

Transcription-dependent Redistribution of the Large Subunit of RNA Polymerase II to Discrete Nuclear Domains

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Abstract. A subpopulation of the largest subunit of RNA polymerase II (Pol II LS) is located in 20–50 discrete subnuclear domains that are closely linked to speckle domains, which store splicing proteins. The speckle-associated fraction of Pol II LS is hyperphosphorylated on the COOH-terminal domain (CTD), and it is highly resistant to extraction by detergents. A diffuse nucleoplasmic fraction of Pol II LS is relatively hypophosphorylated on the CTD, and it is easily extracted by detergents. In transcriptionally active nuclei, speckle bound hyperphosphorylated Pol II LS molecules are distributed in irregularly shaped speckle domains, which appear to be interconnected via a reticular network. When transcription is inhibited, hyperphosphorylated Pol II LS and splicing protein SC35 accumulate in speckle domains, which

are transformed into enlarged, dot-like structures lacking interconnections. When cells are released from transcriptional inhibition, Pol II and SC35 redistribute back to the interconnected speckle pattern of transcriptionally active cells. The redistribution of Pol II and SC35 is synchronous, reversible, and temperature dependent. It is concluded that: (a) hyperphosphorylation of Pol II LS's CTD is a better indicator of its tight association to discrete subnuclear domains than its transcriptional activity; (b) during states of transcriptional inhibition, hyperphosphorylated Pol II LS can be stored in enlarged speckle domains, which under the light microscope appear to coincide with the storage sites for splicing proteins; and (c) Pol II and splicing proteins redistribute simultaneously according to the overall transcriptional activity of the nucleus.

R^{NA} polymerase II transcripts (pre-mRNAs) are co-transcriptionally spliced and packaged into ribonucleoprotein (RNP) particles in diverse eukaryotic nuclei (Sass and Pederson, 1984; Beyer and Osheim, 1988; Fakan et al., 1986; Huang and Spector, 1991; Matunis et al., 1993; Weeks et al., 1993; Baurén and Wieslander, 1994; for review see Beyer and Osheim, 1991). In mammalian cells, many proteins and snRNAs that participate in pre-mRNA splicing are highly concentrated in irregularly shaped intranuclear regions termed “speckle domains” (for review see Spector, 1993). Most nuclei have 20–50 speckle domains, which can be visualized by immunofluorescence microscopy

using antibodies directed against a subset of hnRNPs (Fakan et al., 1984), snRNPs (Lerner et al., 1981; Spector et al., 1983, 1991; Fakan et al., 1984; Nyman et al., 1986; Verheijen et al., 1986; Habets et al., 1989), and non-snRNP splicing proteins of the SerArg (SR) family, such as spliceosome assembly factor (SC35) (Fu and Maniatis, 1990; Spector et al., 1991; Roth et al., 1991; Xing et al., 1993; Carter et al., 1993). Nuclear speckle domains can also be visualized by fluorescent in situ hybridization using oligonucleotide probes complementary to spliceosomal snRNAs (Huang and Spector, 1992; Carmo-Fonseca et al., 1992). At the ultrastructural level, discrete structures termed “interchromatin granule clusters” (ICGCs) are highly enriched with SC35 and several other macromolecules localized in speckles by light microscopic techniques. Less prominent SC35 immunolabeling is present in “perichromatin fibrils” (PCFs), which form a latticework in the nucleoplasm surrounding the ICGCs. The intense SC35 labeling in the ICGCs, combined with the weak SC35 labeling in the surrounding PCF network, correlates well with light microscopic images of SC35-stained speckles, which appear to be interconnected and irregularly shaped (Spector, 1993).

ICGCs contain many proteins and RNPs known to participate directly in pre-mRNA splicing reactions, so it is likely that partially or fully assembled spliceosomes are located in these regions (Spector, 1993; Rosbash and Singer, 1993).

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1. *Abbreviations used in this paper:* CTD, COOH-terminal domain; DPBS, Dulbecco's phosphate buffered saline; DOC, deoxycholate; DRB, 5,6-dichlorobenzimidazole riboside; ICGCs, interchromatin granule clusters; PCF, perichromatin fibrils; Pol II LS, RNA polymerase II large subunit; Pol IIA, hypophosphorylated form (~220 kD) of RNA polymerase II largest subunit; Pol II0, hyperphosphorylated form (~240 kD) of RNA polymerase II largest subunit; RNP, ribonucleoprotein; SR protein, Serine/Arginine family splicing protein; TD buffer, Tris-HCl/Deoxycholate buffer.

ICGCs are also enriched with poly A⁺ RNAs (Carter et al., 1991, 1993), raising the possibility that RNA polymerase II (Pol II)-mediated transcription and/or splicing take place within the ICGCs. However, RNA labeling studies have repeatedly shown that Pol II transcription takes place in nuclear sites which are separate from the ICGCs. For example, the PCFs and nucleoplasm surrounding the ICGCs are labeled strongly and rapidly with [³H]uridine, but the ICGCs are labeled poorly (Fakan and Bernhard, 1971; Fakan et al., 1976; Fakan and Nobis, 1978; Fakan and Puvion, 1980; Spector, 1990). Recent studies using 5' bromouridine 5' triphosphate to label nascent Pol II transcripts revealed 100–500 discrete Pol II transcription foci that are widely distributed in the nucleus (Jackson et al., 1993; Wansink et al., 1993). These focal transcription sites are resistant to nucleolytic treatments which remove ~90% of the chromatin, indicating that Pol II-mediated transcription is associated with a solid phase nuclear structure (Jackson et al., 1993). Furthermore, the transcription foci are separate from the regions most enriched with SC35 (i.e., ICGCs), although weaker SC35 labeling coincides with transcription foci (Wansink et al., 1993). Thus, light and electron microscopic techniques have shown that Pol II transcription takes place outside of the regions that are most enriched with splicing proteins (ICGCs).

Several studies imply that ICGCs are storage/reassembly sites for splicing proteins. ICGCs are comprised of many of the same splicing molecules found in spherical organelles termed "B-snurposomes" in the amphibian germinal vesicle (Gall, 1991; Wu et al., 1991). B-snurposomes are adjacent to transcriptionally active chromatin, but they are not directly connected to the chromosome loops, so these organelles store spliceosomal complexes that are not engaged in splicing. Because B-snurposomes and ICGCs have a very similar composition, and both are located in interchromatin nuclear regions, they are probably analogous structurally and functionally. Perhaps the most compelling evidence that ICGCs are storage sites for splicing proteins is that these structures become enlarged when there is reduction in splicing activity. Thus, inhibition of transcription or splicing induces SR proteins (e.g., such as SC35) to accumulate in enlarged ICGCs, and coincidentally, anti-SR immunolabeling is diminished in the PCFs surrounding the ICGCs (O'Keefe et al., 1994). At the light microscopic level of resolution, SC35-labeled speckles undergo a striking reorganization that reflects the overall transcriptional activity of the nucleus: the irregularly shaped, interconnected speckles of transcriptionally active nuclei are converted into enlarged, round unconnected speckles of transcriptionally inactive nuclei (O'Keefe et al., 1994).

Given that splicing proteins are stored or reassembled in ICGCs, there is presumably a mechanism to dispatch them from these domains to the sites of Pol II transcription. In support of this idea, SC35 redistributes from the ICGCs to Pol II gene templates introduced into the nucleus by viral infection or by plasmid transfection (Bridge et al., 1993; Jiménez-García and Spector, 1993; Rice et al., 1994). Significantly, hnRNPs and the large subunit of RNA polymerase II (Pol II LS) are also recruited to sites of gene transcription, indicating that Pol II transcription and pre-mRNA processing reactions are linked spatially and temporally (Jiménez-García and Spector, 1993). These observations im-

ply that there is a mechanism which coordinately recruits Pol II LS and splicing proteins to genes. Pol II and splicing proteins may be recruited from separate intranuclear compartments. Alternatively, they may be recruited from a common compartment to the sites of Pol II transcription. To distinguish between these two possibilities, it is essential to precisely define the subnuclear distribution of Pol II and splicing proteins during states of transcriptional activity and quiescence.

The present study shows that a subpopulation of the largest subunit of Pol II (Pol II LS) and splicing protein SC35 are located in nuclear speckle domains, and they are tightly associated with a solid phase nuclear structure. When transcription is inhibited, SC35 and Pol II LS accumulate in enlarged speckle domains lacking interconnections. When the cells are released from transcriptional inhibition, Pol II LS and SC35 rapidly and simultaneously redistribute back to an interconnected speckle pattern which is characteristic of actively transcribing nuclei. Throughout the redistribution cycle, Pol II LS and SC35 remain tightly associated with discrete nuclear regions. During states of transcriptional activity and quiescence, the solid phase associated fraction of Pol II LS is hyperphosphorylated on the COOH-terminal domain (CTD). However, the easily extracted and diffusely distributed fraction of Pol II LS is hypophosphorylated to various degrees on the CTD. We conclude that under certain conditions, hyperphosphorylated Pol II LS molecules are stored in speckle domains, and that hyperphosphorylation of Pol II LS's CTD correlates more closely with its distribution to discrete subnuclear domains than its transcriptional activity. The present study shows that the storage sites for Pol II LS are organized in a pattern that is very similar to the storage sites for splicing proteins.

