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Mitotic Repression of RNA Polymerase II Transcription Is Accompanied by Release of Transcription Elongation Complexes

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Nuclear RNA synthesis is repressed during the mitotic phase of each cell cycle. Although total RNA synthesis remains low throughout mitosis, the degree of RNA polymerase II transcription repression on specific genes has not been examined. In addition, it is not known whether mitotic repression of RNA polymerase II transcription is due to polymerase pausing or ejection of transcription elongation complexes from mitotic chromosomes. In this study, we show that RNA polymerase II transcription is repressed in mammalian cells on a number of specific gene regions during mitosis. We also show that the majority of RNA polymerase II transcription elongation complexes are physically excluded from mitotic chromosomes between late prophase and late telophase. Despite generalized transcription repression and stripping of RNA polymerase II complexes from DNA, arrested RNA polymerase II ternary complexes appear to remain on some gene regions during mitosis. The cyclic repression of transcription and ejection of RNA polymerase II transcription elongation complexes may help regulate the transcriptional events that control cell cycle progression and differentiation.

In higher eukaryotes, mitosis is accompanied by dramatic and reversible transformations to the structural organization of both cytoplasm and nucleus (reviewed in references 12, 28, 39, and 40). Mitosis is also accompanied by profound biochemical changes, including a decline in nuclear RNA synthesis. Mitotic repression of transcription was first noted over 30 years ago, in studies analyzing incorporation of RNA precursors during the cell cycle (22, 29, 44, 68, 69). In these studies, it was observed that incorporation of radioactive precursors into nuclear RNA declines in early to mid-prophase and resumes in late telophase. The precise degree of mitotic transcription repression remains uncertain, with some studies detecting mitotic RNA synthesis at 16 to 24% of interphase levels (22, 27, 29, 80). In addition, it is not clear to what degree transcription by each of the three nuclear RNA polymerases (RNAPs) is repressed during mitosis. As approximately 75 to 80% of RNA synthesis in cycling cells is due to RNAP I activity (32, 46, 77), it is possible that some RNAP I, II, or III transcription may escape mitotic repression but be undetectable by pulse-labeling. In addition, pulse-labeling assays measure steady-state RNA levels, which arise through a combination of RNA synthesis and degradation. Therefore, it is possible that part of the loss of labeled steady-state RNA in mitotic cells is due to degradation of nascent labeled RNAs.

Although mitotic repression of RNAP I and III activity has been examined in some detail in the last few years (16, 19, 23, 51, 54, 72, 73), few studies of RNAP II transcription during mitosis have been reported. The in situ hybridization studies of Shermoen and O'Farrell (59) demonstrate that nascent transcripts from the *Drosophila* RNAP II-transcribed gene *Ubx* are aborted and degraded during mitosis. These data suggest that RNAP II transcription complexes may be ejected from mitotic chromatin along with *Ubx* RNA, and that transcription of the *Ubx* gene likely begins anew by reinitiation at the promoter. However, it is also possible that transcripts can be aborted via a rapid degradation mechanism, as chromatin becomes exposed to cytoplasmic nucleases during mitosis, and that RNAP II transcription elongation complexes remain engaged but blocked on mitotic chromatin. Another recent study shows that steady-state *hsp70* mRNA does not accumulate in response to heat shock in mitotic HeLa cells (33). This finding also suggests that RNAP II transcription induction may be absent in mitosis. Some RNAP II transcription factors, but not all, are excluded from mitotic chromosomes (33, 58, 67). However, the ejected transcription factors are not required for transcription elongation, and it is possible that RNAP II transcription elongation complexes persist in a transcriptionally engaged state during M phase.

To understand the mechanisms and regulatory consequences of mitotic transcription repression, it is necessary to determine the activity and location of RNAP II transcription elongation complexes during mitosis. If RNAP II remains transcriptionally engaged during mitosis, mitosis may have few, if any, long-term effects on gene expression. Actively transcribed genes may be earmarked for postmitotic transcription initiation by the presence of blocked but transcriptionally engaged RNAP II elongation complexes that persist throughout mitosis. In this scenario, mitosis would inflict only a transient pause in an unchanging pattern of gene expression. However, if active genes are denuded of both regulatory factors and transcription elongation complexes during chromosome condensation, alternative patterns of transcription and gene expression could be established at the end of M phase.

In this study, we examine the extent of RNAP II transcription repression during mitosis and find that RNAP II transcription is repressed on all genes that we examined. We also find that repression of RNAP II transcription is accompanied by efficient displacement of RNAP II transcription elongation

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complexes from mitotic chromatin; however, some genes may retain transcriptionally engaged RNAP II ternary complexes throughout mitosis. These data support an emerging picture of modification and dislocation of the RNAP II transcription machinery from DNA during mitosis. The cyclic release and reassociation of transcription factors in each cell cycle may have significant consequences for regulation of gene expression during differentiation and the cell cycle.

MATERIALS AND METHODS

Cell culture. HeLa S3 and HeLa CCL2 cells, obtained from American Type Culture Collection, were grown at 37°C as adherent cultures in Dulbecco's modified Eagle medium supplemented with 10% calf serum and antimycotic/antibiotic (from GIBCO BRL). To obtain populations of mitotic cells, HeLa S3 cells were plated at 1×10^4 to 2.5×10^4 cells/cm^2 and incubated for 24 h. The medium was replaced with medium containing 2 mM thymidine, and cells were incubated a further 24 h in order to arrest cells in S phase. The thymidine-containing medium was removed, and the cells were incubated in regular medium for 5 h. Nocodazole {methyl[5-(2-thienylcarbonyl)-1H-benzimidazol-2-yl]} was added at 0.5 μg/ml, and cells were incubated for 10 h to arrest cells in metaphase. Cells were always subconfluent when treated with nocodazole. For reversal experiments, nocodazole-arrested cells were washed free of nocodazole-containing medium and incubated in fresh, prewarmed medium at 37°C for the times indicated.

Flow cytometric analysis. Cells were harvested, resuspended in 0.9% sodium chloride, and fixed by adding 70% ethanol, prechilled to −20°C, dropwise while vortexing. Cells were incubated at 4°C for 30 min and then washed in phosphate-buffered saline (PBS) at 4°C. After centrifugation, cells were resuspended in 1 ml of PBS and stored at 4°C for up to 7 days before fluorescence-activated cell sorting (FACS) analysis. Prior to FACS analysis, propidium iodide was added to the cell suspension at 50 μg/ml, and cells were incubated at room temperature for 30 min. RNase A (100 μl of a 1-mg/ml solution) was added, and cells were incubated at room temperature for 20 min. Flow cytometry was carried out with a Becton Dickinson FACS flow cytometer, and cell cycle kinetics were analyzed by using CellFIT software (Becton Dickinson), employing a sum-of-broadened-rectangles model.

Visual scoring of mitotic index. Cells were harvested and resuspended in 100 μl of hypotonic buffer (20 mM Tris-HCl [pH 7.5], 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM $ZnCl_2$). After 5 min, cells were fixed by adding 10 ml of methanol-acetic acid (3:1 [vol/vol]). The cells were then stained with 50 μg of propidium iodide per ml and treated with RNase A at 100 μg/ml at 30°C for 30 min. Cells were visualized with a Zeiss Axioskop 20 fluorescence microscope under a Plan Neofluor 63× objective lens. Cells were scored by observing nuclear envelope breakdown and condensation of chromatin. Between 300 and 400 cells were scored for each determination.

Cell viability-permeability analysis. Cells were harvested and resuspended in PBS. Aliquots of cell suspension were mixed 1:1 (vol/vol) with a 0.2% solution of trypan blue dye and incubated at room temperature for 2 min. Cells were examined by using a hemocytometer and phase-contrast microscopy. The percentage of cells staining blue was taken as the percentage of cells made permeable or inviable.

Western blot analysis. Proteins were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and transferred to a Hybond-ECL membrane (Amersham). Membranes were blocked for 2 h at room temperature in 3% blocking buffer (PBS, 0.1% Tween 20, 10% [wt/vol] powdered milk, bovine serum albumin), washed in PBS-T (PBS, 0.1% Tween 20), and incubated with primary antibody for 30 min at room temperature. Membranes were washed and incubated with secondary antibody in the same fashion. The membranes were washed, and the secondary antibodies were detected by the enhanced chemiluminescence method (Amersham). Western blots were probed with ARNA-3 (Cymbus Bioscience Ltd., Southhampton, England), an affinity-purified monoclonal antibody that recognizes an epitope on the large subunit of human RNAP II. The secondary antibody was affinity-purified horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G (Jackson ImmunoResearch Laboratories, Inc.).

