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University of Alberta

Regulation of RNAP II Transcription During Mitosis

by

Glenn Gerard Parsons

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science

Department of Biochemistry

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Charlotte Spencer

Dr. Charlotte Spencer

Dr. Colin Rasmussen

Dr. Michael Schultz

Dr. Ross Hodgetts

Date: 21 March, 1997

What did the biologist say when he dropped his slides on the lab floor? Don't step on mitosis.

Anne Michaels
Fugitive Pieces

For Nicholas and Annastasia Flynn
West Bay Centre, Newfoundland

Abstract

Previous studies, employing RNA labelling, have indicated that RNA synthesis is repressed during mitosis. We characterized RNA polymerase (RNAP) II transcription on a variety of genes in mitotic-arrested HeLa cells using run-on transcription assays. During mitosis, transcription of most genes decreases by roughly 80-95%, indicating that the activity or number of engaged RNAP II molecules is decreased. Immunofluorescent localization and protein-DNA cross-linking analysis of RNAP II in mitosis-arrested cells revealed that RNAP II is dispersed from chromatin during mitotic arrest. TBP is also dispersed from mitotic chromosomes, suggesting that the physical stripping of RNAP II general transcription factors may contribute to the repression of RNAP II transcription during mitosis. Also, the RNAP II general transcription factors TBP, RAP74, and TFIIE-α are hyperphosphorylated during mitosis. The relocation and modification of the RNAP II transcriptional machinery during mitosis may have implications for the control of proliferation during early G_1.

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List of Abbreviations

AdMLP	adenovirus major late promoter
ATP	adenosine triphosphate
Å	angstrom
bp	base pair
BSA	bovine serum albumin
CAK	cdk-activating kinase
cdk	cyclin-dependent kinase
Ci	Curie
CsCl	cesium chloride
CTD	carboxy terminal domain
CTP	cytidine triphosphate
DAPI	4'6'-diamidino-2-phenylindole
dCTP	deoxycytidine triphosphate
°C	degree(s) celsius
DHFR	dihydrofolate reductase
DMEM	Dulbecco's Modified Eagles Medium
DNA	deoxyribonucleic acid
DTT	dithiothreitol
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol-bis[β-aminoethyl ether]-N,N,N',N'-tetracetic acid

FACS	fluorescence-activated cell sorting
g	acceleration due to gravity (9.81 $m \cdot sec^{-2}$)
G_0	quiescence
G_1	gap 1 phase
G_1pm	gap1 post-mitosis phase
G_2	gap 2 phase
GTF	general transcription factor
GTP	guanosine triphosphate
h	hour(s)
HEPES	Na-N-[2hydroxyethyl]piperazine-N'-[2ethanesulfonic acid]
HMG	high mobility group
IGS	intergenic spacer sequences
IIa	hypophosphorylated RPB1
IIo	hyperphosphoryated RPB1
Inr	initiator element
kb	kilobase
kDa	kiloDalton
KCl	potassium chloride
L	leucine
L	litre
M	molar
M-phase	mitosis
mg	milligram

ml	millilitre
µg	microgram
µl	microlitre
min	minute(s)
mM	millimolar
mRNA	messenger RNA
MPF	mitosis/maturation-promoting factor
nm	nanometre
NOR	nucleolus-organizing region
NPC	nuclear pore complex
pRb	retinoblastoma protein
R	arginine
R	restriction point
rNTPs	ribonucleoside triphosphates
RNA	ribonucleic acid
rRNA	ribosomal RNA
RNAP	RNA polymerase
RPB1	RNA polymerase II large subunit
P	proline
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PBS-T	tween 20 in PBS

PIC	pre-initiation complex
PMSF	phenylmethyl sulfonyl chloride
PSE	proximal sequence element
§	section
S	serine
S. cerevisiae	*Saccharomyces cerevisiae*
S.pombe	*Schizosaccharomyces pombe*
S-phase	DNA synthesis phase
SDS	sodium dodecyl sulfate
sec	second(s)
SNAPc	snRNA gene activating protein complex
snRNA	small nuclear RNA
SRB	suppressor of RNA polymerase IIB
SRF	serum response factor
T	threonine
TAF	TBP-associated factor
TBP	TATA-binding protein
TEMED	N,N,N',N'-tetramethyl-ethylenediamine
TES	N-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid
TPCK	N-tosyl-L-phenylalanine chloromethyl ketone
Tris	tris[hydroxymethyl]aminomethane
U	units

Ubx	*ultrabithorax*
UBF	upstream binding factor
UCE	upstream control element
UTP	uridine triphosphate
V	volts
v	volume
w	weight
WCL	whole cell lysate
Y	tyrosine

1. INTRODUCTION AND LITERATURE REVIEW

1.1. Transcription

1.1.1. Overview

Transcription is the biochemical reaction whereby RNA is synthesized using a DNA template. This RNA may act as a template for the synthesis of proteins, serve a structural function (as in ribosomes), or participate in other biochemical reactions, such as translation. The diversity of eukaryotic cellular functions involving RNA is reflected in the evolutionary development of three distinct enzymatic systems for the catalysis of transcription. These systems are denoted by the form of RNA polymerase (RNAP) that is central to each. Each system carries out the transcription of a different class of genes: the RNAP I system carries out the synthesis of large ribosomal RNAs (rRNAs), the RNAP II system carries out the synthesis of protein-encoding and most small nuclear RNA (snRNA) genes, and the RNAP III system carries out the synthesis of transfer RNAs (tRNAs), the 5S rRNA and the U6 snRNA (Zawel and Reinberg, 1995).

Despite their differences in structure and function, each of these enzymatic systems catalyzes RNA synthesis in the same basic manner. This mechanism may be dissected into five discrete stages: *pre-initiation complex (PIC) formation*, where a complex of proteins required for the commencement of transcription is assembled onto a promoter; *initiation*, which is demarcated by the formation of the first RNA phosphodiester bond, *promoter clearance*, wherein the RNAP leaves the promoter region and escapes from the PIC; *elongation*, during which the RNAP traverses the gene, and extends the nascent RNA transcript; and *termination*, when RNA phosphodiester bond synthesis ceases and the RNAP is released from the DNA template. For each RNAP system these steps are executed by a different collection of proteins, referred to as general

transcription factors (GTFs), which are required for the transcription of all genes of a given class.

1.1.2. RNAP II

Consistent with its pivotal role in regulating protein expression, transcription by RNAP II in human cells is a complex process, involving roughly sixty to seventy polypeptides (Berk, 1995). Central in this process is RNAP II itself, a twelve-subunit enzyme. The largest subunit of RNAP II (RPB1) possesses a unique carboxy-terminal domain (CTD) that consists, in humans, of 52 heptapeptide repeats of the sequence YSPTSPS (Young, 1991; Woychik and Young, 1990). Biochemical characterization of the other proteins involved in RNAP II transcription has revealed the existence of six major GTF protein complexes, which, in addition to RNAP II, are required for the transcription of class II genes: TFIIA, TFIIB, TFIID, TFIIE, TFIIF, and TFIIH (reviewed in Roeder, 1996; Zawel and Reinberg, 1993). The specific roles that many of these proteins play in orchestrating transcriptional events have been widely investigated, providing an outline of the sequence of biochemical steps constituting RNAP II transcription.

1.1.2.1. Pre-initiation complex formation

Transcription begins with the formation of a PIC on the core promoter of a gene. Class II gene promoters contain either a TATA box, located from 25 to 30 nt upstream of the transcription start site, an initiator (Inr), overlapping the transcription start site, or both of these elements (Roeder, 1996; Zawel and Reinberg, 1993; Young, 1991). The TATA element and Inr sequence are both capable of supporting transcriptional initiation independently, but they can also function synergistically (Concino et al., 1992; Weis and Reinberg, 1992). On TATA-containing promoters, PIC formation must commence with the binding of TFIID to the TATA sequence. TFIID contains the TATA-binding protein (TBP),

which binds specifically to the TATA element (reviewed in Zawel and Reinberg, 1995; Hernandez, 1993). The TFIID complex also contains other peptides, known as TBP-associated factors, or TAFs. These proteins play a role in facilitating the response of the transcriptional apparatus to activators, and some, such as $TAF_{II}150$ of *Drosophila*, interact with DNA (Burke and Kadonaga, 1996; Verrijzer et al., 1994). TAF-DNA contacts may stabilize the TBP-DNA interaction on TATA-containing promoters (Verrijzer et al., 1995). TFIIA, a heterotrimer which binds TBP and interacts nonspecifically with DNA, can also stabilize the TBP-DNA association (reviewed in Jacobson and Tjian, 1996).

PIC formation on TATA-less promoters, which possess Inr elements, may involve several different mechanisms (reviewed in Weis and Reinberg, 1992). TAFs may serve to facilitate TFIID binding on promoters lacking a TATA element by recognizing the Inr element (Martinez et al., 1994; Verrijzer et al., 1994; reviewed in Verrijzer and Tjian, 1996). Alternative mechanisms may include the action of specific Inr binding proteins, which recruit TFIID and other GTFs to the PIC, or direct recognition of the Inr by RNAP II (reviewed in Roeder, 1996; Zawel and Reinberg, 1995; Weis and Reinberg, 1992). Regardless of whether or not a promoter contains a TATA element, TFIID, or at least TBP, must ultimately be recruited to the promoter for a functional PIC to assemble *in vitro* (the adeno-associated virus P5 promoter is the only known exception; see Usheva and Shenk, 1994).

In addition to TFIID, the formation of a full PIC requires the assembly of RNAP II and other GTFs. Precisely how this assembly occurs remains a contentious issue. In reconstituted *in vitro* transcription systems, PIC formation takes place in a stepwise fashion, with TFIIB binding to TFIID, followed by the association of an RNAP II/TFIIF subcomplex, and finally, the sequential addition of TFIIE and TFIIH (reviewed in Buratowski, 1994; Zawel and Reinberg, 1993). *In vivo*, however, this assembly may occur largely in a single step, via the recruitment of a holoenzyme containing RNAP II, GTFs (except

TFIID), and other proteins (reviewed in Koleske and Young, 1995). *In vitro*, the components of the holoenzyme can form a complex independently of DNA (Serizawa et al., 1993b), and purified holoenzyme is capable of specific initiation on class II promoters *in vitro* (Koleske and Young, 1994). Also, the RNAP II holoenzyme contains a subcomplex of proteins, known as the mediator of activation, which associates with the CTD and is required for responsiveness to transcriptional activators (Hengartner et al., 1995; reviewed in Björklund and Kim, 1996). In accordance with a role for this CTD-associated mediator complex in activation of transcription, the CTD of RPB1 is required for the activation of RNAP II transcription by enhancer elements *in vivo* (Gerber et al., 1995). In *S. cerevisiae*, the mediator contains nine SRB (Suppressor of RNA polymerase B) proteins, which are found only in the holoenzyme (Liao et al., 1995). These proteins are essential for RNAP II transcription in *S. cerevisiae* (Thompson and Young, 1995), suggesting that the holoenzyme is essential for transcription on most class II promoters *in vivo*. Also, various components of the holoenzyme, including SRB2, SRB5, and the CTD of RNAP II bind to TBP *in vitro*, suggesting a means by which the holoenzyme could enter TFIID-containing PICs (Thompson et al., 1993; Koleske et al., 1992; Usheva et al., 1992). However, it is important to stress that it has not been definitively proven that the holoenzyme is recruited, as a subcomplex, onto promoters during PIC assembly *in vivo*. Therefore, it remains possible that the holoenzyme is actually a promoter-bound intermediate in stepwise PIC formation (Struhl, 1996) .

If the holoenzyme is the form of RNAP II responsible for initiation *in vivo*, its precise composition has important implications for the mechanisms of transcriptional activation (see §1.1.2.7). However, its makeup is at present uncertain. Young and colleagues purified an RNAP II holoenzyme from *S. cerevisiae* which includes RNAP II, TFIIB, TFIIH, and a mediator subcomplex consisting of TFIIF, nine SRB proteins (Koleske and Young, 1994), the SWI\SNF chromatin remodelling factor, GAL11, and SUG1 (Wilson et al., 1996). In

contrast, another holoenzyme isolated from *S.cerevisiae* contains no GTF except TFIIF (Kim et al., 1994). Two groups have isolated holoenzyme from mammalian cells: one contains all known GTFs, including TFIIB, TFIID, TFIIE, TFIIF, and TFIIH (Carey, 1995; Ossipow et al., 1995), whereas the other lacks TFIIB and TFIID (Maldonado et al., 1996). The source of these discrepancies is not known. Although different methods of isolation may be responsible (Maldonado et al., 1996), it is certainly possible that there are multiple species of holoenzyme (Koleske and Young, 1995; Maldonado and Reinberg, 1995).

On the snRNA genes transcribed by RNAP II, PIC assembly occurs via a different mechanism (reviewed in Zawel and Reinberg, 1995). These genes have promoters which lack a TATA sequence, but which possess a proximal sequence element (PSE) upstream of the transcription start site. This element is recognized by a protein complex, known as SNAPc, or the PSE-binding transcription factor. This complex is composed of four polypeptides and a varying amount of TBP (Sadowski et al., 1996; Yoon and Roeder, 1996; Sadowski et al., 1993). Once bound to the PSE, SNAPc allows RNAP II to associate with the promoter; no other GTFs are required for PIC formation or transcription.

1.1.2.2. Initiation

Before the first phosphodiester bond in the nascent RNA chain can be formed, the PIC must be made "competent" to carry out transcription. This is achieved, at least in part, by an isomerization of the PIC/DNA template complex, resulting in an unwinding of the DNA duplex near the transcription start site to form an "open complex". *In vitro* transcription reactions display a requirement for ATP hydrolysis, and this requirement has been linked to open complex formation (Jiang et al., 1993). TFIIH, which contains two proteins with ATPase/helicase activity, ERCC2 and ERCC3, has been suggested to carry out open complex formation, causing the DNA template to melt between positions -9

and +1 (Holstege et al., 1996). In support of a role for a TFIIH helicase in this process, the *S. cerevisiae* homolog of ERCC3 is essential for transcription (Guzder et al., 1994a; Guzder et al., 1994b; Schaeffer et al., 1994). TFIIE has also been implicated in open complex formation (Holstege et al., 1995), and, at low concentrations, TFIIE stimulates the helicase activity of TFIIH *in vitro* (Serizawa et al., 1994). However, other studies have concluded that the large subunit of TFIIE, TFIIE-α, inhibits ERCC3 *in vitro* (Drapkin et al., 1994; Serizawa et al., 1994). Also, there is evidence that TFIIH and TFIIE are not involved in open complex formation, and instead play an ATP-dependent role during promoter clearance (Goodrich and Tjian, 1994; Maxon et al., 1994; reviewed in Zawel and Reinberg, 1995; Drapkin and Reinberg, 1994). TFIIF has also been suggested to play a role in open complex formation (Robert et al., 1996; Pan and Greenblatt, 1994).

1.1.2.3. Promoter clearance

Upon formation of the open complex, and in the presence of ribonucleotides, RNA synthesis begins upon the single stranded DNA template. The transition from initiation to elongation, or the escape of RNAP II from the promoter region, is referred to as promoter clearance. It has been proposed that phosphorylation of the CTD is involved in this event. Two forms of RPB1 exist *in vivo*: IIa, which possesses a hypophosphorylated CTD, and IIo, which possesses a hyperphosphorylated CTD (IIo and IIa refer to forms of RPB1, whereas IIO and IIA refer to forms of RNAP II; reviewed in Dahmus, 1994). The IIa form of the RNAP II large subunit is found in PICs, while the IIo form is present in elongation complexes, implying that CTD phosphorylation characterizes the transition from initiation to elongation (Payne et al., 1989; Cadena and Dahmus, 1987). In support of this idea, *in vitro* experiments have shown that CTD phosphorylation is essential for promoter clearance on the *dihydrofolate reductase*

(*DHFR*) promoter (Akoulitchev et al., 1995), though this is not the case on all promoters (Serizawa et al., 1993a). On the *DHFR* promoter, CTD phosphorylation is not needed for the formation of the first RNA phosphodiester bond (Akoulitchev et al., 1995), and likely is required to disrupt contacts between RNAP II and other PIC components, such as TBP or mediator proteins. Disruption of these contacts could allow RNAP II to escape the promoter region (Svejstrup et al., 1996; Barberis et al., 1995; Zawel and Reinberg, 1995; Dahmus, 1994; Zawel and Reinberg, 1993; Sigler, 1988). Consistent with this model, RNAP II molecules initiated but stalled on the 5′ end of various *Drosophila* genes have a hypophosphorylated CTD. When those RNAP II molecules pass into the elongation phase, their CTDs become hyperphosphorylated (O'Brien et al., 1994).

The identification of the kinases responsible for CTD phosphorylation has been the objective of much research (reviewed in Dahmus, 1994). Many kinases will phosphorylate the CTD *in vitro*, including mitosis/maturation-promoting factor (MPF) (Cisek and Corden, 1989), DNA-dependent protein kinase (Dvir et el., 1992; Peterson et al., 1992), and c-Abl (Baskaran et al., 1993). CTD phosphorylation can also be carried out by one or more of the RNAP II GTFs (Laybourn and Dahmus, 1990). Given their necessary colocalization with RNAP II in PICs, GTF CTD kinases have been viewed as favoured candidates for *in vivo* CTD kinases involved in transcriptional processes. Though the mediator proteins cdk8 and cyclin C form a cdk/cyclin complex which phosphorylates the CTD *in vitro* (Rickert et al., 1996), the strongest candidate for the GTF CTD kinase responsible for transcription dependent CTD phosphorylation is TFIIH. The cdk7(MO15)/cyclin H/MAT1 subcomplex of TFIIH phosphorylates the CTD of RNAP II *in vitro* (Feaver et al., 1991), and its activity is required for a post-initiation step in transcription on the *DHFR* promoter (Akoulitchev et al., 1995), though not on other promoters (Mäkelä et al., 1995). In *S. cerevisiae*, the homologue of cdk7, Kin28, is essential for normal RNAP II phosphorylation and

transcription (Valay et al., 1995). The presence of RNAP II GTFs, including TFIIE and the mediator, stimulates the CTD kinase activity of TFIIH (Ohkuma et al., 1995; Drapkin et al., 1994; Kim et al., 1994; Ohkuma and Roeder, 1994; Roy et al., 1994; Lu et al., 1992). This suggests that PIC formation is required for TFIIH to function optimally as a CTD kinase, further implicating it as the GTF CTD kinase operating in the context of the PIC

1.1.2.4. Elongation

RNAP II transcription elongation is a discontinuous process, and its rate depends strongly on the DNA sequence being transcribed. Purified RNAPs transcribe *in vitro* at rates far below *in vivo* elongation rates, and stall frequently (Reines et al., 1996; Izban and Luse, 1992). *In vivo*, chromatin structures and DNA binding proteins may impede the action of elongating RNAPs, causing pausing or arrest (Kerppola and Kane, 1991). The current model for the mechanism of RNAP elongation, the "inchworm model", implies that RNAP movement is a discontinuous process in which the catalytic site of the enzyme can move independently of the nascent RNA chain. In this model, pausing occurs when the catalytic site transiently falls behind the 3′ terminus of the RNA, and arrest is the result of a severe dislocation of the two entities, such that 3′ cleavage of the RNA is required for realignment (reviewed in Aso et al., 1995; Chamberlin, 1995).

The modulation of transcript production through control over elongation rate has been documented for several genes, including c-*myc*, c-*fos*, and the *Drosophila hsp70* gene (reviewed in Spencer and Groudine, 1990). Recently, the biochemistry of RNAP II elongation has received much attention, and a number of factors which affect RNAP II elongation in a non-gene-specific manner have been isolated (reviewed in Reines et al., 1996; Aso et al., 1995). Some of these, such as pTEFb and SII, act to prevent RNAP II arrest. These act by distinct

mechanisms: pTEFb may function by affecting CTD phosphorylation (Aso et al., 1995), whereas SII activates a polymerase-associated endoribonuclease activity, which cleaves nascent transcripts in stalled complexes, so as to realign the RNA 3′ terminus with the catalytic site of the enzyme (reviewed in Reines et al., 1996). Others, such as TFIIF, elongin, and ELL are incapable of releasing stalled polymerases, but act to decrease the amount of transient pausing, reducing the probability of arrest. Though their mode of action remains speculative, they have been proposed to act by tethering the 3′ end of the transcript to the catalytic site (Takagi et al., 1995). The *in vivo* importance of these factors has been emphasized by their involvement in several human cancers, such as von Hippel-Lindau syndrome in the case of elongin (Duan et al., 1995; Kibel et al., 1995). Although TFIIH does not associate stably with elongation complexes (Zawel et al., 1995), its kinase activity appears to have a role in elongation (Blau et al., 1996; Yankulov et al., 1995; reviewed in McKnight, 1996; Svejstrup et al., 1996).

1.1.2.5. Termination

In contrast to initiation and elongation, termination of transcription by RNAP II is not well understood biochemically (reviewed in Richardson, 1993; Proudfoot, 1989). Termination on protein-encoding genes occurs after the formation and release of mature transcripts. Once a transcribing RNAP II finds itself downstream of two DNA elements, the poly(A) site and a GT-rich region, the nascent RNA is cleaved at a specific site between these two elements. Both elements are required for transcript cleavage. The transcript is released and then modified at its 3′ end with a poly-adenylic acid (poly-A) tail by the enzyme poly-A polymerase. Polyadenylation may increase mRNA stability and translatability (Lewis et al., 1995). RNAP II continues transcribing until it is dislodged from the DNA template at a heterogenous collection of sites (Maa et al., 1990; Pribyl et al., 1988; Rohrbaugh et al., 1985; Citron et al, 1984;

Hagenbüchle et al., 1984; LeMeur et al., 1984). The action of these termination sequences is typically contingent upon the integrity of the poly(A) site (Edwalds-Gilbert et al., 1993; Miralles, 1991; Connelly and Manley, 1988; Lanoix and Acheson, 1988; Logan et al. 1987; Whitelaw and Proudfoot, 1986; Falck-Pederson et al., 1985). Terminator recognition by RNAP II has been proposed to involve the action of other DNA sequences near the site of termination (Flaspohler et al., 1995; Kerppolla and Kane, 1988; Dedrick et al., 1987) and the bending of DNA (Kerppola and Kane, 1990). Another suggestion as to the mechanism of termination involves the dislocation from the elongation complex, during transcript cleavage, of a component required for stability (Proudfoot, 1989). It has also been suggested that a termination factor may bind to and destabilize the RNAP after transcript release (Richardson, 1993). It is important to note that the termination of RNAP II transcription on snRNA genes occurs by a distinct mechanism (reviewed in Richardson, 1993; Proudfoot, 1989).

1.1.2.6. Re-initiation

An important point regarding the mechanism of RNAP II transcription *in vivo* is the frequency of re-initiation from established PICs. *In vitro* studies using recombinant or highly purified factors indicate that the TFIID-DNA association is very stable (Zawel et al., 1995; Hawley and Roeder, 1987). These studies suggest that once a PIC has formed on a gene *in vivo*, that gene may not require *de novo* PIC formation to undergo another round of transcription (Svaren and Chalkley, 1990). However, other *in vitro* studies suggest that TFIID-DNA complexes remaining after PIC disruption may not be competent for supporting additional rounds of transcription (Van Dyke et al., 1988). Also, the stability of TFIID-DNA complexes *in vivo* may be far less than it is *in vitro* due to the activity of factors, such as the *S. cerevisiae* protein AD1/MOT1, which displace TBP from DNA in an ATP-dependent fashion. Mutations in this factor increase basal

transcription of some genes *in vivo*, suggesting that these factors act to "shut off" RNAP II genes *in vivo* (Auble et al., 1994; Auble and Hahn, 1993; Davis et al., 1992). In fact, *in vitro* transcription reactions in crude *Drosophila* extracts that display competence for re-initiation exhibit complete disassembly of PICs after each round of transcription, implying that each round of transcription requires the removal of TFIID and the formation of a new PIC (Kadonaga, 1990).

1.1.2.7. Regulation

In eukaryotic cells, transcription of protein-encoding genes is a tightly regulated process. Many steps in the process are likely to be affected by the action of sequence-specific transcription factors, which bind to DNA elements (enhancers or silencers) typically located upstream of the promoter region. Each gene possesses a characteristic arrangement of sequence-specific transcription factor binding sites, which dictates when and to what degree the gene will be transcribed (Zawel and Reinberg, 1995). Because of the importance of the activation process *in vivo*, the precise biochemical mechanisms whereby activators control the transcription process have been the subject of much research and controversy.

Some activators of transcription, such as the SWI/SNF complex, act by altering chromatin structure to make DNA binding sites more accessible to other transcription factors (see § 1.4.2) (reviewed in Kingston et al., 1996). An extensive body of *in vitro* evidence suggests that other activators or repressors can affect PIC assembly by binding to GTFs, such as TBP (Klages and Strubin, 1995; reviewed in Lewin, 1990; Ptashne and Gann, 1990), TFIIB (reviewed in Hahn, 1993; Hawley, 1991; Sharp, 1991), TFIIE (Sauer et al., 1995), TFIIF (Joliot et al., 1995), and TFIIH (Tong et al., 1995; Stelzer et al., 1994; Xiao et al., 1994). Once bound to upstream elements, these sequence-specific factors are thought to interact with the promoter region by a looping out of intervening DNA

(reviewed in Lewin, 1990; Sawadogo and Sentenac, 1990). Activator-GTF contacts are thought to facilitate PIC formation by recruiting GTFs to the promoter region. More recently, it has become apparent that other proteins, referred to as cofactors, in addition to the minimal set of factors needed to carry out basal transcription *in vitro* (TBP, TFIIB, TFIIE, TFIIH and RNAP II), are required for activation. Such cofactors include the TAF components of TFIID (Verrijzer and Tjian, 1996; Reese et al., 1994), the mediator complex (reviewed in Björklund and Kim, 1996), the general cofactors (reviewed in Kaiser and Meisterernst, 1996), and TFIIA (reviewed in Jacobson and Tjian, 1996). Since activators may interact with any of these GTFs, and since, as discussed above, the mechanism of PIC assembly is far from clear, the precise mechanisms of transcription activation *in vivo* remain unknown.

1.1.3. RNAPs I and III

Ribosomal RNA constitutes 80% of the RNA present in the cell, and the action of RNAP I accounts for 80% of cellular RNAP activity (Zawel and Reinberg, 1995). In eukaryotic cells, several hundred copies of the rRNA genes are clustered in tandem arrays in genomic regions referred to as nucleolar organizing regions (NORs). In human cells, the 28S, 18S, and 5.8S rRNAs are synthesized together as part of the 45S pre-rRNA. The DNA region encoding the 45S pre-rRNA genes contains 5′ and 3′ external transcribed sequences as well as two internal transcribed sequences, all of which are removed during mature rRNA formation. Tandemly repeated 45S pre-rRNA genes are separated by intergenic spacer sequences (IGS). These regions contain repeated enhancer-like elements, RNAP I promoter sequences, and termination sequences. All of these appear to play a role in the initiation of RNAP I transcription (reviewed in Moss and Stefanovsky, 1995).

RNAP I promoters consist of a roughly 150 bp region containing an upstream control element (UCE) and a core promoter region which encompasses

the transcription start site (reviewed in Moss and Stefanovsky, 1995). The formation of RNAP I PICs on these sequences requires, in addition to RNAP I itself, two components: SL1 and Upstream Binding Factor (UBF) (Bell et al., 1990). Human SL1 is composed of TBP and three RNAP I transcription-specific TAFs of 110, 63 and 46 kDa (Comai et al., 1992). This complex has no DNA binding activity (Learned et al., 1986), and is recruited to promoters by interacting with UBF, which interacts with both the UCE and core sequences in the rDNA promoter (Leblanc et al., 1993; Jantzen et al., 1992). UBF binding induces negative supercoiling in DNA, and may severely alter the structure of the promoter upon binding (Bazett-Jones et al., 1994; Wolffe, 1994). RNAP I enters the PIC by interacting with SL1 (Zawel and Reinberg, 1995). Once RNAP I has joined the PIC, transcription initiation can occur upon the addition of ribonucleotides. Termination of transcription occurs in the IGS via the action of a DNA-binding protein, such as the yeast Rebp1 protein, which binds to a sequence downstream of the termination site, causing the polymerase to pause and facilitating RNAP I release (Lang et al., 1994). The nascent transcript is then cleaved upstream of the arrest site to generate the mature 3′ terminus of the pre-rRNA (Grummt et al., 1985).

RNAP I transcription is affected by many physiological factors which influence cell growth, such as differentiation state, the presence of serum growth factors and nutrient availability (Moss and Stefanovsky, 1995). Regulation of RNAP I transcription may be carried out by the post-translational modification of components of the transcriptional machinery, such as UBF (Labhart, 1994; O'Mahony et al., 1992; Voit et al., 1992) or RNAP I (Mishima et al., 1993), the modification of activity of TIF-IA, a protein required for efficient PIC formation (reviewed in Moss and Stefanovsky, 1995), or the action of repressors which can interfere with RNAP I recruitment into PICs.

RNAP III PIC assembly occurs by a distinct sequence of events on each of the three promoter subclasses which it recognizes (reviewed in Geiduschek and Kassavetis, 1995; Zawel and Reinberg, 1995; Palmer and Folk, 1990). On 5S

rRNA genes, a 40 kDa protein called TFIIIA binds to a DNA sequence, known as the box A element, which is located downstream of the transcription start site. The binding of TFIIIA facilitates the recruitment of another factor, TFIIIC, which is composed of several polypeptides. TFIIIC is in turn recognized by another protein complex, TFIIIB, which binds to the PIC, recruiting RNAP III. TFIIIB in *S. cerevisiae* consists of TBP and two RNAP III transcription-specific TAFs: BRF1 and a 90 kDa protein, TFC5 (Kassavetis et al., 1995). On tRNA genes, the presence of two intragenic promoter sequences, box A and box B, allow TFIIIC to bind directly to the DNA: TFIIIB and RNAP III are then recruited. In contrast, on the human U6 snRNA gene, no intragenic promoter sequences are present. Instead, an upstream PSE recruits a SNAPc complex, as occurs on RNAP II snRNA genes. However, on the human U6 snRNA gene, the presence of a TATA sequence causes RNAP III, instead of RNAP II, to be recruited to the promoter.