Materials and Methods

Immunopurification of Pol II 240 and 140 kD Subunits

The 240-kD and 140-kD subunits of RNA polymerase were immunopurified from the nuclei of 60 rat livers (700 g) using a modification of an established nuclear fractionation procedure (Nickerson et al., 1992). Fresh minced livers were homogenized in a glass vessel with a rotating Teflon pestle in 10 ml/g liver ice cold CSK-100 buffer (100 mM NaCl, 0.3 M Sucrose, 3 mM MgCl₂, 10 mM Pipes, pH 6.8, 1 mM EGTA, and 0.5% Triton X-100) to which was added 1 mM PMSF. The nuclei were collected by centrifugation at 200 g at 4°C, washed with several changes of CSK-100, and resuspended in 1.5 ml/g liver of CSK-50 buffer (50 mM NaCl) and incubated with 110 µg/ml deoxyribonuclease (Sigma Chem. Co., St. Louis, MO; cat. No. DN25) at 22°C for 30 min, and then pelleted at 200 g. The nuclei were then extracted in either of two ways. To maximize the yield of the 240-kD subunit, the nuclear pellet was resuspended in 0.3 vol of TBS (50 mM Tris-HCl 7.4, 150 mM NaCl, 1 mM PMSF), and then 3.3% SDS in TBS was added dropwise to a final concentration of 1% SDS. The suspension was heated in a boiling water bath for 5 min, passed repeatedly through a 19-gauge needle to shear chromosomal DNA, and then diluted with 6 vol of ice cold Py20 lysis buffer (50 mM Tris HCl, pH 7.4, 150 mM NaCl, 0.5% Triton X-100, 0.5% Sodium deoxycholate, and 1 mM PMSF). To immunoprecipitate a complex of proteins including p240 and p140, the nuclear pellet was resuspended in 10 vol of TD buffer (50 mM Tris HCl, pH 7.5, 0.5% Triton X-100, 0.5% Sodium deoxycholate (DOC), 250 mM NaCl, 5 mM EDTA, 50 mM NaF, 1 mM PMSF, 1 mM Sodium orthovanadate, 5 mM β-glycerophosphate; Warren et al., 1992). Insoluble material was removed via centrifugation at 16,000 g at 4°C for 20 min. Pol II was then immunoprecipitated with mAb H5 coupled to protein G Sepharose 4 Fast Flow beads (Pharmacia LKB Biotechnology, Piscataway, NJ). Coupling was performed as follows: 50 µg of IgM mAb H5, 33 µg of rabbit anti-mouse IgM (Zymed Labs, San Francisco, CA) and 66 µl of Protein G beads per rat liver were

incubated in TBS for several hours at 4°C. The mAb H5-coupled beads were pelleted by centrifugation at 250 g and washed with TBS before being added to the 16,000-g supernatant. Immunoprecipitation was performed rocking end over end at 4°C for 16 h, the beads were washed three times with TD buffer, and the immunoprecipitated protein was eluted by incubating with 1.5 times the bead volume of sample buffer (3% SDS, 2 M urea, 62.5 mM Tris HCl, pH 6.9, 2 mM EDTA, and 3.5% (vol/vol) 2-mercaptoethanol) at 100°C for 5 min. The beads were discarded and the eluate was lyophilized and resuspended in 17 μ l per rat liver of 62.5 mM Tris, pH 6.8, 2 mM EDTA, 10% glycerol and 3.5% (vol/vol) 2-mercaptoethanol. The proteins were then resolved by 8% SDS-PAGE (Laemmli, 1970).

Microsequence Determination

The 240- and 140-kD bands were cut out of the gel and submitted to the W. M. Keck foundation Biotechnology Resource laboratory at the Yale School of Medicine for microsequence analysis (Stone and Williams, 1994). Briefly, the two protein samples were digested with trypsin at 37°C for 24 h, and then eluted from the gel by incubating with 2 M urea, 0.1 M NH_4CO_3 at 37°C for another 24 h. The samples were lyophilized, resuspended in H_2O , and then subjected to HPLC. Several tryptic peptides eluted from the reverse phase HPLC column were then subjected to laser desorption mass spectroscopy to assess homogeneity. Subsequently two peptides from the 240-kD protein and one from the 140-kD protein were subjected to microsequence analysis via automated Edman degradation.

Cell Culture

MDCK cells were maintained in monolayer culture with DME supplemented with 7% FBS. HeLa cells were maintained in monolayer culture with RPMI 1640 medium supplemented with 7% FBS and 10 mM glutamine (GIBCO BRL, Gaithersburg, MD). Where indicated, the following transcriptional inhibitors (purchased from Sigma Chem. Co.) were added to the culture media at the indicated final concentrations and times: alpha amanitin (10 μ g/ml, 8 h); 5,6-dichlorobenzimidazole riboside (DRB, 100 μ M, 3 h); actinomycin D (20 μ g/ml, 2 h); *N*-(2[methylamino]ethyl)-5-isouquinolinesulfonamide (H8, 100 μ M, 2 h).

Antibodies

The generation and initial characterization of monoclonal IgM antibodies H5 and H14 are described elsewhere (Warren et al., 1992; Bregman et al., 1994). 8WG16 (Promega, Madison, WI) is a mouse monoclonal IgG antibody that binds to the COOH-terminal domain (CTD) of RNA polymerase II (Thompson et al., 1989). ARNA3 (Research Diagnostics, Flanders, NJ) is a mouse monoclonal IgG antibody that binds to the non-CTD part of RNA polymerase II (Krämer et al., 1980). SC35 (Amer. Type Culture Collection, Rockville, MD) is a mouse monoclonal IgG antibody that binds to a phosphorylated epitope within the highly reiterated Serine/Arginine (SR) domain of spliceosome assembly factor (SC35), a member of the SR family of non-snRNP-splicing proteins (Fu and Maniatis, 1990). mAbs H22 and MOPC 104 are antibody controls. H22 is a mouse monoclonal IgM that binds to an unidentified ~180-kD protein in MDCK cells (unpublished results). MOPC104 (Sigma Chem. Co.) is a mouse monoclonal IgM antibody that binds to α -1, 3 glucoside residues within polysaccharide epitopes.

Immunoprecipitation and Immunoblotting

MDCK protein extracts suitable for immunoprecipitation were prepared using hot "1% SDS lysis buffer" followed by dilution with "0.5% Triton X-100/0.5% deoxycholate dilution buffer" as described previously (Warren et al., 1992). Immunoprecipitations were performed using IgM mAbs H22, H14, and H5 coupled to protein G Sepharose beads via a rabbit anti-mouse IgM linker antibody (see Immunopurification of Pol II subunits above). Whole cell extracts were obtained by scraping cells from nearly confluent culture dishes into hot 2 \times sample buffer and heating the sample to 100°C for 5 min, and then shearing chromosomal DNA with a 19-gauge syringe. Immunoprecipitates as well as whole cell extracts were resolved by 8% SDS-PAGE and transferred to nitrocellulose as described (Laemmli, 1970; Towbin et al., 1979). Nitrocellulose filters were incubated overnight at 4°C in blocking buffer (TBS with 3% [wt/vol] BSA [$>98\%$ pure, Sigma Chem. Co.]), and then incubated 2–6 h at room temperature with 2 μ g/ml H5, 2 μ g/ml MOPC 104 (Sigma Chem. Co.), or 1 μ g/ml 8WG16 (Promega) diluted in blocking buffer or with H14 hybridoma supernatant. Unbound antibody was removed by multiple washes with TBS-0.05% Tween 20 at room

temperature, and then the filters were incubated with horseradish peroxidase-conjugated goat anti-mouse IgM (for H5, MOPC 104, and H14) or horseradish peroxidase-conjugated goat anti-mouse IgG (for 8WG16) (KPL, Gaithersburg, MD) diluted 1:12,500 in blocking buffer for 1 h at room temperature. Unbound antibody was removed by washing with TBS-0.05% Tween 20 and bound antibody was visualized by enhanced chemiluminescence using hyperfilm (Amersham Corp., Arlington Heights, IL).