DNA-protein cross-linking. Protein was cross-linked to DNA by the method of Wrenn and Katzenellenbogen (74). A freshly prepared solution of 11% formaldehyde in 50 mM HEPES (pH 7.5)–0.1 M NaCl–1 mM EDTA–0.5 mM EGTA was added to 2×10^7 to 4×10^7 cells in tissue culture medium, to a final concentration of 0.2%. Cells and formaldehyde were incubated for 8 min at 37°C. Cells were washed twice in cold PBS, counted, and resuspended in 1 ml of nuclear lysis buffer (10 mM HEPES [pH 7.5], 1 mM EDTA, 0.5 mM EDTA, 0.5% Sarkosyl) containing protease inhibitors (20 μg of benzamidine per ml, 2 μg tosylsulfonyl phenylalanyl chloromethyl ketone [TPCK] per ml, 10 μg of leupeptin per ml, and 200 μg of phenylmethylsulfonyl fluoride per ml). Cells and nuclei were lysed by sonication, and nuclear lysis was confirmed by visual inspection under a phase-contrast microscope. Insoluble material was removed by centrifugation at 12,000 × *g* for 10 min at 4°C. Lysates were loaded on CsCl block gradients consisting of 3.8 ml of 1.04-g/ml CsCl, 3.0 ml of 0.67-g/ml CsCl, and 2.5 ml of 0.4-g/ml CsCl in 10 mM HEPES (pH 7.5)–1 mM EDTA–0.5 mM EGTA–0.5% Sarkosyl. Gradients were centrifuged at 77,000 × *g* in a Beckman SW27 rotor for 45 to 48 h at 20°C. Fractions (0.6 ml each) were collected from the bottom of the gradient, using a 20-gauge needle. To identify those fractions containing DNA, 10 μl of each fraction was analyzed by agarose gel electrophoresis. Those fractions containing DNA cross-linked to protein were pooled and dialyzed twice for 3 h each time against 1 liter of dialysis buffer (10 mM Tris-HCl [pH 7.5], 0.1 mM EDTA), prechilled to 4°C. The top three fractions of each gradient, which contained protein not cross-linked to DNA, were treated similarly. Following dialysis, the DNA-containing fractions were ethanol precipitated (0.3 M sodium acetate, 1% SDS, 4 volumes of 95% ethanol) at 20°C overnight, and precipitates were pelleted by spinning at 12,000 × *g* at 4°C for 45 min. The pellet was washed twice with 70% ethanol and air dried. The pellet was resuspended in 1 ml of DNase I treatment buffer (10 mM Tris-HCl [pH 7.5], 1 mM $CaCl_2$, 1 mM $MgCl_2$, 20 μg of benzamidine per ml, 2 μg of TPCK per ml, 10 μg of leupeptin per ml, 100 μg of phenylmethylsulfonyl fluoride per ml. The sample was heated at 95°C for 10 min to reverse formaldehyde cross-linking, and DNA was digested with 200 μg of DNase I, 5 U of micrococcal nuclease, and 1,000 U of S1 nuclease for 30 min at 37°C. Digestion was monitored by analyzing an aliquot by agarose gel electrophoresis and staining with ethidium bromide. Protein from the DNA-cross-linked fractions, as well as the protein from the non-cross-linked fractions, was concentrated to less than 20 μl, using Ultrafree-MC low-binding centrifugal filtrations columns (Millipore Corp.). An equal volume of 2× SDS sample buffer was added to each sample, and samples were analyzed by SDS-polyacrylamide gel electrophoresis and Western blotting.

Indirect immunofluorescence. Immunofluorescence assays were performed on cycling populations of HeLa CCL2 cells. Cells were grown on coverslips and fixed for 10 min in 3.7% formaldehyde in PBS. After fixation, cells were washed in PBS-T (PBS, 0.01% Tween 20), rinsed in distilled water, and then permeabilized for 2 min in cold (−20°C) acetone. Cells were rinsed in PBS-T and distilled water and blocked for 30 min at room temperature in PBS-T with 3% bovine serum albumin. Cells were incubated with the primary antibody ARNA-3 (1:3 dilution in PBS), 8WG16 (1:200 dilution in PBS), or αAP-2 (1:100 dilution in PBS). Antibody 8WG16 is a monoclonal antibody that recognizes epitopes on the carboxy-terminal domain (CTD) of the RNAP II large subunit (47, 70). Antibody αAP-2 is an affinity-purified polyclonal antibody that recognizes the human transcription factor AP-2 (Santa Cruz Biotechnology). Cells were washed in PBS-T and then stained with secondary antibodies rhodamine-conjugated goat anti-mouse immunoglobulin G or rhodamine-conjugated donkey anti-rabbit immunoglobulin G (Jackson ImmunoResearch Laboratories). In some experiments, 4′,6-diamidine-2-phenylindole (DAPI; 5 μg/ml) was added to secondary antibody dilutions. Cells were visualized with a Zeiss Axioskop 20 fluorescence microscope equipped with a Plan Neofluor 63× objective lens.

Whole-cell run-on transcription assays. Run-on transcription assays were performed after permeabilization of whole cells by the method of Miller et al. (35). Asynchronous or mitotic HeLa S3 cells were scraped into cold PBS, pelleted at 200 × *g* at 4°C, and washed twice with cold LYSO A solution [35 mM *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES; pH 7.4), 150 mM sucrose, 80 mM KCl, 5 mM $MgCl_2$, 0.5 mM $CaCl_2$]. Cells were resuspended at 10^7 cells/100 μl of LYSO A solution and transferred to siliconized Eppendorf tubes. One-third volume of a 1-mg/ml solution of lysolecithin (lysophosphatidylcholine) in LYSO A solution was added, and the cells were incubated on ice for 1 min. Cells were pelleted and resuspended in 1 ml of cold LYSO A solution. Permeability was measured by trypan blue exclusion. The percentage of cells permeabilized was always greater than 90%. Cells were pelleted again and resuspended in 210 μl of nuclear freezing buffer (50 mM Tris-HCl [pH 8.0], 5 mM $MgCl_2$, 40% glycerol, 0.5 mM dithiothreitol). Assays were performed immediately on equal numbers of cells per sample.

In a standard reaction, 60 μl of 5× buffer (25 mM Tris-HCl [pH 8.0], 12 mM $MgCl_2$, 750 mM KCl, 1.2 mM ATP, CTP, and GTP) was added, bringing the final KCl concentration to 150 mM. In experiments using Sarkosyl, this reagent was added before the addition of 5× buffer. Radioactive UTP (30 μl of [α-^{32}P]UTP [3,000 mCi/mmol]) was added to the reaction, and tubes were incubated at 30°C for 30 min. The reaction was terminated by addition of 125 μg of DNase I (Worthington) and incubation for 5 min at 30°C. Proteins were digested by addition of 36 μl of 10× SET buffer (100 mM Tris-HCl [pH 7.5], 10% SDS, 50 mM EDTA) and 100 μg of proteinase K and incubation at 45°C for 45 min. RNA was purified by phenol-chloroform-isoamyl alcohol extraction and isopropanol precipitation. The pellet was resuspended in 100 μl of Tris-EDTA, and unincorporated ribonucleotides were removed by Sepharose G-50 column chromatography. RNA was made up to 1 ml with hybridization buffer (10 mM TES [pH 7.4], 1% SDS, 10 mM EDTA, 250 μg of *Escherichia coli* RNA per ml, 0.3 M NaCl, 1× Denhardt's solution, 0.25% powdered milk). Run-on transcription RNA was hybridized to single-stranded DNA probes slot blotted onto GeneScreenPlus filters, and filters were washed as described previously (48, 63). Radioactivity hybridizing to each probe was quantitated with a Fujix BAS100 bioimaging analyzer with MacBAS imaging software.

