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Isolation and Characterization of Monoclonal Antibodies Directed against Subunits of Human RNA Polymerases I, II, and III

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Human nuclei contain three different RNA polymerases: polymerases I, II, and III. Each polymerase is a multi-subunit enzyme with 12–17 subunits. The localization of these subunits is limited by the paucity of antibodies suitable for immunofluorescence. We now describe eight different monoclonal antibodies that react specifically with RPB6 (also known as RPA20, RPB14.4, or RPC20), RPB8 (RPA18, RPB17, or RPC18), RPC32, or RPC39 and which are suitable for such studies. Each antibody detects one specific band in immunoblots of nuclear extracts; each also immunoprecipitates large complexes containing many other subunits. When used for immunofluorescence, antibodies against the subunits shared by all three polymerases (i.e., RPB6, RPB8) gave a few bright foci in nucleoli and nucleoplasm, as well as many fainter nucleoplasmic foci; all the bright foci were generally distinct from speckles containing Sm antigen. Antibodies against the two subunits found only in polymerase III (i.e., RPC32, RPC39) gave a few bright and many faint nucleoplasmic foci, but no nucleolar foci. Growth in two transcriptional inhibitors—5,6-dichloro-1-β-D-ribofuranosylbenzimidazole and actinomycin D—led to the redistribution of each subunit in a characteristic manner.

Key Words: **monoclonal antibody; nuclear structure; RPB6; RPB8; RPC32; RPC39; Sm antigen.**

INTRODUCTION

Eukaryotic nuclei contain three different RNA polymerases (pols) defined by their characteristic elution profiles, nuclear locations, and drug sensitivities [1, 2]. On raising the salt concentration, polymerase (pol) I elutes first from a DEAE–Sephadex column, followed by pols II and III. Pol I is nucleolar and resistant to α-amanitin but sensitive to low concentrations of actinomycin D, pol II is nucleoplasmic and more resistant to actinomycin D but sensitive to low concentrations of α-amanitin, and pol III is nucleoplasmic and inhibited by high concentrations of α-amanitin.

All three pols are multi-subunit enzymes with 12–17 subunits [3, 4]. Many subunits have been characterized in yeast, and the mammalian enzymes are now under intensive study. To date, all human pol II subunits have been identified [5, 6], but little is known about pols I and III. For example, we do not yet even know the composition of human pol I, although the murine complex contains 12 subunits and three associated factors [7–9]. Only four genes encoding murine pol I subunits (i.e., the 2 largest subunits and 2 subunits common to pols I and III) have been cloned [7, 12]. Similarly, a human pol III with >17 subunits has been immunopurified [13], but not all genes encoding them have been cloned [14–16]. Even so, it is clear that all three pols have structural and sequence similarities, with the yeast pols II and III being more closely related to each other than to pol I [17]. Moreover, many human pol II subunits prove to be sufficiently conserved that they complement the corresponding yeast mutants [3, 5, 18].

The current nomenclature has not been standardized and is confusing. Initially, subunits of yeast pols I, II, and III were designated A, B, and C, followed by the appropriate molecular mass (kDa), and human homologues were sometimes named similarly. Recently, the human pol II subunits were renamed hRPB1-11 [19], and yeast homologues can now be referred to as yRPB1-11. Here, we follow common usage and refer to subunits of pols I and III as RPA and C, followed by their molecular mass, and to pol II subunits as RPB1-11. Subunits common to all three pols are named according to the pol II convention, and alternative names are listed in Table I. Yeast, murine, and human subunits are prefixed by "y," "m," and "h" when necessary.

The cellular distribution of mammalian pols has been studied by both light and electron microscopy. For example, pol I has been examined using rabbit polyclonal sera and human autoimmune sera [20–23], and the largest subunit of pol II using antibodies directed

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against the C-terminal domain [24–29]. Many other antibodies directed against the various human subunits are available (e.g., monoclonal anti-RPB5—ref. 30; polyclonal anti-RPB2 and anti-RPB12—ref. 31; monoclonal anti-RPB6 and polyclonal anti-RPB3—ref. 19; polyclonal anti-RPB4 and RPB7—ref. 6).

Monoclonal antibodies are usually selected because they react well against denatured targets in blots. However, experience shows that such antibodies often do not give good immunofluorescence with carefully fixed cells, presumably because their targets are inaccessibly buried in larger protein complexes. Therefore, we set out to obtain monoclonal antibodies that would be useful for immunofluorescence. We now describe some of these reacting specifically with RPB6, RPB8, RPC32, and RPC39. We would expect these antibodies to bind to epitopes exposed on the surface of large complexes and so to immunoprecipitate large protein complexes.

RPB6 and RPB8 (in common with RPB5, RPB10α, and RPB10β) are shared subunits found in all three pols [4]. (RPB6 is also known as RPA20, RPB14.4, or RPC20, while RPB8 is known as RPA18, RPB17, or RPC18.) RPB6 is related to a subunit of the archeal pol and is essential for yeast viability [32]. It interacts with the largest subunit of pol II (RPB1) and may be required for pol assembly [19, 33–35]. Antibodies against yRPB6 inhibit the activity of all pols and this inhibition can be relieved by preincubation with DNA, suggesting that this shared subunit binds to DNA [36–38]. RPB8 has no known bacterial homolog and its function is unknown [32, 39], but it is essential for yeast viability [40] and contains an anti-parallel β-barrel with twofold rotational symmetry plus two oligonucleotide/oligosaccharide binding (OB) folds [41].