Termination of transcription by RNAP III usually occurs at a small run of adenosines on the DNA template (Bogenhogen and Brown, 1981). This event can be carried out specifically *in vitro* in the absence of any proteins other than RNAP III (Cozzarelli et al., 1983), indicating that recognition of these terminator sequences is an intrinsic property of the enzyme itself. However, the pausing of RNAP III at a terminator sequence is not, in itself, sufficient for termination (Campell and Setzer, 1992). Also, the RNA-binding protein La has been shown to facilitate termination *in vitro* (Gottlieb and Steitz, 1989).

1.2. The cell cycle

1.2.1. General description

In eukaryotes, cell growth and division occur via a sequence of physiological events referred to as the cell division cycle, or simply the cell cycle. Cells may be either actively proliferating (in the process of traversing a cell cycle) or in a quiescent state, also referred to as G_0, in which case they have withdrawn from the cell cycle. The cell cycle can be subdivided into several phases: G_1, S, G_2, and M. S phase is the period during which the genome is replicated in preparation for division and M-phase (mitosis) is the period during which the cell divides. The G_1 and G_2 phases follow upon mitosis and S-phase, respectively. In typical cultured animal cells, one cell cycle requires from 12-24 h, with most of this time spent in G_1 and G_2; S phase can take up to 7 h and mitosis itself less than 1 h (Prescott, 1976). However, there are cases where cells utilize cycles varying in the arrangement and duration of these various phases. Cells of the early *Drosophila* embryo, for example, exhibit cell cycles which possess very short G_1 and G_2 phases (Shermoen and O'Farrell, 1991).

Whereas DNA replication and mitosis are discontinuous events which occur only once in each cycle, cell growth is a continuous process which occurs throughout most of the cycle. The G_1 and G_2 phases provide intervals between cell divisions during which rRNA and protein synthesis occurs continuously, so as to generate a parent cell of size sufficient to produce normal-sized progeny (Prescott, 1976). G_1 and G_2 also provide intervals during which the cell assesses its internal state and external conditions and decides whether or not to continue progression through the cell cycle. Early G_1, for instance, provides a period during which the cell decides whether to commit to another round of cell division or exit the cell cycle. During this time, mammalian cells can be induced to enter G_0 in response to a number of conditions, including the removal of growth factors, density-dependent growth inhibition, and the action of

inhibitory growth factors, such as TGF-β (Schneider et al., 1991). At other times in the cycle, so as to ensure the proper execution of cell growth and division, a necessary order is enforced on the progression of events which are not inherently dependent on one another. These times are known as checkpoints, several of which are well documented in mammalian cells (Hartwell and Weinert, 1989). The G_2-M checkpoint ensures that if a cell has not sucessfully replicated its DNA in S phase, or has suffered DNA damage during G_2, it will not progress into mitosis (Murray, 1994; Murray, 1992). The M-phase spindle assembly checkpoint prevents a cell from completing mitotic division if its genetic material is not poised for faithful dissemination to its progeny or if external conditions do not favour commitment to a new cell division cycle. The G_1/S phase checkpoint prevents DNA replication if damage has been incurred by the genome during G_1. In addition, cell size also appears to be monitored by checkpoint controls within the cell (Murray, 1994).

1.2.2. The cell cycle machinery

Despite minor variations in the cell cycle machinery and its operation between different phyla, and even between different types of mammalian cells, all eukaryotic cells appear to regulate their growth and division in the same general manner. Biochemical and genetic studies have demonstrated that this process is executed through the operation of a molecular control system, the cell division cycle machinery, the components of which are among the most well conserved molecules in eukaryotic evolution (O'Farrell, 1992; Nurse, 1990).

The principal components of the cell cycle machinery are cyclin dependent kinases (cdks) (reviewed in Morgan, 1995; Dorée and Galas, 1994). The cdks are present throughout the cell cycle, but become active only when they interact with molecules known as cyclins, many of which are synthesized at specific phases in the cycle. The activities of cdks, which are manifested periodically at specific times of the cell cycle, affect all of the major transitions of

the cell cycle. In mammalian cells, several cdks have been identified, some of which undergo multiple interations with members of the various classes of cyclins. One cdk, Cdc2, complexes with B-type cyclins to produce an enzymatic activity referred to as mitosis or maturation promoting factor (MPF), which initiates the processes of mitosis. Another cdk, cdk2, complexes with E-type cyclin in mid-G_1 and with A-type cyclin during S phase (Pagano et al., 1992; Zindy et al., 1992; Girard et al., 1991). Cdk4 and cdk6 bind to the family of D-type cyclins during G_1 phase (reviewed in Sherr, 1994). It is important to note that the function of some currently known cdks is not clear. Also, it has become apparent that cdks can have roles in processes other than cell cycle regulation. For instance, Pho80 and Pho85, a *S. cerevisiae* cdk and cyclin, respectively, form a complex that modulates phosphate metabolism (reviewed in Cross, 1995). In mammalian cells, cdk7 (MO15) and cyclin H form a complex that apparently functions in RNAP II transcription and DNA repair as well as in the cell cycle, wherein it may act as a cdk-activating kinase (CAK) (Svejstrup et al., 1996).

Though cdks are responsible for triggering the events which drive cell cycle progression, other types of molecules are involved in restricting or facilitating the action of these enzymes. For example, the stimulation of quiescent (G_0) cells to proliferate requires the presence of growth factors and the transmission of their proliferative signals from cell surface receptors to the nucleus, where the transcription program of the cell is affected. Some of the proteins expressed as a result of this process, such as c-Fos, c-Jun and c-Myc, are believed to help set the cell cycle in motion and allow the cell to proceed through early G_1 (Hofbauer and Denhart, 1991). The role of these gene products in facilitating cell cycle progression is highlighted by their oncogenic potential (Hofbauer and Denhart, 1991).

In contrast to gene products which promote cell proliferation, other proteins are involved in arresting it. The *retinoblastoma* gene product, pRb, for example, is present in normal cycling mammalian cells, and serves as part of a

braking mechanism acting on the cell cycle. The phosphorylation state of pRb impacts upon the cell's capacity to proceed into S phase (reviewed in Hollingsworth et al., 1993). In its hypophosphorylated state, pRb complexes with E2F family transcription factors, preventing them from binding DNA. This sequestration of E2F activity by pRb ultimately prevents the expression of genes required for S phase, such as *DHFR* (reviewed in Nevins et al., 1992). When pRb is hyperphosphorylated, it no longer sequesters E2F family transcription factors, and the cell cycle proceeds. There is evidence that pRb is phosphorylated by cdks (reviewed in Sherr, 1994); thus, one way in which cdks may drive cell cycle progression in G_1 is by switching off this braking mechanism. A number of cdk inhibitors have also been discovered (reviewed in Elledge and Harper, 1994), which also play roles in braking cell cycle progression by directly binding to and interfering with the activity of cdks. Other molecules function in restraining cell cycle progression in extraordinary circumstances, as elements of checkpoint controls in response to physiological problems in the cell. The *p53* gene product, for example, responds to DNA damage during G_1 phase by binding DNA and inducing the expression of a cdk inhibitor, p21 (alternatively known as WAF1, Cip1, and Cap20). This protein binds to and inhibits cdk2/cyclin E complexes and thereby arrests the cell cycle at the G_1/S boundary (El-Deiry et al., 1993; Gu et al., 1993; Harper et al., 1993). Unlike pRb, p53 is present only at low levels in normal physiological circumstances, and is induced specifically as part of a checkpoint response to loss of genomic integrity (reviewed in Murray, 1994).

1.2.3. Mitosis

1.2.3.1. General description

The purposes of mitosis are the faithful transmission of genetic information and the physical construction of two complete daughter cells from one parent cell. These are complex tasks that involve dramatic changes in nearly every aspect of cellular physiology (reviewed in Earnshaw and Pluta, 1994). The

sequence of events which constitute mitosis can be divided into six somewhat overlapping phases: prophase, prometaphase, metaphase, anaphase, telophase, and cytokinesis. The commencement of the first stage of mitosis, prophase, is not a clearly demarcated event. The start of prophase is defined by the microscopic detection of chromatin condensation, but this is a process that occurs continuously throughout late G_2. During prophase, the single centrosome of the cell, which contains two centrioles, splits to yield two distinct centromeres which migrate to opposite ends of the cell. The cytoskeletal microtubule arrangement characteristic of interphase breaks down and is rearranged into the mitotic spindle, a bipolar structure radiating from each of the two centrioles. By late prophase, nucleoli have disintegrated, the Golgi apparatus and the endoplasmic reticulum have dispersed into a series of vesicles (Lucocq and Warren, 1987), and membrane trafficking has become arrested (Misteli and Warren, 1995; reviewed in Misteli, 1996; Warren, 1989).

In contrast to prophase, prometaphase begins with a clearly demarcated event, the dissolution of the nuclear membrane, which, in higher eukaryotes, becomes dispersed as a collection of vesicles throughout the cell. Prometaphase is followed by metaphase. At this point, the chromatin is fully condensed into the compacted form of mitotic chromosomes. Each chromosome assembles a protein complex, the kinetochore, on its centromeric region. The kinetochores become attached to a subset of spindle microtubules, known as kinetochore microtubules, which radiate from the poles of the mitotic spindle. These microtubules align the chromosomes along the equatorial line of the cell, engendering a configuration referred to as the metaphase plate. The first part of anaphase, anaphase A, begins with the separation of the sister chromatids of each metaphase chromosome and the migration of one chromatid from each chromosome to each pole of the mitotic spindle. In the second stage, anaphase B, other microtubules in the mitotic spindle, called polar microtubules, elongate, pushing the opposite poles of the mitotic spindle apart and elongating the cell. This process continues into telophase, which commences when the chromatids

arrive at the poles of the spindle. During late telophase, the nuclear envelope reforms around the daughter chromosomes at each pole of the cell, chromatin decondensation begins, and nucleoli reappear. The final stage of mitosis, cytokinesis, commences in anaphase and continues past the end of telophase. Cytokinesis involves the physical division of the cell into two daughter cells. A contractile ring, composed of actin filaments, forms on the plasma membrane at the cell equator, constricting the cell and eventually causing it to pinch in two (reviewed in Alberts et al., 1994).

1.2.3.2. Molecular architecture

In the past ten years, an outline of the molecular processes which underlie the dramatic physiological changes of mitosis has emerged. Indeed, mitosis is the phase of the cell cycle best understood at the molecular level, as biochemical exploration of the cell cycle commenced with investigations into MPF (for a historical review, see Kirschner, 1992). In all eukaryotic cells, mitosis is initiated by the activity of MPF, which is composed of Cdc2 and a B-type cyclin. When MPF becomes active in late G_2, it phosphorylates a number of substrates, initiating complex physiological changes within the cell (Dunphy and Newport, 1988). The list of putative targets for MPF kinase activity is extensive (for review see Nigg, 1993), and includes chromatin-associated proteins such as histones and high mobility group (HMG) proteins (Reeves, 1992), cytoskeletal proteins such as the nuclear lamins and Rab proteins (Nigg, 1992; van der Sluijs et al., 1992; Bailly et al., 1991), transcription factors such as Oct-1 (Segil et al., 1991) and other protein kinases such as casein-kinase II (Mulner-Lorillon et al., 1990). With respect to the protein kinase targets of MPF, it is important to bear in mind that although MPF activity initiates mitosis, some of the particular events of M-phase may be initiated by other kinases which are themselves activated, directly or indirectly, by MPF (Nurse, 1990).

Although Cdc2 must bind a B-type cyclin to become activated, regulation of its activity involves additional mechanisms. These multiple steps of activation

were apparently designed by evolution to confer tight control over entry into mitosis through checkpoint mechanisms that prevent commencement of that event under inappropriate conditions. Cdc2 forms a complex with cyclin B to form pre-MPF, which becomes active only after its phosphorylation state has been adjusted to a certain configuration. Pre-MPF phosphorylation on two residues, Y15 and T14, causes an occlusion of its ATP-binding site and therefore prevents enzyme function (Ferrell et al., 1991; Gould and Nurse, 1989; Draetta et al., 1988). These sites are phosphorylated by kinases, such as the yeast protein Wee1 (reviewed in Morgan, 1995), which antagonize MPF activation. In turn, these kinases are modulated by kinases such as the Nim1 kinase of *S. pombe*, which responds to nutritional conditions in the extracellular milieu and negatively regulates Wee1 activity by phosphorylation (Feillotter et al., 1991). Pre-MPF is converted to active MPF when Y15 and T14 are dephosphorylated by the phosphatase Cdc25. Pre-MPF activation also requires phosphorylation on T161, which is carried out by a cdk-activating kinase (CAK), which may consist of the cdk7/cyclin H/MAT1 complex, the activity of which is constant throughout the cell cycle (Fisher and Morgan, 1994; Poon et al., 1994). MPF activation is autocatalytic, as the enzyme itself inhibits Wee1 and stimulates Cdc25 activity (Morgan, 1995). In *S. pombe*, checkpoint controls over entry into mitosis constrain MPF activation, such that if the genome is not fully replicated in S phase, Y15 phosphorylation is upregulated (Smythe and Newport, 1992; Enoch et al., 1991).

As entry into mitosis is contingent upon the realization of a tightly controlled event, the activation of MPF, so proper exit from mitosis is also dependent upon the achievement of several specific events. The first of these is the activation of a ubiquitin-dependent proteolytic machinery at metaphase. This machinery includes the E3 complex, which in yeast consists of the Cdc16, Cdc23, and Cdc27 proteins (Lamb et al., 1994). During mitosis, the mammalian homologue of this complex localizes to the spindle poles and microtubules

(Tugendrich et al., 1995). In part through the action of E3, the proteolytic machinery carries out the degradation of proteins which inhibit the separation of sister chromatids in metaphase chromosomes, such as the Cut2 protein of *S. pombe* (Funabiki et al., 1996). If this proteolytic machinery is not activated, these proteins are not destroyed and anaphase cannot proceed (Holloway et al., 1993; Surana et al., 1993). The second event which must occur for proper exit from mitosis is the inactivation of MPF, through the degradation of cyclin B, an event carried out by the same proteolytic machinery responsible for facilitating progression into anaphase (Iringer et al., 1995; King et al., 1995). If cyclin B is not destroyed, even though the proteolytic machinery is activated, cells will progress through anaphase but will not complete cytokinesis, decondense their chromosomes, or reform their nuclear envelopes (reviewed in Murray, 1995). The activation of this proteolytic machinery appears to be contingent upon the proper assembly of the mitotic spindle, and is, therefore, the basis of the mitotic spindle assembly checkpoint (Minshull, 1994).

1.3. Interrelation of Transcription and the cell cycle

Given their central roles in so many aspects of cellular function, it is not surprising that transcription and the cell cycle are intimately associated with one another. Several functional connections between transcription and the cell cycle have been uncovered to date. The best understood of these is the transcription of certain genes in a cell cycle phase-specific manner. Many genes are transcribed at a constant rate throughout the cell cycle, but some are transcribed only at certain points due to the regulated action of sequence-specific transcription factors. For instance, when cells enter S phase, they commence transcription of a number of genes required for DNA replication (Müller, 1995; McKinney and Heintz, 1991). This sort of cell-cycle dependent regulation of transcription may occur in a number of ways, including the regulation of production or localization of transcription factors, post-translational modification of transcription factors, alterations in the protein-protein interactions of transcription factors, a feedback of gene products which regulate a gene's own promoter, or the production of repressors which interfere with transcription factor activity (McKinney and Henitz, 1991). Discontinuous transcription events in the cell cycle are not only the consequences of cell cycle progression, but are also the causes of specific cell cycle events. For example, increased transcription, in late G_2, of the genes encoding B-type cyclins leads to an accumulation of these proteins. B-type cyclins then bind to Cdc2, facilitating its activation and, thereby, entry into mitosis.

Other studies have indicated that transcription and the cell cycle are even more intimately related. *In vitro* transcription studies have shown that the general transcription factor machinery for RNAPs II and III is modulated in activity during the different phases of the mammalian cell cycle (White et al., 1995b; Yohana et al., 1995). When quiescent mammalian cells are stimulated with serum, phosphorylation of the CTD of RNAP II is increased (Dubois et al., 1994).

Also, when *S. cerevisiae* cells are arrested by cyclohexamide treatment, they undergo the specific inactivation of components of the RNAP III GTF, TFIIIB (Dieci et al., 1995). These results suggest that the inherent activity of the transcriptional apparatus itself is dependent upon the cell cycle phase of the cell.

$TAF_{II}250$, a component of the RNAP II GTF TFIID, seems to be involved in cell cycle progression. This molecule is required for cell cycle progression through G_1 (Hisatake et al., 1993; Ruppert et al., 1993). As cells deficient in $TAF_{II}250$ do not exhibit global defects in RNAP II transcription, this molecule may be required for the activation of a subset of genes important for G_1 progression. Alternatively, the protein kinase activity of $TAF_{II}250$ may have a more direct role in modulating cell cycle progression (Dikstein et al., 1996).

There are also other examples of RNAP II GTFs with putative roles in facilitating cell cycle progression. TFIIH contains components putatively involved in the cell cycle: cdk7 (MO15), cyclin H, and MAT1. These proteins constitute a complex which is a CTD kinase necessary for transcription on some promoters *in vitro* (Akoulitchev et al., 1995), but which also manifests CAK activity *in vitro* (Shiekhattar et al., 1995; reviewed in Svejstrup et al., 1996). It has therefore been suggested that TFIIH is involved in signalling from the transcriptional machinery to the cell cycle machinery (Svejstrup et al., 1996). Another cdk/cyclin complex, cdk8/cyclin C, is present in the RNAP II mammalian holoenzyme (Maldonado et al., 1996), although its roles in transcription and cell cycle progression are still unclear. Also, the three-dimensional structure of TFIIB is similar to that of cyclins (Brown et al., 1995). This has prompted the speculation that, as part of its role in transcription, TFIIB might bind to and activate a cdk (McKnight, 1996). Emerging from these studies is the fact that the cell cycle and transcription are not easily separable in terms of cellular function: they stimulate and downregulate one another, and even overlap in terms of the molecular machinery they employ (McKnight, 1996).

1.4. Transcriptional repression during mitosis

1.4.1. General description

Although the processes of transcription are intimately linked to those of cell growth and proliferation throughout all of the various cell cycle phases, the most dramatic illustration of this connection occurs during mitosis. Since the early 1960s, investigators have noted an apparent cessation of RNA synthesis as cells traverse M-phase (reviewed in Prescott, 1976; Mitchison, 1971; Prescott, 1964). This phenomenon was first discovered during studies aimed at identifying intracellular sites of RNA production. Investigators hypothesized that RNA was produced from DNA in the nucleus, and that therefore, when this DNA becomes severely condensed in mitosis, RNA production might not occur. These initial investigations into transcription during mitosis involved *in vivo* RNA labelling and bulk RNA quantitation experiments which were aimed at establishing a link between RNA production and the functional state of chromatin. These early studies showed that the production of labelled RNA is drastically decreased in individual cells naturally passing through mitosis and in populations of cells arrested in mitosis. More detailed investigations established that RNA synthesis is disrupted from mid to late prophase (prior to nuclear membrane and nucleolar disintegration) to late telophase (before nucleoli reformation) (Prescott and Bender, 1962; Taylor, 1960). Mitotic repression of RNA synthesis was subsequently documented in a wide variety of cultured animal cell types (Doida & Okada, 1967; King and Barnishel, 1967; Baserga, 1962), including hamster fibroblasts (Konrad, 1963; Prescott and Bender, 1962; Taylor, 1960), HeLa cells (Feinendegen and Bond, 1963; Terasima and Tomach, 1963), chick embryo fibroblasts (Bather & Purdie-Pepper, 1961), grasshopper neuroblasts (Schiff, 1965) and Osgood leukemic cells (Feinendegen et al., 1960). Mitotic repression of transcription was also reported in whole animal tissues (Linnartz-Niklas et al., 1964), *Drosophila* embryos (Edgar & Schubiger, 1986),

many plant cell types (Das et al., 1965; Davidson, 1964; Kusanagi, 1964; Das, 1963; Harris & LaCour, 1963; Van't Hoff, 1963; Taylor, 1958; Taylor, 1956), and cells of lower eukaryotes, such as *Physarum polycephalum* (Fink and Turnock, 1977; Kessler, 1967). Studies on HeLa cells indicate that the uptake of RNA precursors by the cell during mitosis is normal, and that ribonucleotides taken up during mitosis are held and used to synthesize RNA when the cell passes into interphase and resumes transcription (Feinendegen and Bond, 1963).

The precise degree of transcriptional repression is controversial, with some studies claiming that small amounts of RNA (up to 16 % of total interphase levels) continue to be synthesized during mitosis (Zylber and Penman, 1971; Johnson and Holland, 1965; Konrad, 1963). However, this residual RNA labelling has been attributed to failure to perform acid extraction to remove unincorporated precursors (Mitchison, 1971). Such observations have also been ascribed to the detection of RNA produced by transcription in cytoplasmic organelles, such as mitochondria and chloroplasts (Fan & Penman, 1970; Newsome, 1966; Das et al., 1965; Harris and LaCour, 1963). Nonetheless, the synthesis of an acid soluble fraction of low molecular weight nuclear RNA, which would be removed by acid extraction, cannot be ruled out (Levy and Lynt, 1963).

Also, it is not clear from these pulse-labelling studies whether transcription by all three nuclear RNAPs is repressed and to what degree each is repressed. As roughly 80% of RNA synthesis is performed by RNAP I (Love and Minton, 1985; Reeder and Roeder, 1972; C.A. Spencer, unpublished), it is possible that the reduction in total RNA synthesis during mitosis observed in these studies might be primarily due to RNAP I repression.

1.4.2. Chromatin-mediated models

A number of explanations for how transcription is eliminated during mitosis have been presented. Given the highly compact nature of mitotic chromosomes, it is difficult to imagine that chromosome condensation does not influence RNA synthesis during mitosis (Prescott and Bender, 1962). Two models of mitotic transcriptional repression which invoke chromatin condensation as the cause of this phenomenon have been proposed. The stripping model holds that RNAPs are necessarily ejected from the chromatin as it condenses in prophase. Ejection may be a prerequisite for proper chromosome condensation or a consequence of it (Moreno and Nurse, 1990). Only upon decondensation in telophase can RNAPs reassociate with the DNA template. In contrast, the blockage model states that RNAPs are not ejected from metaphase chromosomes, but rather are prevented from catalyzing transcription by the nature of the template (Matsui et al., 1979; Gariglio et al., 1974). Blockage could involve newly initiated polymerases only, with elongating polymerases continuing to transcribe until they terminate. Alternatively, all polymerases, elongating as well as newly initiated, could be blocked (Shermoen and O'Farrell, 1991). This model predicts that if the mitotic DNA template could be altered structurally in such a way as to remove the obstacle to transcription, then transcription complexes could be released, allowing RNA synthesis.

Both of these models insist that the dramatic condensation of the chromosomes during mitosis is incompatible with DNA transcription. In fact, the role of chromatin structure in regulating transcription has been widely studied. The packaging of DNA into nucleosomes does not appear to impede the elongation of RNAPs *in vitro* (Izban and Luse, 1991; Lorch et al., 1987; Losa and Brown, 1987). Mechanistic studies have suggested that the progression of RNAPs through nucleosome-incorporated DNA involves a translocation of nucleosomal core proteins away from the region ahead of the enzyme (Studitsky et al., 1994;

Clark and Felsenfeld, 1992). There are some instances where the arrangement of DNA into nucleosomes actually potentiates transcription, as when a necessary protein-protein interaction is spatially facilitated by the winding of DNA around the nucleosome core (reviewed in Wolffe, 1994). However, in other cases the incorporation of DNA into nucleosomes can have an inhibitory effect upon transcriptional initiation, by occluding the binding sites of transcriptional proteins (Laybourn and Kadonaga, 1991; Lorch et al., 1987; Losa and Brown, 1987; Knezetic and Luse, 1986). A number of transcriptional activators have been shown to alleviate this repressive effect of nucleosomes, presumably by disrupting nucleosome structure around transcriptional protein binding sites (reviewed in Struhl, 1996; Felsenfeld, 1992). The SWI/SNF and NURF protein complexes, for example, are both thought to function by remodelling nucleosomal structure on promoter DNA sequences, allowing the GTFs access to their cognate binding sites (reviewed in Kingston et al., 1996). Further support for the notion that the assembly of DNA into nucleosomes can inhibit transcription comes from the finding that the deletion of histone genes increases the transcription of certain genes in *S. cerevisiae* (reviewed in Croston and Kadonaga, 1993).

Studies of higher level chromatin structure have indicated that the chromatin structure of genes which are being transcribed (active genes) is different from that of non-transcribed (inactive) genes. Microscopic studies on *Xenopus* ooycte lampbrush chromosomes and *Drosophila* polytene chromosomes suggest that active genes possess a less compacted chromatin structure than inactive genes (reviewed in Benbow, 1992). Similarly, studies in mammalian cells have shown that active genes are more easily digested by DNase I than inactive ones, suggesting they have a less condensed arrangement (Croston and Kadonaga, 1993; Weintraub and Groudine, 1976). Further study of active and inactive chromatin regions has revealed several biochemical differences between the two types (reviewed in Ausio, 1992). For example, histone H1 is less tightly

bound to nucleosomes in active chromatin than in inactive chromatin (Bradbury, 1992; Kamakaka and Thomas, 1990). Also, nucleosomes in active chromatin are highly acetylated (Wolffe and Pruss, 1996; Davie and Candido, 1978). Active chromatin is enriched in hv1, a variant of histone H2A (Alliss et al., 1986), and in HMG proteins (Weisbrod and Weintraub, 1979). Although how active chromatin is formed and precisely how it affects transcription is not fully understood, specific DNA sequences have been identified which can confer an active chromatin configuration on downstream regions. These sequences, such as the locus control region of the β-globin gene, confer an active chromatin structure upon large DNA domains. Taken together, these results suggest that the transcription of a gene necessarily involves changes in chromatin structure, and lead to the conjecture that the high degree of chromatin condensation characteristic of mitosis precludes transcription.

However, testing of this hypothesis has been hampered by poor understanding of the structure of mitotic chromosomes. During prophase, chromatin condenses dramatically, resulting in an overall compaction in the length of cellular DNA of roughly 100,000 fold (Alberts et al., 1994). The generally accepted model for how this occurs is the scaffold-loop model (reviewed in Saitoh et al., 1994). According to this hypothesis, nucleosomal DNA, compacted into a 30 nm fiber, is organized into loops of 50-100 kb each. The base of each loop consists of a AT-rich scaffold-associated region (SAR) of DNA which binds to non-histone scaffold proteins. During mitosis, these scaffold proteins aggregate, gathering DNA loops together to produce a condensed structure. Precisely how this packing of the DNA loops takes place is not clear, and several models, including successive helical folding (Sedat and Manvelidis, 1977), radial loop folding (Marsden and Laemmli, 1979), and variations of these schemes (Boy de la Tour and Laemmli, 1988) have been proposed. Some structural components of the mitotic chromosome scaffold have been identified, including the structural maintenance of chromosome (SMC)

proteins (Hirano, 1995) and topoisomerase II, although the role of the latter as a structural component is controversial (Poljak and Käs, 1995; Watt and Hickson, 1994). Overall, the function and organization of these scaffold proteins in the context of the chromosome is not well understood (Hirano, 1995).

Almost as contentious as the manner in which mitotic chromatin is packed are the identities of the molecules involved in carrying out that packing. A role for topoisomerase II has been well established (Adachi et al., 1991; Uemura et al., 1987; reviewed in Hirano, 1995; Watt and Hickson, 1994), although this role seems restricted to the early stages of chromosome condensation (Hirano and Mitchison, 1993). Histone H1 is hyperphosphorylated during mitosis, and the significance of this event with respect to DNA condensation has been widely debated. Early work demonstrated a role for histone H1 phosphorylation in chromosome condensation (reviewed in Bradbury, 1992), but subsequent studies have discredited this notion (Guo et al., 1995; Oshumi et al., 1993). Histone H3 is also phosphorylated during mitosis, and there is circumstantial evidence that this event may be required for proper chromosome condensation (Guo et al., 1995). HMG proteins are components of mitotic chromosomes and are also phosphorylated during mitosis. A role for this modification in producing mitotic chromatin structure has also been suggested (reviewed in Reeves, 1992). In summary, the nature of mitotic chromosomes and the processes which give rise to them are not well defined biochemically, and therefore their impact upon transcription is difficult to estimate.

However, there is indirect evidence to suggest that mitotic DNA condensation does preclude transcription. The mitotic cessation of RNA synthesis temporally coincides with the onset of chromosome condensation in late prophase, and its resumption coincides with the decondensation of chromatin in late telophase. Studies on cells which do not display chromosome condensation as dramatic as that observed in mammalian cells have further strengthened this connection. The yeasts *S. cerevisiae* (Fox et al., 1995; Tauro et al.,

1969; Williamson and Scopes, 1960) and *S. pombe* (Mitchison et al., 1969; Mitchison and Lark, 1962; Mitchison and Walker, 1959), for example, do not exhibit a mitotic repression of transcription. DNA-containing organelles, such as mitochondria and chloroplasts (Fan and Penman, 1970), and the amitotic macronucleus of *Paramecium caudatum* (Rao and Prescott, 1967; Prescott, 1964b) display no such repression during division. Also, prokaryotes, which do not divide by mitosis, show no cessation of RNA synthesis during division (reviewed in Mitchison, 1971). *In vitro* transcription assays using reconstituted chromatin templates have also been used to address the relation between mitotic chromatin structure and transcription. In one study, templates incubated with proteins extracted from S-phase chromosomes were transcribed more efficiently than those incubated with proteins isolated from mitotic chromosomes (Farber et al., 1972).

However, despite the many established correlations between chromatin condensation and reduced transcription, it is important to note that definitive experimental approaches for investigating the relationship of *bona fide* mitotic chromosome structures to *in vivo* transcription are still lacking. Therefore, the blockage and stripping models of mitotic transcriptional repression continue to rest primarily upon circumstantial evidence.