Immunofluorescence Microscopy

Immunofluorescence microscopy and photography was performed essentially as described previously (Warren et al., 1992; Bregman et al., 1994). Briefly, cells grown on coverslips were either fixed with 1.75% paraformaldehyde, and then permeabilized with 0.5% Triton X-100 or permeabilized with 1–1.5% Triton X-100, and then fixed with 1.75% paraformaldehyde. Nonspecific-binding sites were blocked by incubating coverslips with 4% BSA in Dulbecco's phosphate buffered saline (DPBS) followed by incubation with specific antibodies diluted into 0.5% BSA in DPBS. Antibodies employed were H5, H14, 8WG16, and MOPC 104 described under Western blotting above as well as ARNA 3 (Research Diagnostics, Flanders, NJ) and SC35 (Fu and Maniatis, 1990) generously provided by T. Maniatis of Harvard University. Specific antibody binding was visualized by incubating washed coverslips with fluorescein conjugated anti-IgM or rhodamine-conjugated anti-IgG diluted into 0.5% BSA in DPBS (Vector Labs, Burlingame, CA). In Fig. 6 G, MDCK cells grown on glass coverslips were washed and extracted with TD buffer before fixation with 1.75% paraformaldehyde. For this application the EDTA was omitted and the buffer was adjusted to 2 mM MgCl_2 in order to maintain cell-substrate adhesion and nuclear integrity.

Alkaline Phosphatase Treatment

Proteins immunoprecipitated with H14 coupled to protein G Sepharose beads as described above were washed with 20 mM Hepes, pH 7.3, 20 mM MgCl_2 , 40 mM KCl, 0.2 mM PMSF (Rice and Spencer, 1994). The immunoprecipitate was then exposed to calf intestine alkaline phosphatase (New England Biolabs, Beverly, MA) at a concentration of 500 U per ml in a volume of 75 μ l (excluding bead volume) in the presence or absence of the phosphatase inhibitor β glycerophosphate (5 mM) for 5 min at 37°C. The reaction was stopped and the proteins were eluted from the beads by adding 4 \times sample buffer and boiling for 5 min. The eluted proteins were analyzed by SDS-PAGE and Western immunoblot analysis as described above.

Fractionation of Pol II LS

For Western immunoblot analysis, MDCK cells grown on 100-mm dishes were subjected to appropriate drug treatments, washed 3 \times with TBS, and incubated with ice cold TD buffer at 4°C for 15 min. Cell extracts were scraped into 2-ml microcentrifuge tubes, subjected to mechanical disruption by 10 passages through a 23-gauge needle, and centrifuged at 16,000 g for 20 min. The insoluble material was resuspended in 2 ml of 2 \times sample buffer (see immunopurification above) and the supernatant was diluted to 2 ml with 1 ml of 4 \times sample buffer. The two fractions were then subjected to 6% SDS-PAGE followed by immunoblot analysis.

Results

Monoclonal Antibodies that Bind Specifically to the Large Subunit of RNA Polymerase II

Previous studies using mammalian cells showed that mAbs H5 and H14 immunostain nuclear speckles that are enriched with splicing proteins, and that they immunoblot a major protein species at ~240 kD and a minor species at 210 kD (Warren et al., 1992; Bregman et al., 1994). This protein was called "cytostellin" to describe its stellate pattern of immunoreactivity in mitotic cells (Warren et al., 1992). Cytostellin was immunopurified from DNase treated rat liver nuclei using mAb H5 (see Materials and Methods). In some experiments cytostellin was extracted by boiling in 1% SDS, diluted, and then immunoprecipitated with mAb H5 to

yield a single 240-kD band on PAGE (data not shown). In other experiments cytostellin was extracted under non-denaturing conditions, yielding a complex with prominent bands that migrate at 240 kD and 140 kD on PAGE (Fig. 1 A). The p240 and p140 bands were excised, digested with trypsin in situ and subjected to HPLC to resolve the resulting peptides. Amino acid sequences of three peptides derived from p240 exactly match residues 430-444, 919-927, and 1059-1074 of the largest subunit of murine RNA polymerase II (Fig. 1 B). The amino acid sequence of a peptide derived from p140 matches exactly with residues 137-145 of the second largest subunit of RNA Polymerase II (Fig. 1 B). Additional coprecipitating proteins below 45 kD have not been sequenced (Fig. 1 A, *asterisk*). Thus, cytostellin is the largest subunit of RNA polymerase II (Pol II LS).

To confirm that mAbs H5 and H14 recognize Pol II LS, separate aliquots of p240 immunoprecipitated with each antibody were immunoblotted with mAb H5, mAb H14, mAb MOPC 104 (control IgM), and a commercially available anti-Pol II LS mAb, 8WG16 (Fig. 1 C). mAbs H5 and H14 specifically immunoprecipitate the same 240-kD protein (Fig. 1 C, *left two panels*), which is also recognized on immunoblots by the anti-Pol II LS mAb 8WG16 (Fig. 1 C, *far right panel*). Pol II LS that was immunoprecipitated by 8WG16 also binds specifically to mAbs H5 and H14 on immunoblots (data not shown). The control mAb H22 immunoprecipitates a ~180-kD protein (unpublished observations) that is not recognized by mAbs H5, H14, or 8WG16, and the control, mAb MOPC 104, does not bind Pol II LS or the ~180-kD protein (Fig. 1 C).