RPC32 and RPC39 are two subunits found only in pol III. The yeast homolog of RPC32—yRPC31—is essential for viability [42], and mutations in it impair initiation but not elongation [43]. Genetic and two-hybrid interaction assays suggest that it complexes with yRPC82 and yRPC34 and binds to yRPC160 [44]. An analogous human complex containing hRPC62, hRPC39, and hRPC32 dissociates from pol III under partially denaturing conditions [15]. hRPC39 also interacts with hTBP and hTFIIIB90, and pol III lacking the complex is deficient in transcription initiation but not elongation [15].

We show that each antibody detects one specific band in immunoblots of nuclear extracts; each also immunoprecipitates large complexes containing many other subunits. When used for immunofluorescence, antibodies against the subunits shared by all three polymerases (i.e., RPB6, RPB8) gave a few bright foci in nucleoli and nucleoplasm, as well as many fainter nucleoplasmic foci. Antibodies against the two subunits found only in polymerase III (i.e., RPC32, RPC39) gave a few bright and many faint nucleoplasmic foci, but no nucleolar foci.

METHODS

Production of monoclonal antibodies. Proteins tagged with histidine (his) or glutathione *S*-transferase (GST) were expressed in bacteria and purified. A plasmid expressing his-RPB6 was constructed as follows. The *Nhe*I fragment of the vector, pET3a (Novagen; 44), was replaced with the sequence 5′-G CTAGTCACCATCACCATCACCATGCTAGC-3′ to give the pET6His plasmid a unique *Nhe*I site. The coding sequence of hRPB6 [5] was modified by inserting *Nhe*I sites inframe before the ATG and after the stop codon, and the modified coding sequence inserted into the *Nhe*I site of pET6His; this gave an expression vector that directed production of a $(His)_6$ N-terminally tagged RPB6. His-hRPB8 was produced similarly. GST-RPC32, GST-RPC53, and GST-RPC39 were produced as described [15].

Monoclonal antibodies directed against the proteins were isolated essentially as described [46]. Mice were injected five to eight times at 3-week intervals with 20 μg purified protein; primary injections were administered subcutaneously and intraperitoneally, subsequent immunizations were administered intraperitoneally, and final injections were administered intravenously. Spleen cells were recovered, fused with NS1 myeloma cells, and grown in culture; after 10 days, culture supernatants were screened first by enzyme-linked immunosorbent assay (ELISA) using the immunogen and then by indirect immunofluorescence using HeLa cells. Only clones giving bright nuclear fluorescence were selected for further analysis; these clones included 20 clones reactive against RPB6, 6 against RPB8, 18 against RPC32, and 4 against RPC39. After further rescreening by ELISA, immunofluorescence, and immunoblotting, 2 clones reactive against RPB6 (i.e., B6-1, B6-2), 1 against RPB8 (i.e., B8-1), 3 against RPC32 (i.e., C32-1, C32-2, C32-3), and 2 against RPC39 (i.e., C39-1, C39-2) were used for further experiments. Antibodies were purified from supernatants by precipitation with 50% $(NH_4)_2SO_4$ followed by affinity purification on protein–G Sepharose columns (Pharmacia). All were isotyped as IgG1 using a kit (Sigma).

Immunoblotting and immunoprecipitation. Bacteria (BL21-DE3) were transfected with pET-3a (Novagen) or pGEX-2TL+ [47; Pharmacia] vectors encoding recombinant proteins, and expression was induced with isopropylthiogalactoside (IPTG) as described in the manufacturers' handbooks. Nuclear extract was prepared from HeLa cells grown in suspension [48]. Briefly, HeLa nuclei were resuspended in 4 packed vol 20 mM Hepes (pH 7.6), 25% glycerol, 0.42 M NaCl, 5 mM $MgCl_2$, 0.2 mM EDTA, 1 mM DTT, 1 mM PMSF, 1 mM sodium metabisulfite, and protease inhibitors (Boehringer-Mannheim), gently stirred (30 min; 0°C), and spun (21,000*g;* 30 min). After the supernatant was collected, proteins were precipitated with 50% $(NH_4)_2SO_4$, and the pH was adjusted to 7.6 with 10 M KOH. Precipitated proteins were collected by centrifugation (21,000*g;* 15 min), resuspended in 20 mM Hepes (pH 7.6), 20% glycerol, 50 mM KCl, 5 mM $MgCl_2$, 0.2 mM EDTA, 1 mM DTT, 1 mM PMSF, 1 mM sodium metabisulfite, and protease inhibitors, and dialyzed against the same buffer. Insoluble proteins were removed by centrifugation (10,000*g;* 10 min; 0°C) and the supernatant was stored at −70°C. Procedures for resolving proteins in 10 and 15% SDS–polyacrylamide gels and staining gels with Coomassie blue were all as described [49]; proteins were also detected by silver staining using a kit (Daiichi silver stain kit II from Autogen Bioclear). For immunoblotting, proteins were transferred to nitrocellulose (Protran BA 85, Schleicher & Schuell), probed with 1–10 μg/ml monoclonal antibody or a 1/5,000 dilution of a rabbit polyclonal antibody and then a 1/4000 dilution of sheep anti-mouse IgG or donkey anti-rabbit IgG conjugated with horseradish peroxidase (Amersham); finally, bound antibodies were detected using an enhanced chemiluminescence kit (Amersham) and Hyperfilm-ECL (Amersham).