1.4.3. Non-chromatin-mediated models

Two models which account for the mitotic abrogation of transcription without invoking a causal role for chromatin condensation have been proposed. The first holds that RNAPs or other necessary components of the transcriptional machinery are degraded immediately before or during mitosis and rapidly resynthesized after telophase. However, this model has been disproven, as the presence of the protein synthesis inhibitor cyclohexamide throughout mitosis does not prevent the resumption of transcription in late telophase (Simmons et

al., 1974; Fan and Penman, 1971). Also, the overall levels of RNAPs present in the cell during both mitosis and interphase are similar in both HeLa cells (Benicke and Seifart, 1975) and in *Physarum polycephalum* (Hildebrant and Sauer, 1976). Another mechanism, the downregulation model, suggests that specific components of the transcriptional machinery are downregulated in activity by mitotic regulatory events, such as phosphorylation by a regulatory kinase (Hartl et al., 1993), or the action of a repressor (Stein and Farber, 1972). It is important to note that this model and the models described above in relation to chromatin structure are not mutually exclusive. For example, GTFs could be stripped from the chromosomes by condensation, and then downregulated to prevent their reassociation with DNA during mitosis. Also, the stripping model can be incorporated into downregulatory mechanisms of RNAP repression, as well as chromatin-dependent ones. For instance, the DNA-binding activities of GTFs could be downregulated, resulting in a stripping of the transcriptional machinery that is not caused by chromatin condensation. Furthermore, it is possible that each RNAP system may be repressed during mitosis by an independent and/or unique mechanism.

1.4.4. Mechanisms of mitotic transcriptional repression

1.4.4.1. RNAP I

Early studies on transcription during mitosis in plant cells indicated that transcription by RNAP I is the first to resume in late telophase (Morcillio et al., 1976). Consistent with a rapid resumption of RNAP I transcription, a variety of immunocytochemical and biochemical studies have demonstrated that the components of the RNAP I transcription machinery, including RNAP I, UBF, SL1, and DNA topoisomerase I remain on the NORs of chromosomes during mitosis (Jordan et al., 1996; Segil et al., 1996; Roussel et al., 1993; Zatespina et al., 1993; Rendón et al., 1992; Chan et al., 1991; Haaf et al., 1988; Gulner et al., 1986 Scheer & Rose, 1984). Apparently none of these components enters the cytoplasm

in appreciable amounts during mitosis (Roussel et al., 1993). Stoichiometries of the various RNAP I transcription factors are unchanged in active NORs on mitotic chromosomes, indicating that the functional arrangement of these complexes may be preserved in mitosis (Roussel et al, 1996).

Experiments using mitotic cell extracts (Gariglio et al., 1974) or isolated metaphase chromosomes (Matsui et al., 1979; Matsui and Sandberg, 1979) indicate that RNAP I transcription complexes are transcriptionally engaged but blocked during mitosis. Blocked RNAP I complexes can be released, through the application of agents such as the detergent sarkoysl, to facilitate the production of RNA. More recent studies demonstrate that these complexes are present only on those NORs which become active during interphase (Roussel et al., 1996). *In situ* hybridization studies indicate that rRNA transcripts are not detectable in these stalled complexes, an observation which led to the hypothesis that upon the onset of mitosis, RNAP I complexes stall near the promoter region of rRNA genes. In this hypothesis, polymerases already elongating downstream of the promoter region at the time of mitotic onset would quickly run off the gene, leaving a cluster of blocked transcription complexes in the promoter region. These blocked RNAP I complexes would be quickly released in telophase to allow a rapid resumption of rRNA synthesis (Weisenberger and Scheer, 1995). It is also possible, however, that during mitosis RNAP I molecules are stalled throughout the transcription unit, with all nascent transcripts aborted or degraded.

1.4.4.2. RNAP III

In contrast to RNAP I transcription, which may be disrupted in mitosis by a blockage mechanism, RNAP III transcription appears to be abrogated through the downregulation of the activity of a component of the general transcriptional machinery. RNAP III transcriptional repression was initially investigated using extracts of *Xenopus laevis* ooyctes, in which cell cycle progression can be

reproduced *in vitro*. When such extracts enter a mitotic state, transcription of exogenous RNAP III templates is repressed, mimicking the presumed *in vivo* mitotic repression of RNAP III transcription (Hartl et al., 1993). Chromatin condensation is not essential for this *in vitro* repression. In contrast, the kinase inhibitor 6-dimethyl-aminopurine eliminates RNAP III transcriptional repression in this *in vitro* system, suggesting that phosphorylation plays an active role. Further work showed that the *in vitro* repression of RNAP III transcription also occurs in a system reconstituted from purified components, by the addition of purified Cdc2/cyclin B kinase. This repression is alleviated by the addition of TFIIIB purified from interphase cells (Gottesfeld et al., 1994). TFIIIB from mitotic cells is incapable of alleviating repression, unless first treated with a phosphatase. Similar studies using *in vitro* transcription extracts of HeLa cells (White et al., 1995a) also show repression of RNAP III transcription due to a loss of TFIIIB activity. However, in this system, as opposed to the *Xenopus* system, a dominant inhibitor of transcription was not present in mitotic extracts, perhaps indicating a variant mechanism of downregulation. Despite this difference, these studies support the conclusion that downregulation of TFIIIB activity, through phosphorylation by a mitotic kinase, could be responsible for the *in vivo* loss of RNAP III transcription during mitosis.

In keeping with a role for TFIIIB in regulating RNAP III transcription during the cell cycle, a deficiency in TFIIIB activity has also been shown to be responsible for lowered basal RNAP III activity in extracts of HeLa cells in G_1 (White et al., 1995b). To date, the precise target of downregulation within the TFIIIB complex has not been identified. Although TBP is known to be phosphorylated during mitosis (Segil et al., 1996; White et al., 1995a; Gottesfeld et al., 1994), no functional significance has yet been attributed to this modification with respect to RNAP III transcription. Therefore, it appears that the target of downregulation is one of the TAF components of the TFIIIB complex. It is important to note that although biochemical downregulation of

RNAP III GTF activity is an attractive means to explain the loss of RNAP III transcription during mitosis *in vivo*, it remains possible that other factors, such as chromosome condensation, may also contribute to the phenomenon (White et al., 1995a; Hartl et al., 1993).

1.4.4.3. RNAP II

The mechanism of mitotic repression of RNAP II transcription is of particular interest because of its implications with respect to regulation of gene expression and cell cycle progression. However, this phenomenon is less understood biochemically than the repression of transcription by RNAPs I and III. Nonetheless, some aspects of the mechanism have been uncovered. The most provocative study on this subject to date remains that of Shermoen and O'Farrell (1991), who analyzed transcription of the *ultrabithorax* gene in *Drosophila* embryos using *in situ* hybridization of labeled ribonucleotide probes complementary to various regions of nascent *Ubx* transcripts. They found that during mitoses 14 and 15, nascent transcripts of *Ubx* are lost from chromatin. Furthermore, this loss of transcripts occurs progressively over time, from metaphase to telophase, a fact which may reflect a slow process of abortion or, alternatively, a slow dispersal of aborted transcripts. This abortion of nascent transcripts requires progression through prophase, as *Drosophila string* mutants, which arrest in G_2 of mitosis 14 (Edgar and O'Farrell, 1990) do not abort *Ubx* transcription. After mitosis is complete, nascent transcripts of *Ubx* begin to appear from the 5' end of the gene, suggesting that after mitosis, transcription of *Ubx* resumes by re-initiation at the promoter.

To date, however, the mechanism whereby the abortion of nascent transcripts occurs has not been uncovered. It has been suggested that mitotic phosphorylation of the RNAP II general transcriptional apparatus could be responsible for a general disruption of nascent RNAP II transcripts (Shermoen and O'Farrell, 1991). Modification of a component of the transcription machinery

could decrease its activity, disrupting elongation complexes, thereby facilitating the abortion and release of nascent transcripts. In fact, the idea that the activity of the RNAP II basal transcription machinery can be altered in accordance with cell cycle progression has been corroborated by *in vitro* transcription studies (Yonaha et al., 1995; Heintz and Roeder, 1984). In these studies, nuclear extracts from G_1 phase HeLa cells were more active in basal transcription assays than similar extracts made from S or G_2.

One potential target for downregulation via mitotic phosphorylation may be RNAP II itself. The carboxyl-terminal domain (CTD) of the large subunit of this enzyme contains multiple sites for phosphorylation. Furthermore, the phosphorylation state of the CTD has been linked to the transcriptional activity of the enzyme (reviewed in Dahmus, 1994). Also, the CTD of RNAP II can be phosphorylated *in vitro* by some cdk/cyclin complexes, such as the cdk8/cyclin C complex, which is a component of the mammalian RNAP II holoenzyme and which interacts with the RNAP II CTD (Leclerc et al., 1996; Maldonado et al., 1996; Rickert et al., 1996; Tassan et al., 1995b). The cdk8/cyclin C complex is highly homologous to the SRB10/SRB11 cdk/cyclin complex, also a putative CTD kinase, present in RNAP II holoenzymes from *S. cerevisiae* (Liao et al., 1995). Cdk7 (MO15), cyclin H, and MAT1 form a cdk/cyclin subcomplex of TFIIH which also has CTD kinase activity *in vitro* (Shiekhattar et al., 1995; Lu et al., 1992). Most intriguingly, MPF phosphorylates the CTD *in vitro*, and this modification disrupts preformed transcription complexes consisting of RNAP II, TFIID, TFIIA, TFIIB, and TFIIF (Zawel et al., 1993).

However, despite an inaccurate report of the contrary (Reeves, 1992), immunoblotting has not revealed any differences in the phosphorylation state of RNAP II in interphase and mitotic extracts (Greenleaf, 1993; Kolodziej et al., 1990). Given the number of phosphorylation sites in the CTD, and its non-linear changes in migration in response to increasing CTD phosphorylation (Zhang and Corden, 1991), subtle differences, such as changes in the specific sites or

relative amounts of serine/threonine and tyrosine phosphorylation, might not be revealed by such analyses (Greenleaf, 1993). However, even assessments of the levels of phosphotyrosine specifically have failed to reveal any variation in RNAP II post-translational modification during mitosis in HeLa cells (Dahmus, 1994; Wang, 1994). Also, the inactivation of *CDC28*, the *cdc2* homologue of *S.cerevisiae*, fails to have any detectable effect on the phosphorylation state of RNAP II (Koleske et al., 1992), suggesting that MPF may not act as an *in vivo* CTD kinase, or that it is one of several redundant CTD kinases. Furthermore, extracts of mitotic HeLa cells proved to be as competent as those of interphase cells in executing basal transcription of an RNAP II template *in vitro*, suggesting that the activity of the enzyme may not be downregulated during mitosis (White et al., 1995a).

A second potential target for mitotic downregulation is the RNAP II GTF TFIID. Although the TFIID complex remains intact during this phase of the cell cycle, it is defective in activator-dependent transcription in reconstituted *in vitro* systems (Segil et al., 1996). Furthermore, this change in activity during mitosis has been shown to be dependent upon phosphorylation of the TFIID complex. Other *in vitro* experiments examining basal transcription have shown that the activity of TFIID also varies between the G_1, S, and G_2 phases of the cell cycle (Yonaha et al., 1995), although the precise mechanism of these alterations in TFIID activity is not known.

Other recent studies indicate that, in addition to a downregulation of TFIID activity, mitotic repression of transcription is also accompanied by the intracellular relocalization of general and sequence-specific transcription factors. Immunocytochemical studies have revealed that there is a dramatic displacement from the DNA template of both general and gene-specific RNAP II transcription factors during mitosis. Sequence-specific transcription factors which have been shown, by immunofluorescent localization, to undergo stripping from mitotic DNA include HSF1, Oct-1 and Oct-2, B-Myb, E2F-1, Bcl-6,

and Sp1 (Martinez-Balbás et al., 1995). DNA-footprinting analyses of the human *phosphoglycerate kinase 1* promoter in interphase and metaphase chromosomes indicated that sequence-specific transcription factors are disloged from that promoter during mitosis (Hershkowitz and Riggs, 1995). In the case of some sequence-specific transcription factors, such as Oct-1 and Sp1, displacement coincides with a decrease in the DNA binding activity of the protein *in vitro*. For Oct-1, it has been confirmed that this downregulation of DNA binding activity is triggered by mitotic phosphorylation (Segil et al., 1991). However, exclusion of sequence-specific transcription factors from mitotic DNA is not a ubiquitous phenomenon, as AP-2 (Martinez-Balbás et al., 1995), $p67^{SRF}$ (Gauthier-Rouviere et al., 1991), and the *Drosophila* GAGA transcription factor (Raff et al., 1994) do not display this property. Nonetheless, the displacement of transcription factors essential for initiation of transcription from RNAP II promoters during mitosis could play a role in the cell cycle regulation of the expression of some genes.

Displacement of RNAP II GTFs has been less well studied to date than that of sequence-specific RNAP II transcription factors. Immunocytochemical experiments have demonstrated that TBP is largely dislodged from mitotic chromosomes, although some, presumably that portion present in SL1, remains on the DNA (Jordan et al., 1996; Segil et al., 1996). Also, the MAT1 (Tassan et al., 1995a) and cdk7 (MO15) (Tassan et al, 1994) components of TFIIH, and several TAF components of TFIID (Segil et al., 1996) have been shown to be dislodged from chromatin during mitosis. The issue of whether or not RNAP II itself remains on the DNA is of particular importance, as dislodging of the polymerase would provide an attractive mechanism to explain the abortion of nascent transcripts. However, early studies suggest that RNAP II remains on the template during mitosis. For example, Gariglio et al. (1974) found that whole cell extracts of interphase and mitotic 3T3 cells, prepared in the presence of sarkosyl, were equally competent at α-amanatin-sensitive RNA synthesis when exposed to labelled precursors, indicating that equivalent amounts of DNA-bound RNAP II

is present in mitotic and interphase cells. Similarly, when Matsui et al. (1979) isolated metaphase chromosomes and interphase nuclei from Chinese hamster cells and solubilized DNA-bound RNA polymerases, equivalent amounts of RNAP activity sensitive to 0.5 μg/ml α-amanatin were obtained from each. Furthermore, incubation of isolated chromosomes with high amounts of heparin increased endogenous RNAP II activity roughly ten-fold, suggesting that engaged RNAP II complexes are present but stalled on mitotic DNA. However, as these investigators did not physically localize RNAP II molecules, and experimented on cell extracts and isolated chromosomes rather than intact cells, the issue of RNAP II localization during mitosis has not yet been definitively addressed.

It appears, then, that the mitotic repression of RNAP II transcription may be governed by complex mechanisms. There is currently evidence to support a role for each of the three models of mitotic silencing - blockage, stripping, and downregulation - in RNAP II repression. Which of these mechanisms actually function *in vivo*, the nature of their *in vivo* contributions to mitotic repression of RNAP II transcription, and how coexistent mechanisms may be interrelated remain unresolved issues.

Understanding how RNAP II transcription is regulated during mitosis will provide important insights into both transcription and the cell cycle. If various GTFs are post-translationally modified during mitosis, study of these alterations and their effects on RNAP II function may reveal novel biochemical properties of the transcriptional machinery. Given the abundance of the RNAP II GTFs, a better conception of their localization during mitosis may also contribute to an expanded understanding of mitotic chromosome structure. Most importantly, an understanding of how patterns of gene expression are affected by passage through mitosis is essential to our comprehesion of how cell cycle progression is regulated during early G_1 in cycling cells. This area remains

poorly understood, and its advancement will represent an important expansion of our understanding of the regulation of cell growth.

1.5. Aims of the thesis

The severe repression of transcription during mitosis is a major physiological event in the cell. To date, the issue of the relative degree of repression of RNAPs I, II, and III remains uncertain. Also, the mechanisms underlying RNAP II transcriptional repression remain poorly understood. Furthermore, the impact of this phenomenon on gene expression and cell cycle progression are unknown. This thesis describes the results of studies conducted to address the following fundamental questions regarding the nature of the mitotic repression of RNAP II transcription:

1. What is the precise degree of RNAP II transcriptional repression during mitosis?

2. Are RNAP II molecules engaged but stalled on mitotic DNA, or are they ejected from chromosomes during condensation?

3. Are RNAP II GTFs degraded or covalently modified during mitosis?

2. MATERIALS AND METHODS

2.1. Cell culture and mitotic arrest

HeLa S3 and HeLa CCL2 cells (American Type Culture Collection) were grown, at 37°C in 5% CO_2, in Dulbecco's Modified Eagles Medium (DMEM; GIBCO BRL) supplemented with 10% calf serum (GIBCO BRL) and 1% anti-mycotic antibiotic solution (10,000 U/ml penicillin G sodium, 10,000 μg/ml streptomycin sulfate and 25 μg/ml amphotericin B in 0.85% saline; GIBCO BRL). Cell stocks were passaged before reaching confluence by treatment with a solution of 0.05% trypsin and 0.53 mM ethylenediaminetetraacetic acid (EDTA)•4Na (GIBCO BRL) for 5-10 min at room temperature. Typsinized cells were placed in 20 ml of fresh DMEM + 10% calf serum, and pelleted at 200 × g at room temperature for 3 min. The supernatant was aspirated and the cells resuspended in fresh medium and replated.

To obtain synchronized populations of mitotic cells, HeLa S3 cells were plated at 1-2.5 × 10^4 cells/cm^2 and grown overnight. The next day, the medium was replaced with DMEM + 10% calf serum containing 2 mM thymidine (Sigma). After 24 h, the thymidine-containing medium was replaced with regular DMEM + 10% calf serum and cells were grown for an additional 5 h. Nocodazole (methyl[5-(2-thienylcarbonyl)-1H-benzimidazol-2-yl] carbamate; Sigma) was then added to the medium at 0.5 μg/ml and cells were incubated for 8-10 h. Cells were sub-confluent when treated with nocodazole. For reversal experiments, nocodazole-arrested cells were incubated at 37°C in fresh, prewarmed medium for the time indicated.

2.2. Visual scoring of mitotic index

Visual scoring of mitotic index was performed using a modification of the technique of Paulson et al. (1994). Cells (2×10^6) were scraped and collected, then pelleted at 200 × g at room temperature. The pellet was resuspended in 100 µl of a hypotonic buffer (20 mM Tris[hydroxymethyl]aminomethane [Tris]·HCl [pH 7.5], 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM $ZnCl_2$), and incubated at room temperature for 5 min. The cells were then fixed by adding 10 µl of a methanol:acetic acid solution (3:1 v/v). Fixed cells were stained with 0.5 µg of propidium iodide (from a 0.5 mg/ml solution; Sigma) and treated with 10 µg of RNase A (from a preboiled 1 mg/ml solution in 10 mM Tris·HCl [pH 7.5], 15 mM NaCl) at 37°C for 30 min. Aliquots (10 µl) were then visualized using a Zeiss Axioskop 20 fluorescence microscope with a NeoFluor objective lens (63X). Cells were scored for nuclear envelope breakdown and condensation of chromatin. Between three and four hundred cells were scored for each sample.

2.3. Flow cytometric cell cycle phase analysis

Cells were prepared for fluorescence-activated cell sorting (FACS) using the method of Rasmussen (1988), with several modifications. 2×10^6 cells were scraped and collected by centrifugation at 200 × g for 3 min at 4°C. After removal of the supernatant, cells were resuspended in 0.9% NaCl and fixed by adding 70% ethanol (prechilled to -20°C) dropwise while vortexing the cell suspension at low speed (2) on a Vortex-Genie (Scientific Industries, Inc.). Vortexing was necessary to avoid clumping of cells during fixation. Cells were incubated at 4°C for 30 min and centrifuged at 500 × g at 4°C for 10 min. The supernatant was then aspirated from the pellet, and the cells washed in 10 ml of phosphate-buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4 \cdot 7H_2O$, 1.4 mM KH_2PO4, pH 7.4) prechilled to 4°C. After repelleting the

cells and aspirating the supernatant, cells were resuspended in 1 ml of PBS and stored at 4°C for up to seven days before FACS analysis. Thirty minutes prior to FACS analysis, propidium iodide was added to the cell suspension at 50 μg/ml, and the cells were incubated at room temperature. Twenty minutes before analysis, 100 μl of a 1 mg/ml RNase A solution (prepared as described in §2.2) was added. Flow cytometry was carried out using a Becton Dickinson FACS flow cytometer and cell cycle phase distribution was analyzed using CellFIT software (Becton Dickinson).

2.4. Cell viability/permeability analysis

Cells were pelleted at 200 × g and washed with PBS to remove all of the medium. Cells were then resuspended in PBS, and an aliquot of 20 μl was mixed with 20 μl of a 0.2% solution of trypan blue dye (Allied Chemical). After 2 min, cells were examined in a haemocytometer, using phase contrast microscopy (Zeiss ID03). The percentage of cells that stained blue was taken as the percentage of cells permeable or inviable.

2.5. Whole cell run-on transcription assays

Run-on transcription asssays were performed after whole cells were made permeable following the protocols of Contreras and Fiers (1981) and Miller et al. (1978). Cycling or mitosis-arrested HeLa S3 cells were collected, by scraping, into cold PBS and pelleted by spinning at 200 × g at 4°C. The cells were 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 $CaCl_2$), and counted using a haemocytometer. After the second wash, cells were pelleted and resuspended at 10^7 cells/100 μl in LYSO A solution and transfered to siliconized Eppendorf tubes. One-third volume of lysolecithin

(lysophosphatidylcholine) (1 mg/ml in LYSO A solution) was added, and the cells were incubated on ice for 1 min. Cells were then pelleted at 200 × g, the supernatant aspirated, and the pellet resuspended in 1 ml of fresh LYSO A solution. Permeability was assayed by assessing trypan blue exclusion, as described in §2.4. The percentage of cells made permeable 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 [DTT]).

In a standard reaction, 60 µl of a 5× buffer was added such that the final concentration of its components in the 300 µl reaction mixture was: 5 mM Tris·HCl [pH 8.0], 2.5 mM $MgCl_2$, 150 mM KCl, and 0.25 mM ATP, CTP, and GTP. In experiments using N-laurosarcosine (sarkosyl) or α-amanatin, these reagents were added to cells before buffer addition. Thirty microlitres of α-^{32}P-UTP (3000 Ci/mM; Amersham) was added to the reaction mixture and tubes were mixed and incubated at 30°C for 30 min. Every 10 min, the tubes were gently mixed. Samples were then treated with 125 µg of DNase I (Worthington) at 30°C for 5 min. Thirty six microlitres of 10× SET buffer (100 mM Tris·HCl [pH 7.5], 10% SDS, 50 mM EDTA) was then added and the samples were incubated for 45 min at 45°C with 100 µg of Proteinase K (Boehringer Mannheim). Samples were extracted with phenol/chloroform/isoamyl alcohol (25:24:1) and then with chloroform/isoamyl alcohol (24:1), by vortexing and centrifuging at 13,800 × g for 2 min. RNA was precipitated by adding 595 µl of isopropyl alcohol and 200 µl of 7.5 M ammonium acetate and incubating the sample on dry ice for 20 min. The precipitated RNA was collected by spinning at 13,800 × g for 20 min. The supernatant was discarded and the pellet was resuspended in 100 µl of TE (10 mM Tris·HCl [pH 8.0], 1 mM EDTA). Unincorporated nucleotides were removed by passing the RNA through a size exclusion column consisting of a 1 ml syringe packed with G-50 Sepharose (from a 6.35% solution in 10 mM Tris·HCl [pH 7.5], 1mM EDTA). The resuspended RNA was placed on the top of the column and

centrifuged at 380 × g for 5 min at room temperature. The eluate was made up to 1 ml with hybridization buffer (5 mM TES [pH 7.4], 0.5% SDS, 5 mM EDTA, 125 μg/ml *E. coli* RNA, 0.15 M NaCl, 1× Denhardt's solution [1% Ficoll 400, 1% polyvinyl pyrrolidone 360, 1% BSA], and 0.125% powdered milk). This solution was hybridized at 65°C for 48 h to GeneScreenPlus nitrocellulose filters (NEN Research Products), to which were affixed single stranded DNA probes. After hybridization, filters were washed twice in blot wash (0.5% SDS in 1× SSC [150 mM NaCl, 15 mM sodium citrate]) at room temperature, once quickly and then for 15 min. Filters were washed 1 h at room temperature in a solution containing 2 μg/ml RNase A in 2× SSC. Next, filters were washed twice at room temperature, for 5 min each time, in blot wash. They were then washed twice, for 30 min and then for 15 min, at 65°C in a solution of 0.1% SDS in 0.1× SSC. Excess fluid was removed from the filters, and they were sealed in plastic wrapping and exposed either to autoradiographic film (Fuji RX X-ray film) or a phosphoimage plate. After exposure of the phosphoimage plate, radioactivity bound to various probes was quantitated using a Fujix BAS100 bio-imaging analyzer with MacBAS imaging software.

Filters were prepared using a slot blot apparatus (BioDot SF; BIO-RAD). One microgram of each single stranded DNA probe, diluted in 1× SSC, was applied to each filter, and the DNA deposited on the membrane by the application of vacuum suction across the membrane. Filters were ultraviolet light cross-linked, while still damp, using an ultraviolet Stratalinker 2400 (Stratagene). They were then allowed to completely air dry before baking at 80°C for 2 h in a sealed vacuum oven (Fisher Scientific 280). Filters were pre-hybridized with 1 ml of hybridization buffer for 2 h at 65°C prior to incubation with labelled RNA.

Probes used for run-on transcription assays were created by cloning DNA fragments into M13mp18 or M13mp19 vectors. The c-*myc* 5′ probe consisted of a region, from +66 to +511, located in exon 1 of human c-*myc*, and the c-*myc* 3′

probe consisted of a region, from +936 to +1542, located in intron 1. The c-*fos* probes were derived from the human c-*fos* gene: the 5′ probe consisted of the region from -82 to +760 (exon 1 and part of intron 1), whereas the 3′ probe consisted of the region from +1905 to +2396 (exon 4). The γ-*actin* probes were derived from a human γ-*actin* c-DNA: the 5′ probe consisted of the DNA sequence from -100 to +483, which contains exons 1 to 4, and the 3′ probe was the region from +483 to +1083, which contains exons 4 to 6. The *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) probe contained the region from +44 to +1023 of a human *GAPDH* c-DNA, and the *histone H2B* probe consisted of the region from +110 to +412 of the chicken *histone H2B* gene. The *RPB1* 5′ probe was the 471 bp BsaH1-SphI fragment of mouse *RPB1* (-26 to +445), which includes most of exon 1 and a small upstream region. The *RPB1* 3′ probe was the 1.3 kb KpnI-PvuI fragment of mouse *RPB1* (+2107 to +3439), which includes exons 5 to 7 and introns 5 and 6.

2.6. Indirect immunofluorescence

HeLa cells were grown, as described in §2.1, on sterilized coverslips in twelve-well plates. Immunofluorescence was performed on HeLa S3 cells, arrested in mitosis as described in §2.1, and on cycling populations of HeLa S3 and CCL2 cells. In experiments involving HeLa S3 cells, cultures were grown on coverslips coated with poly-L-lysine (Sigma) to increase adherence, and cells were centrifuged onto these coverslips at 200 × g for 5 min prior to fixation. Cells were fixed in 3.7% formaldehyde (Fluka) in PBS for 10 min. After fixation, cells were washed in a 0.05% solution of Tween-20 in PBS (PBS-T) for 5 min, washed briefly in dH_2O ten times, and then made permeable with acetone (prechilled at -20°C) for 2 min. Cells were washed briefly in dH_2O ten times, and washed in PBS-T for an additional 5 min. Cells were blocked with a 3% bovine serum albumin (BSA) solution in PBS-T for 30 min at room temperature before primary

antibody staining. The primary antibodies were ARNA-3 and TBP18, which are described in §2.11, and αAP-2 (CP-18), an affinity-purified polyclonal antibody that recognizes human AP-2 (Santa Cruz Biotechnology). ARNA-3 was used at a 1:3 dilution in PBS, TBP18 at a 1:50 dilution, and αAP-2 at 1:100 dilution. After primary staining, cells were washed three times, for 5 min each time, in PBS-T. Primary antibodies were detected by incubating cells, for 30 min at 37°C, with rhodamine-conjugated goat anti-mouse immunoglobulin G (for ARNA-3 and TBP18) at a 1:200 dilution in PBS-T, or rhodamine-conjugated donkey anti-rabbit immunoglobulin G at 1:150 dilution in PBS-T (for αAP-2) (Jackson ImmunoResearch Laboratories, Inc.). In some experiments, 4'6'-diamidino-2-phenylindole (DAPI) was added to secondary antibody dilutions at 5 μg/ml. Cells were washed three times, for 5 min each time, in PBS-T, and rinsed briefly in dH_2O. Excess fluid was drained off, and coverslips were mounted on a microscope slide using a mounting gel. Slides were cooled at 4°C for 10 min and then observed with either phase contrast or immunofluorescence microscopy, as described in §2.2. Photographs were taken using an MC100 camera (Axioskop) and either Kodak Tmax 400 ISO black and white print film or Kodak Ektachrome Elite 400 ISO slide film.

2.7. Protein-DNA cross-linking

Sub-confluent cells ($2\text{-}4 \times 10^7$) were subjected to protein-DNA cross-linking, according to the protocol of Wrenn and Katzenellenbogen (1990). Formaldehyde (from a freshly made 11% solution in 50 mM Na-N-[2hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid] [HEPES], 1 M NaCl, 1 mM Na-EDTA, 0.5 mM Na-EGTA, pH 7.5) was added to cell culture medium at a final concentration of 0.2%. Cells were then incubated for 8 min at 37°C. After incubation, the medium was removed and cells were scraped and collected in ice cold PBS. Cells were then pelleted at 200 × g and washed once in PBS. Cells were

resuspended in PBS and counted, then pelleted again. The pellet was resuspended in 1 ml of nuclear lysis buffer (10 mM HEPES [pH 7.5], 0.5% sarkosyl, 1 mM Na-EDTA, 0.5 mM Na-EGTA) containing protease inhibitors (20 μg/ml benzamidine ($C_7H_8N_2$), 2 μg/ml N-tosyl-L-phenyalanine chloromethyl ketone [TPCK], 10 μg/ml leupeptin (Ac-L-L-R-A), 200 μg/ml phenylmethyl sulfonyl flouride [PMSF]; all from Sigma). Cells and nuclei were lysed by sonication using a Branson 250 sonicator on output control setting 5 (duty cycle constant) for four 10 sec intervals, each separated by 10 sec incubations on ice. After sonication, plasma membrane and nuclear lysis was confirmed by visual inspection of the lysate using phase contrast microscopy. Insoluble material was removed by centrifugation at 12,000 × g for 10 min at 4°C.