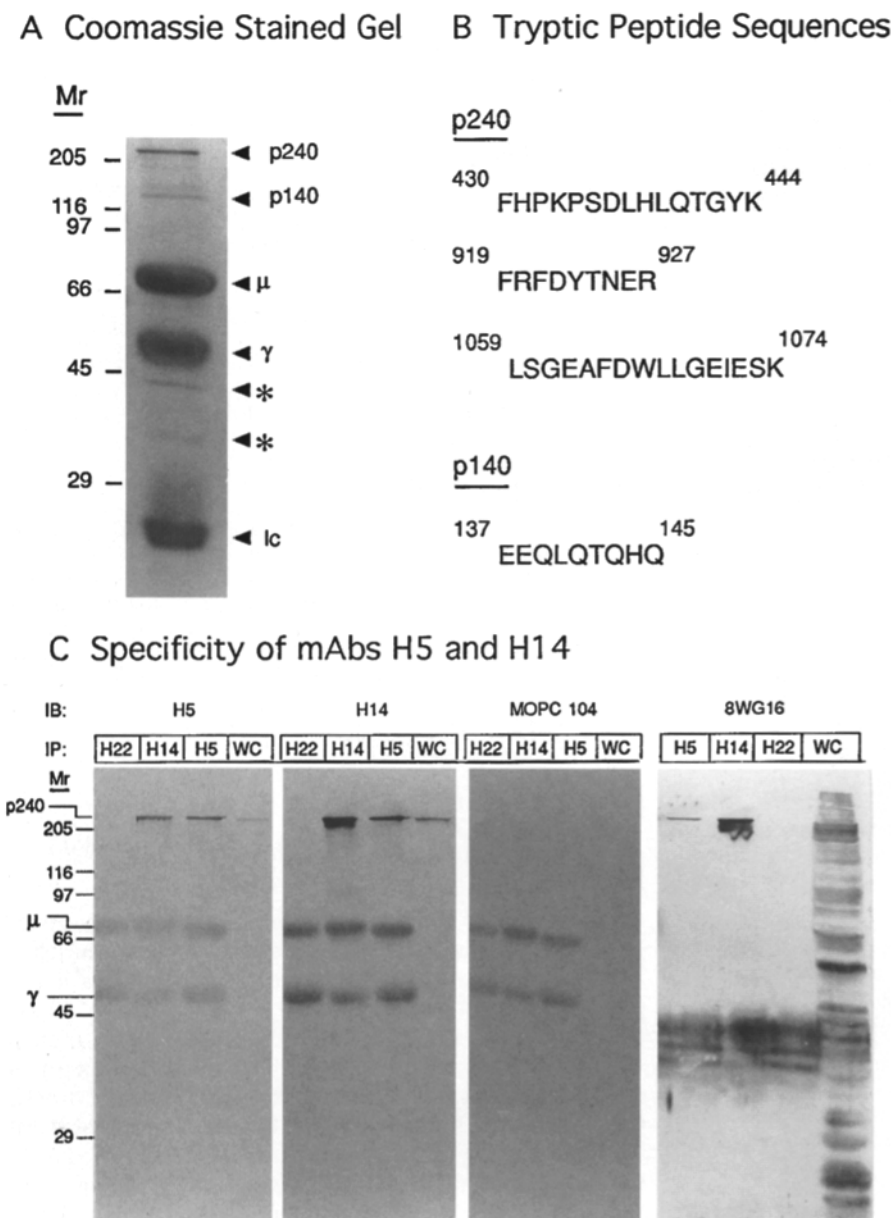


Figure 1. Cytostellin is the largest subunit of RNA Polymerase II. (A) Immunopurification of "cytostellin" and associated proteins. Rat liver nuclear extracts were prepared as described in the Materials and Methods and incubated with mAb H5 overnight. Immune complexes were collected using rabbit anti-mouse IgM linked to Streptococcal protein G-agarose beads. The beads were washed, solubilized in SDS sample buffer and resolved by 8% SDS-PAGE. The p240 and p140 proteins are indicated. μ , mouse IgM heavy chain; γ , rabbit IgG heavy chain; *lc*, immunoglobulin light chains. *Asterisks*, unidentified coimmunoprecipitating proteins. The relative mobilities (M_r) of the molecular weight standards are indicated in kilodaltons (kD). (B) Tryptic Peptide Sequences. Coomassie-stained p240 and p140 bands were excised, trypsin digested, and subjected to HPLC. The amino acid sequences of 3 tryptic peptides derived from p240, and one tryptic peptide from p140 are shown. Superscript numbers indicate amino acid positions of the first and last residue of each peptide as aligned with mouse RNA polymerase II (GenBank). (C) Monoclonal antibodies H5 and H14 bind to RNA polymerase II. Extracts of MDCK cells were immunoprecipitated with mAb H22, mAb H14, or mAb H5 as described in Materials and Methods. Washed immunoprecipitates and whole cell extracts (*WC*) were subjected to SDS-PAGE, transferred to nitrocellulose and immunoblotted with mAb H5, mAb H14, MOPC 104 (control IgM), or 8WG16. Blots were developed using the ECL[®] nonisotopic detection system (Amersham). The 240-kD band (Pol II LS) is indicated at left margin. *IB*, immunoblotting mAbs. *IP*, immunoprecipitating mAbs. μ and γ , immunoglobulin heavy chains. The relative mobilities (M_r) of the molecular weight standards are indicated in kilodaltons (kD).

mAb 8WG16 (Thompson et al., 1989) binds to Pol II LS, but also many additional proteins in whole cell extracts (Fig. 1 C, *far right panel, WC*). In contrast, mAbs H5 and H14 bind exclusively to Pol II LS (Fig. 1 C, *left two panels, WC*). Both of these mAbs bind to a ~240-kD band in whole cell extracts from all eukaryotes tested from human to *C. elegans* (Warren et al., 1992), and they specifically immunoprecipitate and immunoblot a single ~210-kD protein (Pol II LS) from extracts of *S. pombe* and *S. cerevisiae* (unpublished data). Therefore, mAbs H5 and H14 bind to epitope(s) that are present in Pol II LS molecules from a vast evolutionary spectrum of eukaryotic organisms.

mAbs H5, H14, and 8WG16 Recognize Different Phosphorylated Forms of Pol II LS

All known phosphorylation sites on Pol II LS have been mapped to the CTD, which is comprised of 52 tandem heptapeptide repeats similar or identical to the sequence Tyr-Ser-Pro-Thr-Ser-Pro-Ser (for reviews see Corden, 1990; Sawadogo and Sentenac, 1990; Young, 1991; Dahmus and Dynan, 1992; Zawel and Reinberg, 1993). Two major phosphorylated forms of Pol II LS have been defined in mammalian cells: Pol II₀ is hyperphosphorylated predominantly on Ser and Thr residues in the CTD, and it is recognized by a SDS-PAGE mobility of ~240 kD; Pol II_A is hypophosphorylated, and it migrates at ~220 kD (Dahmus, 1981; Bartholomew et al., 1986; Zhang and Corden, 1991; Dahmus and Dynan, 1992). Pol II₀ and Pol II_A are believed to have distinct functional roles in transcriptional initiation and elongation, but the exact function of CTD phosphorylation remains unknown (see Discussion).

Each mAb used in this study binds differently to Pol II₀, Pol II_A, and the multiple intermediate forms of Pol II LS (Fig. 2 A). mAb H5 immunoblots exclusively to Pol II₀ in whole cell extracts (Fig. 2 A). mAb H14 binds well to Pol II₀, Pol II_A (220 kD), and multiple intermediately phosphorylated forms between 220 and 240 kD (Fig. 2 A). mAb 8WG16 binds preferentially to hypophosphorylated forms of Pol II LS, including Pol II_A (Fig. 2 A). To determine whether mAbs H5 and H14 bind to phosphorylated epitopes, Pol II₀ was immunoprecipitated from cells with mAb H5 and incubated with increasing concentrations of alkaline phosphatase (Alk Φ). After incubation, each sample was divided into

three aliquots, which were subjected to PAGE and immunoblotted with mAb H5, mAb H14, or mAb 8WG16 (Fig. 2 B). mAb H14 binds to a series of bands ranging from ~240 kD to ~230 kD. Increasing concentrations of Alk Φ lead to an increased electrophoretic mobility of Pol II LS, and a reduced intensity of mAb H14 binding. Our interpretation of this result is that increasing concentrations of Alk Φ remove increasing numbers of phosphate groups from multiple reiterated H14 epitopes. Presumably, 50 Kunitz units/ml is sufficient to dephosphorylate all of the H14 epitopes, thereby abrogating mAb H14 binding. Increasing concentrations of Alk Φ reduce mAb H5 binding to Pol II₀, but mAb H5 does not detect the hypophosphorylated forms of Pol II LS, which migrate faster than 240 kD. Our interpretation of this result is that mAb H5 binds to a phosphorylated epitope(s) that is less reiterated than the epitopes recognized by mAb H14. mAb 8WG16 binds to a spectrum of phosphorylated forms of Pol II LS, and its binding is increased by Alk Φ treatment, indicating that this mAb binds preferentially to hypophosphorylated forms of Pol II LS (Fig. 2 B). A Ser/Thr phosphatase inhibitor, β -glycerophosphate, blocks all of these effects of Alk Φ (Fig. 2 B, β GP). Rephosphorylation of Pol II LS with casein kinase restores binding of mAbs H5 and H14 to Pol II LS (unpublished results). The above results show that mAbs H5 and H14 bind only to phosphorylated forms of Pol II LS, and indicate that they bind to different epitopes on Pol II LS's CTD, the domain which contains all known Pol II LS phosphorylation sites. Detailed mapping of the H5 and H14 epitopes will be published elsewhere (manuscript in preparation).

A Subpopulation of Pol II LS Is Tightly Associated with Discrete Nuclear Speckles

The ability to detect Pol II LS in speckle domains is influenced by several factors. First, the intensity speckle labeling by mAb H5 fluctuates during the cell cycle. Thus, unsynchronized cell nuclei are stained heterogeneously by mAb H5: most have a diffuse nucleoplasmic Pol II LS-staining pattern (Fig. 3 A, *first panel from left*). However, ~20% of the cells also have 20–50 discrete speckles, which colocalize with splicing protein SC35 and snRNPs of the pre-mRNA splicing class (Bregman et al., 1994). The speckled pattern is most pronounced in early G1 daughter cell nuclei (Breg-

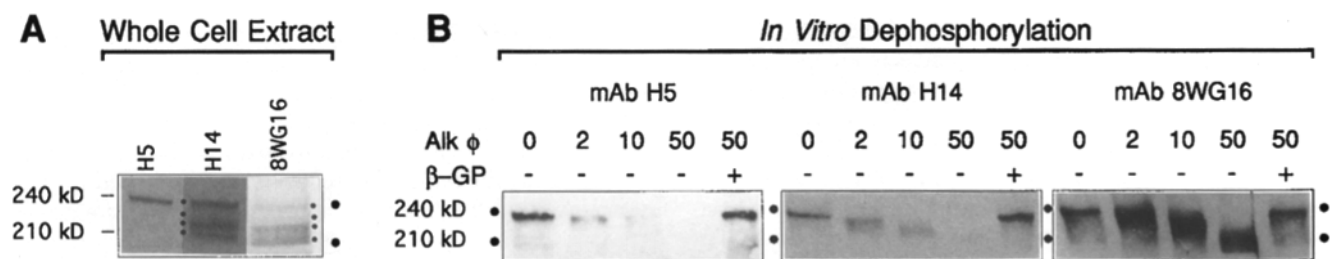


Figure 2. Monoclonal antibodies recognize different phosphorylated forms of Pol II LS. (A) Immunoblot analysis of whole cell extracts MDCK cells were immediately boiled in SDS sample buffer, subjected to 6% PAGE, and immunoblotted with mAbs H5, H14, and 8WG16. mAb H5 binds exclusively to Pol II₀ at ~240 kD. mAb H14 binds to Pol II₀, Pol II_A, and multiple intermediately phosphorylated forms of Pol II LS. 8WG16 binds preferentially to hypophosphorylated forms of Pol II LS including Pol II_A. Dots indicate discrete bands. (B) In vitro dephosphorylation of Pol II LS. Pol II LS was immunoprecipitated from MDCK cells with mAb H5 and divided into three parts, which were incubated for 5 min at 37°C in various concentrations of alkaline phosphatase (Alk Φ), or alkaline phosphatase plus β -glycerophosphate (Alk Φ + β -GP). Samples were then boiled in SDS buffer, subjected to 6% PAGE, and immunoblotted with mAb H5, H14, or 8WG16. Dots indicate positions of Pol II₀ and Pol II_A.