Probes were single-stranded bacteriophage M13 DNAs and were designed to detect either sense or antisense transcription in the gene region of interest. The probes were constructed by cloning the following DNA fragments into either M13mp18 or M13mp19. The c-*myc* exon 1 probe was a 445-bp *Xho*I-*Pvu*II fragment from +66 to +511 of the human c-*myc* gene (63). The c-*myc* intron 1

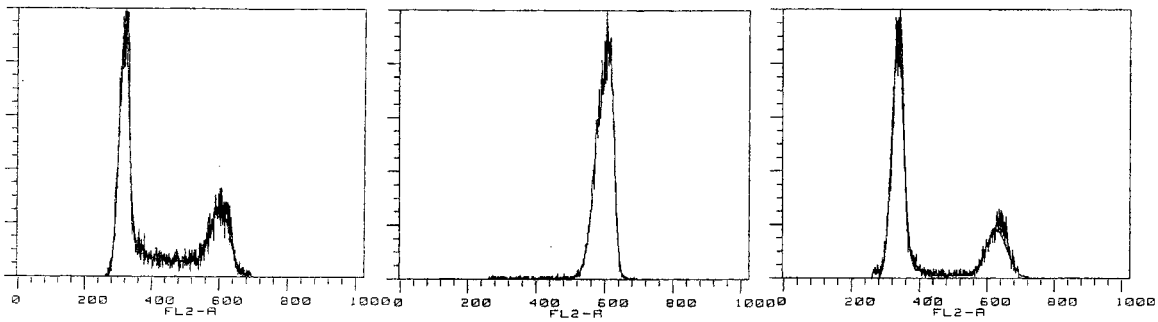

FIG. 1. Flow cytometric analyses depicting relative DNA contents in populations of cycling, mitosis-arrested, and released cells. HeLa S3 cells were untreated (cycling), arrested in mitosis by thymidine-nocodazole treatment (arrested), or released from arrest by incubation in regular medium for 8 h (released) as described in Materials and Methods. Cells were stained with propidium iodide and analyzed by FACS. Below each panel is a quantitative analysis of cell cycle phase distribution, determined by using CellFIT software. The distribution of the arrested population could not be quantitated because too few cells were in G_1 or S phase.

probe was a 606-bp *Sst*I fragment from +936 to +1542 of the human c-*myc* gene (63). The c-*fos* 5′ probe was a 842-bp *Nar*I fragment from −82 to +760 (exon 1 and part of intron 1) of human c-*fos* genomic DNA. The c-*fos* 3′ probe was a 491-bp *Apa*I fragment from +1905 to +2396 (exon 4) of human c-*fos* genomic DNA. The γ-actin 5′ probe was a 583-bp *Bam*HI-*Bgl*I fragment from −100 to +483 (exons 1 to 4) of a human γ-actin cDNA. The γ-actin 3′ probe was a 600-bp *Bgl*I-*Sca*I fragment from +483 to +1083 (exons 4 to 6) of a human γ-actin cDNA (13). The *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase gene) probe was a 979-bp fragment from +44 to +1023 of a human *GAPDH* cDNA (2). The histone H2b probe was a 302-bp *Bst*EII fragment from +110 to +412 of the chicken histone H2b gene (64). The Pol 5′ probe was a 471-bp *Bsa*HI-*Sph*I fragment from −26 to +445 of the mouse RNA polymerase II large subunit gene, encompassing most of exon 1 (1).

Artwork for Fig. 1 to 8. Autoradiographs, phosphorimage plate exposures, color photographs, or black-and-white photographs were scanned with Adobe Photoshop software and images were saved in TIFF. Images were assembled and labeled with Quark Xpress3.0 software on a Macintosh IIfx.

RESULTS

RNAP II transcription in mitotic cells. To examine RNAP II transcription repression during mitosis, we performed run-on transcription assays on interphase and mitotic cells. Traditional nuclear run-on transcription assays involve a brief labeling of nascent RNAs and provide an estimate of the number of RNA polymerase molecules that are transcriptionally engaged on a specific region of DNA at the time of nuclear isolation (32). These assays also allow one to estimate transcription rates across specific gene regions, independent of the effects of RNA processing, and to differentiate transcription by RNAP I, II, and III (4, 31, 56). Because the nuclear membrane disintegrates in prophase and discrete nuclei are absent in mitotic cells, we used a variation of the nuclear run-on transcription assay—the whole-cell run-on transcription assay—to measure RNAP II transcription in interphase and mitotic cells. The whole-cell run-on transcription assay has been used previously to measure transcription in specific gene regions and appears to be as efficient as the nuclear run-on assay (66, 71).

HeLa S3 cells were arrested in M phase with a thymidine-nocodazole block as described in Materials and Methods. Samples of arrested cells were released from the block by washing out the nocodazole and incubating the cells in untreated medium for 8 to 10 h. The proportion of cells arrested in M phase by the thymidine-nocodazole block was estimated by FACS analysis (Fig. 1). In treated cultures, all cells were in G_2 or M, as measured by FACS. In addition, release from arrest was efficient, with approximately 75% of cells reaching G_1 or S phase within 8 h of release. As FACS analysis scores cells by DNA content only, we wished to verify that the G_2/M peak in arrested cells was comprised predominantly of mitotic cells. Visual assessment of mitotic index verified that approximately 95% of cells were arrested in mitosis by the thymidine-nocodazole block and that approximately 20% of the cells remained in M phase following an 8-h release (data not shown). Cell viability after mitotic arrest and release was over 95%, as measured by trypan blue exclusion.

Cycling, mitotic, and released HeLa S3 cells were harvested, treated with lysolecithin, and immediately assayed by whole-cell run-on transcription assay. Assays were performed on equal numbers of cells per sample. As transcription rates are dramatically altered during mitosis, it is not possible to select a constant internal control against which to measure transcription within any specific gene region. DNA and protein content per cell would also be expected to differ between cycling cells, mitotic cells, and cells entering G_1. Therefore, a constant cell number per sample was chosen as the control against which to assess relative transcription levels.

RNAs labeled during run-on transcription assays were hybridized to filters containing single-stranded DNA probes that detect either sense or antisense transcription from various regions of cellular genes, as described in Materials and Methods (Fig. 2). The patterns of transcription in cycling cells were similar to those observed in nuclear run-on transcription assays using the same gene probes and interphase HeLa cell nuclei (65). Sense transcription occurred within the c-*myc*, γ-actin, *GAPDH*, histone H2b, and rRNA genes and at the 5′ end of the c-*fos* gene. The c-*myc* gene displayed a readthrough transcription pattern, indicating that most of the transcription in exon 1 elongated into the intron 1 region. This pattern has been observed in some human cell lines and is correlated with the presence of high levels of c-*myc* steady-state RNA (63). Transcription of the c-*myc* gene is regulated, in part, by controlling the amount of promoter-paused RNAP II that is released into transcription elongation (30, 62). The c-*fos* gene

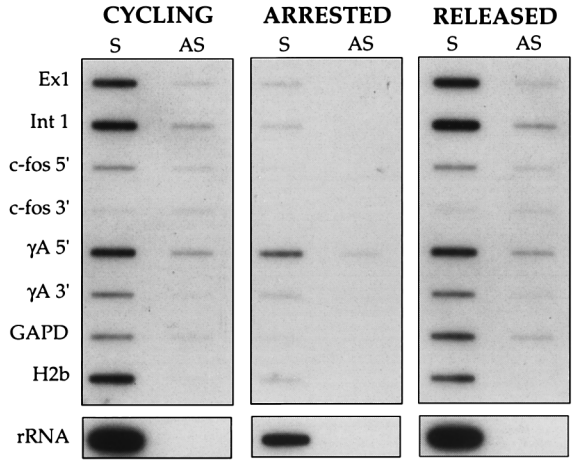

FIG. 2. Whole-cell run-on transcription assays of RNAP II- and RNAP I-transcribed genes. HeLa S3 cells were untreated (cycling), arrested in mitosis by thymidine-nocodazole treatment (arrested), or released from arrest for 8 h (released). Cells were rapidly permeabilized with lysolecithin and incubated in the presence of [α-^{32}P]UTP as described in Materials and Methods. Equal numbers of cells were assayed in each sample. RNA products from each sample were divided into two aliquots: 95% of the RNA was hybridized to filters containing probes that detect transcription from several RNAP II-transcribed genes (top three panels), and 5% was hybridized to filters containing a probe that detects 18S rRNA gene transcription (bottom three panels). The probes were single-stranded M13 DNAs that detect sense (S) or antisense (AS) transcription arising from c-*myc* exon 1 (Ex 1) and intron 1 (Int 1), c-*fos* 5′ and 3′ regions, γ-actin (γA) 5′ and 3′ regions, and *GAPDH*, histone H2b, and 18S rRNA genes.

displayed a typical transcription block pattern, with most of the transcription signal occurring in the 5′ region. Blocks to transcription elongation have been reported in the hamster and murine c-*fos* genes, and release of transcription blockage may be a mechanism for regulating steady-state levels of c-*fos* mRNA (reviewed in reference 62). We have observed increases in transcription readthrough, as well as increases in transcription at the 5′ end of the c-*fos* gene following serum stimulation of quiescent HeLa cells (61). The cells assayed in these experiments were not serum stimulated prior to harvesting and hence show low levels of c-*fos* transcription. The γ-actin gene also showed a transcription block pattern, with higher levels of transcription at the 5′ end than at the 3′ end. We have previously observed variations in the amount of readthrough transcription in the γ-actin gene (65); hence, this gene may also be subject to transcription regulation by release of promoter-paused RNAP II complexes. The *GAPDH* and histone H2b probes detect transcription throughout these transcription units. Transcription signals over the 18S rRNA gene probe were approximately 1,300-fold higher than those over the histone H2b signal. The inclusion of 2 μg of α-amanitin per ml in run-on transcription assays abolished transcription on all probes (except the rRNA probe), indicating that transcription of these genes was carried out by RNAP II (data not shown). Run-on transcription assays conducted in the presence of 0.5% Sarkosyl (which inhibits reinitiation of transcription) indicated that the transcription that we observed was produced by transcriptionally engaged RNAP II complexes and was not due to reinitiation during the assay (data not shown).

