunit composition is similar to that recently reported by Hanada *et al.* and Song *et al.* (12,13), using conventional biochemical purification techniques. The polypeptides migrating at 67 and 44 kDa (denoted by asterisks) are contaminating proteins not associated with RPI since they are also present in FLAG-affinity purified extracts from cells not expressing FLAG-A127 (results not shown). The two polypeptides with apparent molecular masses of 33 and 37 kDa (denoted by arrows) do not correlate with previously identified subunits of RPI and are likely to represent novel RPI-associated factors.

The Holo-RPI complex purified in the presence of 200 mM NaCl (Holo-RPI, Fig. 6C, lane 4) consisted of a considerable number of polypeptides in addition to the core RPI subunits

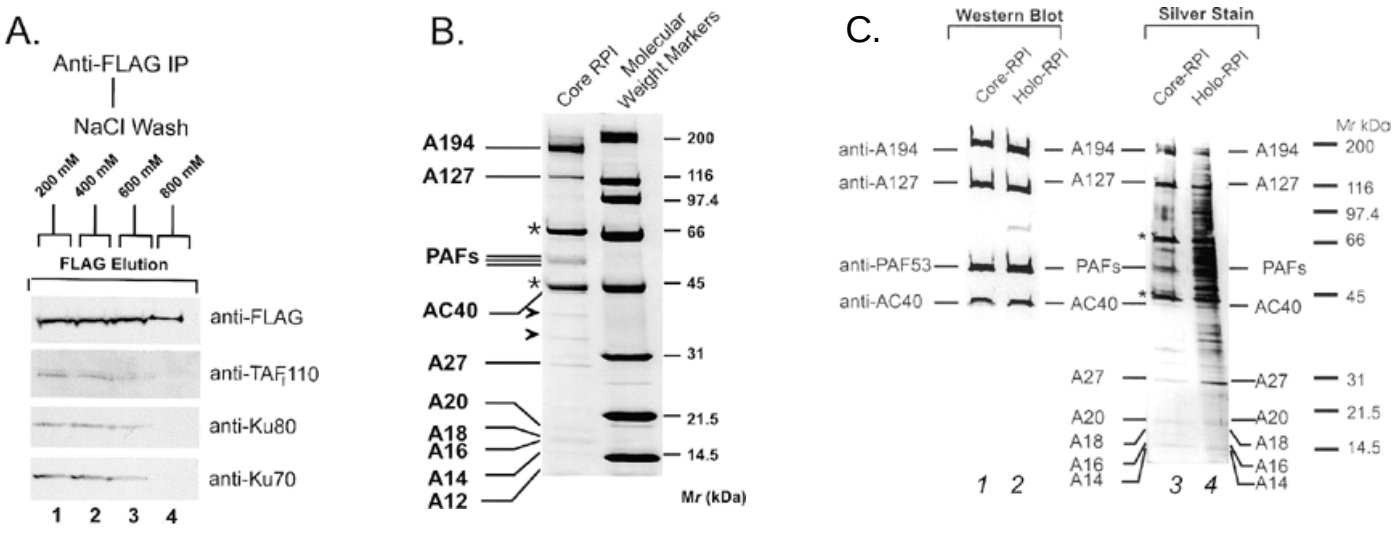

Figure 6. The holoenzyme components remain associated with RPI under relatively 'high salt' conditions. (**A**) Nuclear extracts of N1S1C3 cells were fractionated by chromatography over FLAG-affinity resin in four separate experiments (lanes 1–4) as described in Figures 1A and 2A. Prior to elution with the FLAG peptide, the columns were washed with buffer containing either 200 (lane 1), 400 (lane 2), 600 (lane 3) or 800 (lane 4) mM NaCl. The fractions containing the FLAG-eluted proteins from each column were pooled and subjected to SDS–PAGE and western analysis with the antisera indicated as described in Materials and Methods. (**B**) Silver stain of the Core-RPI complex affinity purified in the presence of 800 mM NaCl. Nuclear extracts of N1S1C3 cells were fractionated by chromatography over FLAG-affinity resin as described above. Prior to elution with the FLAG peptide, the column was washed with buffer containing 800 mM NaCl (100 column vol). The fractions containing the FLAG-eluted proteins were pooled and an aliquot (60 µl) was subjected to SDS–PAGE and silver staining. Arrows denote two novel RPI-associated polypeptides not identified by conventional RPI purification schemes. *, cross-reacting proteins not associated with RPI. (**C**) Comparison of the polypeptides present in the Core- and Holo-RPI preparations. Nuclear extracts of N1S1C3 cells were fractionated by chromatography over FLAG-affinity resin in two parallel experiments as described above. Prior to elution with the FLAG peptide, the columns were washed with buffer containing either 800 (lanes 1 and 3, Core-RPI) or 200 (lanes 2 and 4, Holo-RPI) mM NaCl. The fractions containing the FLAG-eluted proteins from each column were pooled and an aliquot (50 µl) was subjected to SDS–PAGE and either transferred to a nylon membrane and blotted with the indicated antibodies (Western Blot, left, lanes 1 and 2) or silver stained (Silver Stain, right, lanes 3 and 4). *, cross-reacting proteins not associated with RPI.