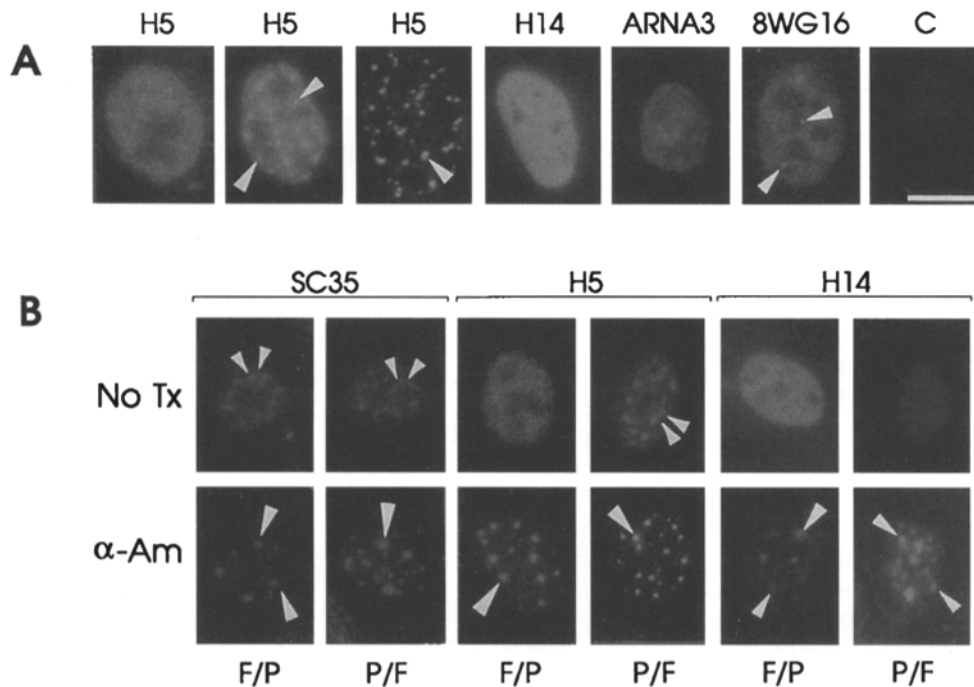


Figure 3. Immunolocalization of Pol II LS in control and α -amanitin-treated cells. (A) Different patterns of intranuclear Pol II LS immunoreactivity. Exponentially growing MDCK cells were fixed and immunostained with control IgM (C), mAb H14, mAb H5, or mAb 8WG16 as described in Materials and Methods. The third panel from the left was preextracted in 1.5% Triton X-100 before fixation, and then immunostained with mAb H5. All anti-Pol II LS mAbs stain the diffuse nucleoplasmic compartment, but in \sim 20% of cell nuclei mAb H5 stains an additional fraction of Pol II LS localized in 20–50 “speckles” (arrowheads, 2nd and 3rd panels from left). If the cells are extracted with Triton X-100 before fixation and permeabilization, nearly

all cell nuclei have a speckled distribution of Pol II LS as determined by mAb H5 immunostaining (see Fig. 3 B). mAb 8WG16 stains 2–5 “dots” in over 50% of cell nuclei (arrowheads, fifth panel from left). (B) Control cells (No Tx) and α -amanitin-(α -Am) treated cells were prepared for immunostaining by two protocols. In the preextraction/fixation protocol (P/F), cells were extracted with 1% Triton X-100, and then fixed with 1.75% paraformaldehyde. In the fixation/permeabilization protocol (F/P), cells were fixed with 1.75% paraformaldehyde and permeabilized with 0.5% Triton X-100 (see Materials and Methods). Subsequently, cells were immunostained with antibodies directed at SC35 (SC35) or Pol II LS (H5 or H14), and then visualized using goat anti-mouse IgM or IgG secondary antibodies linked to fluorescein isothiocyanate (FITC). Arrowheads in control panels (No Tx) indicate irregularly shaped nuclear speckles with apparent interconnections. Arrowheads in α -amanitin (α -Am) panels indicate enlarged round speckles without apparent interconnections. Bar, 10 μ m.

man et al., 1994); an example of this pattern is shown in Fig. 3 A, second panel from left. Second, growth factor stimulation induces a rapid “burst” of intranuclear mAb H5 immunoreactivity which facilitates visualization of Pol II LS in the speckles (Bregman et al., 1994). Third, speckles are more easily visualized when the diffuse nucleoplasmic fraction of Pol II LS is extracted with nonionic detergents before fixation and mAb H5 immunostaining; an example of a nucleus preextracted with 1.5% Triton X-100 is shown in Fig. 3 A, the third panel from the left. In fact, if a population of cells is preextracted with 1.5% Triton X-100, and then fixed and stained with mAb H5, nearly all nuclei have an interconnected speckled pattern (see Fig. 3 B). Taken together, the above results show that Pol II LS is detected in the speckles using mAb H5, but only under certain conditions.

The speckle bound subpopulation of Pol II LS is difficult to detect using mAbs that bind to other Pol II LS epitopes. mAb H14 stains speckle domains in transcriptionally inhibited cells (Fig. 3 B, H14, α -Am), but under ordinary conditions it strongly labels the diffusely distributed fraction of Pol II LS without revealing speckle domains (Fig. 3 A). A commercially available anti-Pol II LS mAb, ARNA3 (Krämer et al., 1980), weakly stains Pol II LS in the diffuse nucleoplasmic compartment, but not in the speckles (Fig. 3 A). mAb 8WG16 stains the diffuse nucleoplasmic compartment, but in over 50% of nuclei it also stains \sim 3–5 intranuclear dots (Fig. 3 A, arrowheads). The above study shows that different anti-Pol II LS antibodies immunostain nuclei with different patterns.

α -Amanitin Induces a Detergent-resistant Fraction of Pol II LS to Redistribute to Enlarged Speckle Domains

α -Amanitin, a compound that completely blocks Pol II-mediated transcription (Lindell et al., 1970), induces a striking accumulation of SC35 in multiple intranuclear dots, which lack apparent interconnections (O’Keefe et al., 1994). These dots have the ultrastructural characteristics of enlarged ICG clusters; their expansion appears to result from the recruitment of splicing proteins from the PCFs to the ICGCs (O’Keefe et al., 1994). The redistribution of SC35 from PCFs to ICGCs correlates with the loss of interconnections between the speckles, which is evident at the light microscopic level of resolution. To distinguish the dot-like structures in transcriptionally inhibited cells from the irregularly shaped speckle domains in transcriptionally active control cells, the former are referred to as “enlarged speckle domains,” and the latter are referred to as “interconnected speckle domains.”

The finding that a detergent-resistant fraction of Pol II LS colocalizes with SC35 in control cells (Bregman et al., 1994) prompted us to ask whether this fraction of Pol II LS redistributes with SC35 to enlarged nuclear speckles in response to α -amanitin (Fig. 3 B). To ascertain whether or not Pol II LS molecules are resistant to detergent extraction, cells were prepared for antibody staining using two protocols: (a) a standard fixation/permeabilization protocol (F/P), and (b) a 1% Triton X-100 preextraction/fixation protocol (P/F), which

removes the diffusely distributed fraction of Pol II LS before fixation (see Materials and Methods). Control (No Tx) and α -amanitin-treated (α -Am) cells were processed by each protocol and immunostained with mAbs directed against SC35 or Pol II LS.

SC35 is distributed in speckles that appear to be interconnected in control cell nuclei prepared by either protocol (Fig. 3 B, *SC35, No Tx*). α -Amanitin induces SC35 to redistribute to enlarged speckles lacking interconnections (Fig. 3 B, *SC35, α -Am*). The enlarged speckle pattern of SC35 is revealed by P/F and F/P protocols. In contrast, the pattern of Pol II LS immunoreactivity is different in control cells prepared by the two protocols. All cells prepared by the F/P protocol have a diffuse nucleoplasmic pattern Pol II LS immunoreactivity when stained with mAb H14. When cells prepared by F/P protocol are stained with mAb H5, ~80% have a diffuse nucleoplasmic pattern immunoreactivity and ~20% have a speckled pattern (not shown here). The P/F protocol extracts the diffuse fraction of Pol II LS, so when these cells are immunostained with mAb H14, little if any Pol II LS is detected (Fig. 3 B, *H14, No Tx*). Pol II LS in the extract and residue can be easily detected by immunoblotting with mAb H14, so the loss of staining is not due to proteolysis or dephosphorylation (data not shown). Significantly, when cells prepared by the P/F protocol are immunostained with mAb H5, nearly every nucleus has an interconnected speckle pattern (Fig. 3 B, *H5, No Tx*). This speckle bound fraction of Pol II LS colocalizes with SC35 at the light microscopic level of resolution (Figs. 4 and 5).

