2 RNAPII: A Specific Target for the Cell Cycle Kinase Complex

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1 Introduction

Transcription in plants, as in other eukaryotes, is catalyzed by three RNA polymerases (RNAPs). Catalytically active forms of RNAPs were first isolated by Roeder and Rutter (1969) and designated as RNAPI(A), II(B) and III(C). RNAPI transcribes rRNA genes, RNAPII synthesizes the precursors of mRNAs and RNAPIII is involved in the transcription of 5S RNA and tRNA genes. In contrast to prokaryotes in which a single RNA polymerase, consisting of $\beta\beta'\alpha_2$ subunits and associated σ factors (Yura and Ishihama 1979; Helmann and Chamberlin 1988), is sufficient for promoter recognition, the assembly of transcriptionally active initiation complexes in eukaryotes requires specific interactions of RNAPs with multiple transcription factors (TFs) and promoter-specific activator proteins. Studies of the regulation of transcription were started by characterization of the subunit composition of RNAPs.

2 Structure and Function of Eukaryotic RNA Polymerases

A comparison of subunit composition of RNAPs purified from lower eukaryotes, plants, insects, amphibians and mammals was initially obtained by biochemical and immunological methods (for review, see Lewis and Burgess 1982; Guilfoyle 1983; Sentenac 1985). Isolation of yeast cDNAs encoding individual RNAP subunits (Young and Davis 1983; Riva et al. 1986) opened the way to genetic approaches, such as site-specific mutagenesis and epitope tagging of RNAP subunits (for review, see Sentenac 1985; Sawadogo and Sentenac 1990; Young 1991). Phosphorylation, inhibitor binding and chemical cross-linking studies contributed to a comprehensive picture of the structural and functional organization of eukaryotic RNAPs. The yeast system is used as a reference for comparison because most data on RNAP genes and proteins are derived from studies with this organism

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(Table 1; Guilfoyle and Dietrich 1987; Geiduschek and Tocchini-Valentini 1988; Woychik and Young 1990; Young 1991).

Essential domains involved in DNA binding and RNA synthesis are carried by the two largest subunits of RNAP enzymes which are related in size and sequence in RNAPI, II and III, and show homology to the ββ′ subunits of eubacterial and archaebacterial RNA polymerases (Allison et al. 1985; Sweetser et al. 1987; Mémet et al. 1988; Pühler et al. 1989). The B44.5 subunit of RNAPII(B), and the common subunits AC40 and AC19 of RNAPI(A) and RNAPIII(C) enzymes in yeast are homologous to the α subunit of eubacterial RNA polymerases. Yeast RNAPs contain five common subunits: ABC27, ABC23, ABC14.5, ABC10α and ABC10β. In plants and other eukaryotes three common subunits are shared by all RNAPs.

Thus, RNAPI, II and III of wheat contain identical subunits of 20, 17.8 and 17 kDa, whereas common subunits of 25, 19 and 17.5 kDa are present in RNAPs of cauliflower and other dicotyledonous plants (Guilfoyle et al. 1984; Guilfoyle and Dietrich 1987). In addition, an identical subunit of 38 kDa is present in RNAPI and III, and another subunit of 25 kDa is shared by RNAPII and III in wheat (Table 1).

RNAPI in *S. cerevisiae* contains 13 subunits, whereas in *S. pombe*, plants and mammals it consists of only 6–7 subunits. In *S. pombe* the "core" RNAPI subunits A190(β′), A135(β), AC40(α), and AC19(α) form a complex with the "common" subunits ABC27, ABC23 and ABC14.5 (Sentenac 1985; Woychik et al. 1990). This simple structural organization of RNAPI reflects a conserved feature of transcription factors that regulate the expression of rRNA genes of higher eukaryotes in a similar fashion (Bell et al. 1990; Reeder 1990; Comai et al. 1992).

The ability of RNAPII to recognize TATA-box containing promoters is used as a didactic argument to differentiate between RNAPI and RNAPII. RNAPIII may be considered an "intermediate" form of RNAPs because it initiates transcription on promoters that either contain or lack a TATA element. A functional similarity between RNAPII and III is indicated by the observation that related transcription factors, Oct-1 and Oct-2, regulate the transcription of snRNA and mRNA genes (Tanaka et al. 1992). Nucleotide exchanges in the TATA sequence or alteration of the spacing between the TATA box and upstream activator regions convert the RNAPIII promoters into promoters recognized by RNAPII (for review, see Gabrielsen and Sentenac 1991). In contrast, upstream control elements are not exchangable between RNAPII and RNAPI promoters (Schreck et al. 1989). Transcription factors that interact with the TATA box and upstream activator factors thus direct selective binding of appropriate RNA polymerases to diverse promoters.

Genes encoding most RNAPIII subunits in yeast have been cloned, and all of them were found to be unique and essential for viability. Properties of RNAPIII subunits and their role in transcription have been reviewed recently (Gabrielsen and Sentenac 1991). Due to its pivotal role in transcription

Table 1. Subunit composition of RNA polymerase enzymes purified from yeast and wheat germ (Lewis and Burgess 1982; Guilfoyle et al. 1984; Sentenac 1985; Woychik et al. 1990; Carles et al. 1991; Young 1991)