As seen in Fig. 2, transcription in mitotic cells was repressed on all RNAP II-transcribed genes, as well as on the 18S rRNA probe. Phosphorimage analysis showed that transcription of RNAP II-transcribed genes was repressed to levels between approximately 6% (histone H2b) and 26% (c-*fos* 5′) of cycling levels. Transcription of the 18S rRNA gene was repressed to 13% of cycling levels. Transcription in the γ-actin 5′ region declined to approximately 41% of cycling levels. Transcription levels returned to normal, or higher than normal, following release from mitotic arrest. Total incorporation of label during the run-on transcription assay declined to 11.4% of cycling levels in the mitotic cells, providing an estimate of the combined activities of RNAP I, II, and III in mitotic cells during the run-on transcription assay.

In summary, these assays indicate that both RNAP II and RNAP I transcription declines in mitotic arrested cells. However, the extent of RNAP II repression may not be uniform for all RNAP II-transcribed genes. The presence of high transcription signals on some gene regions (such as γ-actin 5′) may indicate either transcriptional activity in that region during mitosis or the presence of transcriptionally engaged but arrested RNAP II ternary complexes that are released during the run-on assay. Transcriptional activity resumes within 8 h of release from mitosis.

Absence of functional RNAP II transcription elongation complexes during mitosis. An important question regarding the repression of RNAP II transcription during mitosis is whether the repression is due to transient arrest of RNAP II elongation complexes or to complete dissociation of these complexes. It has previously been hypothesized that transcription may be repressed during M phase because condensed chromatin inhibits the elongation of RNAP complexes (19, 59).

To determine whether RNAP II remains in functional ternary complexes on specific genes during mitosis, we attempted to release paused RNAP II complexes by using high salt and Sarkosyl. Both of these agents strip many chromatin proteins from DNA and disrupt protein-protein interactions but leave RNAP II elongation complexes stable and capable of elongation (7, 17, 20, 21, 53). As a result, high salt and Sarkosyl can confer two simultaneous and apparently contradictory effects on transcription elongation, depending on the context of the RNAP II elongation complex. First, Sarkosyl and high salt can release RNAP II complexes that are engaged but blocked on the promoter regions of some genes such as *hsp70* and c-*myc* (30, 41, 50). This results in a stimulation of transcription at the 5′ ends of these genes during nuclear run-on transcription assays. In addition, Sarkosyl and high salt can improve the overall transcription efficiency of some in vitro transcription systems (24). Second, Sarkosyl and high salt slow the rate of elongation of RNAP II ternary complexes that are not blocked. This may be due to the dissociation of elongation factors such as TFIIF from the elongation complexes (24). We reasoned that Sarkosyl or high salt may stimulate RNAP II transcription in mitotic cells, if the low transcriptional activity in these cells was due predominantly to blockage of RNAP II elongation complexes by chromatin.

HeLa S3 cells were harvested from cycling populations or mitotic populations and then permeabilized with lysolecithin, and whole-cell run-on transcription assays were performed. Assays were performed on equal numbers of cells per sample and in the presence of 15, 75, or 300 mM KCl. In cycling cells, transcription on all probes was maximal at 75 mM KCl (Fig. 3, top panels), and increasing the salt concentration from 15 to 75 mM increased transcription signals on all probes, from between 1.3- and 2.1-fold. Higher salt concentrations (300 mM) depressed transcription signals on all probes. Although transcription levels were low in mitotic cells, increasing the salt concentration from 15 to 75 mM did not induce increases in transcription on any probes except γ-actin 3′ and histone H2b, which showed increases of 1.6- and 2.0-fold, respectively (Fig. 3, bottom panels). Therefore, RNAP II transcription in mitotic

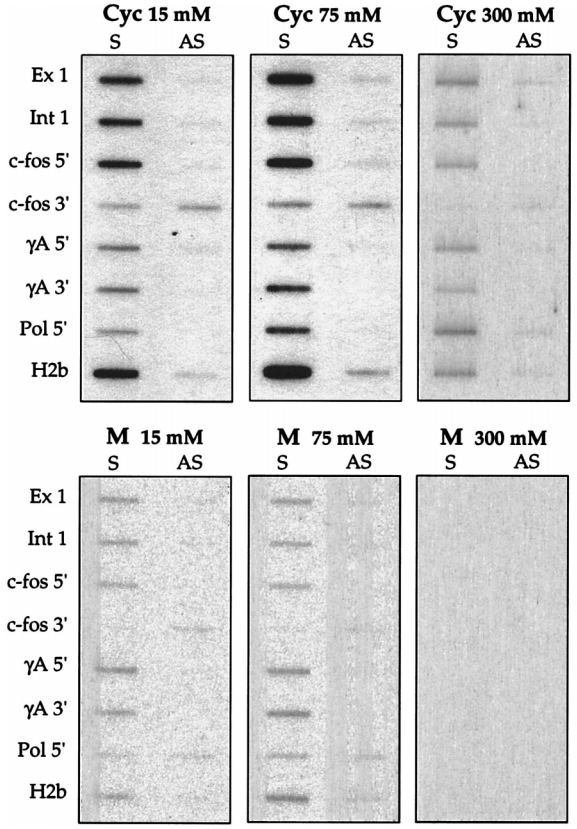

FIG. 3. RNAP II transcription in the presence of various salt concentrations. Whole-cell run-on transcription assays were performed on HeLa S3 cells that were cycling (top three panels) or arrested in mitosis by thymidine-nocodazole treatment (bottom three panels). Assays were carried out in the presence of 15, 75, or 300 mM KCl, as indicated. Equal numbers of cells were assayed in each sample. Probes were the same as those described in the legend to Fig. 1 except for the Pol 5′ probe, which detects sense (S) or antisense (AS) transcription in exon 1 of the RNAP II large subunit gene. Two of the top three panels (Cyc 15 mM and Cyc 75 mM) are autoradiographic images of the filters detecting transcription in cycling cells. The bottom three panels and the Cyc 300 mM panel are phosphorimager-generated images of filters from the remainder of the experiment.

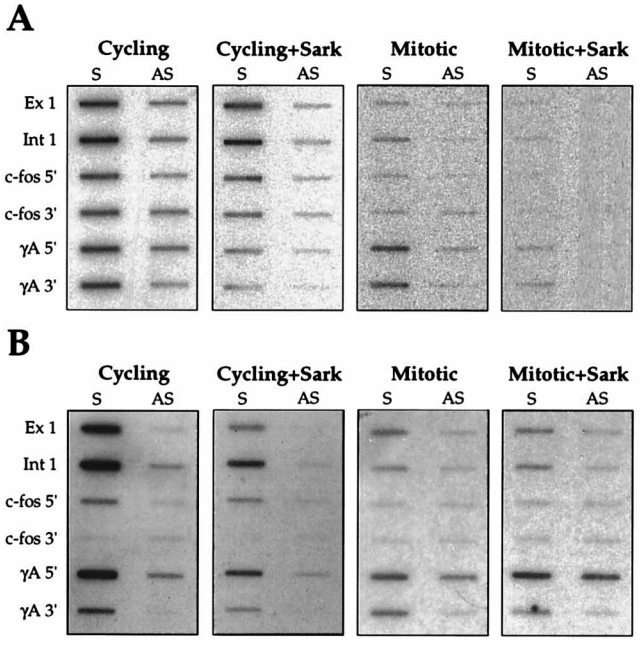

FIG. 4. RNAP II transcription in the absence or presence of 0.5% Sarkosyl. Whole-cell run-on transcription assays were performed on HeLa S3 cells that were either cycling or arrested in mitosis by thymidine-nocodazole, as indicated. Run-on transcription assays were carried out in the presence of 15 mM KCl (A) or in the presence of 75 mM KCl (B) and in the presence or absence of 0.5% Sarkosyl (Sark). Equal numbers of cells were assayed in each sample. Probes are described in the legend to Fig. 2. (A) Phosphorimager-generated images; (B) autoradiographic images of run-on transcription filters.

cells could not be restored by salt concentrations up to 300 mM.