Pol II LS redistributes to enlarged speckle domains in α -amanitin-treated cells, as revealed by mAbs H5 and H14, which recognize different epitopes in Pol II LS's CTD (Fig. 3 B, *H5 and H14, α -Am*). Significantly, the redistribution of Pol II LS is revealed using the P/F and F/P protocols, indicating that: (a) the pattern of Pol II LS immunoreactivity induced by α -amanitin is not distorted by the preextraction step; and (b) Pol II LS is tightly associated with the enlarged speckle domains. Pol II LS located in the enlarged speckles of α -amanitin-treated cells is not detected by anti-Pol II mAb ARNA3 or mAb 8WG16. A possible explanation for this finding is offered in the Discussion. Finally, other nuclear proteins including hnRNP A1 and C do not redistribute to speckles in response to α -amanitin (unpublished data). In summary, there are two populations of Pol II LS, which can be distinguished by differences in their intranuclear distribution, detergent solubility properties and accessibility to monoclonal antibodies.

Multiple Transcriptional Inhibitors Induce Pol II LS and SC35 to Redistribute to Enlarged Speckles

α -Amanitin inhibited transcription involves direct binding of this compound to Pol II LS (for a review see Roeder, 1976), raising the possibility that drug-bound, inactivated Pol II LS precipitates in the enlarged speckles. Therefore, we sought evidence that Pol II LS and SC35 redistribute to enlarged speckles in response to other transcriptional inhibitors, including compounds which inhibit Pol II indirectly. To test this idea, SC35 and Pol II LS were simultaneously immunolocalized in cells treated with four agents that arrest Pol II-mediated transcription. In Fig. 4, control and transcriptionally inhibited cells were double stained with SC35,

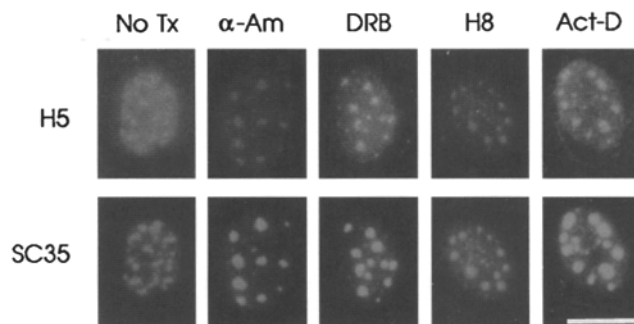


Figure 4. Diverse Pol II transcriptional inhibitors induce SC35 and Pol II LS to redistribute to an enlarged speckled pattern. Cells were extracted with 1% Triton X-100, fixed with 1.75% paraformaldehyde, and double immunostained with mAbs H5 and SC35. Pol II mAb H5 was visualized with a goat anti-mouse IgM-fluorescein isothiocyanate (FITC) conjugate (*upper panels*) and mAb SC35 was visualized using goat anti-mouse IgG-rhodamine conjugate (*lower panels*). Note that control cell nuclei (*No Tx*) have irregularly shaped speckles with apparent interconnections, whereas transcriptionally inhibited cells have enlarged round speckles lacking apparent interconnections. Essentially the same redistribution is observed when cells were fixed with 1.75% paraformaldehyde, and then permeabilized with 0.5% Triton X-100. Bar, 10 μ m.

which is detected by a rhodamine-linked secondary antibody (*lower panels*), and Pol II LS, which is detected by a fluorescein-linked antibody (*upper panels*). Treatments include transcriptional inhibitors with different mechanisms of action: α -amanitin (α -Am), DRB (Tamm et al., 1976; Sehgal et al., 1976; Zandomeni et al., 1986; Stevens and Maupin, 1989; Chodosh et al., 1989), *N*-(2-[methylamino]ethyl)-5-isoquinoline-sulfonamide (H8; Serizawa, 1993) and actinomycin-D (Act-D; Perry and Kelley, 1970). All of the above agents induce SC35 and Pol II LS to redistribute to the enlarged speckles; in each case the interconnections between the enlarged speckles have disappeared or diminished (Fig. 4). All agents induce this effect in ~95% of interphase cells. Significantly, Pol II LS and SC35 are tightly associated with the enlarged speckles as indicated by their resistance to detergent extraction. The redistribution is observed in cells prepared by the F/P protocol, indicating that the preextraction step does not lead to artifactual staining patterns (unpublished data). In summary, transcriptional inhibitors acting by different mechanisms can induce Pol II LS and splicing proteins to relocate to enlarged speckle domains.

Intranuclear Redistribution of Pol II LS and SC35 Is Reversible and Temperature Dependent

DRB is an adenosine analogue that blocks Pol II transcription in part by its ability to inhibit protein kinases which phosphorylate Pol II LS's CTD, a putative regulatory region of Pol II LS (Zandomeni et al., 1986; Stevens and Maupin, 1989; Chodosh et al., 1989; also see below). DRB rapidly penetrates cell membranes, and it can be rapidly washed out to reverse the transcriptional block (Tamm et al., 1976). Using DRB washout experiments, the redistribution of Pol II LS and SC35 are shown to be reversible and temperature dependent (Fig. 5). After a 3-h DRB treatment, Pol II LS and SC35 redistribute from their usual speckled pattern to en-

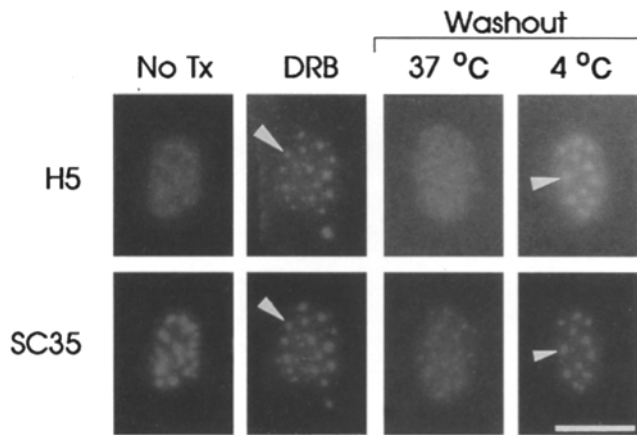


Figure 5. Intranuclear redistribution of Pol II LS and SC35 is reversible and temperature dependent. Three dishes of MDCK cells were treated with DRB (DRB) for 3 h. In two of the dishes, DRB-containing medium was thoroughly washed out with complete medium; one dish was incubated for an additional hour at 37°C, and the other at 4°C before fixation and double immunostaining with mAbs SC35 and H5. SC35 was visualized with rhodamine-conjugated goat anti-mouse IgG (*lower panels*), and mAb H5 was visualized with goat anti-mouse IgM conjugated to FITC (*upper panels*). Note the irregular multifocal mAb H5 immunostaining in the control cell nucleus (No Tx, H5), which overlaps with the interconnected speckled pattern of SC35 (No Tx, SC35). Arrowheads indicate enlarged speckles that are coimmunostained by mAbs H5 and SC35 (DRB, H5, and SC35; DRB washout at 4°C, H5, and SC35). Enlarged speckles are not observed 1 h after DRB washout at 37°C. Bar, 10 μ m.

larged speckles lacking interconnections (Fig. 5, *No Tx vs DRB*). Separate dishes of cells were subjected to the same DRB treatment, followed by DRB washout and incubation at 37°C or 4°C for an additional hour (Fig. 5, *Washout*). The cells were preextracted with 1% Triton X-100, fixed, and immunostained with mAb H5 or SC35 so that the speckle bound fraction of Pol II LS could be optimally visualized. SC35 and Pol II LS are sequestered in the enlarged speckles when the cells are maintained at 4°C (Fig. 5, *Washout, 4°C, arrowheads*). However, when the cells are incubated at 37°C, Pol II LS and SC35 relocate from the enlarged speckles to a distribution that is similar to untreated cells (Fig. 5, *Washout, 37°C*). Time course experiments have shown that Pol II LS and SC35 redistribute simultaneously, beginning ≤ 15 min after the DRB is removed (unpublished data). The temperature dependent, synchronous intranuclear redistribution of Pol II LS and SC35 suggest that these proteins are shuttled by a common mechanism that requires energy.