Lysates were loaded on block gradients composed 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 (all in 10 mM HEPES [pH 7.5], 1 mM Na-EDTA, 0.5 mM ethylene glycol-bis[β-aminoethyl ether]-N,N,N',N'-tetracetic acid [EGTA], 0.5% sarkosyl). These gradients were centrifuged at 77,000 × g (24,000 revolutions per minute in a Beckman SW-27 rotor) in a Beckman L-70 ultracentrifuge for 45-48 h at 20°C. Fractions (0.6 ml each) were collected from the bottom of the gradient through a 20 guage needle. To identify those fractions containing DNA, 10 μl of each fraction was loaded onto a 1% agarose gel which was run at 50 V for 1-1.5 h. Those fractions containing DNA cross-linked to protein, as detected by ethidium bromide staining of this gel, were pooled and dialyzed at 4°C in SpectraPor2 dialysis tubing (MWCO 10,000; Spectrum) against 1 L of dialysis buffer (10 mM Tris·HCl [pH 7.5], 0.1 mM EDTA) for 3 h, with the buffer replaced after 1.5 h. The top three fractions of each gradient, which contained protein not cross-linked to DNA, were treated similarly. After dialysis, the DNA-containing sample was precipitated at -20°C overnight, after adding sodium acetate (pH 5.2) to 0.3 M, SDS to 1%, and 4 volumes of 95% ethanol prechilled to -20°C.

Precipitated DNA (with any cross-linked protein) was pelleted by spinning at 12,000 × g for 45 min at 4°C. The pellet was washed twice with 70% ethanol (prechilled to -20°C) and air dried. The pellet was resuspended in 1 ml of DNase I treatment buffer (10mM Tris•HCl [pH 7.5], 1 mM $CaCl_2$, 1 mM $MgCl_2$, 20 μg/ml benzamidine, 2 μg/ml TPCK, 10 μg/ml leupeptin, 200 μg/ml PMSF). The sample was then heated at 95°C for 10 min to reverse formaldehyde cross-linking, and DNA was digested for 30 min at 37°C with 200 μg of DNase I (Worthington), 5 U of micrococcal nuclease (Sigma), and 1000 U of S1 nuclease (BRL). Aliquots of these samples were assayed for digestion by running on a 1% agarose gel for 1-1.5 h at 50 V and staining with ethidium bromide. Protein from this sample, as well as that from the dialysed sample containing protein not cross-linked to DNA, was then concentrated using an Ultrafree-MC Low Binding Regenerated Cellulose centrifugal filtration column (Millipore; MWCO 30,000) by centrifugation at 3000 × g at 4°C until the volume was less than 20 μl. One volume of 2× SDS-PAGE sample buffer (1×: 60 mM Tris•HCl [pH 6.8], 1% β-mercaptoethanol, 1% SDS, 10% glycerol, 0.01% bromophenol blue) was added to each sample, and the mixture was heated at 95°C for 10 min. Samples were separated by SDS polyacrylamide gel electrophoresis (SDS-PAGE) and analyzed by Western blotting, as described in §2.10 and §2.11.

2.8. Agarose gel electrophoresis

Agarose (molecular biology grade; BIO-RAD) was dissolved in TAE (40 mM Tris•HCl [pH 7.5], 40 mM glacial acetic acid, 2 mM $Na_2EDTA•2H_2O$) to the appropriate concentration. Before loading, samples were mixed with one-tenth volume of gel dye (0.25% bromophenol blue, 0.25% xylene cyanol, 30% glycerol). Gels were run in TAE buffer, in the presence of 0.5 μg/ml ethidium bromide, using a commerical electrophoresis apparatus (Tyler Research Instruments). Gels were visualized using a 312 nm variable intensity trans-illuminator

(FisherBiotech), and photographed with 3000 ISO black and white film (Polaroid).

2.9. Separation of free from DNA-bound RNAP II

Free and DNA-bound RNAP II molecules were separated according to the method of Rangel et al. (1987). HeLa S3 cells (2×10^7) were pelleted and resuspended in hypotonic lysis buffer (25 mM Tris·HCl [pH 7.9], 2 mM EDTA, 1 mM DTT, and 0.2 mM PMSF). Nuclei were isolated by centrifugation at 8700 × g for 10 min at 4°C on a discontinuous sucrose gradient consisting of 4 ml of 0.8 M sucrose in gradient buffer (20 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1 mM DTT, 0.2 mM PMSF), 2 ml of 0.5 M sucrose in gradient buffer with 0.25% Triton X-100, and 2 ml of 0.3 M sucrose in gradient buffer. The nuclei were resuspended in 200 µl of 0.34 M sucrose and incubated on ice for 15 min. Nuclei were then pelleted at 700 × g for 10 min at 4°C. The supernatant (free RNAP II) was collected and the nuclear pellet was resuspended in 200 µl of buffer (10 mM Tris-HCl [pH 7.9], 1 M sucrose, 5 mM $MgCl_2$, 1mM DTT, 0.2 mM PMSF). Two hundred microlitres of 2× SDS-PAGE sample buffer (described in §2.7) was added to each sample; both were heated at 95°C for 10 min and analyzed via SDS-PAGE and Western blotting, as described in §2.10 and §2.11.

2.10. SDS-PAGE

To prepare whole cell lysates, cells were scraped, collected in ice cold PBS, counted using a haemocytometer, and pelleted at 200 × g. Cells were resuspended and lysed in 1X SDS sample buffer (see §2.7) at 10^7 cells/ml and heated at 95°C for 10 min before storage at -20°C. These extracts were separated via SDS-polyacrylamide gel electrophoresis (SDS-PAGE), according to the basic protocol of Laemmli (1970). Gels were cast and run using a Mini-Protean II Dual

Slab Cell apparatus (BIO-RAD). Separating gels consisted of 0.1% v/v N,N,N',N'-tetramethyl-ethylenediamine (TEMED), 0.045% ammonium persulfate (from a freshly made 3% stock solution), 375 mM Tris•HCl (pH 8.8), 0.1% SDS, and enough of a 30% acrylamide/0.8% bis-acrylamide solution to bring the level of acrylamide to the required percentage. Separating gels were layered with water-saturated isobutanol and allowed to polymerize for 1 h before pouring of the stacking gel, which contained 0.1% TEMED (v/v), 0.045% ammonium persulfate, 125 mM Tris•HCl (pH 6.8), 0.1% SDS, 3.8% acrylamide and 0.1% bis-acrylamide. Stacking gels were allowed to polymerize for 45 min.

Gels were run in a buffer consisting of 125 mM Tris•HCl, 0.96 M glycine, and 0.5% SDS (overall pH 8.3). Kaleidoscope protein molecular weight standards (BIO-RAD) (5 μl/gel) were used to monitor separation. All samples were heated at 95°C for 10 min prior to loading. The gel was run at 100 V for 15 min, then at 200 V until the desired marker distribution was achieved. For phosphatase treatment experiments, 10 μl of lysate was treated with 9 U of calf intestinal alkaline phosphatase (GIBCO BRL) at 37°C for 30 min prior to SDS-PAGE.

In some experiments SDS-PAGE gels were silver stained to detect proteins using a commercial silver staining kit (BIO-RAD). Briefly, gels were fixed once in 40% methanol/10% acetic acid for 30 min, then twice in 10% ethanol/5% acetic acid, for 15 min each time. They were then placed in oxidizer solution for 5 min, and given three washes in water of 5 min each. Next they were placed in silver reagent solution for 20 min, after which they were washed in water for 5 min. Gels were then placed in developer solution until the desired level of staining was achieved. Development was stopped by placing the gel in a solution of 5% acetic acid. Stained gels were photographed using Polaroid black and white ISO 3000 film.

2.11. Western blot analysis

Proteins separated via SDS-PAGE were transferred to a HYBOND ECL nitrocellulose membrane (Amersham) using a Mini Trans-Blot Electrophoretic Transfer Cell (BIO-RAD). Transfer was carried out at 20 V overnight in a buffer (prechilled to 4°C), consisting of 25 mM Tris, 192 mM glycine, 0.05% SDS, and 20% methanol. The membrane was then blocked for 2 h at room temperature with PBS-T (0.1%), containing 10% powdered milk (w/v), and 3% BSA. After blocking, the membrane was washed five times with 10 ml rinses of PBS-T: twice quickly, once for 15 min, and twice for 5 min. The membrane was then incubated for 30 min at room temperature with the primary antibody diluted in PBS-T. The membrane was washed as described above, and then incubated with secondary antibody in the same fashion. After secondary antibody incubation, the membrane was washed again, with two additional 5 min washes. Secondary antibodies were detected using the Enhanced Chemiluminescence detection kit (Amersham), and membranes exposed to X-ray film (Fuji RX).

Antibodies used in immunoblotting are described below. ARNA-3 is a monoclonal antibody, affinity-purified using protein A beads, which recognizes the 220 kDa subunit of human RNAP II (RPB1) (Cymbus Bioscience Limited). ARNA-3 was used at a 1:50 dilution. TBP18 is a monoclonal antibody raised against human TBP (gift of Dr. Nancy Thompson, University of Wisconsin), and was used at a 1:1000 dilution. αRAP74, αTFIIE-α, and αTFIIB are affinity-purified rabbit polyclonal antibodies which recognize the following human proteins: the RAP74 subunit of TFIIF, the 56 kDa subunit of TFIIE, and TFIIB respectively (gifts of Dr. Jack Greenblatt, University of Toronto). αRAP74 was used at a 1:300 dilution, αTFIIE–α was used at a 1:500 dilution, and αTFIIB was used at a 1:3000 dilution. αK35 and αp32 are polyclonal rabbit sera that recognize cdk8 and the MAT1 subunit of TFIIH, respectively. These sera were used at a 1:1000 dilution. MO-1.1 is a mouse monoclonal antibody that

recognizes the cdk7 (MO15) subunit of TFIIH (gifts of Dr. Erich Nigg, University of Geneva). This antibody was used at a 1:10,000 dilution. Secondary antibodies were affinity-purified horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G, and affinity-purified horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (Jackson ImmunoResearch Laboratories, Inc.). Secondary antibodies were applied at a 1:100,000 dilution.

3. SYNCHRONIZATION AND CHARACTERIZATION OF MITOTIC HELA CELL POPULATIONS

To study transcription during mitosis in intact cells it was necessary to obtain synchronized cultures. Although mitotic cells may be selectively harvested from asynchronous cultures of HeLa cells by mitotic shakeoff, this method does not yield large numbers of cells, since only roughly 3% of cells in such cultures are in mitosis at any time (see Table 1, panel A). Therefore, it was necessary to use a chemical agent to arrest cells in mitosis. For our mitotic synchronizations we employed the drug nocodazole (5-[2-thienylcarbonyl]-1H-benzimidazol-2yl] carbamate), which interferes with cellular microtubule dynamics at low (nM) concentrations and depolymerizes microtubules completely at higher (mM) concentrations (Jordan et al., 1992; De Brabander et al., 1976). Since the arrangement of microtubules into a mitotic spindle is necessary for progression into anaphase, nocodazole treatment arrests cells at this spindle assembly checkpoint. Treated cells possess the fully condensed chromosomes characteristic of metaphase, but do not align these chromosomes on a metaphase plate. Treatment with nocodazole, at concentrations from 0.04 to 0.5 μg/ml, for time periods of up to 24 h, efficiently arrests populations of many cell types, including HeLa cells, in mitosis (Downes et al., 1994; Chou and Omary, 1993; Zalvide et al., 1992). These treatments apparently do not adversely affect cell metabolism (Zieve et al., 1980).

To obtain mitotic synchronization, we first tested nocodazole treatment, at 0.5 μg/ml, as described in Materials and Methods, §2.1. The proportion of cells in mitosis (mitotic index) was monitored by swelling cells in a hypotonic buffer, staining DNA with propidium iodide, and scoring for condensation of chromosomes using an immunofluorescence microscope. The absence of a nuclear envelope was also scored. To ensure that treatments were not toxic to the cell, cell viability was also assayed, by measuring trypan blue exclusion. We also removed nocodazole-containing medium from arrested cell cultures and

allowed cells to grow in regular media, so as to determine their ability to escape mitotic arrest and proceed into a normal cell cycle.

Nocodazole, applied at 0.5 μg/ml for 8 h, did not produce greater than 60% arrest in sub-confluent cultures of HeLa S3 cells (Table 3.1, panel A). After arrested cells were placed in regular medium for 3 h, only 21% of the population remained in M-phase, indicating that cells were escaping arrest. Viability remained high throughout nocodazole treatment and release.

To improve the efficiency of mitotic arrest, we presynchronized cells with 2 mM thymidine for 24 h. This concentration of thymidine inhibits the enzyme ribonucleotide reductase, leading to a dCTP deficiency that prevents cells from completing DNA replication. Hence, they become arrested in S phase (reviewed in Adams, 1990). This treatment has been widely used for the synchronization of mammalian cultured cells, apparently does not harm cellular physiology, and is fully reversible (Bootsma et al., 1964).

Thymidine treatment for 24 h decreased the mitotic index from 3.5 to 0.7 %, suggesting that it was arresting cells in S phase (Table 3.1, panel B). When thymidine-arrested cells were grown in regular media for 5 h and then treated with 0.5 μg/ml nocodazole for 4 h, 63.1% of the population arrested in mitosis. This treatment did not substantially reduce cell viability and was reversible after 3 h, but did not produce significantly better synchrony than 8 h treatment with nocodazole alone. Therefore, we combined thymidine presynchronization with nocodazole treatments of 8 h. This method yielded 82.5% synchronization in mitosis. We conjectured that our method of removing thymidine, by rinsing the cultures with regular medium, might not be removing all of the drug, and so could be causing some cells to remain arrested in S phase. Therefore, we tried performing a more thorough removal of the drug by trypsinizing the cells and washing them in regular media before replating them. However, this method did not improve upon the 82% synchronization produced by 8 h nocodazole treatment. In fact, when the method of removing thymidine involving

trypsinization was performed before 4 h nocodazole treatement, it reduced the mitotic index of the synchronized population from 63.1 to 24.8 %, indicating that trypsinization was impeding the progress of cells into mitosis. For further experiments, therefore, we employed our initial method of thymidine removal.

When cells were pretreated with thymidine for 24 h, grown in normal medium for 5 h, and then treated with nocodazole at 0.5 μg/ml for 10 h, synchronizations of greater than 90% were consistently obtained. This procedure did not substantially reduce cell viability, and was fully reversible after removal of nocodazole and growth in regular medium for 12 h. The ability of over 95% of cells to reverse arrest indicates that the basic integrity of the cells was not compromised by arrest conditions.

To verify that our method of scoring mitotic index visually was accurate, the cell cycle distribution of these populations (arrested by pretreatment with thymidine, grown in regular medium for 5 h, and then treated with nocodazole for 10 h) was also analyzed by fluorescence-activated cell sorting (FACS) analysis (Figure 3.1). As described in Materials and Methods (§2.3), cells were fixed in ethanol, stained with propidium iodide, treated with RNase A and subjected to flow cytometry to measure the DNA content of each cell. FACS verified that arrested populations were nearly 100% mitotic and, after nocodazole-containing media was replaced with regular media, escaped into G_1 phase. Therefore, in all subsequent experiments requiring mitotic synchronization, this arrest protocol was employed.

Table 3.1 Synchronization of HeLa S3 cells. Mitotic index was determined by swelling of cells and visualization of propidium iodide-stained DNA under an immunofluorescence microscope, as described in Materials and Methods, §2.2. Percentage viability was assessed by measuring trypan blue exclusion, as described in Materials and Methods, §2.4. Cycling, sub-confluent populations of cells were synchronized with either nocodazole (*panel A*) or thymidine and nocodazole (*panel B*), as described in Materials and Methods, §2.1. Five hours elapsed between the removal of thymidine-containing medium and addition of nocodazole. In most cases, after thymidine treatment the cultures were rinsed and then incubated with pre-warmed regular medium. Where indicated, cells were trypsinized and pelleted after thymidine treatment, then washed in regular medium, repelleted and replated. ND - not determined * average of two determinations ** average of three determinations

A

Synchronization Conditions	Mitotic Index	Percent Viability
asynchronous	3.5	96.8
8h nocodazole	58.9	95.3
8h nocodazole + 3h release	21.3	97.3

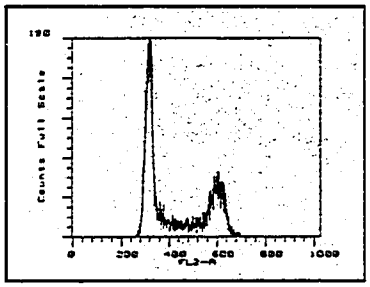
B

Synchronization Conditions	Mitotic Index	Percent Viability
thymidine	0.7	98.3
thymidine + 4h nocodazole	63.1	96.4
thymidine (trypsin) + 4h nocodazole	24.8	ND
thymidine + 4h nocodazole + 1h release	55.0	86.6
thymidine + 4h nocodazole + 3h release	8.93	78.5
thymidine + 8h nocodazole	82.5 *	ND
thymidine (trypsin) + 8h nocodazole	82.8	ND
thymidine + 8h nocodazole + 12 h release	1.1	97.7
thymidine + 10h nocodazole	93.2 **	96.2
thymidine + 10h nocodazole + 8h release	20.1	ND
thymidine + 10h nocodazole + 10h release	7.1	95.4
thymidine + 10h nocodazole + 12h release	2.1	94.2

Figure 3.1 FACS analysis of cell cycle arrest of HeLa S3 cells. 2×10^6 cells were fixed in ethanol, stained with propidium iodide, treated with RNase A and analyzed by flow cytometry, as described in Materials and Methods, §2.3. Shown are histograms of the DNA content of cells which were either actively cycling (*panel A*), mitosis-arrested (*panel B*), or mitosis-arrested and then released (*panel C*). DNA content is represented on the x-axis, and relative cell number on the y-axis. Mitosis-arrested populations were produced by treating sub-confluent cultures with 2 mM thymidine for 24 h, then growing them in regular medium for 5 h, as described in Materials and Methods, §2.1. Nocodazole was then added to the medium at 0.5 μg/ml, and cells grown for 10 h. Released cells were generated by removing the nocodazole-containing medium at the end of this period and growing cells in regular medium for an additional 8 h. *Panel D* contains a quantitative analysis of cell cycle phase distribution of the cycling, mitosis-arrested, and released populations, determined using CellFIT software. Note that the distribution of the arrested population could not be quantitated because too few cells were in G_1 or S phase: however, visual inspection of FACS data indicates that nearly 100% of arrested cells possessed a G_2/M DNA content.

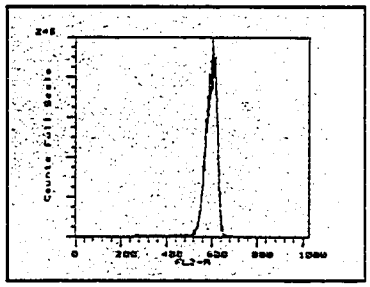
A

Cycling



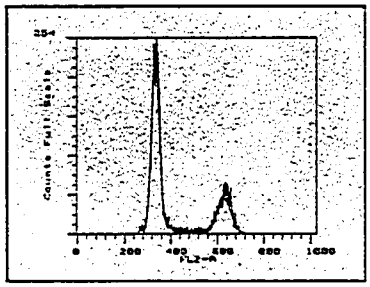
B

Mitosis-Arrested



C

Released



D

	cycling	mitosis-arrested	released
G1	48.5	0	66.7
S	27.3	0	8.1
G2 + M	24.2	100	25.2

4. REPRESSION OF RNAP II TRANSCRIPTION DURING MITOSIS

Previous studies which investigated the mitotic repression of RNAP II transcription employed RNA labelling methods, which measure steady state levels of RNA. In such experiments, a decrease in detectable RNA can indicate either a reduction in transcription, an increase in RNA degradation rates, or both. In addition, in these labelling assays it is difficult to clearly distinguish between transcription by the different RNAPs. Therefore, in order to directly examine RNAP II transcription during mitosis, we used run-on transcription assays.

Run-on transcription assays are typically performed with isolated nuclei, to facilitate the uptake of ribonucleotides into the nucleus. To perform run-on transcription assays in mitotic cells, which do not possess an intact nuclear membrane, we first made whole cells permeable by treatment with lysolecithin, as described in Materials and Methods, §2.5 (Contreras and Fiers, 1981; Miller et al., 1978). Lysolecithin treatment consistently resulted in permeability in over ninety percent of treated HeLa S3 cells, as assayed by trypan blue exclusion (see Materials and Methods, §2.4). After lysolecithin treatment, run-on transcription assays were carried out by a method similar to that used for nuclear run-on transcription assays (see Materials and Methods, §2.5). Using this modified run-on transcription assay, we monitored the production of sense and corresponding antisense RNA from regions of the following genes: *histone H2B, glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*), c-*myc*, c-*fos*, γ-*actin*, and the gene encoding the largest subunit of RNAP II, *RPB1* (for a detailed description of the probes used, see Materials and Methods, §2.5). HeLa S3 cells displayed patterns of transcription on these gene regions similar to patterns observed in nuclear run-on transcription assays (Figure 4.1, panel A; Spencer et al., 1997).

HeLa S3 cells arrested in mitosis by treatment with thymidine and nocodazole displayed reduced levels of transcription in whole cell run-on assays

(Figure 4.1, panel A). Phosphoimage quantitation indicated that in mitotic cells, transcription of the various genes examined was reduced to anywhere from 5.8 to 22.7% of the levels in cycling populations (Table 4.1). Exceptions were the 5′ end of the γ-actin gene, the transcription of which was reduced to 36.7% of its normal level, and the 3′ end of c-*fos*, which was reduced to undetectable levels in mitotic cell populations. Removal of the nocodazole-containing medium and growth in regular medium for 8h resulted in a restoration of transcription to levels similar to those of untreated cells (Figure 4.1, panel A; Table 4.1). This return to normal transcription levels after mitotic release supports the conclusion that cell cycle arrest produced by thymidine and nocodazole treatment does not result in permanent physiological damage to the cell.

To understand the mechanism of the loss of RNAP II transcription during mitosis, it was necessary to know if the labelled RNA we detected in whole cell run-on transcription assays was produced by the elongation of nascent transcripts alone, or if some of it was due to initiation of new transcripts during the assay period. Though signals in nuclear run-on transcription are generally taken to reflect the number of engaged RNAPs on a given segment of a gene, reinitiation may contribute to the signal obtained, especially on promoter-proximal regions (Turcotte et al., 1985). Since transcription in run-on assays typically does not proceed farther than about 500 nucleotides, (Hofer et al., 1981; Weber et al., 1977; Cox, 1976; C.A. Spencer, unpublished results), reinitiation events would be expected to contribute more to the transcription of 5′ regions than 3′ ones. Detergents, such as heparin and sarkoysl, have been widely used to determine the presence of reinitiation events in such assays. At low concentrations, these agents block RNAP II initiation, but not elongation (Bentley and Groudine, 1986). In particular, when run-on transcription assays are performed in the presence of 0.5% sarkosyl, a decrease in transcription from the 5′ segment of a gene, relative to its 3′ end, indicates that reinitiation events are contributing to 5′ transcription (DesJardin et al., 1993; Turcotte et al., 1985).

When we assayed transcription in cycling HeLa S3 cells in the presence of 0.5% sarkosyl, transcription on 5′ regions of two genes, c-*fos* and *γ-actin*, was not decreased substantially more than that on 3′ regions (Figure 4.1; Table 4.2). For c-*fos*, transcription at the 3′ end of the gene was abrogated completely, whereas transcription at the 5′ end was reduced only 54.5%. In the case of *γ-actin*, transcription at the 5′ end was reduced 65.3% and that at the 3′ end 58.0%. This indicates that although some reinitation may be occuring on the 5′ end of the γ-actin gene, it does not contribute most of the signal we observe (i.e. more than 17%). For c-*myc*, however, 5′ transcription was reduced roughly 20% more at the 5′ end than at the 3′ end, suggesting that transcripts initiated during the whole cell run-on transcription assay may constitute a significant part (up to 34%) of the signal we observe on the 5′ region of this gene.

These results suggest that although part of the loss of RNAP II transcription in mitotic cells might be due to a loss of the capacity for reinitiation in mitotic cells, most of it must be due to a decrease in the number or activity of engaged RNAP II molecules on mitotic chromosomes. To verify that the transcription we observe was indeed being carried out by RNAP II, we carried out whole cell run-on transcription assays on cycling cells which were incubated with 2 μg/ml α-amanatin prior to the addition of rNTPs. At this concentration, α-amanatin selectively inhibits RNAP II, whereas at higher concentrations it will also inhibit RNAP III (Stott, 1991). Transcription in our whole cell run-on assay was sensitive to 2 μg/ml α-amanatin (Figure 4.2), indicating that it was indeed carried out by RNAP II.

One explanation for decreased RNAP II transcription during mitosis is that RNAP II molecules remain associated with the DNA template but are unable to catalyze transcription. It has been suggested that the condensation of chromatin during mitosis precludes productive transcription elongation by engaged RNAPs (Matsui et al., 1979; Garigilio et al., 1974). To test whether any RNAP II elongation complexes were present but stalled on mitotic

chromosomes, we sought to release any arrested RNAP II molecules by treatment with salt and sarkosyl.

High concentrations of salt disrupt protein-DNA interactions and can alter chromatin structure, releasing stalled RNAP II elongation complexes and allowing productive elongation and an increase in transcription signal in run-on transcription assays (Rougvie and Lis, 1988; Cai and Luse, 1987). We carried out whole cell run-on transcription assays in the presence of low (15mM), moderate (75mM) and high (300 mM) concentrations of potassium chloride (KCl), using both cycling and mitotic HeLa S3 cells (Figure 4.3). Increasing the salt concentration from 15 to 75 mM increased transcription of observed genes in cycling cells by anywhere from 40 to 143% (Table 4.3). However, similar changes in salt concentration did not increase transcription in mitotic cells to interphase levels (Figure 4.3). In fact, transcription of most genes decreased, although minor increases in transcription of the 3′ region of *γ-actin* and of the *histone H2B* gene were noted (Table 4.3). At high salt concentrations (300 mM), transcription was severely compromised in both populations of cells. Thus, treatment of cells with high salt concentrations in run-on transcription assays did not reveal the presence of large numbers of stalled RNAP II complexes on mitotic DNA.

Sarkoysl (0.5-0.6%) has been shown to strip roughly 80% of chromatin-associated proteins, including histones, from DNA (Scheer, 1978). In the presence of 0.5% sarkosyl, chromatin structure is severly disrupted, but RNAP II-DNA complexes which have synthesized at least one RNA phosphodiester bond remain stable (Hawley and Roeder, 1987; Gariglio, 1976; Green et al., 1975; Garigilo et al., 1974). Therefore, sarkosyl, like high concentrations of salt, can facilitate the release of RNAP II elongation complexes and cause an increase in transcription signal in run-on transcription assays (DesJardin et al., 1993; Krumm et al., 1992; Rougvie and Lis, 1988).

To determine whether RNAP II elongation complexes were stalled on mitotic DNA due to the effects of chromatin condensation, we performed whole

cell run-on transcription assays in the presence of sarkosyl (Figures 4.4 and 4.5). The addition of 0.5% sarkosyl during whole cell run-on transcription assays (carried out at low salt concentration [15 mM]), using mitosis-arrested or cycling HeLa S3 cells, did not cause an increase in the transcription of any gene regions we assayed (Figure 4.4). In fact, transcription levels were universally decreased in the presence of sarkosyl (Figure 4.4). Other studies have observed similar decreases in total RNA synthesis in run-on assays in the presence of 0.5% sarkosyl, and have attributed this to the dissociation of elongation factors from the RNAP II complex by the detergent (Groudine et al., 1981). In support of this hypothesis, *in vitro* transcription studies have demonstrated that sarkosyl lowers the elongation rate of RNAP II (Kephart et al., 1992), and inhibits the association of the elongation factor RAP74 with elongating RNAP II complexes (Zawel et al., 1995).

As our intitial attempt at releasing stalled RNAP II complexes with sarkosyl (Figure 4.4) was carried out at a low salt concentration (15mM), we also applied sarkosyl in our assay at a higher salt concentration (150 mM) (Figure 4.5). However, transcription in mitotic cells was not restored to interphase levels under these conditions. The transcription of most genes in fact declined slightly, although an increase was noted on the 5′ region of γ-actin, perhaps indicating the presence of a small number of engaged RNAP II complexes on this gene in mitotic cells (see Discussion, §7).

Thus, we were not able to release significant numbers of stalled RNAP II complexes in mitotic cells, although we employed a variety of conditions known to disrupt chromatin and facilitate the elongation of such complexes. This suggests that either large numbers of stalled, transcriptionally engaged RNAP II elongation complexes are not present on mitotic chromosomes, or such elongation complexes cannot be detectably released by our salt or detergent treatments.

In summary, whole cell run-on transcription assays reveal that during mitosis RNAP II transcription is repressed on all genes we observe, although the degree of repression varies from gene to gene. Our results also indicate that a low level of RNAP II transcription may be occuring in certain gene regions on mitotic chromosomes (*e.g.* the 5′ region of γ-actin). However, our salt and sarkosyl release experiments are consistent with the hypothesis that large numbers of RNAP II molecules are not transcriptionally engaged during M-phase.