TABLE I

The Different Antibodies

Target subunit (pols)	Alternative names	Size (kDa)	Immunogen	Antibody
RPB6 (I, II, III)	RPA20, RPB14.4, RPC20	14.4 (19)	his-RPB6	B6-1, B6-2
RPB8 (I, II, III)	RPA18, RPB17, RPC18	17 (17)	his-RPB8	B8-1
RPC32 (III)		27 (30)	GST-RPC32	C32-1, C32-2, C32-3
RPC39 (III)		36 (35)	GST-RPC39	C39-1, C39-2

Note. Sizes are calculated from the DNA sequence, and the size observed in gels is shown in parentheses.

For immunoprecipitation, 200 μg antibody was coupled with 50 μl packed protein–G Sepharose beads in a total volume of 100 μl using dimethylpimelimidate [50]. NaCl, NP-40, and Tris (pH 8.0) were added to 200 μl nuclear extract to give 150 mM, 0.1%, and 50 mM, respectively, and a final volume of 0.5 ml. Then, this was incubated (2 h; 4°C) with 60 μl 50% protein–G Sepharose plus 60 μg normal mouse IgG (Pierce) and spun (10,000*g;* 1 min; 4°C) to remove proteins that bind nonspecifically. Then, the supernatant was incubated (2 h; 4°C) with 60 μl 50% protein–G Sepharose coupled to 60 μg monoclonal antibody. After washing 3× with 150 mM NaCl, 0.1% NP-40, and 50 mM Tris (pH 8.0) and 1× with 0.5 ml 50 mM Tris (pH 8.0), proteins were eluted with 30 μl 100 mM glycine (pH 2.5), resolved in 10 or 15% gels, and either silver-stained or blotted. A monoclonal antibody against the C-terminal domain of RPB1 (i.e., 8WG16; ref. 51; Babco) and normal mouse IgG were used as positive and negative controls for immunoprecipitation. Blots were probed with (i) the monoclonal antibodies described here, (ii) monoclonal antibodies against RPB1 (as above) and RPB5 (i.e., 4H7; ref. 30), and (iii) rabbit antibodies against RPC53, RPC62, and RPC82 [15].

Indirect immunolabeling and fluorescence microscopy. Cells were grown overnight on glass coverslips and fixed (30 min; 0°C) in 4% paraformaldehyde in 250 mM Hepes (pH 7.4), and reactive aldehydes quenched with 0.3 M glycine in PBS; then, cells were permeabilized (30 min; 0°C) with 0.1–0.5% Triton X-100 in PBS and incubated (30 min) in PBS+ (PBS containing 40 mM glycine, 1% BSA, 0.2% gelatin). Pol subunits were detected by indirect immunolabeling using antibodies diluted in PBS+. Fixed cells were incubated (1 h) with 1–10 μg/ml primary monoclonal antibody, washed 5× in PBS over 1 h, incubated (1 h) with 1 μg/ml donkey anti-mouse IgG conjugated with Cy3 (Jackson Immunoresearch), washed 5× in PBS over 1 h, counterstained with 20 μM TOTO-3 (Molecular Probes) in PBS, washed 3× in PBS over 30 min, and mounted in Vectashield (Vector Laboratories). The largest subunit of pol II was detected using a monoclonal antibody, 7C2, directed against the C-terminal domain of the largest subunit of RNA polymerase II [52]. Pol subunits and Sm antigens were doubly labeled as above using a mixture of a monoclonal antibody and a human autoimmune serum (1/10,000 dilution; anti-nuclear antigen reference human serum No. 5; Center for Disease Control, Atlanta; ref. 53) during the first incubation and a mixture of the anti-mouse IgG conjugated with Cy3 (as above) and 10 μg/ml donkey anti-human IgG conjugated with FITC (Jackson) during the second incubation.

Digital images were collected on a Bio-Rad MRC 1000 confocal laser scanning microscope equipped with an argon/krypton laser (running under Comos 7.0a software) and coupled to a Nikon Diaphot inverted microscope. Images were collected using a 60X PlanApo objective (NA 1.4), a pinhole aperture of 0.7 mm, a minimum laser power that filled the entire gray scale using the low signal and slow scan mode, and Kalman filtering of 5–10 images. "PIC" files were converted to "TIFF" files using Confocal Assistant v4.02 (T. C. Brelje). Then, images were contrast stretched to fill the entire gray scale using Adobe Photoshop v4.0, assembled in Powerpoint, and printed using a Tetronix Phaser 440 dye sublimation printer.

RESULTS

Generation and Characterization of Monoclonal Antibodies

Our aim was to select monoclonal antibodies useful for immunofluorescence. Pol subunits tagged with his or GST were expressed in bacteria, purified, and used to immunize mice; after production of hybridomas, supernatants were screened first by ELISA, and positive clones rescreened by indirect immunofluorescence using HeLa cells. Only clones giving bright nuclear staining were selected for further analysis (Table I).