Speckle-Bound Pol II LS Is Hyperphosphorylated on the COOH-Terminal Domain

The immunolocalization studies with mAb H5 (a Pol II0-specific antibody) indicate that the subpopulation of Pol II LS which remains in the speckle domains after Triton X-100 extraction is hyperphosphorylated on the CTD (Fig. 3 A). This observation prompted us to explore the possibility that Pol II LS's resistance to detergent extraction and its localization to speckle domains are correlated with a high degree of

CTD phosphorylation. First, we established extraction conditions that optimally fractionate Pol II0 from Pol IIA and other hypophosphorylated forms of Pol II LS. None of the conditions we tried yielded fractions containing exclusively one phosphorylated form of Pol II LS; however, Pol IIA and intermediately hypophosphorylated species of Pol II LS are more readily extracted than Pol II0 in 0.5% Triton/0.5% deoxycholate (TD buffer; see Materials and Methods). Therefore, TD-soluble and TD-insoluble fractions were analyzed by immunoblotting with mAbs H5, H14, and 8WG16 to compare the solubility properties of Pol II0, Pol IIA, and intermediately phosphorylated forms of Pol II LS. In addition, TD-extracted nuclear residues were immunostained with mAb H5 to visualize the subnuclear distribution of speckle-bound Pol II0 in control and transcriptionally inhibited cells.

TD supernatant (S) and pellet (P) fractions prepared from control cells were immunoblotted with mAbs H14, H5, and 8WG16 (Fig. 6, A-C). Various hypophosphorylated forms of Pol II LS between ~ 240 and 220 kD are almost completely extracted, as indicated by mAbs H14 and 8WG16. A similar spectrum of hypophosphorylated forms is detected in whole cell extracts prepared by immediate boiling in SDS sample buffer with phosphatase inhibitors, indicating that hypophosphorylation does not take place during extraction with TD buffer (Fig. 2 A). Significantly, the TD pellets contain the Pol II LS molecules that have the most retarded electrophoretic mobility (Fig. 6, A and B; *Control*), whereas the TD supernatants contain Pol II LS molecules that have a spectrum of electrophoretic mobilities ≤ 240 kD. Note that Pol II LS molecules in the pellet fraction have a more retarded electrophoretic mobility than the slowest migrating Pol II LS molecules in supernatant fractions (Fig. 6, A and B; *Control*).

DRB is a CTD kinase inhibitor *in vitro* and *in vivo* (Zandomeni et al., 1986; Stevens and Maupin, 1989; Chodosh et al., 1989; Dubois et al., 1994). Exposure of cells to 100 μ M DRB for 3 h results in the appearance of multiple partially hypophosphorylated forms of Pol II LS (Dubois et al., 1994). The DRB-induced, hypophosphorylated forms of Pol II LS are nearly completely extracted in TD buffer as shown in the blots probed with mAbs H14 and 8WG16. mAb H5 does not recognize these forms in the supernatant fraction (Fig. 6 B; *DRB*). After a 3-h treatment in 100 μ M DRB, some Pol II0 remains; significantly, nearly all of this Pol II0 resists TD extraction (Fig. 6, A and B; *DRB*).

DRB induces dephosphorylation of the CTD in a fraction of Pol II LS molecules; simultaneously, mAb H5 immunostaining disappears from the diffuse compartment and intensifies in the enlarged speckle domains (Figs. 4 and 5). One possible explanation is that hypophosphorylated Pol II LS molecules redistribute from the diffuse compartment to the enlarged speckle domains. According to this idea, hypophosphorylated Pol II LS molecules may be sequestered in enlarged speckle domains with the spliceosomal proteins that are not engaged in splicing. This seems reasonable, especially given that Pol II0 has been identified as the actively elongating form of Pol II LS *in vivo* (O'Brien et al., 1994). However we do not favor this hypothesis because (a) the enlarged speckle domains in DRB-treated cells are visualized by mAb H5, a Pol II0-specific mAb (Figs. 4 and 5); (b) Pol IIA and the other hypophosphorylated forms of Pol II LS re-

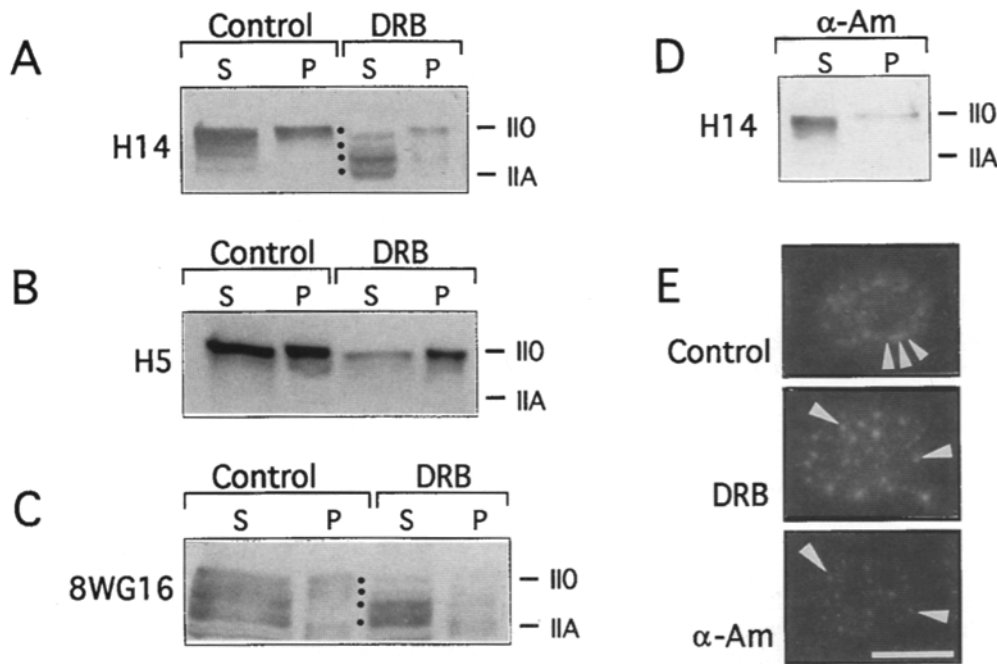


Figure 6. Speckle-bound Pol II LS is hyperphosphorylated on the CTD. (A–D) Equal numbers of control, DRB, and α -amanitin-treated MDCK cells were fractionated in TD buffer into soluble (S) and insoluble (P) fractions as described in Materials and Methods. Supernatant (S) and pellet (P) fractions were solubilized in SDS sample buffer, subjected to 6% PAGE and immunoblotted with mAbs H14 (A and D), H5 (B), and 8WG16 (C). In all cases, the pellet fraction contains the most hyperphosphorylated form(s) of Pol II LS that migrate as a sharp band at 240 kD. The soluble fraction is comprised of a spectrum of Pol II LS forms phosphorylated to various degrees. Dots in A, four partially hypophosphorylated species

of Pol II LS. (E) In Control, DRB and α -amanitin-(α -Am) treated cells, Pol II0 remains in the TD resistant nuclear residue in a speckled distribution. After TD extraction, residues were fixed and immunostained with the Pol II0-specific mAb H5. A goat anti-mouse IgM conjugated to fluorescein isothiocyanate (FITC) was used to visualize the distribution of Pol II0. Bar, 10 μ m.

main in the diffuse nucleoplasm as indicated by immunostaining DRB-treated cells with 8WG16, which preferentially recognizes hypophosphorylated Pol II LS molecules (unpublished data); and (c) hypophosphorylated Pol II LS species accumulate in the TD supernatant fraction of DRB-treated cells (Fig. 6 C; compare S fraction in Control and DRB). Thus, neither the immunolocalization data or the biochemical data supports the hypothesis that Pol II LS moves from the diffuse nucleoplasmic compartment (TD supernatant) to the speckle compartment (TD residue) in response to DRB. These data suggest that DRB induces dephosphorylation of Pol II LS in the diffuse nucleoplasmic compartment, and that it independently induces a reorganization of the detergent insoluble fraction of Pol II0: the subpopulation of Pol II0 in an interconnected speckled pattern appears to redistribute to an enlarged speckled pattern during states of transcriptional inhibition.

α -Amanitin inhibits Pol II-mediated transcription by a mechanism that does not involve dephosphorylation of the CTD. As expected, Pol II LS extracted from control and α -amanitin-treated cells has an indistinguishable PAGE mobility (unpublished results). When α -amanitin-treated cells are extracted in TD buffer, Pol II0 fractionates with the residue (Fig. 6 D, P) and less phosphorylated forms of Pol II LS are completely soluble (Fig. 6 D, S). Thus, in control, DRB and α -amanitin-treated cells, the TD pellet contains exclusively Pol II0, whereas the TD supernatant contains multiple phosphorylated forms of Pol II LS.

To visualize the tightly bound fraction of Pol II0, TD extracted nuclear residues (i.e., TD pellet fraction) from control, DRB-treated and α -amanitin-treated cells were immunostained with mAb H5. Pol II0 remains attached to the speckle domains, thereby corroborating the biochemical

data (Fig. 6 E, DRB). Interestingly, Pol II0 is distributed in speckles lacking interconnections in the nuclear residues of α -amanitin- and DRB-treated cells, but it is distributed in an interconnected speckle pattern in control nuclear residues (Fig. 6 E, α -Am and DRB). Double immunofluorescence shows that Pol II0 and SC35 resist extraction by 0.5% Triton X-100/0.5% DOC, and they colocalize in the speckles of control, DRB-treated, and α -amanitin-treated cells (unpublished observations).