We next examined the effects of 0.5% Sarkosyl on RNAP II transcription in cycling and mitotic cells. HeLa cells were harvested from cycling or arrested populations and permeabilized with lysolecithin, and run-on transcription assays were performed. Assays were performed in the presence of either 15 mM KCl (Fig. 4A) or 150 mM KCl (Fig. 4B). With the exception of γ-actin 5′ transcription in mitotic cells at 150 mM KCl (Fig. 4B), the addition of 0.5% Sarkosyl during whole-cell run-on transcription assays decreased transcription, or did not change transcription, in each gene region examined. The γ-actin 5′ sense and antisense transcription signals both increased approximately 1.1-fold in the presence of Sarkosyl at 150 mM KCl (Fig. 4B). This finding suggests that paused or arrested RNAP II ternary complexes may be present in this region during mitosis and that these arrested complexes can be released to a modest degree by Sarkosyl but not by high salt (Fig. 3). We do not know the nature of antisense transcription in this region of the γ-actin gene. The γA5′ probe is a cDNA encompassing exons 1 to 4 of the human γ-actin gene. Therefore, the antisense signal could originate anywhere within this region. Whatever the nature of the antisense signal, it responds to Sarkosyl similarly to the sense signal. Phosphorimager analysis of the cycling and cycling-plus-Sarkosyl filters shown in Fig. 4A showed that the ratio of antisense to sense signal was higher than expected on the c-*myc* exon 1, γ-actin 5′, and histone H2b regions (1.6- to 2.1-fold higher) but lower than expected on the other three probes (0.5- to 0.8-fold lower). We do not know the reason why some antisense/sense transcription ratios were higher, and some lower, in this experiment.

In summary, these data suggest that the majority of RNAP II ternary complexes either are absent from mitotic chromosomes or cannot be stimulated by agents that disrupt chromatin and facilitate elongation of blocked ternary complexes. The observation that transcription in the γ-actin 5′ region could be enhanced by the addition of Sarkosyl in the presence of 150 mM KCl supports the conclusion that some functional but arrested RNAP II ternary complexes may remain on some gene regions during mitosis.

Indirect immunofluorescence localization of RNAP II during mitosis. The preceding data suggest that most RNAP II molecules may be physically absent from mitotic chromatin during mitosis. To detect exclusion of RNAP II from chromosomes during M phase, we examined RNAP II localization during interphase and mitosis. Cycling HeLa CCL2 cells were grown on coverslips, fixed, and stained with monoclonal antibody ARNA-3, which reacts with an epitope on the body of the large subunit of RNAP II (Fig. 5). ARNA-3 detects all phosphorylation variants of the large subunit of RNAP II (47). Cells were simultaneously stained with DAPI in order to visualize DNA. In interphase cells, RNAP II staining was nuclear with exclusion from nucleoli, as observed previously (47, 48). During prophase, RNAP II staining declined on condensing

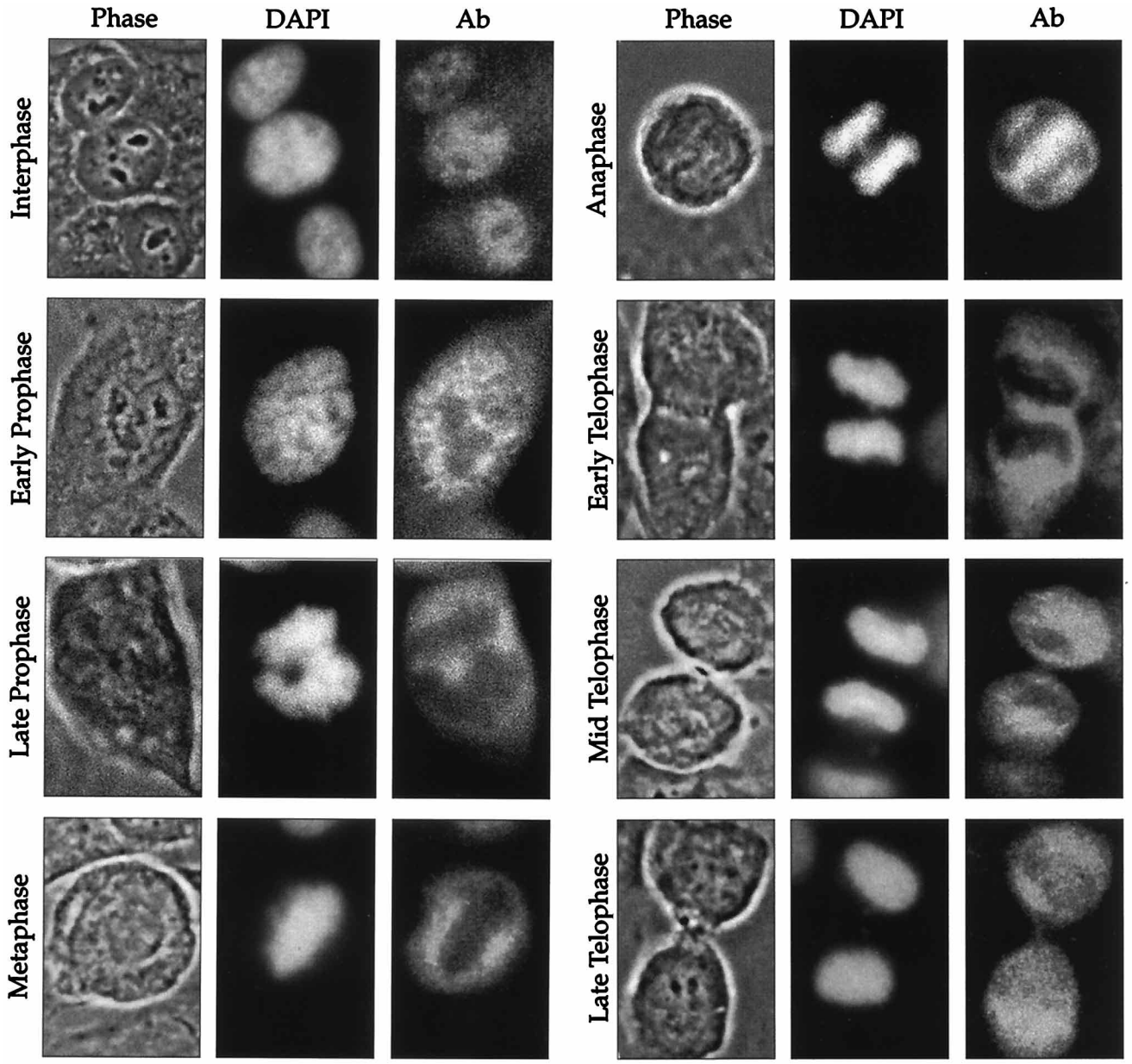

FIG. 5. Subcellular localization of the large subunit of RNAP II during interphase and several stages of mitosis. Cycling HeLa CCL2 cells were grown on glass coverslips, fixed, and stained with ARNA-3, an affinity-purified monoclonal antibody which recognizes an epitope on the body of the large subunit of RNAP II. Cells were counterstained with DAPI to detect DNA. Cells were visualized with phase-contrast (phase) or fluorescence microscopy to reveal DNA or antibody (Ab) staining, as indicated. In early prophase, nucleoli and nuclear membranes were less distinct than in interphase. In late telophase, nuclear membranes and nucleoli were detectable.

chromatin and increased in the cytoplasm, even prior to complete nucleolar dissolution (Fig. 5). In metaphase, anaphase, and early telophase, RNAP II large subunit staining remained cytoplasmic and excluded from chromosomes (5). As telophase progressed, RNAP II staining was more uniform between cytoplasm and chromatin. However, even in late telophase, with nucleoli and nuclear membranes re-forming, some exclusion of RNAP II large subunit staining from chromatin was detectable. It has recently been reported that RNAP II localizes with splicing factors in granule-like clusters in mitotic cytoplasm (26). However, our immunofluorescence analysis did not show an obvious punctate distribution (Fig. 5).