Selected antibodies were first characterized by immunoblotting. A typical experiment is illustrated in Figs. 1A and 1B. Proteins of uninduced bacteria, bacteria expressing his-RPB6, the purified his-RPB6 used as an immunogen, and a HeLa nuclear extract (NE) were resolved in a gel and stained with Coomassie blue (Fig. 1A). After induction, a band corresponding to his-RPB6 was present (Fig. 1A, lane 2). This band was labeled after blotting and probing with an antibody (i.e., B6-1) raised against his-RPB6 (Fig. 1B, lanes 2 and 3). A band of lower molecular weight in the nuclear extract was also labeled; this had the size expected of the immunogen lacking the his tag (Fig. 1B, lane 4). [Like its yeast counterpart [40], RPB6 runs more slowly than expected [54].] As no other proteins were labeled, this shows that antibody B6-1 reacts specifically with a protein with the size of RPB6. We performed similar analyses with each of the antibodies listed in Table I, with the expected results (not shown), and representative blots are illustrated in Fig. 1C; all antibodies reacted specifically with a single nuclear protein of the expected size.

We next investigated the ability of the antibodies to immunoprecipitate pols from nuclear extract. Immunoprecipitated proteins were resolved in 10 and 15% gels and then stained with silver (Figs. 2A and 2B) or blotted and the blots probed with various antibodies specific for different subunits (Fig. 2C). A control antibody—a mouse IgG fraction—coupled to beads nonspecifically immunoprecipitated the immunoglobulin heavy and light chains and various other proteins

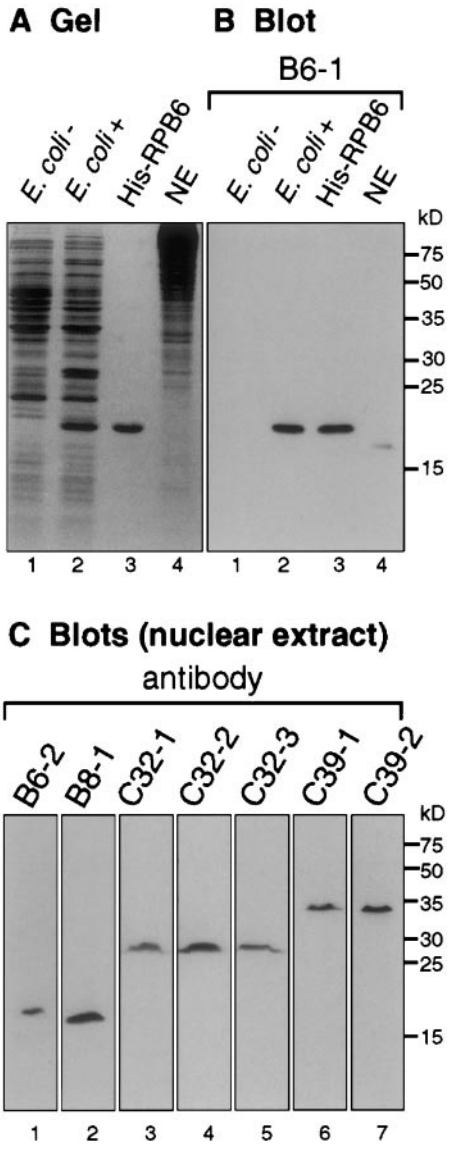

FIG. 1. Characterizing antibodies by immunoblotting. (A) Gel. Proteins of uninduced (lane 1) and induced (lane 2) *Escherichia coli*, purified his-RPB6 (lane 3), and HeLa nuclear extract (NE; lane 4) were resolved in a 15% polyacrylamide gel and stained with Coomassie blue. Positions of marker proteins are indicated. (B) Blot. Proteins in the gel shown in (A) were blotted and probed with antibody B6-1 raised against his-RPB6. (C) Blots. Blots like those in (B) were probed with various antibodies; only blots of lanes containing nuclear extract are shown.

(Figs. 2A and 2B, lane 10); these proteins were seen in all lanes.

As expected, B6-1—which was raised against a shared subunit—pulled down proteins that migrated in the positions expected of subunits specific to pol II (RPB1) and pol III (RPC155, RPC135, RPC82, RPC62, and RPC39), as well as the shared subunits (RPB5, RPB6, RPB8; Figs. 2A and 2B, lane 1). The presence of most of these subunits (i.e., RPB1, RPC82, RPC62, RPC39, RPB5, and RPB8) was confirmed by immunoblotting (Fig. 2C, lane 1). Blotting also showed that RPC53—which was probably obscured by the heavy