Table 4.1 Repression of RNAP II transcription during mitosis. Results of whole cell run-on transcription assays (Figure 4.1, panel A) were quantitated using a phosphoimager. Transcription levels are represented in arbitrary units, after subtraction of phosphoimaging plate background. Transcription levels for each gene region during mitotic-arrest are also expressed as a percentage of levels in cycling cells.

Probe	Cycling	Mitotic Arrest	Released	Percent Transcription in Mitosis
c-myc 5′	8.1	1.1	10.8	13.6
c-myc 3′	11.4	0.8	15.3	7.0
c-fos 5′	2.2	0.5	2.4	22.7
c-fos 3′	0.6	0	0.8	0
γ-actin 5′	10.1	3.7	10.0	36.7
γ-actin 3′	3.6	0.7	4.5	19.4
GAPDH	3.2	0.4	6.6	12.5
histone H2b	12.1	0.7	6.2	5.8

Table 4.2 Analysis of reinitiation in whole cell run-on transcription assays. Results of whole cell run-on transcription assays conducted on cycling HeLa S3 cells in the presence of 0.5% sarkosyl (Figure 4.1, panel B) are compared to results obtained in the absence of the detergent (Figure 4.1, panel A). Results were quantitated using a phosphoimager, and levels of transcription are expressed in arbitrary units, after subtraction of background due to the phosphoimaging plate. The decrease in transcription levels for each gene region caused by the presence of sarkosyl is also indicated as a percentage of transcription levels in cycling cells.

Probe	Cycling	Sarkosyl Treated	Percent Reduction by Sarkosyl
c-*myc* 5′	8.1	0.9	88.9
c-*myc* 3′	11.1	3.6	67.6
c-*fos* 5′	2.2	1.0	54.5
c-*fos* 3′	0.6	0	100
γ-actin 5′	10.1	3.5	65.3
γ-actin 3′	3.6	1.5	58.0

Table 4.3 Effect of varying salt concentration on RNAP II transcription. Results of whole cell run-on transcription assays (see Figure 4.3) conducted on HeLa S3 cell populations which were either cycling (*panel* A) or arrested in mitosis by thymidine and nocodazole treatment (*panel B*). Whoie cell run-on transcription assays were performed at low (15 mM), moderate (75 mM), and high (300 mM) concentrations of KCl. Transcription signals on each probe were quantitated using a phosphoimager. Levels of transcription are expressed in arbitrary units, after subtraction of non-specific hybridization to DNA probes (for each individual filter, the amount of signal on the least radioactive probe was taken as the level of non-specific hybridization). The amount of transcription of each gene obtained at moderate salt concentration is also expressed as a percentage of the amount of transcription observed at low salt concentration (value in brackets). Note that at 300 mM KCl, transcription in mitosis-arrested cells was undetectable.

A

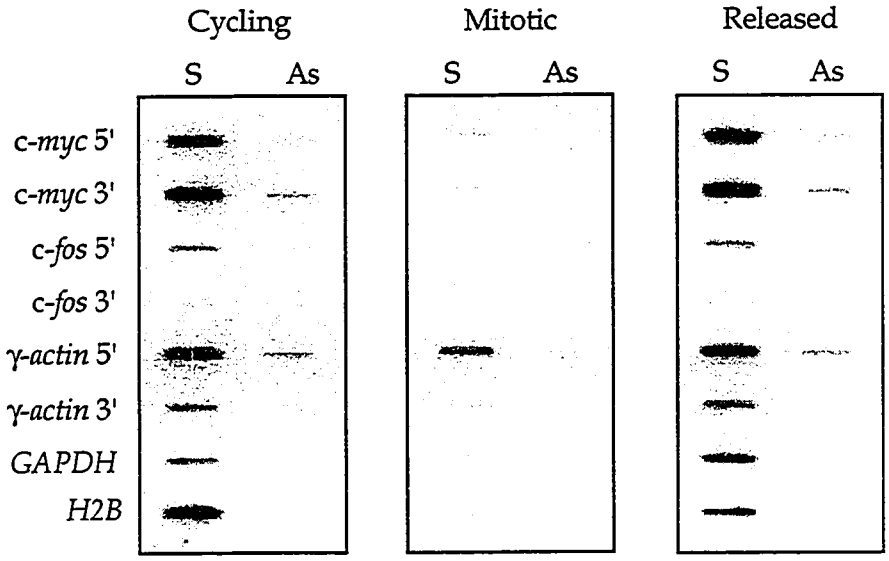
Probe	15 mM	75 mM	300 mM
c-myc 5′	994.0	1832.1 (184.3)	112.0
c-myc 3′	953.9	1611.7 (169.0)	69.4
c-fos 5′	1033.8	1716.3 (166.0)	48.1
c-fos 3′	255.8	364.0 (142.3)	13.9
γ-actin 5′	478.1	671.0 (140.3)	70.3
γ-actin 3′	520.6	910.0 (175.0)	48.8
RPB1 5′	367.9	896.6 (243.7)	169.7
histone H2b	1812.9	3856.9 (212.7)	110.2

B

Probe	15 mM	75 mM	300 mM
c-myc 5′	40.7	32.1 (78.9)	ND
c-myc 3′	38.4	25.2 (65.6)	ND
c-fos 5′	31.1	19.8 (63.7)	ND
c-fos 3′	11.9	0	ND
γ-actin 5′	67.6	63.7 (94.2)	ND
γ-actin 3′	42.3	73.5 (173.8)	ND
RPB1 5′	15.0	17.4 (116.0)	ND
histone H2b	31.5	80.9 (256.8)	ND

Figure 4.1 RNAP II transcriptional repression in mitosis-arrested cells. Shown are autoradiographs of whole cell run-on transcription assays carried out as described in Materials and Methods, §2.5. Briefly, cells were made permeable by treatment with lysolecithin, and incubated for 30 min at 30°C in the presence of α-^{32}P-UTP. RNA was isolated and hybridized to nitrocellulose filters containing single stranded DNA probes that detect sense and antisense transcription from the indicated genes. Equal numbers of cells were assayed in all samples. *Panel A.* RNAP II transcription in populations of HeLa S3 cells which were either cycling, arrested in mitosis by thymidine and nocodazole treatment, or arrested and then released for 8 h (see Materials and Methods, §2.1). *Panel B.* RNAP II transcription in a population of cycling cells treated with 0.5% sarkosyl prior to the addition of rNTPs. Note that this autoradiograph has been exposed longer than those in panel A (see Table 4.2 for a direct comparison of transcription levels in untreated and sarkosyl treated cells). The run-on transcription assay and isolation of RNA were performed by C.A. Spencer.

A


Cycling
Mitotic
Released
S
As
S
As
S
As
c-myc 5'
c-myc 3'
c-fos 5'
c-fos 3'
γ-actin 5'
γ-actin 3'
GAPDH
H2B

B

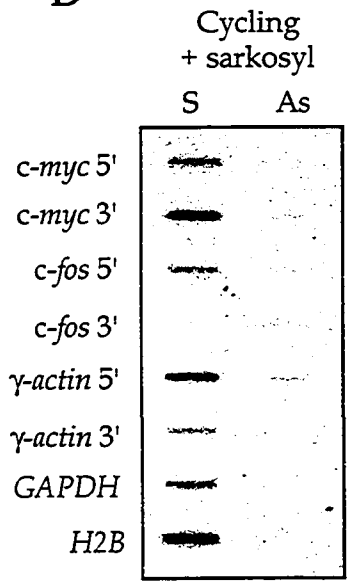

Cycling
+ sarkosyl
S
As
c-myc 5'
c-myc 3'
c-fos 5'
c-fos 3'
γ-actin 5'
γ-actin 3'
GAPDH
H2B

Figure 4.2 Effect of α-amanatin on whole cell run-on transcription assays. Shown is a computer-generated image of a phosphoimage plate exposed to filters hybridized to whole cell run-on transcription assay samples. Transcription assays were carried out on populations of cycling cells as described in Materials and Methods, §2.5. RNA was isolated and hybridized to nitrocellulose filters containing single stranded DNA probes that detect sense and antisense transcription from the indicated genes. An equal number of cells were assayed in each sample. In the sample indicated, α-amanatin was added at a concentration of 2 μg/ml before the addition of rNTPs.

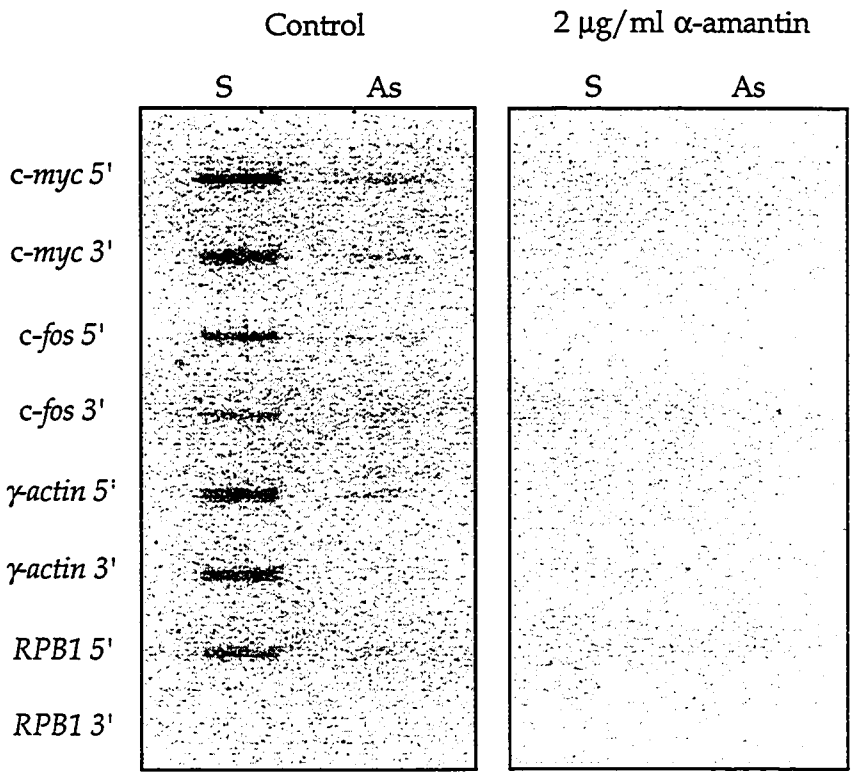

Control
2 µg/ml α-amantin
S
As
S
As
c-myc 5'
c-myc 3'
c-fos 5'
c-fos 3'
γ-actin 5'
γ-actin 3'
RPB1 5'
RPB1 3'

Figure 4.3 Effect of varying salt concentration on RNAP II transcription during interphase and mitotic-arrest. Shown is a computer-generated image of a phosphoimage plate exposed to filters hybridized to whole cell run-on transcription assay samples. Transcription assays were carried out as described in Materials and Methods, §2.5. Populations of cells were either cycling (*panel A*) or arrested in mitosis by thymidine and nocodazole treatment (*panel B*), as described in Materials and Methods, §2.1. Whole cell run-on transcription assays were performed in the presence of the indicated concentration of KCl. RNA was isolated and hybridized to nitrocellulose filters containing single stranded DNA probes that detect sense and antisense transcription from the indicated genes.

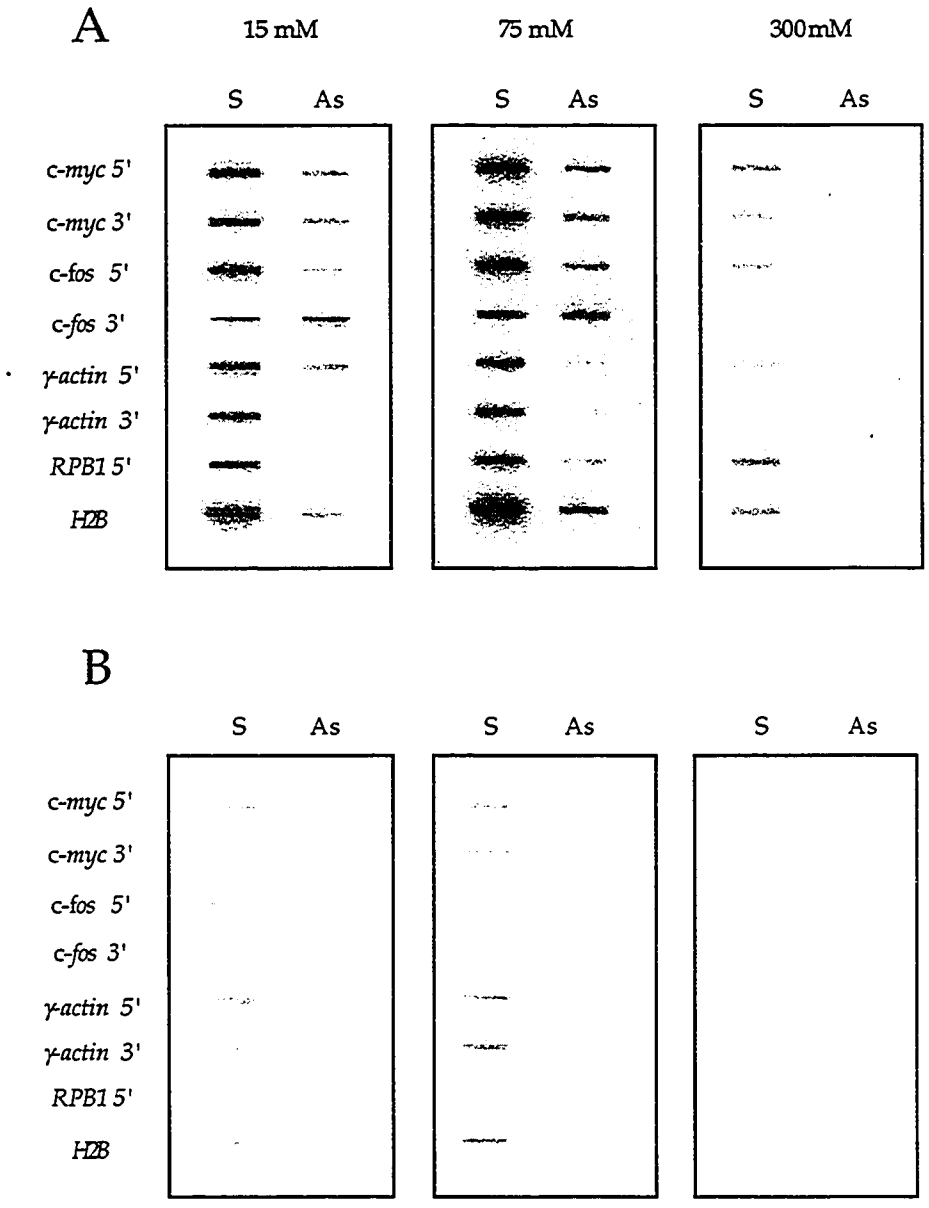

A
15 mM
75 mM
300 mM
S
As
S
As
S
As
c-myc 5'
c-myc 3'
c-fos 5'
c-fos 3'
γ-actin 5'
γ-actin 3'
RPB1 5'
HZB
B
S
As
S
As
S
As
c-myc 5'
c-myc 3'
c-fos 5'
c-fos 3'
γ-actin 5'
γ-actin 3'
RPB1 5'
HZB

Figure 4.4 Effect of sarkosyl on RNAP II transcription at low salt concentration during interphase and mitotic-arrest. Shown is a computer-generated image of a phosphoimage plate exposed to filters hybridized to whole cell run-on transcription assay samples. Transcription assays were carried out as described in Materials and Methods, §2.5, except that the KCl concentration was reduced to 15 mM. Where indicated, cells were incubated with 0.5% sarkosyl prior to the addition of rNTPs. Populations of cells were either cycling (*panel A*) or arrested in mitosis by thymidine and nocodazole treatment (*panel B*), as described in Materials and Methods, §2.1. RNA was isolated and hybridized to nitrocellulose filters containing single stranded DNA probes that detect sense and antisense transcription from the indicated genes. Note that different exposure times are represented in panels A and B.

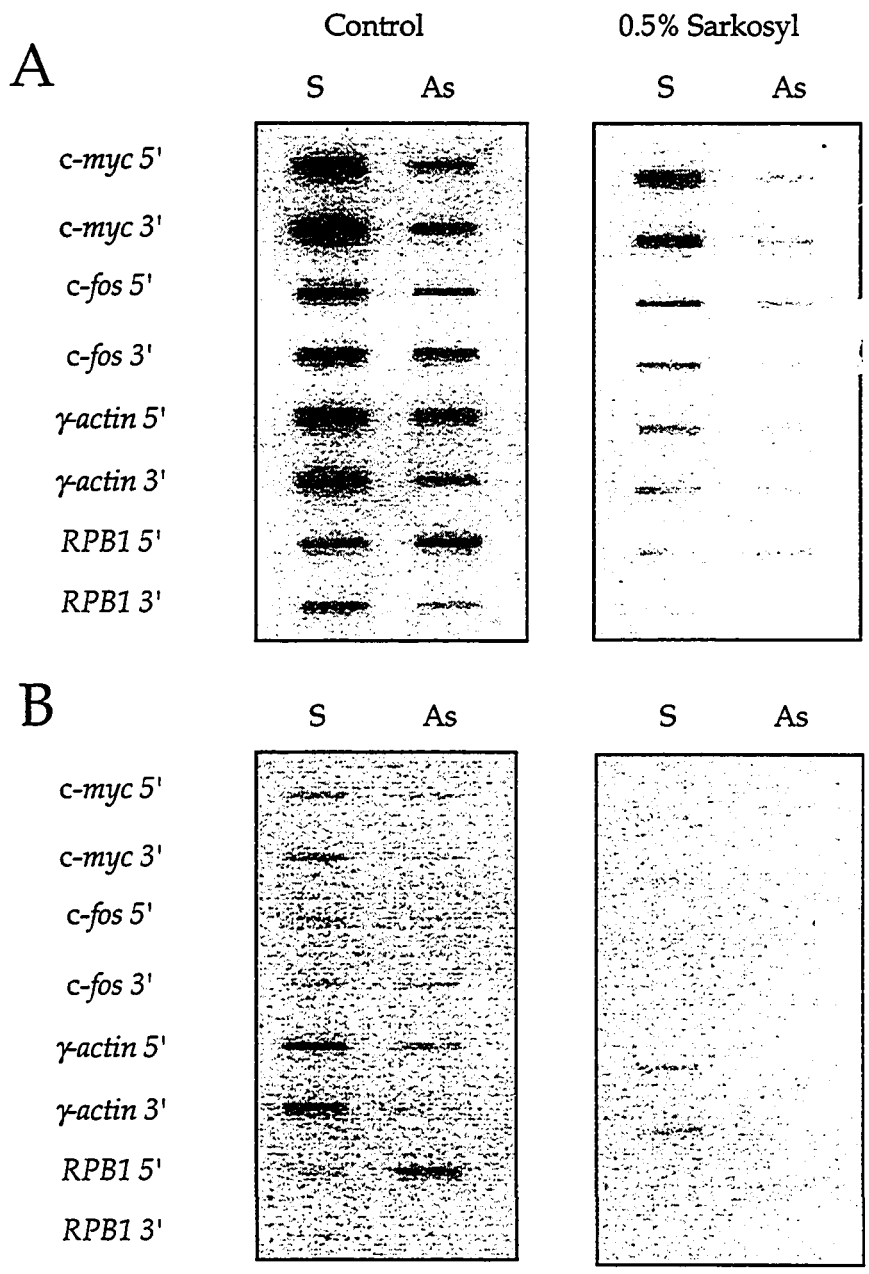
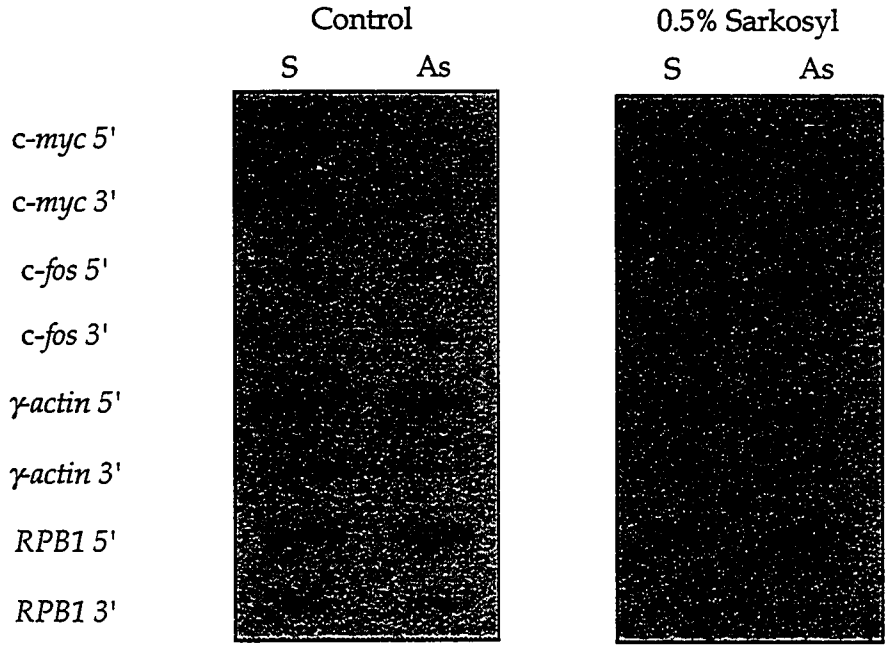

A
Control
0.5% Sarkosyl
S
As
S
As
c-myc 5'
c-myc 3'
c-fos 5'
c-fos 3'
γ-actin 5'
γ-actin 3'
RPB1 5'
RPB1 3'
B
S
As
S
As
c-myc 5'
c-myc 3'
c-fos 5'
c-fos 3'
γ-actin 5'
γ-actin 3'
RPB1 5'
RPB1 3'

Figure 4.5 Effect of sarkosyl on RNAP II transcription at moderate salt concentration during mitotic-arrest. Shown is an autoradiograph of filters hybridized to whole cell run-on transcription assay samples. Transcription assays were carried out as described in Materials and Methods, §2.5, in the presence of 150 mM KCl. Where indicated, cells were treated with 0.5% sarkosyl prior to the addition of rNTPs. Populations of cells were arrested in mitosis by thymidine and nocodazole treatment, as described in Materials and Methods, §2.1. RNA was isolated and hybridized to nitrocellulose filters containing single stranded DNA probes that detect sense and antisense transcription from the indicated genes.


Control
0.5% Sarkosyl
S
As
S
As
c-myc 5'
c-myc 3'
c-fos 5'
c-fos 3'
γ-actin 5'
γ-actin 3'
RPB1 5'
RPB1 3'

5. CHANGES IN RNAP II GTF LOCALIZATION DURING MITOSIS

5.1. RNAP II

5.1.1. RNAP II localization during mitosis

5.1.1.1. Immunofluorescent localization

Knowledge of the physical location of RNAP II in mitotic cells is of central importance to understanding the mechanism of RNAP II transcriptional repression during mitosis (see §1.4.4.3). Our run-on transcription assays failed to detect stalled RNAP II molecules on mitotic DNA. Therefore, to determine whether RNAP II molecules were associated with DNA during mitosis, we visualized the large subunit of the enzyme (RPB1) using indirect immunofluorescence. This technique has proven useful in determining whether sequence-specific transcription factors (Martinez-Balbás et al., 1995) and other RNAP II GTFs (Segil et al., 1996) remain associated with mitotic chromosomes.

HeLa cells were fixed in 3.7% formaldehyde and made permeable in acetone. They were incubated with primary antibodies recognizing RPB1, washed, and then incubated with fluorochrome-conjugated secondary antibodies and DAPI. Cells were then observed using immunofluorescence or phase-contrast microscopy. HeLa S3 cells arrested in mitosis by thymidine and nocodazole treatment do not display a typical metaphase plate chromosome arrangement; instead, the chromosomes are located in a irregular cluster near the cell centre (Figure 5.1, panel B, DAPI). In these cells, RPB1 detected with the ARNA-3 monoclonal antibody, which recognizes all known phosphorylation variants of the protein (IIo, IIa, and IIi), was found to be dispersed throughout the cytoplasm, and excluded from the chromosomes. (Figure 5.1 panel B, Immunofluorescence). This is in marked contrast to the RPB1 distribution in interphase cells, in which the enzyme is localized predominantly in the nucleus (Figure 5.1 panel A, Immunofluorescence). Similar results were obtained using

the 8WG16 monoclonal antibody, which recognizes a different epitope of RPB1 and detects the IIa form only (Figure 5.1 panel B, Immunofluorescence). Also, cells stained with secondary antibody alone displayed very little detectable signal in photographs taken with equivalent exposure times (Figure 5.1, panel C), indicating that the observed fluorescence patterns were generated by the RNAP II large subunit-specific antibodies.

As these experiments utilized cells which were nocodazole-arrested, we wished to confirm that our results were not artifacts of this drug treatment. Therefore, we observed RPB1 localization in naturally occuring mitotic cells. To do this, we performed indirect immunofluorescence on populations of cycling HeLa CCL2 cells (Figure 5.2). This cell line was used, instead of the HeLa S3 line, because it displayed increased adherence to cover slips, facilitating the attainment of numerous mitotic figures. The use of cycling populations rather than synchronized ones also allowed the observation of RPB1 localization during each of the subphases of mitosis. Cells in early prophase, which displayed a coarseness in their chromatin appearance as visualized by DAPI staining, did not show discernible exclusion of RPB1 from chromatin, although some cytoplasmic dispersion of RPB1 was apparent. Cells in prometaphase, whose chromatin possessed a severely clumped character, did show exclusion of RPB1 from DNA, in sharp contrast to the strongly nuclear localization of RPB1 during interphase (Figure 5.1, panel A). In fact, in prometaphase cells, the areas which stained with antibodies against RPB1 coincided precisely with those regions of the cell not stained by DAPI. Cells in metaphase showed the most dramatic exclusion of RPB1 from chromatin, but cells in early and late anaphase also displayed this phenomenon. Interestingly, cells in late telophase, which had all but completed cytokinesis, and which had reformed nuclear envelopes and nucleoli, also showed a small degree of RPB1 exclusion from the nucleus, with most signal remaining dispersed in the cytoplasm (Figure 5.2, bottom panels).

To be certain that any failure to visualize RPB1 on mitotic DNA was not caused by a general exclusion of all antibodies from mitotic chromatin, we

attempted to detect the AP-2 transcription factor, which is not excluded from chromatin during mitosis in HeLa S3 cells (Martinez-Balbás et al., 1995). Indirect immunofluorescent localization using an affinity-purified polyclonal antibody directed against AP-2 demonstrated that AP-2 was not excluded from metaphase chromosomes (Figure 5.3). This indicated that the observed exclusion of RPB1 from mitotic DNA was not caused by a general inability of antibodies to recognize epitopes associated with condensed chromosomes. Furthermore, the observed exclusion of RPB1 from mitotic chromatin was not caused by the staining of the DNA with DAPI, since exclusion occurred even in the absence of DAPI (Figure 5.4).

5.1.1.2. Protein-DNA cross-linking analysis.

Our immunofluorescence data (see previous section) suggested that RNAP II is excluded from chromatin during mitosis, and is dispersed throughout the cytoplasm. In order to corroborate these data using a different method, we attempted to cross-link chromatin-associated RNAP II to DNA in both cycling and mitosis-arrested populations of HeLa S3 cells, using a modification of the formaldehyde cross-linking protocol of Wrenn and Katzenellenbogen (1990) (Figure 5.5). These investigators succesfully employed this protocol to assay changes in RPB1-DNA associations between different populations of MCF-7 human breast cancer cells.

Formaldehyde produces protein-nucleic acid and protein-protein cross-links with a much greater efficiency than ultraviolet light (Wrenn and Katzenellenbogen, 1990). It is capable of bridging distances as small as 2 Å through the formation of methylene bridges between lysine residues of proteins or between lysine residues and the amino or imido groups of DNA bases (Jackson, 1978). Formaldehyde has been widely employed in the study of histone-DNA contacts, and will cross-link to chromatin only those proteins intimately associated with it (Solomon et al., 1988; Solomon and Varshavsky, 1985). Furthermore, alterations in protein-DNA contacts detected using this

technique correlate with changes monitored by other techniques, suggesting that formaldehyde measures *in vivo* protein-DNA associations (Wrenn and Katzenellenbogen, 1990; Solomon et al., 1988). Formaldehyde-induced protein-DNA cross-links are also reversible under mild conditions, allowing the characterization of cross-linked proteins (Jackson, 1978).

Briefly, cycling or mitosis-arrested cells were treated with formaldehyde and lysed. The DNA, along with any proteins cross-linked to it, was then purified on a CsCl gradient, isolating it from all non-cross-linked proteins. On these gradients, free DNA migrates to the bottom of the gradient, DNA cross-linked to protein runs in the middle of the gradient, and non-cross-linked protein runs in the top fractions of the gradient (Wrenn and Katzenellenbogen, 1990). Fractions were collected from the bottom of the gradient and those containing DNA were identified by running an aliquot of each fraction on a 1% agarose gel and staining with ethidium bromide, allowing visualization of high molecular weight DNA. In fractionated lysates derived from cells not treated with formaldehyde, DNA migrated to the bottom of the gradient (Figure 5.6). In contrast, DNA in fractionated lysates derived from cells that were treated with formaldehyde migrated towards the center of the gradient (Figure 5.6). This change in mobility suggested that the DNA was being cross-linked to protein by formaldehyde, as also observed by Wrenn and Katzenellenbogen (1990). From visual inspection of ethidium bromide staining of aliquots of various CsCl gradients (*e.g.* Figure 5.6), it was also observed that formaldehyde treatment resulted in the isolation of greater amounts of DNA, perhaps by inactivating nuclease activities which may have digested DNA during lysate preparation or CsCl gradient centrifugation.

We wished to confirm that the high molecular weight, ethidium-bromide staining material apparent in agarose gel electrophoresis of aliquots of our CsCl gradients was indeed DNA. Therefore, aliquots of DNA-containing fractions from the center of CsCl gradients performed using lysates of formaldehyde-

treated cycling HeLa S3 cells were dialyzed to remove salts. Subsequently, these aliquots were treated with either DNase I or RNase A for 30 min at 37°C, or merely incubated for 30 min at 37°C. Aliquots were then visualized by performing agarose gel electrophoresis in the presence of ethidium bromide. The high molecular weight material we observed on CsCl gradients was apparently DNA, since, after dialysis to remove salts, it was sensitive to DNase I but not RNase A (Figure 5.7).