Discussion

New Anti-Pol II LS Reagents Detect a Cryptic Population of Pol II LS in Speckle Domains

The present study establishes that mAbs H5 and H14 bind exclusively to Pol II LS, and that a subpopulation of Pol II LS molecules is “hidden” in speckle domains. mAbs H5 and H14 bind to a major band at \sim 240 kD and a minor band at \sim 210 kD in extracts prepared from mammalian, avian, amphibian, fish, insect, and nematode cells; furthermore, both mAbs bind to a \sim 210-kD protein in *S. pombe* and *S. cerevisiae* (Warren et al., 1992 and unpublished results). In retrospect, the electrophoretic mobility and striking evolutionary conservation of this protein might have provided an early clue that it is Pol II LS. However, before this protein was identified as Pol II LS, it had been localized to nuclear speckles which harbor multiple-splicing proteins (Bregman et al., 1994), and to the axis of amphibian lampbrush chromosome loops—a pattern that is distinct from the “matrix” staining pattern of many pre-mRNA processing proteins (Joseph Gall, personal communication). Previous studies had not detected Pol II LS in speckle domains or B-snur-

posomes (Clark et al., 1991; Jiménez-García and Spector, 1993; for review see Spector, 1993), so the finding that this protein is Pol II LS was quite surprising. The data presented here show that the speckle domains, which have been known for several years to harbor spliceosomal proteins, also harbor a key transcriptional protein, Pol II LS.

Speckle-associated Pol II LS molecules are detected only by a subset of anti-Pol II LS mAbs, and only under certain conditions. A logical explanation of these results is that the speckle-bound Pol II LS molecules are partially masked by interactions with other macromolecules. Speckle domains are densely packed with ribonucleoprotein particles (RNPs), so it is expected that some epitopes of the constituent proteins are masked, and therefore inaccessible to antibodies. Consistent with this idea, the mAbs used in the present study bind to widely separated sites within the Pol II LS molecule. ARNA3 binds to an epitope in the non-CTD part of Pol II LS (Krämer et al., 1980). 8WG16 binds to a nonphosphorylated site in the NH₂-terminal half of the CTD (Thompson et al., 1989; and our unpublished results). mAbs H5 and H14 bind to one or more phosphorylated sites within the CTD (Fig. 2 and our unpublished results). It is possible that certain epitopes in the CTD may be more exposed than other regions of Pol II LS, particularly when the molecules are tightly attached to the speckle domains.

Pol II0 Is Stored in Enlarged Speckle Domains during States of Transcriptional Inhibition

Speckles immunostained by SC35 lose their interconnections and become enlarged during states of transcriptional inhibition (O'Keefe et al., 1994; Spector et al., 1991); therefore, enlarged unconnected speckle domains are thought to be storage and/or reassembly sites for splicing proteins. The present study shows that in cells inhibited by diverse transcriptional inhibitors, Pol II LS and SC35 are core-distributed to the enlarged speckle domains. Thus, it appears that transcriptionally dormant Pol II LS can be stored in speckle domains. In transcriptionally active nuclei at certain stages of the cell cycle, a substantial fraction of Pol II LS is located in storage/reassembly sites, while another fraction is located at sites of Pol II transcription in the adjacent nucleoplasm. The implication is that Pol II LS and splicing proteins can be recruited from these storage sites to the sites of Pol II gene expression as they are needed.

Implications for the Organization of RNA Polymerase II-mediated Transcription In Vivo

Several studies indicate that the phosphorylation state of Pol II LS's CTD correlates with its transcriptional activity in vitro (for reviews see Dahmus and Dynan, 1992; Corden, 1993; Greenleaf, 1993). Hypophosphorylated Pol II LS (Pol IIA) is efficiently recruited to transcription initiation complexes in vitro (Lu et al., 1991; Kang and Dahmus, 1993), but the elongation phase of transcription is heralded by phosphorylation of Pol II's CTD to yield Pol II0 (Bartholomew et al., 1986; Chestnut et al., 1987; Cadena and Dahmus, 1987; Payne et al., 1989; Laybourn and Dahmus, 1990). In vivo, paused polymerases are primarily Pol IIA, but they are converted to Pol II0 as they enter a stable elongation phase (O'Brien et al., 1994). In addition, highly active genetic loci on *Drosophila* polytene chromosomes are enriched with Pol

II0 (Weeks et al., 1993). These data imply that Pol II LS's CTD undergoes a phosphorylation/dephosphorylation cycle which regulates (or reflects) Pol II's cycle of transcriptional initiation, elongation, and termination.

The present study shows that Pol IIA and intermediately phosphorylated forms of Pol II LS are diffusely distributed in the nucleus, whereas Pol II0 is tightly associated with discrete domains. The differential localization of Pol IIA and Pol II0 seems to be functionally significant, given the evidence cited above that these forms of Pol II LS have distinct roles in transcription. However, Pol II0-rich domains labeled by mAb H5 do not necessarily represent clusters of transcriptionally elongating Pol II. Indeed, an important conclusion of present study is that a subset of Pol II0 molecules cannot be engaged in transcriptional elongation. First, Pol II0 is concentrated in enlarged round speckles during pharmacologically induced states of transcriptional inhibition. Transcription is unlikely to take place in the enlarged round speckles—especially in the presence of α -amanitin, DRB, or actinomycin D. Second, Pol II0 is most prominent in speckle domains when transcription is quiescent—during the M/G1 and G2/M cell cycle transition points (Bregman et al., 1994). Third, Pol II0 molecules are bound to multiple dot-like structures that are widely distributed throughout mitotic cells, which are transcriptionally inactive. Finally, the level of Pol II0 is the same throughout the cell cycle, even though overall transcriptional activity fluctuates markedly during the cell cycle (Bregman et al., 1994). These findings do not contradict data showing that Pol II0 is the actively transcribing form of Pol II LS, but they do indicate the existence of a transcriptionally dormant fraction of Pol II0. In fact, if one considers the total population of Pol II LS molecules, hyperphosphorylation of the CTD correlates better with Pol II LS's detergent extractability properties and its intranuclear localization than its transcriptional activity.

Recent studies indicate that each nucleus has 100–500 discrete foci enriched with nascent Pol II transcripts; these “transcript foci” were shown to be tightly associated with a solid phase nuclear structure, and they did not colocalize with nuclear regions that are most highly enriched with SC35 (Wansink et al., 1993; Jackson et al., 1993). The present study does not directly address the question of where actively transcribing Pol II LS is localized in the mammalian cell nucleus. However, recent immunoelectron microscopic localization studies of transcriptionally active cell nuclei reveal intense mAb H5 labeling in multiple discrete foci; some of these Pol II LS Rich Foci (PRFs) appear to be in direct contact with ICGCs, while others are separate from the ICGCs (Spector, D., personal communication). The PRFs were identified by mAb H5 labeling, so they contain Pol II0. We are currently testing the idea that one set of these PRFs are sites of transcription, and another set of PRFs are sites of Pol II LS storage.

The present study also has potentially significant implications for cotranscriptional splicing. Pre-mRNAs are cotranscriptionally spliced and packaged into RNP particles in diverse eukaryotic nuclei (references given in Introduction). Pol II LS, SC35, and other pre-mRNA processing molecules are coordinately recruited to Pol II gene templates introduced into the nucleus by transfection or infection (Bridge et al., 1993; Jiménez-García and Spector, 1993; Rice et al., 1994). The latter studies showed that splicing proteins are

recruited from speckle domains to the gene templates, but they did not reveal the subnuclear origin of the recruited Pol II LS molecules. Here, we have demonstrated that Pol II LS is stored in discrete sites that are very near the storage sites for splicing proteins; furthermore, Pol II LS and SC35 redistribute between an enlarged speckle pattern and an interconnected speckle pattern coincident with changes in the overall transcriptional activity of the nucleus. The finding that the storage sites of Pol II LS and splicing proteins are organized similarly in mammalian nuclei suggests the possibility that Pol II LS and splicing proteins may be recruited simultaneously from these storage compartments to Pol II genes; this would allow spliceosomes to gain direct and immediate access to nascent pre-mRNA transcripts.

We gratefully acknowledge the technical assistance of Stephen Ribisi and Xun Sun. We also thank David Spector at the Cold Spring Harbor Laboratory, and Joan Steitz at the Yale School of Medicine, for helpful discussions.

This work was supported by the March of Dimes Birth Defects Foundation, the Donaghy Medical Research Foundation, the Council for Tobacco Research and the National Institutes of Health (NIH) to S. L. Warren and by the NIH to D. B. Bregman.

Received for publication 9 September 1994 and in revised form 20 December 1994.

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