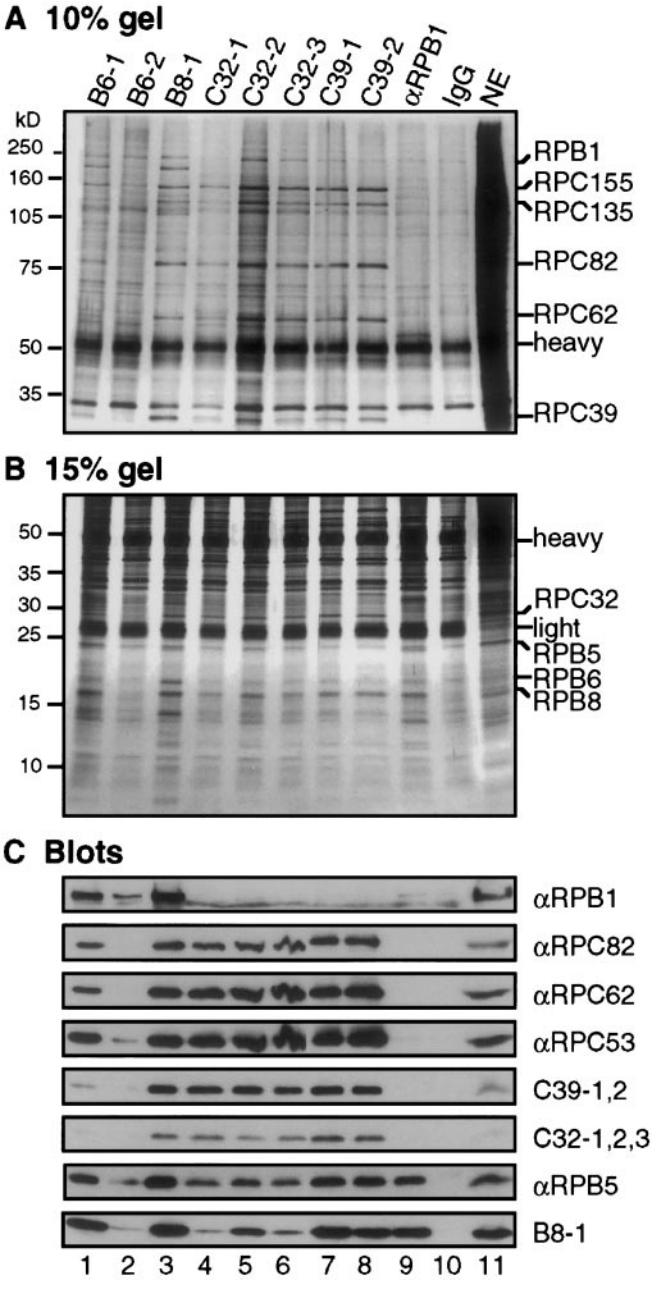

FIG. 2. Immunoprecipitating polymerase subunits from nuclear extracts. (A and B) Gels. Subunit-specific (lanes 1–9) or nonspecific (lane 10) IgGs were bound to protein G–Sepharose, incubated with NE, and pelleted; then, proteins in the pellet were eluted with glycine (pH 2.5) and resolved in 10 or 15% polyacrylamide gels. Nuclear extract was applied to lane 11. Positions of marker proteins, selected subunits, and heavy and light immunoglobulin chains are indicated. (C) Blots. After blotting gels like those in (A) and (B), blots were probed with antibodies shown on the right. Only relevant regions of blots are shown.

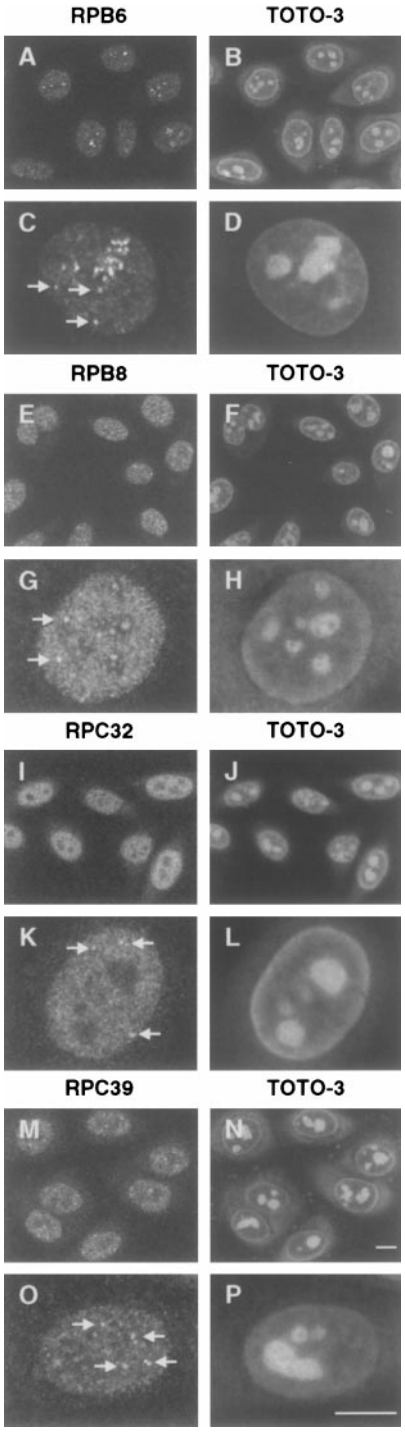

FIG. 3. The distribution of various subunits in interphase HeLa cells. The subunits were indirectly immunolabeled, nucleic acids counterstained with TOTO-3, and optical sections through the center of nuclei collected by confocal microscopy. Representative low- and high-power sections of a field (subunits on left; nucleic acids on right) are shown. Bars, 10 μm. (A–D) Shared subunit RPB6 detected using B6-1. Many small foci and a few brighter ones (arrows) are seen in the nucleoplasm, and some large bright foci in most nucleoli. (E–H) Shared subunit RPB8 detected using B8-1. The pattern is generally similar to that given by B6-1, although nucleoplasmic foci are often brighter and nucleolar foci fainter. (I–L) Pol III subunit RPC32 detected using C32-2. Many nucleoplasmic foci are seen against a diffuse background, and the few bright foci (arrows) are less distinctive. The cytoplasm is weakly labeled. (M–P) Pol III subunit RPC39 detected using C39-2. The many nucleoplasmic foci are more discrete than those labeled by C32-2, and the brighter foci are more intensely labeled. Again, the cytoplasm is weakly labeled.