To confirm that formaldehyde was cross-linking protein to DNA, we examined protein-DNA interactions in cycling populations of HeLa S3 cells which were either treated with formaldehyde or untreated (Figure 5.8). Lysates of each population were fractionated on CsCl gradients. Three DNA-containing fractions from the bottom of a gradient prepared using a lysate of untreated cells (lane UD) were pooled, as were three DNA-containing fractions from the center of a gradient prepared using a lysate of formaldehyde-treated cells (lane FD). After dialysis, DNA precipitation and DNA digestion, the proteins in each of these samples were isolated. Additionally, for both the untreated and formaldehyde-treated gradient, three fractions migrating between DNA and free protein were also pooled and the proteins in them isolated in a similar manner (lanes UX and FX). The amount of RPB1 present in each sample was then analyzed using SDS-PAGE and Western blotting with ARNA-3 antibodies. These experiments demonstrated that when cells were not treated with formaldehyde, very little RPB1 copurified with DNA after CsCl gradient subcellular fractionation (lane UD). After formaldehyde treatment, however, a substantial amount of RPB1 IIo copurified with DNA (lane FD), though it was not found in other gradient fractions which migrated between the DNA and non-cross-linked protein (lane FX). This suggests that formaldehyde covalently cross-links DNA and RPB1 IIo molecules, and does not merely alter the mobility of RPB1 IIo on CsCl gradients. Also, only the IIo form of RPB1 was found to copurify with the DNA (Figure 5.8, lane FD). Since the IIo form is present in elongation complexes, whereas the IIa form is the species which enters PICs

from solution (reviewed in Dahmus, 1994), this result demonstrates that our cross-linking technique is specific for DNA-associated proteins.

As this technique offered a means of comparing the relative levels of protein-DNA association in different populations of cells, we employed it to determine whether the same amount of RPB1 is bound to DNA in mitosis-arrested cells as in interphase cells. We exposed cycling or mitosis-arrested HeLa S3 cells to protein-DNA cross-linking by formaldehyde. Lysates were prepared and then fractionated on CsCl gradients. DNA was then precipitated and subsequently digested, and cross-linked proteins were analyzed via SDS-PAGE and Western blotting. Much less RNAP II was cross-linked to DNA in populations of mitosis-arrested cells than in interphase populations (Figure 5.9, panel A).

The paucity of cross-linked RPB1 IIo obtained from mitosis-arrested cells was probably not due to the selective proteolysis of RPB1 from mitotic cells during our isolation procedure, as similar amounts of non-cross-linked RPB1 were isolated from the mitosis-arrested and interphase cell lysates used in this experiment (Figure 5.9, panel B). We noted that RNAP IIo was relatively more abundant in samples of non-cross-linked protein isolated from mitosis-arrested cells than in those isolated from cycling cells (Figure 5.9, panel B). This alteration in the relative amount of RPB1 IIo was not caused by formaldehyde treatment, as it was observed in lysates of non-cross-linked cells that were subjected to similar DNA purification procedures (data not shown). Given the increased kinase activity characteristic of mitosis, it seems likely that this increase in RNAP IIo is caused by RNAP IIa phosphorylation, after cell lysis, by mitotic kinases. Based on comparison to the amount of RPB1 in the free protein found at the top of the gradient, we estimate the amount of RPB1 cross-linked to DNA in the cycling population to be approximately 10% of total cellular IIo. Thus, our cross-linking procedure was less efficient than that of Wrenn and Katzenellenbogen (1990), who cross-linked from 20-50% of cellular IIo to DNA in interphase MCF-7 cells.

Our failure to detect RPB1 in Western blot analysis of DNA-cross-linked proteins from mitosis-arrested HeLa S3 cells did not seem to be caused by an inability of our DNA digestion conditions to degrade mitotic DNA. This circumstance could result in a failure of proteins to migrate normally in SDS-PAGE. Agarose gel electrophoresis indicated that the DNA from both interphase and mitosis-arrested cells was reduced to a low molecular weight form by treatment with nucleases (Figure 5.10). Therefore, if any RPB1 was cross-linked to DNA in mitosis-arrested cells, it should have migrated normally in SDS-PAGE (see also below, and Figure 5.11).

Also, the lack of detectable RPB1 in the DNA-containing fractions of fractionated mitosis-arrested cell lysates was not caused by a general failure of formaldehyde to cross-link DNA-associated proteins to chromatin in mitosis-arrested cells. We performed protein-DNA cross-linking on populations of mitosis-arrested and cycling HeLa S3 cells and made lysates from each. Lysates were fractionated by CsCl gradient centrifugation. DNA-containing fractions were pooled, dialyzed, and treated with nucleases. Proteins in the DNA-containing fractions were then detected by SDS-PAGE and silver staining. This experiment revealed that much more total protein was cross-linked to DNA in mitosis-arrested cells than in interphase cells (Figure 5.11). This result further highlights the selectivity of our cross-linking technique, since the amount of RPB1 IIo cross-linked to DNA does not increase in mitosis-arrested cells, as opposed to interphase cells, although the total level of protein-DNA cross-linking increases dramatically.

5.1.3. RNAP II localization during interphase

The results of our formaldehyde cross-linking studies (see previous section), together with the results of our run-on transcription assays (see §4) and immunofluorescence studies (§5.1.1.1), suggest that RNAP II is not present on mitotic chromosomes. If the RNAP II GTF machinery is ejected from DNA during chromosome condensation, this could be responsible for the cessation of

transcription during mitosis (Moreno and Nurse, 1990). However, if the amount of RNAP II GTFs bound to the DNA during interphase is very low, then the dramatic exclusion of GTFs which we observe by immunofluorescence during mitosis might not represent an ejection of transcription factors from the DNA, but merely a dispersal into the mitotic cytoplasm of soluble factors due to nuclear envelope breakdown. To address the question of whether RNAP II is actually ejected from DNA upon entry into mitosis or merely diffuses out of the disintegrating nucleus, we attempted to determine the amount of RNAP II which is associated with DNA during interphase (Figure 5.12).

Briefly, nuclei were isolated, purified on a sucrose gradient, and then resuspended in 0.34 M sucrose. This procedure has been shown to facilitate the solubilization of a proportion of RNAP II activity from nuclei into the supernatant solution (Yu, 1976). The RNAP II activity of the supernatant can only function using exogenous DNA templates, suggesting that it is constituted by free, or uninitiated, RNAP II. Conversely, RNAP II activity remaining in the nuclei after solubilization is capable of transcribing only endogenous DNA, suggesting that it is composed of DNA-bound, transcriptionally engaged RNAP II molecules. Since we were concerned with the physical distribution of RNAP II between DNA and the nucleoplasm, we used SDS-PAGE and Western blotting to determine how much RNAP IIo could be solubilized by this technique. We found that all RPB1 IIo remained in the nuclei, whereas all the RPB1 IIa detected was found outside the nuclei (Figure 5.12). Since RNAP IIO constitutes at least half of the total RNAP II in HeLa S3 cells (Dahmus, 1994; Rice et al., 1994; see also Figure 5.8), this result is consistent with roughly fifty percent of total RNAP II being bound to DNA during interphase. This figure agrees with previous estimates made using this technique but based solely on RNAP II activity assays (Yu, 1976). Thus, in light of our immunofluorescent localization studies (see above) this result suggests that substantial amounts of RNAP II must be ejected from DNA upon entry into mitosis.

5.2. TBP

The finding that RNAP II association with DNA is dramatically reduced during mitosis (see §5.1.1.1 and §5.1.1.2) suggests that other RNAP II GTFs may also be stripped from mitotic DNA. Therefore, we also examined the localization of TBP during mitosis using indirect immunofluorescence, as described in Materials and Methods, §2.6. Briefly, cells were fixed in 3.7% formaldehyde and made permeable by incubation in acetone. They were incubated with TBP18 monoclonal antibodies, washed, and then incubated with fluorochrome-conjugated secondary antibodies and DAPI. TBP distribution was then examined using an immunofluorescence microscope.

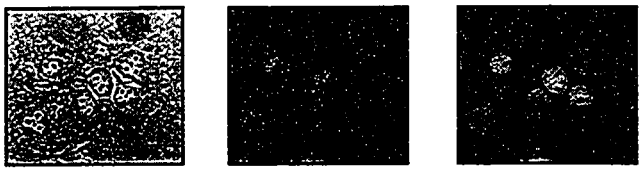
Like RNAP II, TBP is predominantly a nuclear protein during interphase in HeLa S3 cells (Figure 5.13, panel A, Immunofluorescence). In mitosis-arrested cells, however, TBP displays a pattern of exclusion from chromosomes similar to that observed for RPB1 in mitosis-arrested cells (Figure 5.13, panel B, Immunofluorescence). In control experiments conducted in the absence of primary antibodies, little staining of mitosis-arrested cells was observed (Figure 5.13, panel C), indicating that immunofluorescent signal specifically indicates the presence of TBP18. In cycling populations of HeLa CCL2 cells, TBP is gradually excluded from DNA during mitosis in a fashion similar to that observed for the RNAP II large subunit. This exclusion commences in late prophase and persists until late telophase (Figure 5.14). These results confirm the observations of Segil et al. (1996) and Jordan et al. (1996), and suggest that stripping of multiple GTFs from chromosomes may be an important aspect of the mechanism of the repression of RNAP II transcription during mitosis.

Figure 5.1. Immunofluorescent localization of RPB1 during interphase and mitotic-arrest. Shown are interphase HeLa S3 cells from cycling populations (*panel A*) and cells arrested in mitosis by thymidine and nocodazole treatment, as described in Materials and Methods, §2.1 (*panels B and C*). Indirect immunofluorescence was performed as described in Materials and Methods, §2.6. Briefly, cells were grown on glass coverslips, fixed in 3.7% formaldehyde and made permeable by treatment with acetone. After washing in PBS, they were incubated with either ARNA-3 or 8WG16 monoclonal antibodies (*panels A and B*), or with PBS alone (*panel C*). After further washing, cells were incubated with rhodamine-conjugated secondary antibodies and DAPI. Rhodamine and DAPI fluorescence were then detected by immunofluorescence microscopy. Cells were also visualized using phase contrast microscopy. Note that equivalent exposure times are represented in the immunofluorescence photographs in panels B (ARNA-3) and C.

A

Phase Contrast | Immuno-fluorescence | DAPI

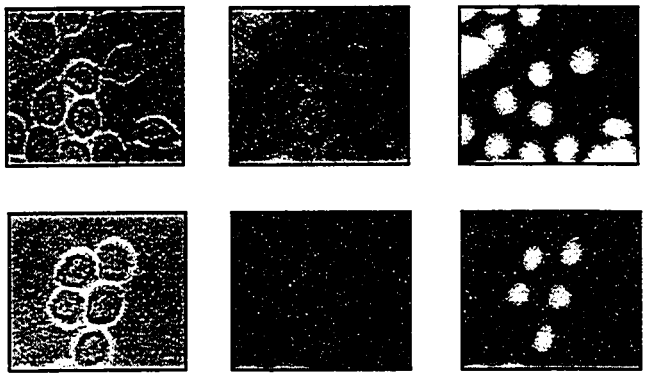
ARNA-3



B

8WG16

ARNA-3



C

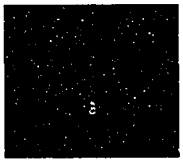


Figure 5.2. Immunofluorescent localization of RPB1 during mitosis. Indirect immunofluorescence was performed as described in Materials and Methods, §2.6. Briefly, HeLa CCL2 cells were grown, at sub-confluence, on glass coverslips, fixed in 3.7% formaldehyde and made permeable by treatment with acetone. After washing in PBS, they were stained with ARNA-3 monoclonal antibodies. After further washing, cells were incubated with rhodamine-conjugated secondary antibodies and DAPI. Rhodamine and DAPI fluorescence were then detected by immunofluorescence microscopy. Cells were also visualized using phase contrast microscopy.

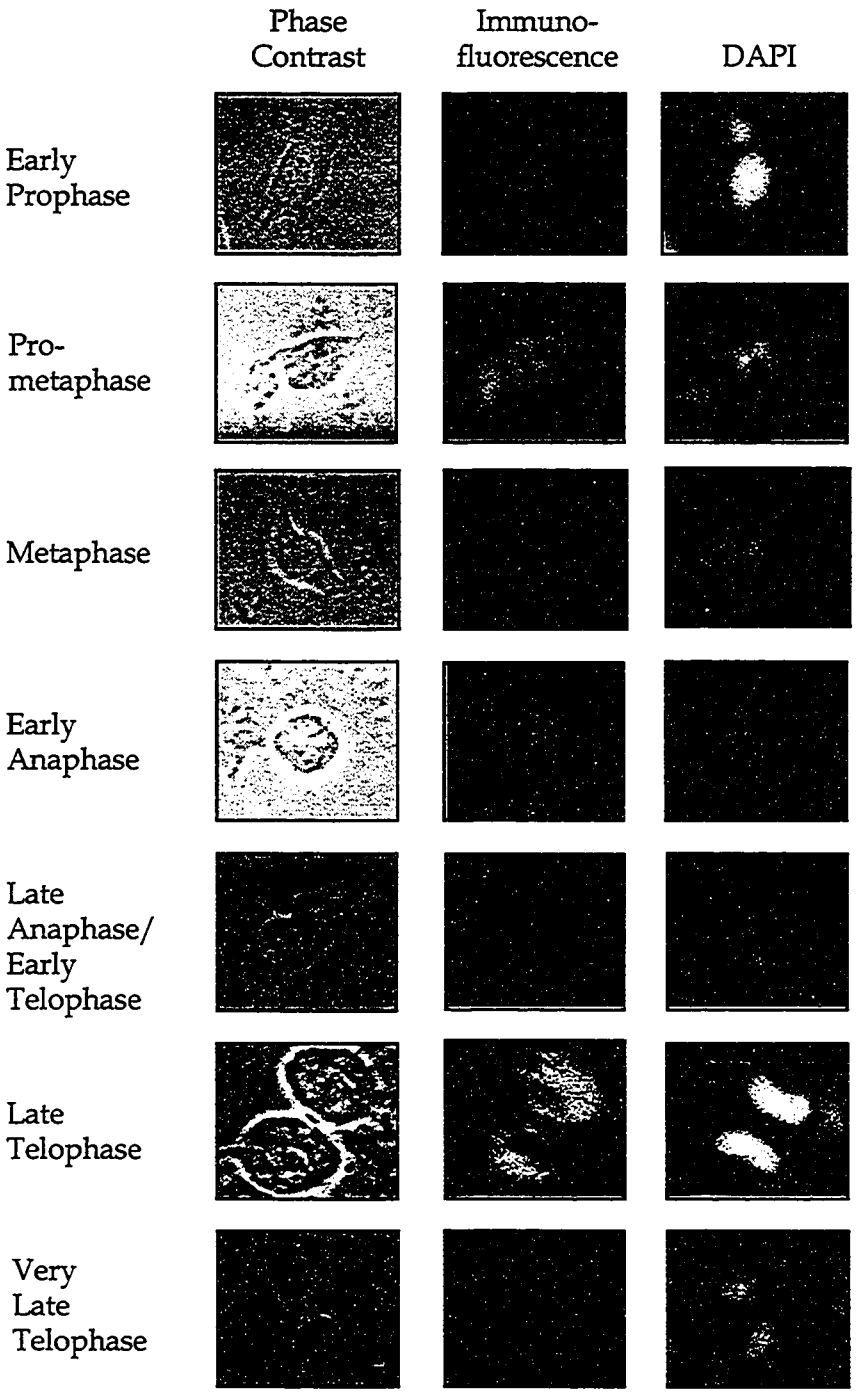

Phase Contrast
Immuno-fluorescence
DAPI
Early Prophase
Pro-metaphase
Metaphase
Early Anaphase
Late Anaphase/ Early Telophase
Late Telophase
Very Late Telophase

Figure 5.3. Immunofluorescent localization of AP-2 during mitosis. Indirect immunofluorescence was performed as described in Materials and Methods, §2.6. Briefly, HeLa S3 cells were grown on glass coverslips, fixed in 3.7% formaldehyde and made permeable by treatment with acetone. After washing in PBS, they were stained with affinity-purified α-AP-2 polyclonal antibodies. After further washing, cells were then incubated with rhodamine-conjugated secondary antibodies and DAPI. Rhodamine and DAPI fluorescence were then detected by immunofluorescence microscopy. Cells were also visualized using phase contrast microscopy. Shown is a metaphase cell.

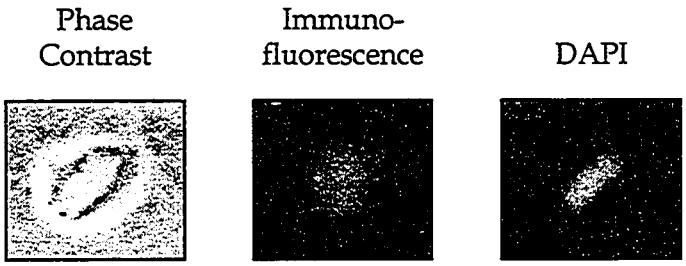
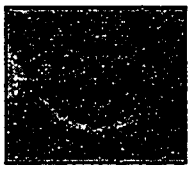

Phase Contrast
Immuno-fluorescence
DAPI

Figure 5.4. Immunofluorescent localization, in the absence of DAPI, of RPB1 during mitosis. Indirect immunofluorescence was performed as described in Materials and Methods, §2.6. Briefly, HeLa S3 cells were grown on glass coverslips, fixed in 3.7% formaldehyde and made permeable by treatment with acetone. After washing in PBS, they were stained with ARNA-3 monoclonal antibodies. After further washing, cells were incubated with rhodamine-conjugated secondary antibodies. Rhodamine fluorescence was then detected by immunofluorescence microscopy. Cells were also visualized using phase contrast microscopy. Shown is a metaphase cell.

Phase Contrast



Immuno-fluorescence

Figure 5.5. Method of measuring changes in protein-DNA associations *in vivo* via formaldehyde cross-linking. For a full description of this protocol, see Materials and Methods (§2.7). Briefly, 0.2% formaldehyde was added to the cell culture growth medium, and the cultures then incubated for 8 min at 37°C. Cells were then scraped and collected by centrifugation. Cell pellets were lysed and sonicated. These lysates were layered onto discontinuous CsCl gradients and centrifuged at 77,000 × g for 45-48 h at 20°C. Fractions containing DNA were dialyzed to remove salts, and the DNA, along with any proteins cross-linked to it, was precipitated. The samples were then heated at 95°C for 10 min to reverse cross-linking, DNA was digested, and proteins were then concentrated. In some experiments, fractions from the top of the gradient, which contain free protein, were also pooled, dialyzed to remove salts, and concentrated. Proteins were analyzed via SDS-PAGE and Western Blotting, as described in Materials and Methods, §2.10 and §2.11.

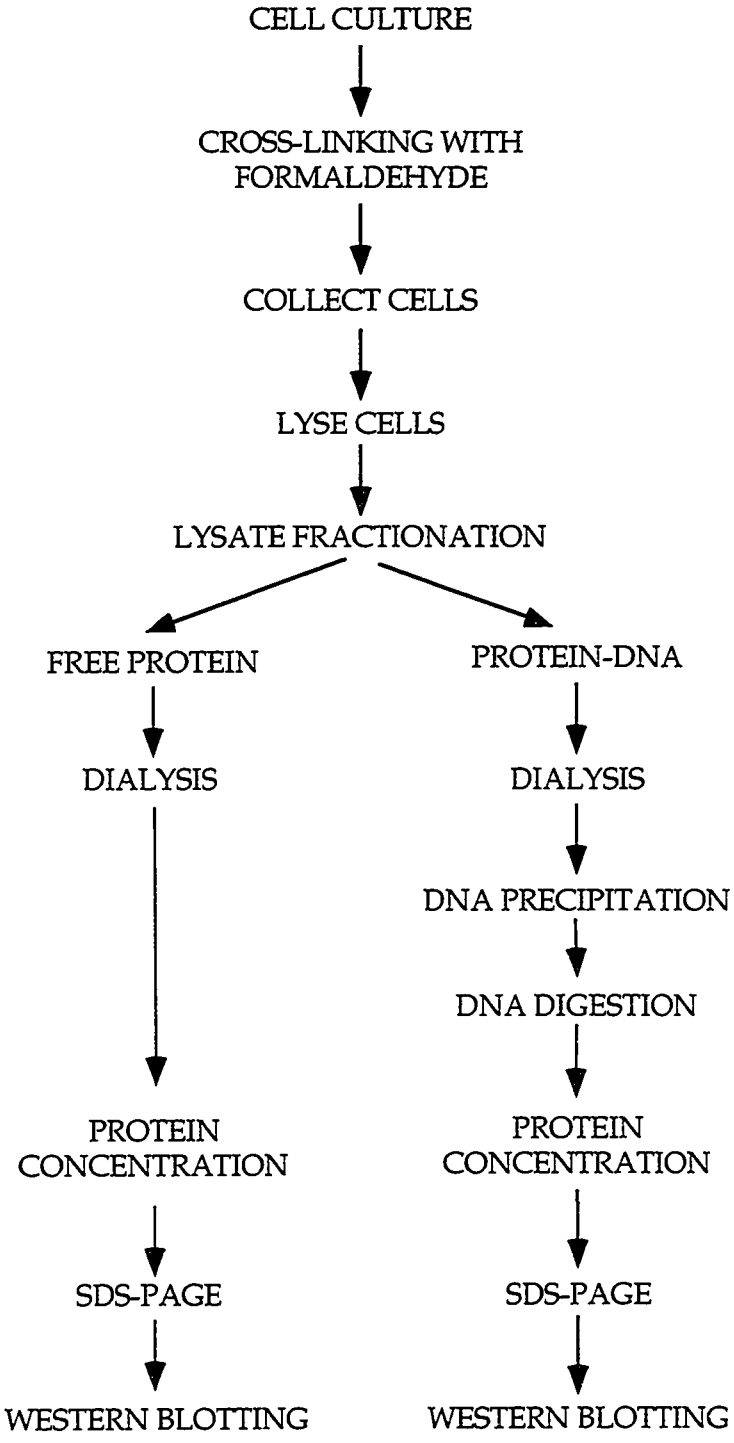

CELL CULTURE
CROSS-LINKING WITH FORMALDEHYDE
COLLECT CELLS
LYSE CELLS
LYSATE FRACTIONATION
FREE PROTEIN
PROTEIN-DNA
DIALYSIS
DIALYSIS
DNA PRECIPITATION
DNA DIGESTION
PROTEIN CONCENTRATION
PROTEIN CONCENTRATION
SDS-PAGE
SDS-PAGE
WESTERN BLOTTING
WESTERN BLOTTING

Figure 5.6. Effect of formaldehyde treatment on the distribution of DNA in CsCl gradient separation of whole cell lysates. Cycling or mitosis-arrested HeLa S3 cells were either untreated (control) or treated with 0.2% formaldehyde, lysed, and fractionated on CsCl gradients as described in Materials and Methods, §2.7. Ten microlitres of each fraction was then run on a 1% agarose gel at 50 V for approximately 1-1.5 h in the presence of ethidium bromide, as described in Materials and Methods, §2.8. Fraction 1 was taken from the bottom of the gradient, and only the twelve bottom-most fractions were analyzed. Fractions from the top of the gradient, which contained free protein, did not stain with ethidium bromide (data not shown).

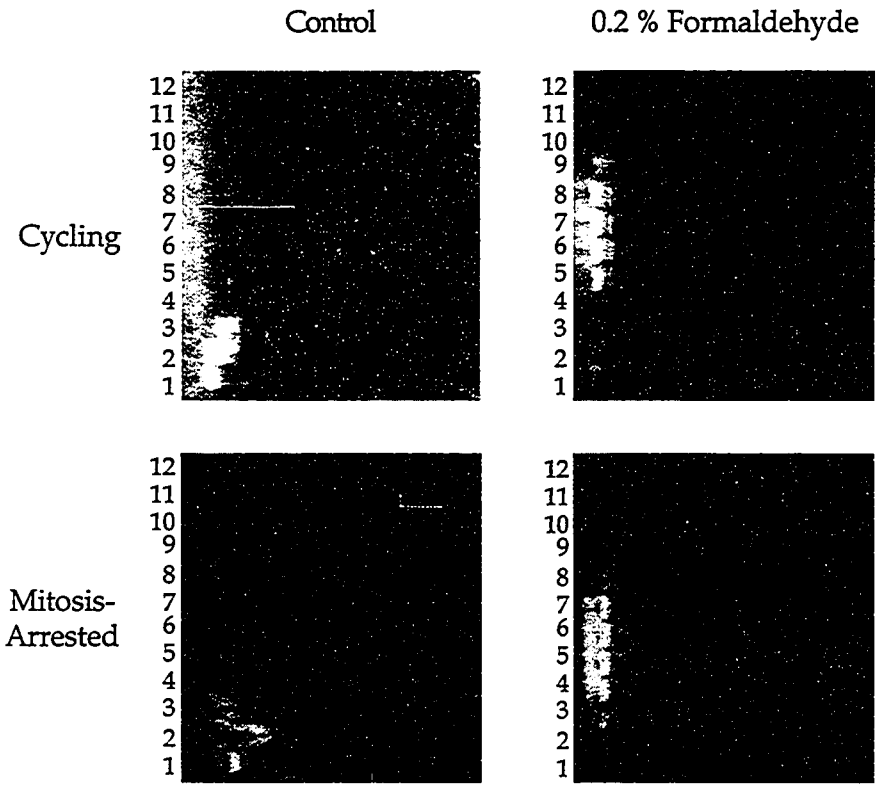

Control
0.2 % Formaldehyde
Cycling
Mitosis-
Arrested
12
11
10
9
8
7
6
5
4
3
2
1

Figure 5.7. Characterization of DNA purified by CsCl gradient separation from whole cell lysates. Cycling HeLa S3 cells were treated with 0.2% formaldehyde, lysed, and fractionated on CsCl gradients as described in Materials and Methods, §2.7. Material from a pool of the DNA-containing fractions was then either untreated or dialysed to remove salts. Ten microlitre aliquots of the dialyzed samples were either untreated, digested with DNase I (2 µl of a 5 mg/ml solution), or digested with RNase A (2 µl of a 10 mg/ml solution) for 10 min at room temperature. Samples were then run on a 1% agarose gel at 50 V for 1-1.5 h in the presence of ethidium bromide, as described in Materials and Methods, §2.8.


Dialysis + RNase A
Dialysis + DNase I
Dialysis
Non-dialysed

Figure 5.8. Specificity of protein-DNA cross-linking in HeLa S3 cells. Cycling cells which were either untreated (U) or treated with 0.2% formaldehyde for 8 min at 37°C (F) were assessed for protein-DNA interactions as described in Materials and Methods, §2.7. Briefly, cells were lysed, sonicated, and then fractionated on CsCl gradients. Three of the DNA containing fractions (D) were pooled, as were three fractions (X) migrating between the DNA-containing fractions and the top three fractions of the gradient. Pooled fractions were subjected to DNA precipitation, and then DNA degradation. Proteins remaining in each sample were concentrated and analyzed, along with whole cell lysate (WCL) from cycling HeLa S3 cells, via SDS-PAGE and Western blotting using ARNA-3 monoclonal antibodies (as described in Materials and Methods, §2.10 and §2.11).

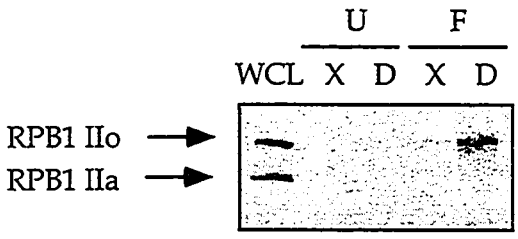

U
F
WCL X D X D
RPB1 IIo
RPB1 IIa

Figure 5.9. Cross-linking analysis of RPB1-DNA association during interphase and mitotic-arrest. Equal numbers of HeLa S3 cells were prepared, which were either cycling (C) or arrested in mitosis by thymidine and nocodazole treatment (M), as described in Materials and Methods, §2.1. These cultures were subjected to formaldehyde treatment. Subsequently, proteins cross-linked to DNA were isolated, as described in Materials and Methods, §2.7. The amount of RPB1 cross-linked to DNA was assessed using SDS-PAGE separation of total cross-linked protein and Western blotting with ARNA-3 monoclonal antibodies (*panel A*). To provide a standard for the mobility of RPB1, whole cell lysate (WCL) from cycling HeLa S3 cell cultures was also examined. The mobilities of the two phosphorylation variants of the RNAP II large subunit, IIo and IIa, are indicated. Also, proteins not cross-linked to DNA were isolated, as described in Materials and Methods, §2.7 (*panel B*). The amount of free RPB1 in 7% of the total non-cross-linked protein was assessed using SDS-PAGE separation of total cross-linked protein and Western blotting with ARNA-3 monoclonal antibodies, as described in Materials and Methods, §2.10 and §2.11.