(compare the polypeptide compositions of the complexes in Fig. 6C, lanes 3 and 4). Even so, the high molecular weight RPI subunits, A194 and A127, and many of the smaller subunits, including A27, A20, A18, A16 and A14, could be identified (Fig. 6C, lane 4). Moreover, western blot analysis of those same samples, run in parallel, demonstrated that the bands identified as A194, A127, PAF53 and AC40 in both the core and holoenzyme preparations are in fact those same proteins (Fig. 6C, lanes 1 and 2).

Taken together these results suggest that the components of the RPI holoenzyme are associated *in vivo* and are not artifacts of the affinity purification process. However, exposure of this complex to salt concentrations >600 mM NaCl will dissociate various components and result in an extremely low yield of holoenzyme. This may explain why the holoenzyme complex has not previously been identified during purification of RPI on classic chromatography, which often employs wash and elution steps in excess of 600 mM salt.

The above experiments describe the affinity purification of RPI complexes using highly selective affinity matrices, i.e. immobilized anti-FLAG. Since the purification schemes used rely upon the immunoaffinity purification of RPI itself, it is unlikely that we have co-purified complexes that do not contain RPI along with the RPI holoenzyme. However, this does not eliminate the possibility that we have isolated more than one RPI complex. For example, the presence of both SL-1 and TTF-1 in the preparation raises the possibility that we may have isolated an initiation complex as well as a termination complex.

The presence of SL-1 in association with RPI fulfills the operational definition of a holoenzyme, in the sense that the purified complex contains all of the components sufficient to initiate transcription accurately *in vitro*. There have been previous reports that RPI might exist as a holoenzyme (34,35,38,39). However, there have been considerable differences in the composition of the purified RPI complexes. For example, a putative RPI holoenzyme immunopreciptated from mouse extracts was not self-sufficient for promoter-dependent transcription, but the *X.laevis* holoenzyme was. Interestingly, our affinity-purified rat holoenzyme was self-sufficient for promoter-dependent transcription**.**

A further difference between the immunopurified mouse RPI complex and the affinity-purified rat holoenzyme is that the mouse complex contained significant amounts of UBF whereas the rat holoenzyme is devoid of this factor. In agreement with our findings in these studies we have previously reported that UBF does not co-purify with either endogenous RPI during immunoprecipitation experiments using anti A194 antibodies or with epitope-tagged RPI during ligand affinity purification (8). Given the excess of UBF over the other components of the

holoenzyme, such as SL-1 and RPI, it is tempting to speculate that the UBF in the mouse complex may represent a contaminant. Our results do not rule out the formal possibility that UBF may transiently interact with the holoenzyme under the appropriate conditions. In fact, we have previously reported that a UBF–SL-1 complex can be immunoprecipitated (21). However, a number of other observations lead us to suspect that UBF is in fact not a component of the RPI holoenzyme: (i) in complementary experiments we have not been able to demonstrate co-purification of RPI and UBF by immunoprecipitaion using either anti-UBF antibodies or antibodies directed towards an epitope-tagged UBF (21; R.D.Hannan and L.Rothblum, unpublished data); (ii) UBF is not present in RPI holoenzyme affinity purified following gel filtration chromatography (results not shown); (iii) a putative *X.laevis* RPI holoenzyme complex purified by sequential column chromatography did not contain detectable amounts of UBF (39).

If UBF is not part of an autonomously initiating RPI enzyme, then how does one explain the ability of UBF to activate transcription *in vivo* (41,42). One possibility is that UBF might contribute to the regulation of transcription initiation, at least in part, by facilitating recruitment of the holoenzyme to the rRNA genes. This model would be consistent with the observation that UBF is associated with rRNA genes that may be transcribed, but is not associated with inactive rRNA genes (36). In apparent agreement with this, we have demonstrated that UBF stimulates transcription by the RPI holoenzyme. Future studies will need to examine the potential role of activators such as UBF in the organization of the rRNA chromatin and recruitment of the polymerase holoenzyme to the rDNA promoter *in vivo*.

ACKNOWLEDGEMENTS

The authors would like to thank Dr David Carey for his helpful comments on this manuscript. We thank L. Lei and Z. Burton for anti-RAP30 and anti-RAP74 antibodies, J.-M. Egly and A. Fery for anti-p62 and anti-p44 (TFIIH) antibodies, K. Yankulov and D. Bentley for anti-cycH and anti-MAT1 antibodies, T. Bessho and A. Sancar for anti-ERCC1 antibodies, I. Grummt for the initial samples of anti-TTF, J. Hurwitz for anti-RPA and anti-RFC antibodies, C. Glover for anti-CK2 antibodies, E. Nigg for anti-CDK8 and anti-CK2 antibodies and G.C. Li for anti-Ku70 and anti-Ku80 antibodies. R.D.H. was supported by an American Heart Association Grant-in-Aid and by a Fellowship from the Australian Foundation for High Blood Pressure Research. This work was supported in part by National Institute of Health Grant GM46991 and an award from the Geisinger Foundation to L.I.R.

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