Cells stained with ARNA-3 alone (and not with DAPI) also showed the same exclusion of RNAP II large subunit from mitotic chromatin. Therefore, exclusion was not due to the presence of DAPI staining on DNA (data not shown). Staining with secondary antibody alone did not yield detectable fluorescence at these same exposures (data not shown). We also stained interphase and mitotic cells with the affinity-purified polyclonal antibody αAP-2, which has previously been shown to uniformly stain mitotic cells and not to be excluded from mitotic chromatin (33). The staining of both chromatin and cytoplasmic regions of metaphase HeLa CCL2 cells with αAP-2 indicates that exclusion of antibody is not a general

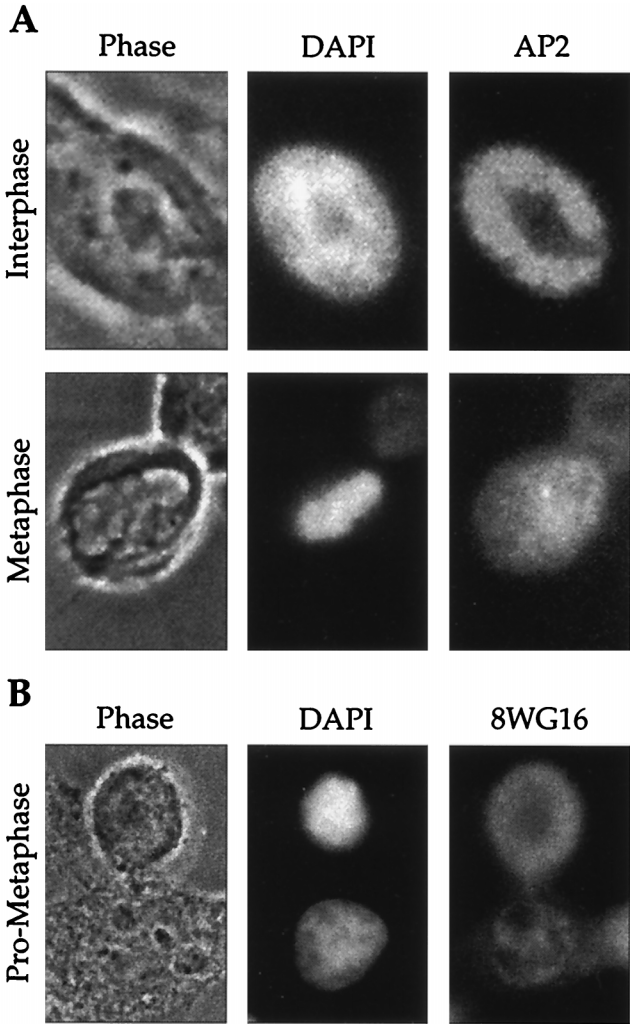

FIG. 6. (A) Subcellular localization of transcription factor AP-2 during interphase and metaphase. Cycling HeLa CCL2 cells were grown on glass coverslips, fixed, and stained with αAP-2, an affinity-purified polyclonal antibody that recognizes the RNAP II transcription factor AP-2. Cells were also stained with DAPI to visualize DNA. (B) Subcellular localization of RNAP II in interphase (bottom cell) or pro-metaphase (upper cell). HeLa S3 cells were grown on glass coverslips, fixed, and stained with monoclonal antibody 8WG16, which recognizes epitopes on the CTD of the RNAP II large subunit. Cells were counterstained with DAPI to visualize DNA. Cells were photographed by using phase-contrast or fluorescence microscopy to detect DNA or antibody, as indicated.

property of mitotic chromosomes (Fig. 6A). We also stained interphase and mitotic cells with antibody 8WG16, which recognizes epitopes on the CTD of the RNAP II large subunit (70). This antibody preferentially reacts with nonphosphorylated and intermediately phosphorylated forms of the RNAP II large subunit (47). As seen in Fig. 6B, the staining patterns with 8WG16 were similar to those with ARNA-3 (Fig. 5). Antibody 8WG16 stains nuclei of interphase cells (Fig. 6B, bottom cell), with exclusion from nucleoli. In the pro-metaphase cell (Fig. 6B, top cell), staining is predominantly cytoplasmic, with exclusion from condensed chromatin at the center of the cell. Thus, our immunofluorescence data suggest that RNAP II is physically dissociated from mitotic chromatin. RNAP II appears to remain predominantly cytoplasmic from late prophase to late telophase.

One possible explanation for the apparent exclusion of RNAP II from mitotic chromosomes is that the majority of RNAP II molecules are free within the interphase nucleus and simply disperse following nuclear membrane breakdown in prophase. However, we and others have shown by fractionation assays that the majority of the hyperphosphorylated (IIO) form of RNAP II in HeLa cells is bound to cellular chromatin (43, 45) and that approximately 50% of RNAP II in HeLa cells is of the IIO form (see Fig. 8A and reference 65). In addition, RNAP IIO has been shown to be the transcriptionally engaged form of RNAP II in vivo and in vitro (3, 6, 65). Therefore, even if all of the nonphosphorylated and transcriptionally unengaged RNAP II (RNAP IIA) dispersed into the cytoplasm following nuclear membrane breakdown, approximately 50% of RNAP II would be expected to remain associated with chromatin. The degree of exclusion of RNAP II that we observe in these immunofluorescence assays suggests that the majority of RNAP II is absent from mitotic chromosomes.

RNAP II is physically excluded from mitotic chromosomes. Both the inability to release paused RNAP II elongation complexes by salt or Sarkosyl and the exclusion of RNAP II staining from mitotic chromosomes suggest that RNAP II transcription complexes are disengaged from DNA during mitosis. To verify this conclusion, we performed in vivo formaldehyde cross-linking assays on intact interphase and mitotic cells.

Formaldehyde cross-linking assays have been used to demonstrate that the estrogen receptor associates with chromatin both before and after binding to ligand (74) and that histone H4 remains bound to the *Drosophila hsp70* gene promoter both before and after heat shock (60). The RNAP II large subunit also cross-links to chromatin with this method, and specific cross-linking of RNAP II to the *hsp70* gene increases following heat shock (60, 74). Cross-linking is selective for proteins in direct or close contact with DNA, when formaldehyde concentrations and treatment times are below those leading to extensive protein-protein cross-linking (74). Formaldehyde is added directly to the culture medium, where it rapidly penetrates cells and forms protein-protein and protein-DNA linkages. In vivo formaldehyde cross-linking appears to be more efficient than UV cross-linking of proteins to chromatin, allowing its use as an in vivo probe for low-abundance chromatin-associated proteins in mammalian cells (74). In addition, formaldehyde cross-linking is reversible, facilitating analysis of intact polypeptides.

Cycling or mitotic HeLa S3 cells were untreated or treated with 0.2% formaldehyde for 8 min. Cells were lysed, the cell lysates were briefly sonicated, and free proteins were separated from cross-linked DNA-protein by CsCl gradient centrifugation. To verify that proteins were cross-linked to DNA, resulting in differential migration of DNA in CsCl gradients, we analyzed fractions from the gradients by agarose gel electrophoresis (Fig. 7). DNA migrated to the bottom of the gradient in the absence of formaldehyde cross-linking (Fig. 7A and C). DNA from cells treated with formaldehyde migrated to the middle of the gradient (Fig. 7B and D). To confirm that the material migrating to the center of the gradients contained DNA, aliquots of fractions from the center of the gradient (Fig. 7B) were dialyzed and treated with either DNase I or RNase A (Fig. 7E). DNase I digestion eliminated all ethidium bromide staining of fractions from the middle of the CsCl gradient. Free protein at the top of the gradient did not stain with ethidium bromide.

We next examined the RNAP II content in the gradient fractions from formaldehyde-treated cells. DNA-containing fractions from the center of each gradient were pooled and concentrated, as were fractions from the top of the gradient, which contain free proteins (74). Pooled fractions were treated with nucleases, and the large subunit of RNAP II was analyzed

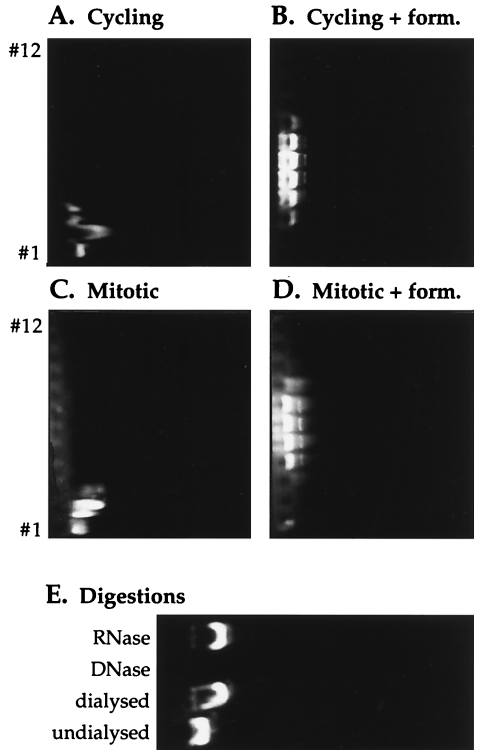

FIG. 7. Analysis of DNA-containing fractions from formaldehyde cross-linking gradients. Cycling or mitosis-arrested HeLa S3 cells were untreated (A and C) or treated with 0.2% formaldehyde (B and D) in vivo. Cells were lysed, and extracts were fractionated on CsCl gradients as described in Materials and Methods. Aliquots of each fraction were analyzed by agarose gel electrophoresis and ethidium bromide staining (A to D). Fractions 1 were from the bottom of the gradient, and fractions 12 were from the top of the gradient. Fractions 13 to 16 did not contain DNA in untreated or formaldehyde (form.)-treated samples and are not shown. (E) DNA-containing fractions from a gradient of cycling cells treated with formaldehyde were pooled and either dialyzed (top three lanes) or untreated (bottom lane). Dialyzed fractions were treated with either DNase I or RNase A, as indicated, and analyzed by agarose gel electrophoresis and ethidium bromide staining.