chain in the silver-stained gel (Figs. 2A and 2B, lane 1)—was present in the precipitate (Fig. 2C, lane 1). B6-1 did not appear to pull down any pol I subunits (Fig. 2, lane 1), even though it detects pol I in fibrillar centers (see below). This may be due to the low concentration of pol I in the nuclear extract. B6-2—a different antibody raised against the same immunogen—did not perform as well; low levels of a few subunits were pulled down and detected in blots (Fig. 2, lane 2). B8-1—which was raised against a different shared subunit—pulled down RPB1, RPC155, RPC135, RPC82, RPC62, RPC39, RPC32, RPB5, RPB6, RPB8, and some smaller subunits of <15 kDa (Fig. 2, lane 3). As expected, C32-1, C32-2, and C32-3—which were directed against the pol III subunit RPC32—all immunoprecipitated roughly the same set of pol-III-specific subunits (i.e., RPC155, RPC135, RPC82, RPC62, RPC39, RPC32, and RPB8; Figs. 2A and 2B, lanes 4–6). RPC82, RPC62, RPC53, RPC39, RPC32, RPB5, and RPB8 were also detected by immunoblotting (Figs. 2A–2C, lanes 4–6). Subunits found only in pols I and II did not appear to be pulled down by these antibodies, and RPB1 could not be detected by immunoblotting (Fig. 2C, lanes 4–6). C39-1 and 39-2—which were directed against the pol III subunit RPC39—pulled down roughly the same set of subunits as C32-1 and its sisters (Fig. 2, lanes 7 and 8). An antibody (obtained commercially) that was directed against the C-terminal domain of RPB1 pulled down RPB1, RPB5, RPB6, and RPB8 (Fig. 2C, lane 9; not shown). [Unlike the smaller subunits, most RPB1 remains bound to the Sepharose beads under our conditions, but can be detached using SDS (not shown).]

These results show that all antibodies except B6-2 should be useful for immunopurification and that the ones directed against the shared subunits pull down pols II and III, whereas those against pol III subunits pull down only pol III subunits.

Distributions of Subunits Revealed by Immunofluorescence

Figure 3 illustrates the distributions of four subunits after indirect immunolabeling. Under low power, B6-1—raised against the shared subunit, RPB6—gave a few bright foci in nucleoli and nucleoplasm, as well as many fainter foci in the nucleoplasm (Fig. 3A). Under

high power, several bright foci were clearly visible in the nucleoplasm (Fig. 3C, arrows). Faint cytoplasmic labeling and similar nuclear foci were detected with B6-2 (not shown); this—and the results obtained by immunoblotting and immunoprecipitation—suggests that B6-2 might have a lower affinity. B8-1—directed against the shared subunit, RPB8—gave a similar pattern to B6-1, although nucleoplasmic foci were generally brighter and nucleolar foci fainter (Figs. 3E–3H). C32-2—raised against the pol III subunit RPC32—gave many faint foci and a few brighter ones in the nucleoplasm (arrows); however, a diffuse background was also present, so both appeared less distinctive (Figs. 3I–3L). As expected, nucleoli were not labeled (Figs. 3I–3L). C32-1 and C32-3 gave similar patterns (not shown). Nucleoplasmic foci detected by C39-2—raised against the pol III subunit RPC39—were more discrete than those detected by C32-2, and the brighter foci (arrows) were more intensely labeled (Figs. 3M–3P). C39-1 gave a similar pattern (not shown).

Distribution of Subunits and Sm Antigens

As splicing factors carrying Sm antigens are concentrated in large speckles and many smaller nucleoplasmic foci [e.g., 55, 56], we investigated their distribution relative to those of the subunits. After immunolabeling, subunits were pseudocolored red and Sm antigens green; then, yellow indicates overlap. Red RPB6 foci were found in the complete absence of any green in nucleoli, and most nucleoplasmic foci appeared either red or green, indicating little overlap (Fig. 4A). The brightest yellow regions were generally found at the edges of large Sm foci (Fig. 4A, arrow and inset)—which are probably coiled bodies [55]. RPB8 had a roughly similar pattern (Fig. 4B), except that the red nucleoplasmic foci were more distinct, consistent with the results seen in Figs. 3E and 3G. After labeling RPC32 and Sm, the nucleoplasm had a yellowish tinge, showing that at this level of resolution the distributions of the two antigens partially overlapped. As before, the brightest yellow was seen at the edge of a large Sm focus (Fig. 4C, arrow and inset). RPC39 gave more discrete red nucleoplasmic foci (Fig. 4D).