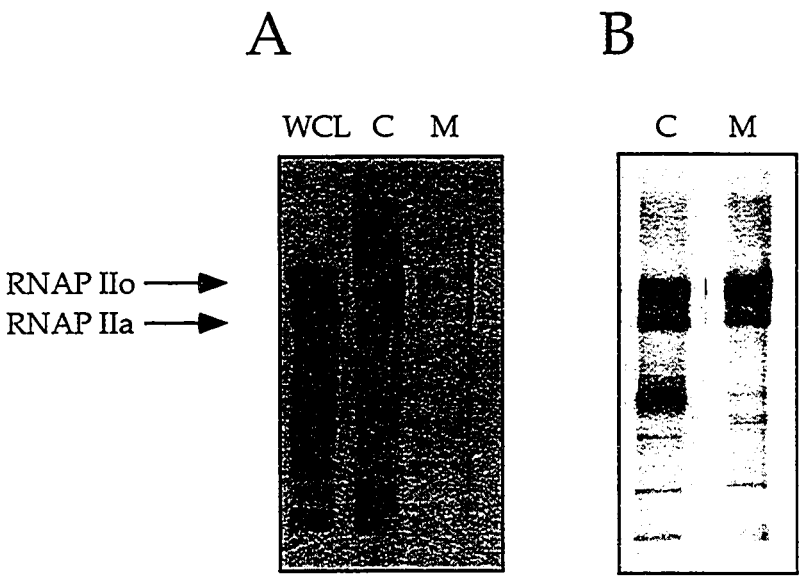

A
B
WCL C M
C M
RNAP IIo
RNAP IIa

Figure 5.10. Digestion of DNA purified from formaldehyde-treated cells. HeLa S3 cell cultures were either cycling (C) or arrested in mitosis by thymidine and nocodazole treatment (M), as described in Materials and Methods, §2.1. Each of these cultures was subjected to formaldehyde treatment, and, subsequently, lysis and fractionation as described in Materials and Methods, §2.7. Fractions containing DNA were pooled and dialysed to remove salts. The samples were either untreated (U) or heated to 95°C for 10 min to reverse cross-linking and then digested (D), as described in Materials and Methods, §2.7, with a combination of DNase I, micrococcal nuclease, and S1 nuclease for 30 min at 37°C. One percent of each sample was then loaded onto a 1% agarose gel, which was run at 50 V for 30 min in the presence of ethidium bromide, as described in Materials and Methods, §2.8. Hind III-digested lambda phage DNA was also run as a molecular weight standard (S).

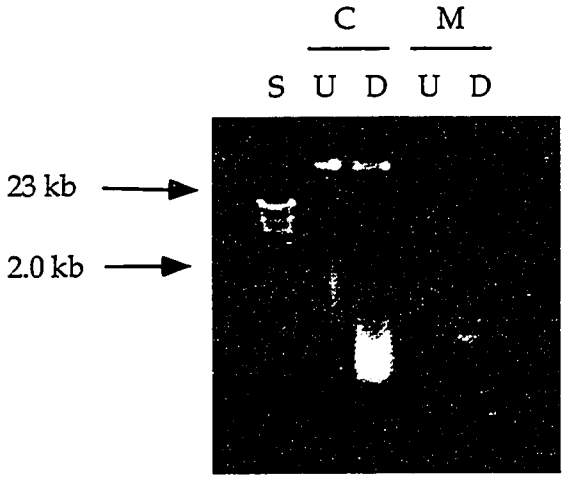

C
M
S U D U D
23 kb
2.0 kb

Figure 5.11. Total protein-DNA cross-linking in cycling and mitosis-arrested cells. HeLa S3 cell cultures were either cycling (C) or arrested in mitosis by thymidine and nocodazole treatment (M), as described in Materials and Methods, §2.1. These cultures were subjected to formaldehyde treatment, lysis, and CsCl gradient fractionation. Subsequently, proteins cross-linked to DNA were isolated, as described in Materials and Methods, §2.7. The amount of total protein cross-linked to DNA was assessed by SDS-PAGE separation of total cross-linked protein and silver staining, as described in Materials and Methods, §2.10 and §2.11. Note that, upon longer exposure, a greater number of bands could be visualized in the sample derived from cycling cells.

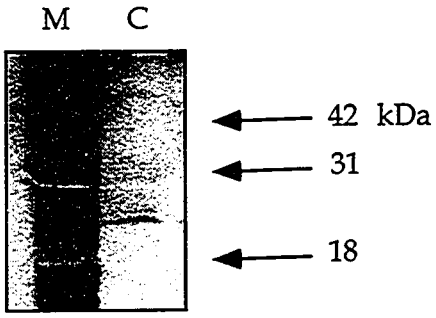

M
C
42 kDa
31
18

Figure 5.12. Separation of free from DNA-bound RPB1 molecules in isolated nuclei. Cycling HeLa S3 cultures were scraped and pelleted and then lysed. As described in Materials and Methods, §2.9, nuclei were isolated by centrifugation of the whole cell lysate on a discontinuous sucrose gradient. Nuclei were resuspended in 0.34 M sucrose and incubated on ice for 15 min. Nuclei were then pelleted by centrifugation and the supernatant collected. The nuclear pellet and supernatant were each dissolved in Laemmli buffer and the proteins in each separated via SDS-PAGE, as described in Materials and Methods, §2.10. Shown is a measure of the amount of RPB1 in an equivalent amount (5%) of each sample, as assessed by Western blotting with ARNA-3 monoclonal antibodies, as described in Materials and Methods, §2.11.

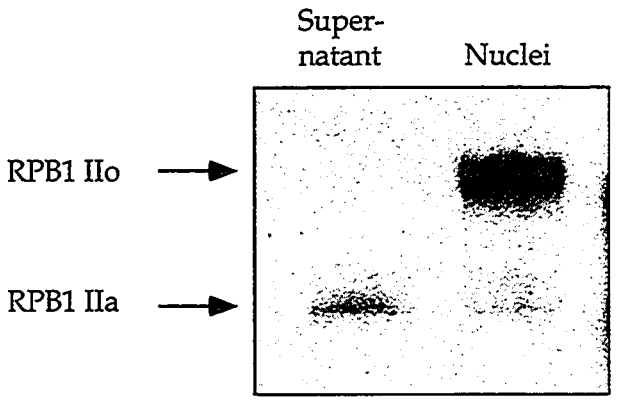

Super-
natant
Nuclei
RPB1 IIo
RPB1 IIa

Figure 5.13. Immunofluorescent localization of TBP during interphase and mitotic-arrest. Shown are interphase HeLa S3 cells from cycling populations (*panel A*) and cells arrested in mitosis by thymidine and nocodazole treatment, as described in Materials and Methods, §2.1 (*panels B and C*). Indirect immunofluorescence was performed as described in Materials and Methods, §2.6. Briefly, cells were grown on glass coverslips, fixed in 3.7% formaldehyde and made permeable by treatement with acetone. After washing in PBS, they were incubated with TBP18 monoclonal antibodies (*panels A and B*) or with PBS alone (*panel C*). After further washing, cells were incubated with rhodamine-conjugated secondary antibodies and DAPI. Rhodamine and DAPI fluorescence were then detected by immunofluorescence microscopy. Cells were also visualized using phase contrast microscopy.

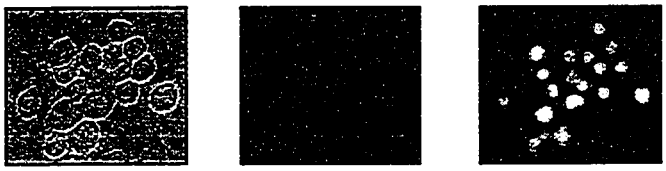
A

Phase Contrast

Immuno-fluorescence

DAPI

B



C

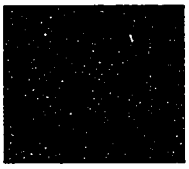
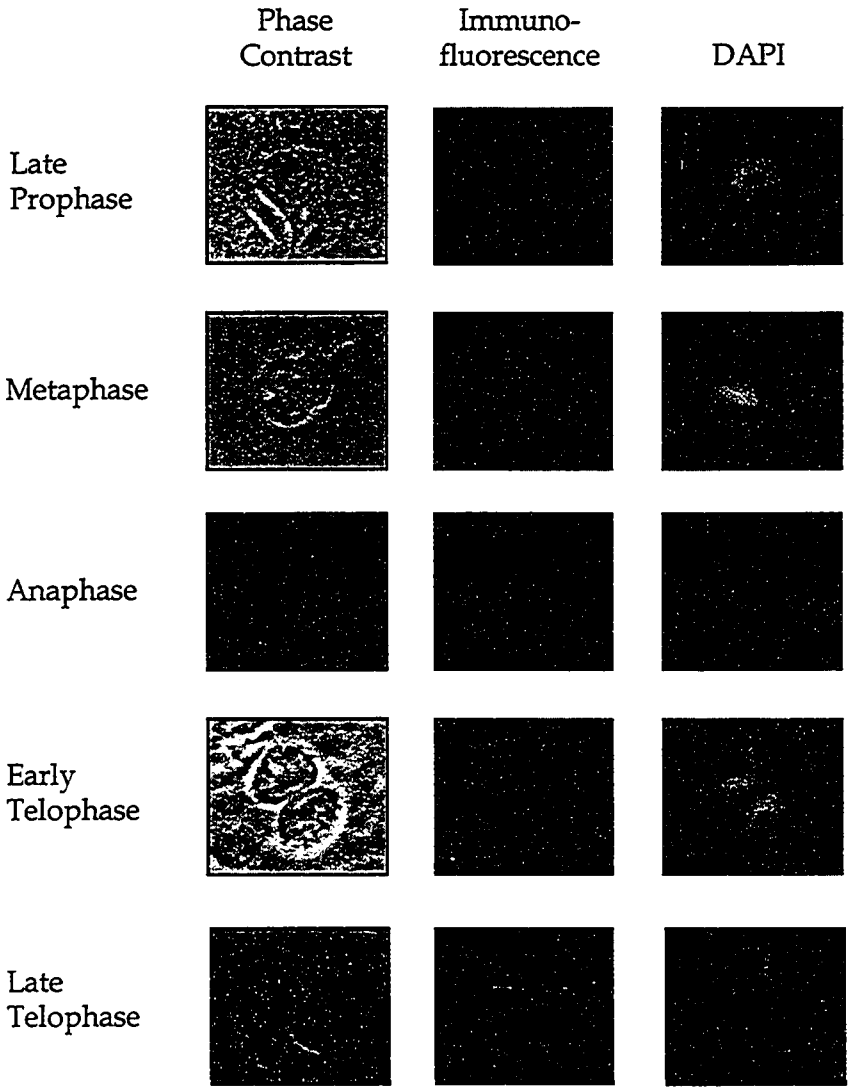


Figure 5.14. Immunofluorescent localization of TBP during mitosis. Indirect immunofluorescence was performed as described in Materials and Methods, §2.6. Briefly, HeLa CCL2 cells were grown on glass coverslips, fixed in 3.7% formaldehyde and made permeable by treatment with acetone. After washing in PBS, they were incubated with TBP18 monoclonal antibodies. After further washing, cells were incubated with rhodamine-conjugated secondary antibodies and DAPI. Rhodamine and DAPI fluorescence were then detected by immunofluorescence microscopy. Cells were also visualized using phase contrast microscopy.


Phase Contrast
Immuno-fluorescence
DAPI
Late Prophase
Metaphase
Anaphase
Early Telophase
Late Telophase

6. MODIFICATION OF RNAP II GTFs DURING MITOSIS

Our previous results suggest that RNAP II transcriptional repression during mitosis may involve stripping of RNAP II and TBP from DNA (see §4 and §5). However, it is also possible that the downregulation of GTF activity also contributes to the repression of RNAP II transcription during mitosis. Transcription by RNAP III appears to be downregulated during mitosis through the specific inactivation, by phosphorylation, of an essential GTF (Gottesfeld et al., 1994; Hartl et al., 1993). Also, some sequence-specific RNAP II transcription factors, such as Oct-1, are subject to mitotic phosphorylation which decreases their DNA-binding activities (Segil et al., 1991). Most importantly, enhanced phosphorylation of TFIID components has recently been shown to downregulate TFIID activity in mitosis-arrested HeLa cells (Segil et al., 1996). Given these precedents for the regulation of RNAP II transcription by mitotic phosphorylation, we sought to identify other RNAP II GTFs which might be downregulated, through enhanced phosphorylation, in mitosis-arrested HeLa cells.

To determine if components of the RNAP II GTF machinery are covalently modified during mitosis, Laemmli extracts were made from HeLa S3 cells which were either freely cycling, arrested in mitosis, or arrested and then released by growth in regular medium for 8 h (as described in Materials and Methods, §2.1). Following separation of these extracts via SDS-PAGE, Western blotting was performed to determine the mobility of various RNAP II GTFs including RPB1, TBP, MO15, MAT1, cdk8, TFIIB, TFIIE-α, and RAP74 (Figure 6.1).

The phosphorylation state of the CTD of RPB1 is thought to play an important role in determining the functional state of RNAP II (reviewed in Dahmus, 1994; see also §1.1.2.3). MPF has been shown to phosphorylate the CTD *in vitro* (Cisek and Coren, 1989). Furthermore, this phosphorylation disrupts

PICs *in vitro* (Zawel et al., 1993). In light of these facts, it has been suggested that the phosphorylation of RPB1 by MPF during mitosis may account for the loss of RNAP II trancriptional activity (Zawel et al., 1995; Cisek and Corden, 1989). However, no evidence has yet been adduced to support this hypothesis. Here we confirm that the phosphorylation state of RPB1 is not altered in extracts of mitosis-arrested HeLa cells (see Figure 6.1), at least insofar as it can be assessed by immunoblotting. It is interesting that hyperphosphorylated forms of RPB1 remain abundant during mitotic arrest, when cells are transcriptionally repressed and when RNAP II appears to be predominantly cytoplasmic. It has been shown that RNAP IIO is the form of the enzyme involved in transcription elongation (see §1.1.2.3); however, our results (see §4 and §5) suggest that in mitotic cells, this form is not transcriptionally engaged. The presence of RNAP IIO in mitotic cells has also been observed by others (Kim et al., 1997), and this observation has been used to argue that the state of CTD phosphorylation does not necessarily correlate with the transcriptional activity of the enzyme. However, as the mobility of RPB1 does not increase linearly upon its increased phosphorylation, the RPB1 IIo observed in mitosis-arrested cells may not be identical to the RPB1 IIo which carries out transcription elongation in interphase cells. In any case, previous studies on CTD phosphorylation (reviewed in Dahmus, 1994) would suggest that the RNAP IIO in mitotic cells is inactive in initiation, and thus must be converted to RNAP IIA before it can engage in transcription upon commencement of G_1.

TBP plays an essential role in all three eukaryotic transcription systems (reviewed in Hernandez, 1993; Rigby, 1993; see also §1.1.2.1-1.1.2.4). In the context of RNAP II transcription, TBP not only binds to promoter TATA sequences, nucleating PIC assembly, it also likely plays a major role in PIC architecture, engaging in interactions with multiple GTFs. TBP is apparently not phosphorylated during interphase (Segil et al., 1996; Figure 6.2). However, we observed that in extracts of mitosis-arrested HeLa cells, roughly 50% of total TBP

possessed a SDS-PAGE mobility slower than that of TBP from cycling cell extracts (Figure 6.1). Also, the altered mobility of this fraction of TBP in mitosis-arrested cell extracts could be converted to the mobility found in cycling cell extracts upon incubation of extracts with calf intestinal alkaline phosphatase (CIAP) (Figure 6.2). Other investigators have also reported the phosphorylation of TBP during mitosis. White et al. (1995a) found that most TBP in mitotic HeLa cell extracts has a decreased mobility, similar to that which we observe. Segil et al. (1996), however, reported that most TBP in mitotic cells possesses an even slower electrophoretic mobility than that observed in this study and in that of White et al. (1995a). Segil et al. (1996) detected multiple forms of phosphorlyated TBP in mitosis-arrested cell extracts. The discrepancies in the results of these studies may be attributable to various degrees of dephosporylation during extract preparation.

Cdk7 (MO15), cyclin H and MAT1 form a subcomplex of TFIIH (Adamczweski et al., 1996; Serizawa et al., 1995; Shiekhattar et al., 1995; Feaver et al., 1994; Roy et al., 1994) which likely is the CTD-kinase activity of that GTF. *In vitro,* TFIIH containing kinase-inactive cdk7 does not phosphorylate RNAP II (Akoulitchev et al., 1995; Mäkelä et al., 1995). Also, antibodies directed against cdk7 interfere with RNAP II transcription (Roy et al., 1994). Finally, the *S. cerevisiae* homologue of cdk7, Kin28, is essential for RNAP II transcription *in vivo* (Cismowski et al., 1995) and *in vitro* (Valay et al., 1995) and is a CTD-kinase *in vitro* (Cismowski et al, 1995; Valay et al., 1995).

Cdk7/cyclin H/MAT1 complexes also exhibit CAK activity. After a cdk binds its cyclin partner, cdk7/cyclin H can phosphorylate the cdk on the residue corresponding to T160 of $p34^{cdc2}$ (Fisher and Morgan, 1994; reviewed in Clarke, 1995; Solomon, 1994). The cdk7/cyclin H/MAT1 complex exists both as a subcomplex of TFIIH and as an independent complex (Drapkin et al., 1996; Sheikhattar et al., 1995; Fisher and Morgan, 1994; Tassan et al., 1994), suggesting that discrete subpopulations of cdk7/cyclin H/MAT1 may have different

functions. Consistent with this picture, free cdk7/cyclin H does not phosphorylate the CTD in PICs (Drapkin et al., 1996; Shiekhattar et al., 1995). Cdk7/cyclin H complexes within TFIIH possess CAK activity, a fact which has fueled speculation that TFIIH is involved in relaying information from the transcriptional machinery to the cell cycle control system (Shiekhattar et al., 1995; Serizawa et al., 1995). However, the *in vivo* relevance of cdk7/cyclin H CAK activity is controversial, since Kin28 has no CAK activity *in vitro* (Cismowski et al., 1995; Valay et al., 1995; Feaver et al., 1994; Solomon, 1994), or *in vivo* (Cismowski et al., 1995), and reports of other mammalian CAK activities are emerging (see Svejstrup et al., 1996). TFIIH also functions in nucleotide excision repair (reviewed in Svejstrup et al., 1996). However, the cdk7/cyclin H subcomplex is not involved in this process, and during excision repair is apparently either released from TFIIH or inactivated (reviewed in Svejstrup et al., 1995).

Cdk7 is phosphorylated *in vivo* (Tassan et al., 1994), and in particular, phosphorylation of T170 in human cdk7, and its correlate amino acid T176 in *Xenopus* MO15, is required for CAK activity (Fisher and Morgan, 1994; Labbé et al., 1994; Poon et al., 1994). Human cdk7 is apparently phosphorylated on other sites as well (Fisher and Morgan, 1994) and *Xenopus* cdk7 is also phosphorylated on S170 (Labbé et al,. 1994). Human cdk7 is a nuclear protein throughout interphase, and is dispersed from mitotic chromosomes (Tassan et al., 1994). *Xenopus* cdk7 apparently has to enter the nucleus to become active as CAK (Labbé et al,. 1994). Cdk7/cyclin H CAK activity does not vary during the cell cycle (Poon et al., 1994).

MAT1 is a 32 kDa protein which associates with MO15/cyclin H complexes *in vivo* (Adamczewski et al., 1996; Devault et al., 1995; Tassan et al., 1994) and *in vitro* (Tassan et al., 1995). The function of MAT1 is unclear. It apparently stabilizes cdk7/cyclin H complexes *in vitro* (Tassan et al., 1995a), and, perhaps through this stabilization, stimulates the CTD-kinase activity of

cdk7/cyclin H complexes (Adamczewski et al., 1996). However, MAT1 is necessary neither for complex formation, nor for the production of its CAK or CTD-kinase activities (Adamczewski et al., 1996; Fisher and Morgan, 1994). Also, neither MAT1 levels (Tassan et al., 1995a) nor TFIIH composition (Adamczewksi et al., 1996) fluctuate across the cell cycle. MAT1 has been suggested to act as a determinant of cdk7/cyclin H substrate specificity (Clarke, 1995; Solomon, 1994). It has also been proposed that the putative zinc finger domain of MAT1 may play a role in the function of cdk7/cyclin H CTD-kinase activity (Adaczweski et al., 1996).

Previous studies showed that in mouse 3T3 cells, cdk7 phosphorylation and protein levels did not change upon entry into mitosis (Tassan et al., 1994; Poon et al., 1994). In agreement with these results, we found that cdk7 levels and SDS-PAGE mobility were not altered in M-phase extracts of HeLa cells (Figure 6.1). Also, in agreement with a previous study in HeLa cells, levels of MAT1 did not change upon commencement of mitosis (Tassan et al., 1995a). We have also determined that MAT1 does not undergo a change in mobility or abundance during mitosis (Figure 6.1).

We also examined the SDS-PAGE mobility of cdk8, a 53 kDa protein which is the mammalian homologue of the *S. cerevisiae* SRB10 mediator protein. Cdk8 interacts *in vitro* and *in vivo* with cyclin C (Leclerc et al., 1996; Tassan et al., 1995b), and these two molecules are components of a mammalian RNAP II holoenzyme (Maldonado et al., 1996). Cdk8 and cyclin C are also found in a smaller 170 kDa complex (Rickert et al., 1996). The role of cdk8/cyclin C complexes remains obscure. Cdk8 interacts with RPB1 *in vitro* (Leclerc et al., 1996). Immunoprecipitations performed using antibodies directed against cdk8 possess CTD-kinase activity, suggesting that the kinase activity of cdk8 may be involved in RNAP II transcription (Leclerc et al., 1996). This conjecture is supported by the demonstration that yeast holoenzymes lacking SRB10 or SRB11 are deficient in CTD phosphorylation (Liao et al., 1995). However, neither cdk8

nor SRB10 has yet been shown to directly phosphorylate the CTD. Also, *SRB10* is not an essential gene in yeast (reviewed in Svejstrup et al., 1995). In this study, we demonstrated that cdk8 levels are not altered in M-phase-arrested HeLa cell extracts. Also, cdk8 mobility is not altered in these extracts (Figure 6.1).

TFIIB interacts with TBP, promoter DNA and other RNAP II GTFs in PICs. It is necessary for the recruitment of RNAP II-TFIIF complexes into PICs *in vitro* (Buratowski et al., 1989), and its role in facilitating transcriptional activation has been widely studied (reviewed in Hahn, 1993). TFIIB has also been shown to be involved in determining the transcriptional start site (Li et al., 1994; Pinto et al., 1992). To date, TFIIB phosphorylation has not been investigated with respect to the cell cycle. We observed that the mobility of TFIIB in extracts of mitosis-arrested HeLa cells was not different from that in extracts of interphase cells (Figure 6.1).

TFIIE is composed of two subunits, TFIIE-α and TFIIE-β, which have been proposed to form a heterodimer (Conaway et al., 1991) or an $\alpha_2\beta_2$ heterotetramer (Ohkuma et al., 1990; Inostroza et al., 1991) *in vivo*. The α subunit of TFIIE is a 439 amino acid protein with a molecular weight of 49.5 kDa (Peterson et al., 1991). Current understanding of the function of TFIIE is closely tied to our understanding of TFIIH. TFIIE from *S. cerevisiae* can only function in an *in vitro* transcription system reconstituted from *S. pombe* factors if *S. cerevisiae* TFIIH is also added (Li et al., 1994). This result underscores the importance of the functional interaction of these molecules in RNAP II transcription.

TFIIE recruits TFIIH to PICs *in vitro* (Maxon et al., 1994; Flores et al., 1992), and stimulates the CTD-kinase activity of TFIIH (Ohkuma et al., 1995; Drapkin et al., 1994; Ohkuma and Roeder, 1994; Lu et al., 1992). An oligomeric form of recombinant TFIIE-α is capable of stimulating the TFIIH CTD-kinase, whereas monomeric TFIIE-α is not, although whether such oligomeric forms of TFIIE-α exist *in vivo* is not known (Serizawa et al., 1994). TFIIE has also been proposed to influence template melting (Hostege et al., 1996) or promoter

clearance (Goodrich and Tjian, 1994) by affecting the helicase activity of TFIIH. TFIIE-α binds directly to ERCC-3 *in vitro* (Drapkin et al., 1994; Maxon et al., 1994). However, biochemical studies on the effect of TFIIE on the helicase activity of TFIIH conflict. Although one study found that TFIIE-α could inhibit ERCC3 helicase activity (Drapkin et al., 1994), another found that it stimulates helicase activity (Serizawa et al., 1994). TFIIE-α also binds *in vitro* to RNAP IIA, TBP, and TFIID (Maxon et al., 1994). Functional domains of TFIIE-α responsible for stimulating CTD phosphorylation by TFIIH, for binding TFIIE-β, and for binding TFIIH have been identified (Ohkuma et al., 1995). *In vitro* transcription systems have revealed that TFIIE is released from PICs before the formation of the tenth phosphodiester bond in the nascent RNA, prior to the release of TFIIH (Zawel et al., 1995).

The phosphorylation state of TFIIE has not been investigated. We observed that in extracts of mitosis-arrested populations of HeLa S3 cells, roughly 50% of TFIIE-α has a slower mobility than TFIIE-α in cycling cell extracts (Figure 6.1). Treatment of mitosis-arrested cell extracts with CIAP indicated that this change in mobility is due to hyperphosphorylation of TFIIE-α (Figure 6.2) Furthermore, treatment of cycling cell extracts with CIAP revealed that TFIIE-α is a phosphoprotein in interphase cells (Figure 6.2).

RAP74 is a 517 amino acid protein, with a molecular weight of 58 kDa, which associates with RAP30 to form TFIIF (Aso et al., 1992; Finkelstein et al., 1992). However, the nature of this interaction is controversial, with some investigators claiming it is heterodimeric (Kitajima et al., 1990; Flores et al., 1990; Price et al., 1989) and others claiming it is heterotetrameric (Conaway and Conaway, 1990). TFIIF functions in both initiation and elongation of RNAP II transcription. Both RAP74 (Wang and Burton, 1995) and RAP30 (Buton et al., 1988; Sopta et al., 1985) interact with RNAP II, and the TFIIF-RNAP II association facilitates RNAP II recruitment into PICs *in vitro* (reviewed in Buratowski, 1994). The TFIIF-RNAP II interaction also appears to prevent RNAP

II from binding non-specifically to DNA (Killeen and Greenblatt, 1992; Conaway and Conaway, 1990). TFIIF can also decrease the rate of RNAP II pausing *in vitro* (reviewed in Reines et al., 1996). However, neither the *Drosophila* homologue of TFIIF, F5 (Price et al., 1989), nor human RAP74 (Zawel et al., 1995) associates stably with elongating RNAP II molecules, indicating that this effect is mediated through a transient interaction.

Much biochemical work has been directed towards understanding which subunits of TFIIF, and which domains of each subunit, are involved in these different functions. Based on its primary sequence, RAP74 has been suggested to consist of three domains: a globular N-terminal domain (amino acids 1-179), a charged central region (180-356), and a globular C-terminal domain (357-517) (Finkelstein et al., 1992). A deletion analysis of human RAP74 indicated that amino acids 73-205 and 356-517 are essential for basal transcription (Yonaha et al., 1993). In contrast, similar studies on human RAP74 (Joliot et al., 1995; Wang and Burton, 1995) and on the *Drosophila* RAP74 homolog, F5a (Kephart et al., 1994), indicated that the entire N-terminus is essential for the basal transcriptional activity of RAP74. Also, activation of *in vitro* transcription by serum response factor (SRF) requires amino acids 206-291 of RAP74. SRF and RAP74 engage in a protein-protein interaction between this region of RAP74 and the activation domain of SRF (Joliot et al., 1995). RAP74 interacts *in vitro* with the $TAF_{II}80$ (Hisatake et al., 1995) and $TAF_{II}250$ (Ruppert and Tjian, 1995) subunits of TFIID. The RAP74-$TAF_{II}250$ interaction is apparently essential for cell viability, suggesting that it plays a critical role in PIC formation or function (Ruppert and Tjian, 1995). Also, RAP74 binds to RNAP II through amino acids 363-444 (Wang and Burton, 1995), and contacts TFIIB though amino acids 358-517 (Fang and Burton, 1996). The latter contact is apparently important in PIC architecture *in vivo*, as mutations in the RAP74 homologue of *S. cerevisiae* can suppress TFIIB mutations which alter the site of transcriptional intitiation (Sun and Hampsey, 1995).

Despite the extensive biochemical characterization of RAP74, its precise functions remain controversial. Cross-linking studies have localized RAP74 to a region of the DNA template from -19 to -5 (Robert et al., 1996), leading to the speculation that it plays a role in melting the template upstream of the initiation site (Pan and Greenblatt, 1994). Consistent with this hypothesis, some *in vitro* transcription studies have suggested that both RAP74 and RAP30 are required for both stable PIC formation and early phosphodiester bond synthesis (Tan et al., 1994). On the other hand, other such studies imply that RAP74 has no role in PIC assembly or promoter melting, but functions rather in promoter clearance (Chang et al., 1993). RAP74 has also been shown to stimulate the activity of a CTD-phosphatase, with amino acids 358-517 being required for this function (Chambers et al., 1995).

Our results confirm previous observations that RAP74 in interphase HeLa cells is a phosphoprotein (Figure 6.2; Kitajima et al., 1994; Sopta et al., 1989; Sopta et al., 1985). We also observe that in mitosis-arrested HeLa cell extracts, all detectable RAP74 possesses a slower SDS-PAGE mobility than RAP74 in interphase HeLa cells (Figure 6.1). CIAP treatment revealed that this difference in mobility is a result of hyperphosphorylation of RAP74 in mitosis-arrested cells (Figure 6.2).

In summary, some RNAP II GTFs (RNAP II, MO15, MAT1, cdk8, TFIIB) appear similar in abundance and electrophoretic mobility in interphase and mitosis-arrested cells. Other GTFs (RAP74, TFIIE-α, and TBP), however, display an unusual migration pattern in mitosis-arrested cell extracts. In these extracts, roughly 50% of total TBP and TFIIE-α appeared as a distinct species with a slower mobility than that found in cycling cell extracts. The RAP74 subunit of TFIIF was present entirely as a distinct, more slowly migrating species in mitosis-arrested cell extracts. Treatment of the mitosis-arrested cell extracts with CIAP before SDS-PAGE restored the mobility pattern of all three of these proteins to that characteristic of cycling cell extracts treated with CIAP (Figure

6.2). This result demonstrates that mobility differences seen in mitosis-arrested cell extracts were caused by phosphorylation of these proteins. Furthermore, treatment of cycling cell extracts with CIAP produced a qualitative change in the mobility of TFIIE-α and RAP74, indicating that these proteins are also phosphorylated during interphase.