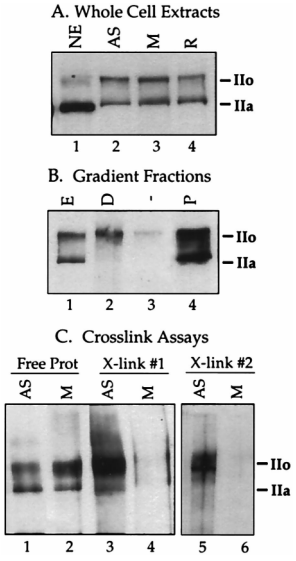

FIG. 8. Formaldehyde cross-linking of RNAP II large subunit to interphase and mitotic DNA. All blots were probed with monoclonal antibody ARNA-3, which reacts with an epitope on the large subunit of RNAP II and recognizes all phosphorylation variants of the large subunit of RNAP II. (A) Western blot analysis of whole-cell extracts prepared from cycling (AS, lane 2), mitosis-arrested (M, lane 3), or released (R, lane 4) HeLa S3 cells. An aliquot of a HeLa cell nuclear extract (NE, lane 1) contains predominantly the nonphosphorylated (IIa) form of the RNAP II large subunit, as seen previously (47, 48, 65). Whole-cell extracts contain a mixture of phosphorylated (IIo) and nonphosphorylated (IIa) RNAP II large subunits. (B) Western blot analysis of fractions from a gradient prepared from cycling HeLa S3 cells treated with 0.2% formaldehyde. Shown is the RNAP II large subunit content of DNA-containing fractions from the center of the gradient (D, lane 2), of free protein at the top of the gradient (P, lane 4), and of fractions located between the DNA-containing fractions and free protein (−, lane 3). An aliquot of whole-cell extract is shown for comparison (E, lane 1). (C) Western blot analysis of RNAP II large subunit content in cross-linked and free protein fractions from cycling and mitotic cells. Equal numbers of cycling (AS) or mitosis-arrested (M) HeLa S3 cells were treated with 0.2% formaldehyde in vivo. Cells were lysed, extracts were fractionated on CsCl gradients, and material from the DNA-containing fractions (X-link) was pooled, as was material from free protein fractions (Free Prot). Pooled fractions were treated with nucleases, and proteins were analyzed by Western blotting. Lanes 3 and 4 and lanes 5 and 6 contain the entire pooled material from the DNA-containing cross-linked fractions. Lanes 1 and 2 contain 7% of the total free protein from the gradients. Lanes 1 to 4 show fractions from one experiment; lanes 5 and 6 show DNA-cross-linked fractions from another experiment.

by Western blotting. A Western blot of whole-cell lysates prepared from cells not treated with formaldehyde is shown for comparison (Fig. 8A). Both RNAP IIA and RNAP IIO (as indicated by the presence of the IIa and IIo large subunits) were present in whole cell lysates of cycling, mitotic, and released HeLa S3 cells (Fig. 8A, lanes 2 to 4). Interestingly, hyperphosphorylated (IIO) forms of RNAP II were present in mitotic cells, even though little transcription occurs in these cells. Cycling, formaldehyde-treated cells also contained both RNAP IIA and IIO in the free protein fractions (Fig. 8B, lane 4) and predominantly RNAP IIO in the cross-linked fractions (Fig. 8B, lane 2). Only minor amounts of RNAP II appeared in fractions taken from between the center and top of the gradient (Fig. 8B, lane 3). These data show that the RNAP IIO form of RNAP II cross-links to DNA in cycling cells, confirming previous data that show RNAP IIO to be the transcriptionally engaged form of the enzyme in vivo (3, 6, 65). Although RNAP IIO cross-links to DNA in cycling cells (Fig. 8C, lanes 3 and 5), little if any RNAP II cross-links to DNA in mitotic cells (Fig. 8C, lanes 4 and 6). Both RNAP IIA and IIO are present in the free protein fractions from both cycling and mitotic cross-linked cells (Fig. 8C, lanes 1 and 2). The amount of hyperphosphorylated RNAP II appears to be enhanced in mitotic free protein compared to cycling free protein or whole-cell extracts (compare Fig. 8C, lane 2, with Fig. 8C, lane 1, and with Fig. 8A, lanes 2 and 3). It is possible that kinases present in the mitotic cell extracts hyperphosphorylate the RNAP II large subunit during the lysate and gradient preparation.

We confirmed that formaldehyde cross-linking to DNA was as efficient in mitotic cells as in cycling cells by silver staining an SDS-polyacrylamide gel containing cross-linked samples from mitotic and cycling cells (data not shown). The amount of cross-linked protein was as great, or greater, in mitotic DNA-cross-linked fractions as in cycling DNA-cross-linked samples. Therefore, we conclude that RNAP II fails to cross-link to mitotic chromatin because the majority of the enzyme is not in close contact with chromatin during mitosis.

In summary, our run-on transcription experiments indicate that RNAP II transcription is repressed on a number of specific gene regions during mitosis in human cells. We also conclude that the repression of RNAP II transcription is accompanied by the stripping of the majority of RNAP II transcription elongation complexes from DNA, as reflected in the reduction of RNAP II cross-linking to DNA, the inability to release engaged RNAP II transcription complexes with high

salt or Sarkosyl, and the visual exclusion of RNAP II from mitotic chromosomes.

DISCUSSION

RNAP II transcription is repressed during M phase. In this study, we find that run-on transcription signals on all RNAP II-transcribed genes that we assay is reduced in mitotic HeLa S3 cells. The degree of reduction varies, with transcription signals ranging from 6% of interphase levels in the case of the histone H2b gene to 41% of interphase levels in the case of the 5′ region of the γ-actin gene. Some of the residual transcription signal in mitotic cells may be attributed to the presence of unarrested cells, which constitute approximately 5% of the population, as assessed by visual scoring of mitotic index. However, the higher levels of mitotic transcription signal in some gene regions, such as γ-actin 5′, may reflect the presence of transcriptionally engaged RNAP II elongation complexes on these regions during mitosis. This raises the possibility that some RNAP II transcribed genes either escape mitotic silencing or remain associated with transcriptionally engaged RNAP II ternary complexes during mitosis.

As we have observed varying degrees of transcription readthrough in the γ-actin 5′ and 3′ regions (65), it is possible that the γ-actin gene is regulated by a conditional block to transcription elongation, similar to those observed in the *Drosophila hsp70* gene (50) and the human c-*myc* gene (30). It is possible that the γ-actin gene is maintained in a transcriptionally engaged state, primed for rapid reexpression following mitosis, by the presence of a paused RNAP II complex at the promoter. Although the γ-actin gene may remain associated with an initiated but paused RNAP II throughout M phase, this does not appear to be the case for the c-*myc*, c-*fos*, *GAPDH*, and histone H2b genes. Both c-*myc* and c-*fos* are regulated in part through release of promoter-proximal paused RNAP II complexes (reviewed in reference 62); however, we were unable to detect significant amounts of run-on transcription signals at the 5′ ends of these genes during mitosis under normal, high-salt, or Sarkosyl conditions. This suggests that these two growth-related genes do not remain in a transcriptionally engaged state through M phase and that transcription may need to reinitiate at these promoters as cells enter G_1. It is possible that promoter-proximal paused RNAP II complexes are not present on the c-*myc* promoter in HeLa cells, as these cells exhibit constitutive readthrough transcription in the c-*myc* gene (reference 65 and Fig. 1).

Despite the possibility that some genes retain functional RNAP II elongation complexes during M phase, our transcription analyses support the conclusions of others that the majority of RNAP II transcription is repressed during mitosis (5, 11, 14, 18, 27, 29, 36, 37, 44, 55, 68). Because run-on transcription assays measure the activity of both transcriptionally active and engaged but paused RNA polymerases, we cannot determine how much of the run-on transcription signals that we observe during mitosis represents active rather than potentially active transcription. Recent data from Shermoen and O'Farrell (59) and Rothe et al. (49) show that nascent transcripts are aborted during mitosis and also support the conclusion that RNAP II transcription is repressed during mitosis. Based on data from our study, we conclude that the abortion of transcripts observed by Shermoen and O'Farrell and by Rothe et al. is accompanied by abortion of RNAP II ternary complexes.