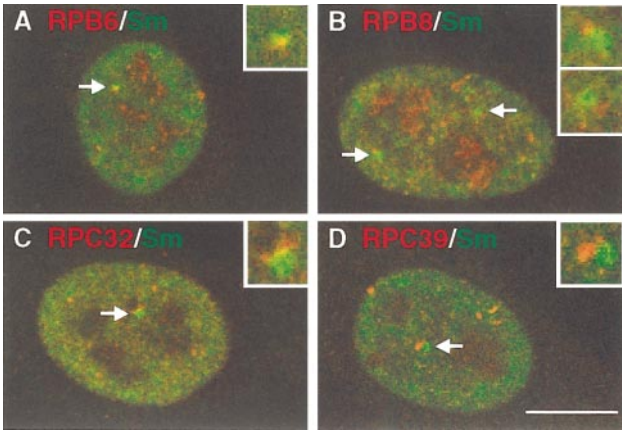

FIG. 4. Distribution of RPB6, RPB8, RPC32, and RPC39 relative to Sm antigen in HeLa cells. A pol subunit and Sm antigen were indirectly immunolabeled, images through the center of nuclei collected by confocal microscopy, and pseudocolored images of the pol subunit (red) and Sm antigen (green) merged. Insets show arrowed foci at 2.8-fold magnification. (A) Shared subunit RPB6 detected with B6-1. In nucleoli, RPB6 (red) is found in the absence of Sm. In the nucleoplasm, most foci appear either red or green, indicating little overlap. Part of the large (green) Sm focus (arrow)—which is probably a coiled body—contains RPB6 and so appears yellow (inset). (B) Shared subunit RPB8 detected with B8-1. The pattern is roughly similar to that given by B6-1, except that the red nucleoplasmic foci are more distinct, consistent with the results seen in Figs. 3E and 3G. (C) Pol III subunit RPC32 detected with C32-2. Nucleoli contain little RPC32 or Sm. The nucleoplasm has a yellowish tinge, showing that the diffuse nucleoplasmic distribution of RPC32 partially overlaps that of Sm. Part of the large Sm focus—which is probably a coiled body—contains RPC32 and so appears yellow (inset). (D) Pol III subunit RPC39 detected with C39-2. Red and green nucleoplasmic foci are more distinct than those given by C32-2. Again, RPC39 is found next to the large Sm focus (arrow and inset). Bar, 10 μm.

The Effect of Transcriptional Inhibitors on Subunit Distribution

We next investigated the effects of two transcriptional inhibitors on subunit distribution. The adenosine analogue 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) inhibits pol II activity through its effect on a protein kinase that phosphorylates the largest subunit of pol II, RPB1 [57, 58]; it has little immediate effect on transcription by pols I or III, even though they contain phosphorylated subunits (e.g., RPB6; 59, 60). Previous studies have also shown that DRB causes chromosome domains to decondense, nucleoplasmic polymerases to concentrate in larger foci, nucleoli to disperse, and fibrillar centers to split into strings of tiny foci that look like "beads-on-a-necklace" [27, 56, 61–64]. We find it has similar effects on nuclear structure (Fig. 5B), aggregating nucleoplasmic RPB1, RPB8, and RPC32 (Figs. 5E, 5K, and 5N) and reducing the overall fluorescence in the nucleoplasm (Figs. 5E, 5K, and 5N). It also has the expected effects on nucleoli. RPB6—a component of pol I—is detected efficiently by antibody B6-1 in fibrillar centers (Fig. 3C); DRB disperses it to give the beads-on-a-necklace appearance (Fig. 5H). RPB8—another component of pol I—is labeled sufficiently weakly in fibrillar centers (Fig. 3G) that the DRB-induced dispersal eliminates most nucleolar labeling (Fig. 5K).

A high concentration of the intercalating agent, actinomycin D, inhibits all polymerases, and causes chromatin domains to condense, nucleoli to compact, and polymerases to concentrate in larger foci [e.g., 22, 63, 65, 66]. We found that it affected nuclear structure similarly (Fig. 5C) and caused the nucleoplasmic forms