Figure 6.1. Modification of RNAP II GTFs during mitotic-arrest. Laemmli extracts were prepared using cell populations which were either cycling (C), arrested in mitosis via thymidine and nocodazole treatment (M), or arrested and then released by growth in regular medium for 8 h (R). Cells were arrested and released as described in Materials and Methods, §2.1. Laemmli extracts, along with HeLa cell nuclear extract (Promega) (NE), were separated by SDS-PAGE and the electrophoretic mobility of the indicated GTFs was analyzed via Western blotting, as described in Materials and Methods, §2.10 and §2.11. In some experiments, 10 ng of recombinant RAP74 and TFIIB (gifts of Dr. Jack Greenblatt, University of Toronto) (P) were also analyzed. The antibodies used are fully described in §2.11.

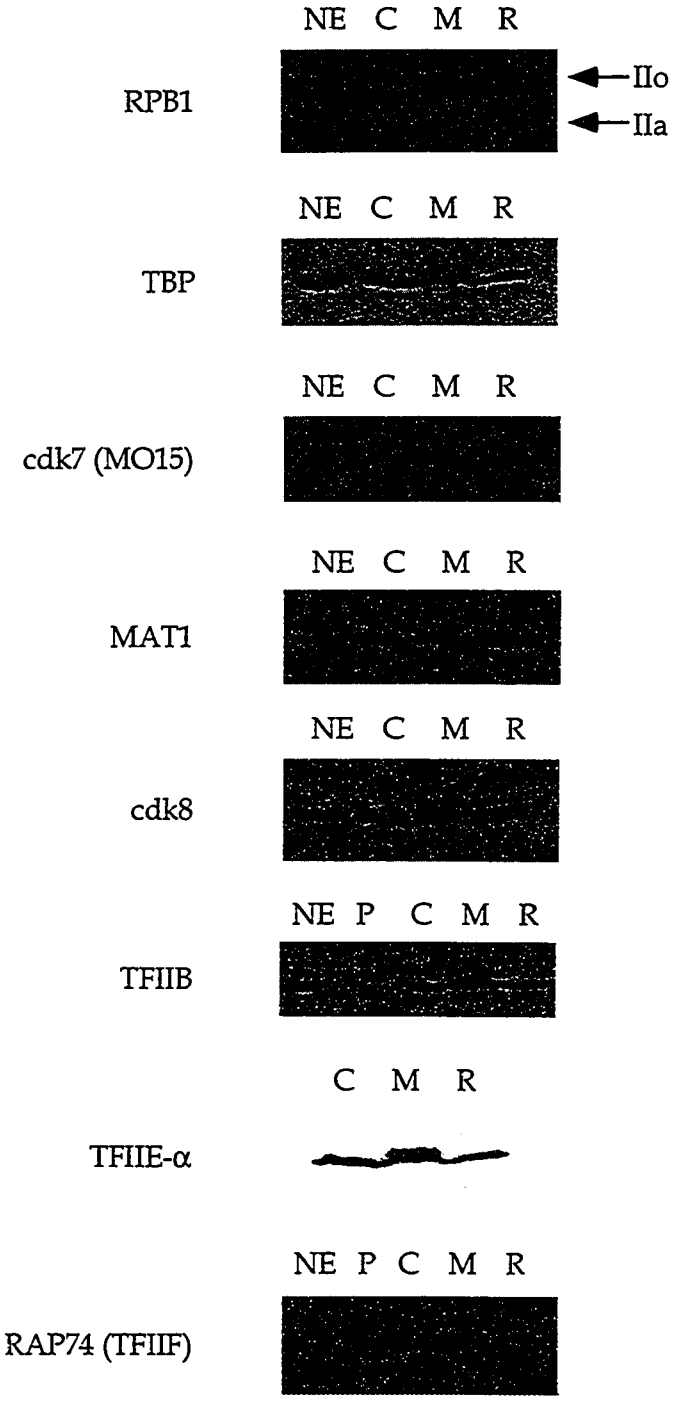
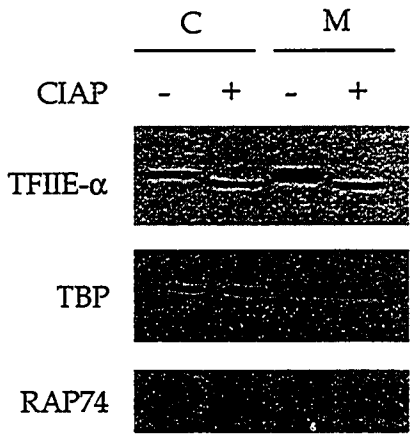

NE C M R
RPB1
IIo
IIa
NE C M R
TBP
NE C M R
cdk7 (MO15)
NE C M R
MAT1
NE C M R
cdk8
NE P C M R
TFIIB
C M R
TFIIE-α
NE P C M R
RAP74 (TFIIF)

Figure 6.2. CIAP treatment of Laemmli extracts of interphase and mitosis-arrested cells. Laemmli extracts were prepared using cell populations which were either cycling (C) or arrested in mitosis via thymidine and nocodazole treatment (M), as described in Materials and Methods, §2.1. Ten microlitre aliquots of these extracts were either incubated with 9 U of CIAP for 30 min at 37°C or mock-treated by incubation for 30 min at 37°C. The aliquots were then run on SDS-PAGE, and the mobility of various GTFs was analyzed via Western blotting, as described in Materials and Methods, §2.10 and §2.11. The antibodies used are fully described in §2.11.


C
M
CIAP
-
+
-
+
TFIIE-α
TBP
RAP74

7. DISCUSSION

A number of studies employing *in vivo* RNA labelling methods have demonstrated that RNA synthesis severely decreases during mitosis (reviewed in Mitchison, 1971). However, these methods do not clearly differentiate between RNA synthesis carried out by RNAPs I, II, and III. In addition, studies which assess total precursor incorporation into RNA cannot assay the transcription of specific genes. This study is the first to directly examine RNAP II transcription of specific gene regions during this phase of the mammalian cell cycle. We find that in HeLa S3 cells synchronized in mitosis by treatment with thymidine and nocodazole, transcription of all RNAP II transcribed-genes that we assay is reduced (Figure 4.1). The degree of this repression varies, with transcription being reduced to anywhere from 5.8%, in the case of the *histone H2B* gene, to 36.7%, in the case of the 5′ region of the *γ-actin* gene, of the levels in cycling cell populations (Table 4.1).

Some of the transcription we observe in mitosis-arrested cells can be attributed to the presence of unarrested cells, which may constitute up to 7% of the population (Table 3.1). However, the higher levels of transcription in mitosis-arrested populations of other gene regions, such as the 5′ regions of *γ-actin* and of *c-fos*, are difficult to ascribe to the action of this unarrested subpopulation. It is possible that in the unarrested subpopulation, exposure to thymidine and/or nocodazole alters gene expression in such a way as to produce elevated transcription of these genes. It seems more plausible, however, that our results reflect the presence of small numbers of RNAP II transcription complexes on some genes in mitosis-arrested cells. This may be reflective of a small amount of RNAP II transcription during mitosis *in vivo*. In any case, our studies support the widely held belief that most RNAP II transcription ceases during mitosis. Control experiments conducted in the presence of 0.5% sarkosyl indicate that most of the transcription which we observe in interphase cells using our run-on

assay is produced by engaged RNAP II molecules (Figure 4.1, panel B; Table 4.2). This indicates that most of the repression of RNAP II transcription that we observe is caused by a decrease in the number or activity of DNA-bound RNAP II molecules in mitosis-arrested cells.

We wished to understand the mechanism whereby RNAP II transcription is downregulated during mitosis. A central issue concerning this mechanism is whether or not RNAP II itself remains bound to DNA during mitosis. Estimates made in other studies, and this one (Figure 5.12), suggest that, in interphase cells, up to fifty percent of the total RNAP II is associated with DNA, presumably either in pre-initiation or elongation complexes. Therefore, the repression of RNAP II transcription during mitosis might occur if much of this DNA-bound RNAP II is displaced from the template during chromatin condensation. On the other hand, if the RNAP II transcriptional machinery is not displaced from the DNA, then some other mechanism must be invoked to explain its lack of RNA synthesis during mitosis.

We investigated the localization of RNAP II during mitosis using three different methods: run-on transcription assays, indirect immunofluorescence, and a chemical cross-linking assay. We reasoned that if RNAP II elongation complexes were present, but stalled, on mitotic DNA during our run-on transcription assays, then the use of conditions such as high concentrations of salt (Figure 4.3), the presence of the detergent sarkosyl (Figure 4.4), or both (Figure 4.5), might release them by disrupting chromatin structure. However, none of these conditions result in substantial increases in RNAP II transcription. Although this result suggests that large numbers of RNAP II elongation complexes are not present on mitotic DNA, it does not provide a physical localization of the enzyme. Also, our sarkosyl and salt release conditions may have not been capable of releasing stalled polymerases on mitotic chromosomes. Therefore, we sought to confirm the notion that RNAP II is not present on mitotic chromosomes using independent methods. To this end, we visualized RPB1, the large subunit of RNAP II, in mitosis-arrested HeLa S3 cells using

indirect immunofluorescence (Figure 5.1). In these cells, RPB1 is excluded from mitotic chromatin and dispersed throughout the cytoplasm. To confirm this result, we assessed the level of RNAP II large subunit-DNA associations in our mitosis-arrested cells, as opposed to interphase cells, using protein-DNA cross-linking (Figure 5.9). This experiment indicated that much less RPB1 is associated with DNA during mitotic arrest in these cells.

However, it is conceivable that our mitotic-arrest protocol produced these results by causing an artifactual displacement of RNAP II from mitotic chromosomes. Therefore, we subjected HeLa CCL2 cells naturally undergoing mitosis to indirect immunofluorescence, to determine the localization of the large subunit of RNAP II. Since the transcription factor AP-2 is not excluded from mitotic chromosomes in these cells when visualized using our indirect immunofluorescence technique (Figure 5.3), we are presumably capable of detecting any RPB1 associated with mitotic DNA. However, we observed a dramatic exclusion of RPB1 from the DNA, commencing in late prophase and continuing until late telophase (Figure 5.2). In fact, cells which had all but completed telophase, and which had reformed nuclei and nucleoli, still disperse most of their RPB1 throughout the cytoplasm.

In contrast to these results, previous studies have concluded that the amount of RNAP II bound to mitotic DNA is roughly equivalent to that bound to DNA in interphase cells (Matsui et al., 1979; Gariglio et al., 1974). However, these investigators did not physically locate the polymerase, as we have done here, but instead relied on the indirect method of assessing RNA synthesis activity to define the presence or absence of the enzyme. This method is complicated by the necessity of discriminating between RNAP I and II activity using α-amanitin treatment. Furthermore, these experiments were not performed on whole cells, but on whole cell extracts (Gariglio et al., 1974) or isolated metaphase chromosomes (Matsui et al., 1979). It is possible that free RNAP II might reinitiate on DNA during these procedures, particularly if DNA

is nicked or sheared during chromosome preparation. However, it is important to note that although we did not observe detectable levels of RPB1 on mitotic DNA using our indirect immunofluorescence technique, small amounts of the enzyme might still be present. In fact, this would agree with the results of our run-on transcription assays, which suggest that some RNAP II molecules remain on certain genes in functional elongation complexes during mitosis (Table 4.1; see also Figures 4.4 and 4.5).

Our evidence that RNAP II is stripped from mitotic chromatin agrees with the results of Shermoen and O'Farrell (1991). Using *Drosophila* embryos, these investigators demonstrated that nascent RNAP II transcripts are aborted and then ejected from DNA during mitosis. The ejection of elongating RNAP II molecules from DNA during mitosis could provide the mechanism whereby transcript abortion occurs. We also observe that TBP, a component of the RNAP II GTF TFIID, is largely displaced from mitotic DNA (Figures 5.13 and 5.14). Others have documented similar observations (Jordan et al., 1996; Roussel et al., 1996; Segil et al., 1996). In fact, all RNAP II GTFs whose subcellular localization during mitosis has been determined using immunocytochemical techniques ($TAF_{II}20/15$, $TAF_{II}31$, $TAF_{II}80$ [Segil et al., 1996], MO15 [Tassan et al., 1994], and MAT1 [Tassan et al., 1995]), with the exception of cyclin C (Leclerc et al., 1996), appear to be largely excluded from the chromosomes. Taken together, these results argue that the repression of RNAP II transcription during mitosis involves the stripping of a large portion of the general RNAP II transcriptional machinery from the chromosomes.

However, it is likely that additional mechanisms are involved in the repression of RNAP II transcription during mitosis. The studies of Segil et al. (1996) indicate that the activity of TFIID is downregulated during mitosis. Therefore, the cessation of RNAP II transcription at this time may involve specific downregulation of GTF activity. The downregulation of TFIID activity is contingent upon the enhanced phosphorylation of its components which occurs

during mitosis. We confirm reports that TBP is phosphorylated during mitosis (Segil et al., 1996; White et al., 1995a). In addition, we observe that TFIIE-α and the RAP74 subunit of TFIIF are hyperphosphorylated during mitosis (Figures 6.1 and 6.2). RAP74 has previously been shown to be phosphorylated in interphase cells (Yonaha et al., 1992; Flores et al, 1989; Sopta et al., 1989; Burton et al., 1988), in agreement with our results (Figure 6.2). Furthermore, its *in vitro* transcriptional initiation and elongation activities are modulated by this modification (Kitajima et al., 1994).

Although we do not know the functional significances of these mitotic modifications to RNAP II GTFs, it is intriguing to speculate that they may function in a manner similar to those documented for TFIID, downregulating GTF activity. As RAP74 has been shown to prevent the pausing of elongation complexes, its inactivation could destabilize RNAP II elongation complexes and contribute to the stalling and/or dislocation of the enzyme. It is also noteworthy that TFIIF and TFIIE both exist as multiprotein complexes. Although TFIID (Segil et al., 1996) and TFIIH (Adamczewski et al., 1996) apparently remain intact during mitosis, the integrity of TFIIE and TFIIF during this phase of the cell cycle has not been examined. Phosphorylation of a component of these GTFs could interfere with proper complex formation, and hence disrupt activity. Similarly, GTF phosphorylation could disrupt protein interactions which are necessary for RNAP II holoenzyme formation, serving to reduce or eliminate transcription initiation during mitosis. A further possibility is that these physical modifications cause GTF stripping from DNA. In relation to this point, Segil et al. (1996) observe that mitotic cells contain subpopulations of differentially phosphorylated TBP. They propose that the phosphorylation state of a given subpopulation could cause it to localize to a specific subcellular area (*e.g.* chromosomes or cytoplasm).

It is also possible, however, that these modifications do not have a regulatory function (White et al., 1995a). Phosphate incorporation in cytoplasmic

and nuclear non-histone proteins increases roughly eight to ten fold during mitosis (Sahasrabuddhe et al., 1984), and it is conceivable that some RNAP II GTFs are modified during this time merely as a fortuitous consequence of increased kinase activity in the cell and the exposure of nuclear proteins to cytosolic factors. If such modifications do not affect cell physiology, they may not have been selected against during the course of evolution.

The kinases responsible for modifying RNAP II GTFs during mitosis are currently unknown. It has been noted that $TAF_{II}250$ phosphorylates RAP74 *in vitro* (Dikstein et al., 1996). However, this modification appears to increase the mobility of the recombinant protein on SDS-PAGE, whereas the phosphorylation we document produces a reduction in mobility (Figure 6.1). It is intriguing that TFIIH phosphorylates TFIIE-α, RAP74, and TBP *in vitro* (Ohkuma and Roeder, 1994). However, since this study assessed phosphorylation through an analysis of phosphate incorporation, rather than electrophoretic mobility, the similarity of these *in vitro* modifications to the phosphorylation events we describe here cannot be determined.

The picture, emerging from this study and others, of dislocation from the DNA and modification of the RNAP II GTF machinery during mitosis seems likely to have important consequences for our understanding of both transcription and the cell cycle. As regards the former, the stripping of RNAP II, other RNAP II GTFs, and nascent mRNA transcripts from the DNA during mitosis would necessitate that transcription must reinitiate at promoters at the start of G_1. The currently accepted model of transcriptional initiation involves the action of a preformed RNAP II holoenzyme complex (reviewed in Koleske and Young, 1994). As conjectured above, it is possible that the phosphorylation of RNAP II GTFs disrupts holoenzyme complexes during mitosis. If this is the case, then before transcription can reinitiate, a new set of holoenyzme complexes must reassemble. The resumption of transcription would then be contingent upon proper dephosphorylation events at the mitosis/G_1 transition.

RPB1 and TBP are still largely dispersed throughout the cytoplasm when the nuclear membrane reassembles after telophase (Figures 5.2 and 5.14). As interphase cells are uniformly observed to have a strongly nuclear localization of these molecules, this indicates that they must be rapidly taken up into nascent nuclei, through nuclear pore complexes (NPCs), at the start of interphase. The facilitated movement of TBP into the nucleus is carried out by NPCs in an ATP-dependent manner (Bustamante et al., 1995). Interestingly, TBP also interacts with the cytoplasmic face of NPCs *in vitro*, and can modulate their activity through this interaction (Bustamante et al., 1995). These results suggest that the relocalization of RNAP II GTFs during mitosis may be important in the regulation of cellular functions other than transcription.

The stripping of RNAP II GTFs and nascent mRNA transcripts from DNA during mitosis also has important consequences in regards to gene expression in early development. In *Drosophila*, for example, early embryonic cell cycles possess short G_1 and G_2 phases. As mRNA transcripts are aborted during each mitosis, if the time required to transcribe a gene is greater than the length of the cell cycle, that gene will not be expressed. The stripping of RNAP II and mRNA transcripts from mitotic DNA thus serves to make the expression of genes with long transcription units, such as *Ubx*, contingent on the length of the cell cycle, which increases during development (Shermoen and O'Farrell, 1991).

A pertinent question regarding the significance of the downregulation of RNAP II transcription is whether it serves to regulate cell cycle progression and cellular proliferation. One possible answer is that this event has no effect on cell cycle progression. According to this view, RNAP II GTF stripping and downregulation are simply necessary correlates of chromosome condensation during mitosis and do not furnish the cell with an opportunity to check its proliferation. Although the precise structural nature of mitotic chromatin is not well understood, several of the RNAP II GTFs, such as $TAF_{II}250$ and RNAP II, are at least as large as a nucleosomal particle. Perhaps the presence of large

amounts of these proteins, or of nascent mRNA transcripts, interferes with proper chromosome condensation. Thus, it is not difficult to imagine reasons why the RNAP II transcription machinery should have to be removed from DNA during mitosis. Nonetheless, it does not necessarily follow that this event is exploited by the cell to serve as a cell cycle progression regulatory mechanism. In other words, transcription may re-start after mitosis in a pre-determined manner, regardless of the external environment or internal state of the cell. A prediction of this hypothesis is that if downregulation of RNAP II GTFs did not occur (*e.g.* by a modification of mitotic chromosome structure), the character of subsequent cell cycle progression would not be affected (*i.e.* it would be subject to the same requirements and checkpoint mechanisms as a normal cell cycle).

However, studies on early G_1 progression in fibroblasts provide some indications that the dramatic changes in RNAP II transcription which take place in mitosis may play a role in cell cycle regulation. Cells which have just completed mitosis require the presence of serum for roughly 3.5 h, until they reach a point in G_1 referred to as the restriction point (R). After R, cells are autonomous with respect to serum growth factors, and do not require their presence to proceed through S phase and cell division (Pardee, 1974; Temin, 1971). However, if serum is withdrawn at any time during the 3.5 h between mitosis and R, a period referred to as G_1pm (G_1 phase post-mitosis), cells immediately leave the cell cycle and enter a quiescent state (G_0) (Zetterberg and Larson, 1985). Therefore, this entire period of time represents a cell cycle checkpoint "window", during which the cell monitors the presence of growth factors. This monitoring function appears to involve the regulation of expression of the cdk inhibitor $p27^{kip1}$ (Coats et al., 1996). Cells that have left G_1pm to enter G_0 can re-enter G_1pm upon serum stimulation, and do so at the same point in that phase at which they left it (Zetterberg et al., 1995). Furthermore, kinetic studies have demonstrated that G_1pm is always the same length, further indicating that it consists of an ordered series of events which must be

completed in order for cells to achieve growth factor autonomy at R (Zetterberg et al., 1995).

There are some clues as to the nature of these events. Growth factor autonomy has been suggested to be, in part, the product of the phosphorylation, and consequent inactivation, of pRb by cdks. As the induction of G_0 produced by the withdrawal of serum during G_1pm can also be caused by protein synthesis inhibitors, the expression of certain gene products during G_1pm may be an important part of the development of growth factor autonomy by contributing to the phosphorylation and inactivation of pRb at R (reviewed in Weinberg, 1995; Zetterberg et al., 1995). In support of this suggestion, it has been observed that Rb -/- fibroblasts cannot be caused to enter G_0 by cyclohexamide treatment during G_1pm (Herrera et al., 1996). Therefore, it seems likely, then, that the ordered sequence of events which occurs in G_1pm and which leads to growth factor autonomy may consist, at least in part, of the sequential activation of various genes in response to growth factors (Figure 7.1). These might include genes such as *cyclin D1* and *cyclin D2*, the overexpression of which shortens G_1 and reduces serum dependence (Quelle et al., 1993). In agreement with this idea, studies in cycling 3T3 fibroblasts have demonstrated that the expression of a number of proliferation-associated genes, including c-*myc*, c-*fos, and* c-*jun*, varies during the G_1pm phase of the cell cycle (Cosenza et al., 1994; Cosenza et al., 1991). Furthermore, inhibition of c-*fos* or c-*jun* expression during G_1pm, using antisense strategies, impedes cell cycle progression (Cosenza et al., 1994)..

Such a sequential activation of proliferation-associated genes during G_1pm could be profoundly affected by mitotic repression of RNAP II transcription. It has been shown that in addition to RNAP II GTFs, many sequence-specific RNAP II transcription factors are also dislodged from DNA during mitosis (Hershkowitz and Riggs, 1995; Martinez-Balbás, 1995). If sequential gene activation in G_1pm involves the regulation of transcription, then this promoter stripping during mitosis may be required in order to allow the

sequential activation of these genes. For example, if a gene located late in this temporal cascade did not have its promoter stripped during mitosis, its transcription during G_1pm might be independent of growth factors or prematurely responsive to such factors. In either case, the activation of transcription of upstream genes in the cascade would not be required for the execution of this step. This would eliminate part of the G_1pm checkpoint controls, and would favour proliferation under inappropriate conditions (*i.e.* in the absence of normally requisite growth factors).

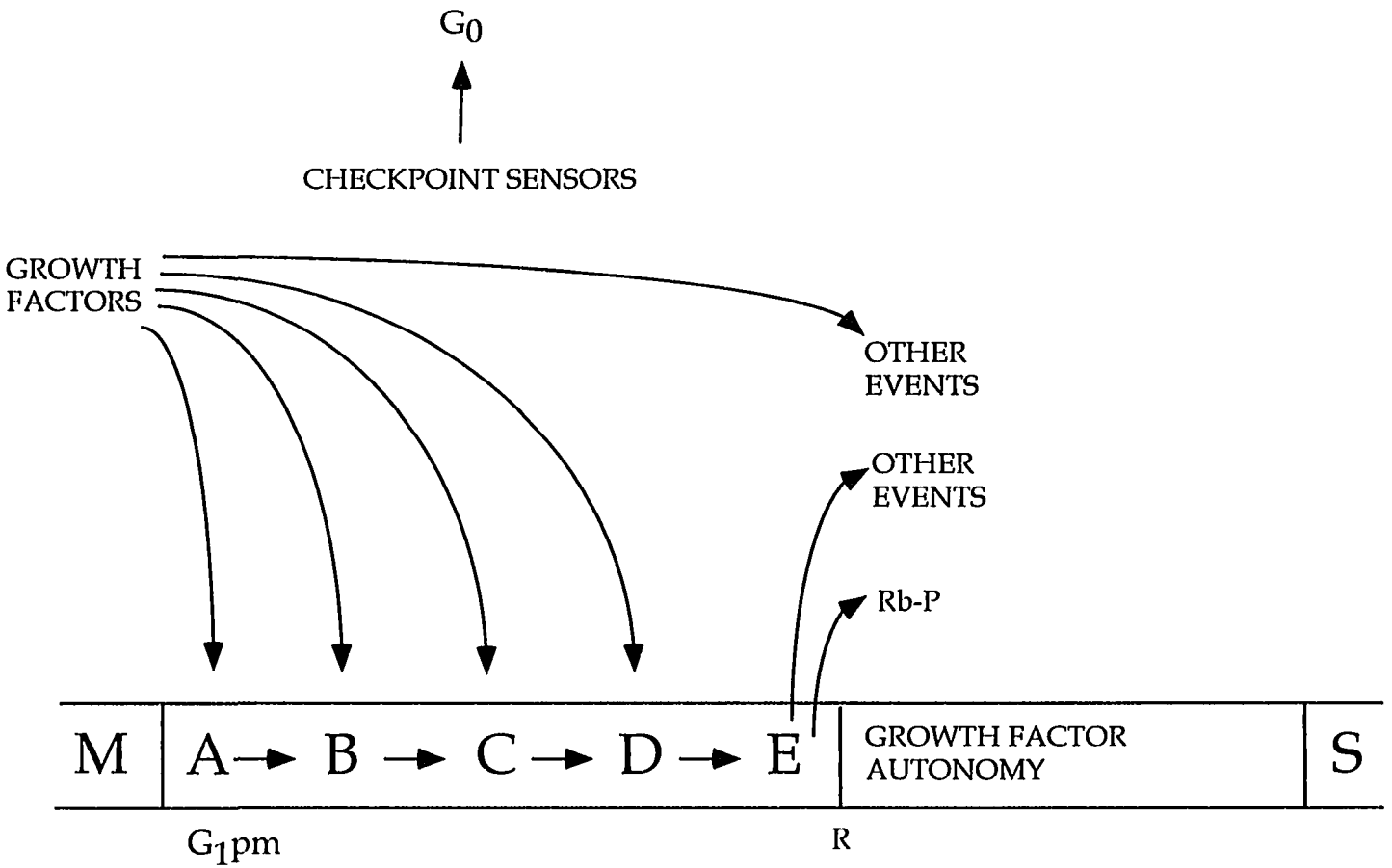
It has also been previously proposed that the constant length of G_1pm may be due in part to the gradual nature of chromosome decondensation (Zetterberg et al., 1995), a process which takes place progressively throughout early G_1 (Belmont and Bruce, 1994). Therefore, it is possible that the sequential activation of promoters required for the achievement of growth factor autonomy could involve, in addition to promoter stripping during mitosis, chromosome unfolding events in G_1 which are necessary to provide transcription factor access to promoter DNA. However, recent results imply that the interrelation between RNAP II transcription and chromosome decondensation may be more complex than this simple model suggests. The treatment of cells with specific inhibitors of RNAP II disrupts the topological organization of interphase chromatin (Haaf and Ward, 1996). This result is not caused by a loss of protein synthesis, and suggests that RNAP II transcription is not only dependent upon chromatin structure but may also function in the production and maintenance of that structure *in vivo*.

Another implication of mitotic promoter stripping involves the maintenance of patterns of transcription from one cell generation to the next. Since active chromatin regions maintain a hypersensitivity to DNase I even in metaphase chromosomes (Kerem et al., 1983; Gazit et al., 1982), it has been suggested that transcription factors remain associated with promoter regions of active genes during mitosis (Hershkowitz and Riggs, 1995). In this fashion, genes

expressed in the parental cell would be "programmed" for expression in early G_1 of its progeny cells. However, DNA footprinting experiments on the *phosphoglycerate kinase* gene, the expression pattern of which is heritable, failed to reveal any transcription factor association with promoter DNA during mitosis (Hershkowitz and Riggs, 1995). This result, combined with other reports of sequence-specific transcription factor stripping during mitosis (Martinez-Balbás et al., 1995), suggests that other mechanisms are involved in maintaining patterns of transcription on expression-heritable genes during cell division.

In summary, a comprehensive understanding of the implications of the repression of RNAP II transcription during mitosis will require further investigation of both its mechanisms and its consequences. As regards mechanisms, the biochemical effects of RNAP II GTF phosphorylation during mitosis, as well as the kinases and phosphatases responsible for affecting these modifications, need to be identified. Also, a better biochemical understanding of the relationship of condensed chromatin structures, such as those characteristic of mitosis, to transcription factor binding is required to explain the stripping of some transcription factors from mitotic DNA. With respect to the consequences of RNAP II transcriptional repression during mitosis, the integrity of RNAP II GTF and holoenzyme complexes during mitosis needs to be assessed. Also, in light of the potential role of TBP in NPC function, the potential roles of dispersed GTFs in other cellular functions during mitosis seems worthy of consideration. Finally, a detailed analysis of the temporal pattern of resumption of transcription of various genes implicated in contributing to the achievement of growth factor autonomy at R is required. Such studies may identify a role for mitotic RNAP II promoter stripping and transcriptional repression in the checkpoint mechanisms which regulate cell cycle progression during G_1pm.

Figure 7.1. Model of early G_1 progression checkpoints requiring RNAP II transcriptional repression during mitosis and transcriptional reprogramming during G_1pm. Temporal progression through the cell cycle is represented by the bar at the bottom of the figure. In G_1pm, a series of genes, indicated by letters A to E, must be transcriptionally reprogrammed in sequence. This process depends on the presence of growth factors, either at its commencement only, or alternatively, at multiple points during G_1pm. The culmination of this sequential gene activation is the phosphorylation of pRb, and perhaps other events as well. Besides this sequential gene activation, serum is required throughout G_1pm for the induction of other events which contribute to the achievement of growth factor autonomy at the restriction point (R). The events of G_1pm, including sequential gene activation, are monitored by checkpoint sensors, which can detect either a failure to complete part of the sequential gene activation, or the absence of other serum-induced events. When activated, the G_1pm checkpoint controls cause the cell to enter G_0.


G_0
CHECKPOINT SENSORS
GROWTH FACTORS
OTHER EVENTS
OTHER EVENTS
Rb-P
M
A → B → C → D → E
GROWTH FACTOR AUTONOMY
S
G_1pm
R

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