RNAP II is released from mitotic chromatin and enters the cytoplasm. A number of explanations for how transcription is repressed during mitosis have been presented (19, 59). These hypotheses fall into two general categories: blockage hypotheses and ejection hypotheses. In one scenario, RNAP II complexes remain transcriptionally engaged on DNA during mitosis but are blocked, perhaps due to the inhibitory effects of condensed chromatin. Nascent transcripts may be degraded during the block period. In the second scenario, either engaged RNAP II complexes are actively ejected from DNA or initiation is repressed, allowing elongating complexes to run off or to terminate normally. In this second scenario, actively transcribed genes are denuded of RNAP II transcription complexes at each mitosis.

Our data support the second (ejection) hypothesis. Our DNA-protein cross-linking studies show that the RNAP II large subunit does not cross-link to DNA in mitotic cells. As the large subunit of RNAP II is the site of DNA and nascent RNA binding (reviewed in reference 75), loss of large subunit binding to DNA argues that the enzyme itself is not in contact with mitotic chromatin. These cross-linking studies, in combination with the salt and Sarkosyl transcription release studies and indirect immunofluorescence, lead to the conclusion that the RNAP II transcription elongation machinery is disengaged from mitotic chromosomes during mitosis. A number of RNAP II basal and regulatory transcription factors also appear to dissociate from DNA during mitosis. These include the human heat shock factor HSF1, Oct-1, Oct-2, Ets-1, B-Myb, c-Fos, E2F-1, Sp1, and Bcl-6 (33) and the basal factors TFIID and the MO15 subunit of TFIIH (58, 67). Some of these factors, in their mitotic state, show reduced activity or reduced ability to bind DNA (33, 57, 58). These data suggest that RNAP II transcription initiation may be repressed during mitosis through loss of DNA-binding transcription factors from promoter regions. Our data do not address the question of whether the loss of RNAP II from mitotic chromatin is due to the active ejection of elongating RNAP II transcription complexes or to normal termination events that follow cessation of transcription initiation. In either case, the repression of RNAP II transcription during mitosis likely involves dissociation of a large proportion of the RNAP II initiation and elongation machinery from mitotic chromatin.

Our finding that the majority of RNAP II is absent from mitotic chromosomes contradicts two earlier studies that concluded that the majority of RNAP II remained transcriptionally engaged on isolated metaphase chromatin (15, 34). Although we cannot definitively state the reason why conclusions from these studies contradict ours, it is possible that chromatin isolation procedures lead to initiation of free RNA polymerases.

Mechanism of mitotic repression of RNAP II transcription. The mechanism by which RNAP II transcription is repressed during mitosis is unknown, although it has been suggested that both chromatin modifications and mitotic phosphorylation events can trigger dissociation of basal and regulatory factors from DNA (33, 38). Hyperphosphorylation of both basal and regulatory transcription factors has been detected in mitotic cells (16, 19, 57, 58, 73). We have also observed hyperphosphorylation of TATA-binding protein, RAP74, and TFIIEα in mitosis-arrested cells (43). Mitotic phosphorylation of some transcription factors interferes with their DNA-binding and biochemical activities (33, 57, 58). In addition, phosphorylation of the CTD of the RNAP II large subunit by maturation-promoting factor disrupts preformed transcription complexes in vitro (76). It is not yet clear, however, whether mitotic phosphorylation of RNAP II transcription factors in vivo precipitates transcription repression or is a consequence of the heightened phosphorylation activity in mitotic cells (52) and follows events in early mitosis that trigger the dissociation of transcription factors and RNAP II.

Our Western blot analyses reveal no major differences in the phosphorylation profiles of the RNAP II large subunit in cycling and mitotic cells. Given the number of phosphorylation sites on the CTD, and the nonlinear changes in migration of the large subunit of RNAP II in response to increasing CTD phosphorylation (79), subtle differences such as changes in the specific sites or relative amounts of serine, threonine, or tyrosine phosphorylation may not be revealed by these analyses. The presence of RNAP IIO in mitotic cells has been observed by others (26) and led to the conclusion that hyperphosphorylation of the CTD does not correlate with the transcriptional activity of the enzyme, because transcription is repressed in mitosis. It is also possible that the hyperphosphorylated IIO-like forms present in mitotic cells are not identical to the hyperphosphorylated IIO forms that accompany transcription elongation. Mitotic RNAP IIO may arise through the actions of one or more kinases or phosphatases present in the cytoplasm of mitotic cells. In support of this view, we have observed an increase in the relative abundance of RNAP IIO during the 45- to 48-h CsCl gradient preparation of formaldehyde-cross-linked mitotic extracts. Regardless of its origins, the mitotic hyperphosphorylated forms of RNAP II may be deficient in transcription initiation during mitosis, as RNAP IIO must be converted to RNAP IIA in order to initiate transcription (reviewed in reference 10).

The effects of mitotic phosphorylation on transcription factor activities are still not clear, and results of experiments addressing this issue appear to depend on the experimental method used. Whole-cell extracts prepared from mitotic HeLa cells appear to be as competent as those from cycling cells in executing basal transcription of an RNAP II promoter in vitro, suggesting that the activity of the soluble enzyme and its basal factors may not be downregulated during mitosis (73). In contrast, mitotic TFIID, purified in the presence of phosphatase inhibitors, is unable to support activator-dependent transcription in vitro (58).

As the repression and later reactivation of transcription coincide with the condensation and decondensation of mitotic chromosomes, it is also possible that the two events are functionally linked (33). There may be a requirement to strip DNA-binding proteins and large transcription elongation complexes from DNA in order to condense chromosomes. However, the mechanisms whereby chromatin condensation could lead to ejection of the RNAP II transcription machinery await investigation.

Mitotic repression of RNAP II transcription and regulatory checkpoints. The cyclic stripping and reassociation of RNAP II and transcription factors at each mitosis could have several regulatory consequences. First, as Shermoen and O'Farrell point out, the rapid mitotic cycles of early embryos could preclude expression of long transcription units, as nascent RNAs would be truncated at each mitosis and transcription would need to reinitiate anew at the end of each cleavage cycle (42, 59). Expression of long transcription units (such as *Ubx*) would be precluded until cell cycles were long enough to allow full-length transcription. This could be one mechanism that operates during early development to regulate the expression of long transcription units (42, 49).

Second, if transcription complexes and activators are lost from actively transcribed genes at each mitosis, and if these same genes are to be expressed following mitosis, mechanisms would need to exist to tag actively transcribed genes for reexpression. These mechanisms would need to remain stable throughout the dramatic structural and biochemical transformations that occur to mitotic chromatin. It is tempting to speculate that this tagging mechanism may involve aspects of chromatin structure that are independent of transcription factor binding. Evidence supporting this speculation comes from studies showing that DNase I sensitivity patterns characteristic of actively transcribed genes persist on mitotic chromosomes (25). Similarly, mechanisms would need to exist to maintain transcriptional silencing on those genes that are not to be expressed at the end of mitosis.

Third, the cell may take advantage of the cyclic stripping of RNAP II transcription factors and elongation complexes from actively transcribed genes, in order to alter gene expression. These altered patterns of gene expression could facilitate cell cycle arrest and differentiation. Interestingly, the decision to remain in the cell cycle, or to exit into quiescence (G_0), is made within a 3.5-h period immediately following exit from mitosis (78). Cells exiting mitosis require the presence of serum growth factors throughout this time in order to proceed into G_1/S. If serum is withdrawn at any time during this period, cells leave the cell cycle and enter G_0. Therefore, this critical postmitotic period represents a cell cycle checkpoint during which the cell monitors the presence of cues that signal proliferation or quiescence. It is conceivable that the selective and sequential transcriptional activation of specific subsets of genes during this postmitotic phase could determine the outcome of this checkpoint. In agreement with this idea, it has been shown that the immediate-early competence genes c-*fos* and c-*jun* are induced immediately after mitosis (9). In addition, a number of proliferation-associated genes, such as c-*myc* and the ornithine decarboxylase and p53 genes, are sequentially induced at the end of mitosis in a pattern similar to that in cells emerging from quiescence (8). It is possible that mitotic transcriptional silencing plays an important role in the M-G_1/G_0 cell cycle checkpoint by allowing labile gene products of proliferation-associated genes to decay to basal levels. In addition, the stripping of transcription initiation and elongation factors from these genes would prevent their rapid reexpression at the end of mitosis. In this way, the mitotic repression of transcription could reset the cell to a proliferation ground state from which it could decide to reenter the cell cycle or to enter quiescence.

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