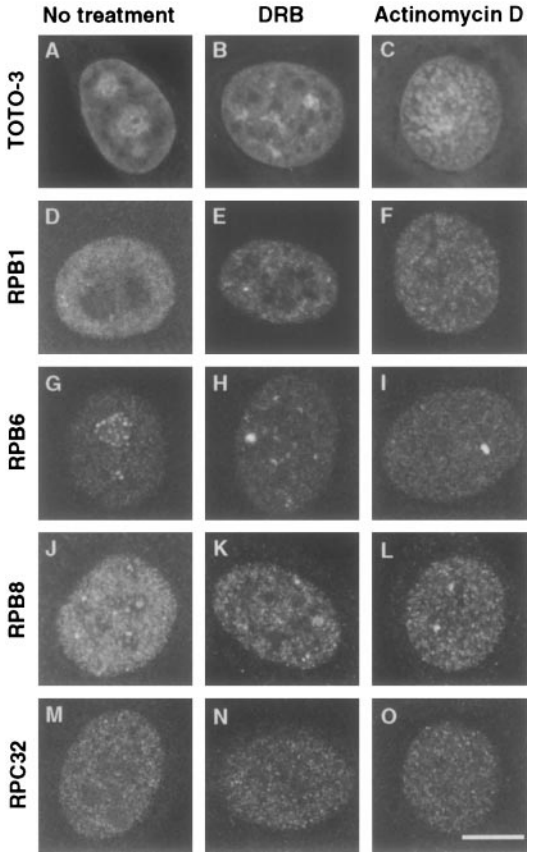

FIG. 5. The effects of actinomycin D and DRB on the distribution of polymerase subunits. HeLa cells were grown (3 h) without and with 100 μM DRB or 5 μg/ml actinomycin D, indirectly immunolabeled, and counterstained with TOTO-3, and an image through the center of a nucleus was collected by confocal microscopy. (A–C) Nucleic acid staining; nucleoli fragment in DRB, and chromatin condenses in actinomycin. (D–F) RPB1, the largest subunit of pol II. RPB1 aggregates in both inhibitors, while the overall fluorescence becomes less intense. (G–I) Shared subunit RPB6 detected with B6-1 (an antibody that labels fibrillar centers in nucleoli more than nucleoplasmic sites). In DRB, nucleoli disperse, and fibrillar centers split to give strings of tiny foci that look like "beads-on-a-necklace"; in actinomycin D, fibrillar centers have fused into one large aggregate. (J–L) Shared subunit RPB8 detected with B8-1 (an antibody that labels nucleolar and nucleoplasmic sites equally). In DRB, nucleoplasmic RPB8 aggregates and—as nucleoli fragment—nucleolar RPB8 can no longer be distinguished. In actinomycin, nucleoplasmic RPB8 aggregates, and the overall fluorescence becomes less intense; nucleolar RPB8 also aggregates, often at the nucleolar surface. (M–O) Pol III subunit RPC32 detected with C32-1. This subunit aggregates in both inhibitors, and fluorescence becomes less intense. Bar, 10 μm.

of RPB1, RPB8, and RPC32 to aggregate while reducing the overall intensity of fluorescence (Figs. 5F, 5L, and 5O).

DISCUSSION

A number of different antibodies directed against the various subunits of human RNA polymerases are available (see Introduction). We have screened a number of these and found that most perform well during immunoblotting (not shown), presumably because they bind efficiently to the denatured targets found after treatment with SDS. However, we also found that most of these antibodies did not give good immunofluorescence in carefully fixed cells (not shown), presumably because their targets were inaccessibly buried in larger complexes. Therefore, we set out to obtain monoclonal antibodies that would be useful for immunofluorescence and now describe ones that react specifically with RPB6, RPB8, RPC32, and RPC39. (Alternative names for the subunits are listed in Table I.) We would expect such antibodies to bind to epitopes exposed on the surface of large complexes and so to be useful for immunoprecipitation.

RPB6 is a shared subunit found at the heart of all three polymerizing complexes [reviewed in ref. 31]. One antibody raised against it (i.e., B6-1) pulled down shared subunits (i.e., RPB5, RPB6, RPB8), those specific to pols II (i.e., RPB1) and III (i.e., RPC155, PRC135, RPC82, RPC62, and RPC39; Figs. 2A–2C, lane 1). When used for immunofluorescence, it gave a few bright foci in nucleoli and the nucleoplasm, as well as many fainter nucleoplasmic foci (Figs. 3A–3D). The bright foci were generally distinct from speckles containing Sm antigen (Fig. 4A), although some were found around the large Sm foci that were probably coiled bodies [55]. Most bright foci detected by this (and the other) antibodies are probably inactive stores. In nucleoli, the bright foci colocalized with UBF (not shown) and so mark fibrillar centers; even here, only a minority of pol I at the surface of these dense structures is active [67, 68]. The major concentrations of pol II in the nucleoplasm are also inactive [e.g., ref. 64], as the active fraction is either at their surface or distributed throughout the fainter foci [e.g., ref. 69].

An antibody (i.e., B8-1) raised against a different shared subunit—RPB8—behaved much like B6-1. It pulled down various subunits (i.e., RPB1, RPC155, RPC135, RPC82, RPC62, RPC39, RPC32, RPB5, RPB6, RPB8, plus some smaller subunits of $<$15 kDa; Fig. 2, lane 3). It also gave bright foci in the nucleoplasm and nucleoli, although the nucleoplasmic foci were generally brighter—and nucleolar foci fainter (Figs. 3E–3H and 4B)—than those found with B6-1. It remains to be established whether such differences in intensity result from different protein concentrations or differences in accessibility to the target epitopes.

RPC32 and RPC39 are two subunits found only in pol III. Three antibodies (i.e., C32-1, C32-2, C32-3) directed against RPC32 all immunoprecipitated roughly the same set of pol-III-specific subunits (i.e., RPC155, RPC135, RPC82, RPC62, RPC53, RPC39, RPC32, RPB5, and RPB8), but no subunits found solely in pols I or II (Fig. 2C, lanes 4–6). Two antibodies (i.e.,

C39-1, C39-2) raised against RPC39 pulled down the same set of subunits (Fig. 2, lanes 7 and 8). All these antibodies gave roughly the same general patterns—many faint foci and a few brighter ones in the nucleoplasm, but no nucleolar foci (e.g., Figs. 3I–3P). However, each pattern was characteristic, presumably due to differences in protein concentration and/or accessibility.

Active RNA pols are concentrated in discrete sites or "factories" that contain a fraction of the polymerase, so the highest pol concentrations probably represent inactive stores [68, 70]. Thus, pol I is concentrated in fibrillar centers in nucleoli with only that on the surface being active [67, 71], and considerable amounts of pol II are stored in large speckles [e.g., ref. 27, 64], with the active fraction being dispersed throughout thousands of nucleoplasmic factories [72, 73]. Presumably, inhibiting transcription by DRB or actinomycin D leads to the relocation of the active component to these stores (Fig. 5; 27, 56, 64).

In summary, we have generated eight different monoclonal antibodies that react specifically with RPB6, RPB8, RPC32, and RPC39; we hope these will be useful for immunofluorescence and immunoprecipitation.

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