Yeast			Wheat germ			
PNAPI(A)	PNAPII(B)	RNAPIII(C)	RNAPI	RNAPII	RNAPIII	
190	***220***	***160***	***200***	***220***	***150***	**β′**
135	***150***	***128***	***125***	***140***	***130***	**β**
					94	
		82				
					55	
		53				
49						
	44.5 (α)					
43						
				42		
40 (α)		**40 (α)**		40		
			38		**38**	
		37				
34.5						
		34				
	32					
		31				
					30	
					28	
27	***27***	***27***		27		
		25		**25**	**25**	
				24	24.5	
23	***23***	***23***				
				21	21.5	
			20	***20***	***20***	
19 (α)		**19 (α)**			19.5	
			17.8	***17.8***	***17.8***	
			17	***17***	***17***	
	17			16.3		
				16		
14.5	***14.5***	***14.5***				
14				14		
	13*					
12.2						
*10**	*10**	*10**				

* Subunits represented by two unrelated proteins of identical molecular mass. α and β mark subunits homologous to prokaryotic and archaebacterial RNAP proteins. Using the nomenclature introduced by Sentenac (1985), the RNAP subunits are designated according to their molecular mass. Subunits of RNAPI(A) are marked by A, subunits of RNAPII(B) and RNAPIII(C) are labelled by B and C, respectively. Common subunits of the RNAP enzymes are designated by the combination of A, B and C letter codes (i.e., AC40 or ABC23). In the nomenclature of Young (1991) the RNAP subunits are designated according to their mobility on SDS-PAGE. Thus, RNAPI(A) subunits are labelled as RPA1, RPA2, etc., whereas RNAPII(B) subunits are designated as RPB1, RPB2, etc. Genes corresponding to these subunits are marked accordingly, such as *RPB1*, *RPB2*, etc. (Young 1991). To avoid confusion, both nomenclatures are used simultaneously in the text. Homologous (i.e., common) subunits of RNAP enzymes are indicated by italic boldface numbers, whereas subunits common between either RNAPI(A) and RNAPII(B) or RNAPII(B) and RNAPIII(C) are labelled by boldface numbers.

of protein coding genes, RNAPII is one of the best-studied enzymes in eukaryotes (Sentenac 1985; Sawadogo and Sentenac 1990; Woychik and Young 1990; Young 1991). Twelve RNAPII subunits and corresponding genes have been identified in yeast (see Table 1; Carles et al. 1991; Young 1991). The subunit organization of RNAPII enzymes isolated from yeast, plants, insects and mammals is very similar. Although RNAPII is thought to perform a more complex function than RNAPIII, genetic studies indicate that mutations in several RNAPII subunit genes (*RPB7*, *RPB4* and *RPB9*) do not cause lethality, whereas the loss of any RNAPIII subunit function is lethal in yeast. The RPB4(B32) and RPB7(B17) subunits of RNAPII form a subcomplex which can dissociate from the enzyme. *RPB4* mutants are temperature-sensitive and lack the RPB7(B17) subunit. RPB4 is homologous to the σ^{70} subunit of prokaryotic RNA polymerases, and together with RPB7 stimulates the formation of preinitiation complexes. Subunit RPB9(B13a), a zinc-binding protein, is only essential for growth at temperature extremes. Gene *RPB11* encodes a second 13-kDa subunit which is required for viability in yeast. Common subunits ABC10α and β are essential zinc-binding proteins, whereas RPB5(ABC27), RPB6(ABC23) and RPB8(ABC14.5) have been suggested to determine nuclear localization and DNA binding (Woychik and Young 1990). RBP6(ABC23) is phosphorylated, and may thus function as a common regulatory protein for all three RNAPs. RBP5(ABC27) is a basic protein, two copies of which are present per enzyme molecule. RNAPII also contains two RBP3(B44.5) subunits with sequence homology to the α-subunit of prokaryotic RNAP enzymes. In prokaryotes the α-subunit regulates the assembly of ββ′ subunits. Mutations affecting either the α, β or β′ subunit all result in defective assembly. Mutations abolishing the assembly of RPB1(B220), RPB2(B150) and RPB3(B45) subunits were similarly localized in the RNAPII subunit genes *RPB1*, *RPB2* and *RPB3* in yeast (Kolodziej et al. 1990; Woychik et al. 1990; Carles et al. 1991; Young 1991). The largest subunit of RNAPII carries a unique C-terminal domain that is absent from homologous subunits of RNAPI and III enzymes, and may regulate RNAPII activity and/or specificity.

3 Functional Domains of the Largest Subunit of RNAPII

In RNAPII enzymes isolated from diverse organisms, three forms of the largest RPB1 subunit, IIa (220 kDa), IIb (180 kDa) and IIo (240 kDa), were detected. Immunological and peptide mapping studies revealed that these RPB1 forms are closely related (Dahmus 1983; Kim and Dahmus 1986). Biochemical analysis indicated that the IIb protein is an artifact that results from protease digestion of the C-terminal domain of RPB1 forms IIa and IIo during enzyme purification (for review, see Lewis and Burgess 1982; Guilfoyle 1983). The IIo form of RBP1 was shown to result from phosphorylation of the C-terminal domain of form IIa (Dahmus 1981). Elegant

biochemical and genetic studies revealed that the RPB1(β′) and RPB2(β) subunits are involved in binding of DNA, nucleotides, and amatoxin inhibitors of RNA chain elongation and pyrophosphate exchange reactions (for review, see Sentenac 1985; Riva et al. 1987).

To genetically identify the amatoxin binding RNAPII subunit, stable, conditionally lethal and thermosensitive mutants conferring α-amanitin resistance were isolated in mouse and *Drosophila* (Ingles 1978; Greenleaf et al. 1979). Following genetic mapping of α-amanitin resistance mutations, the $RPII^{C4}$ locus encoding the largest subunit of RNAPII was cloned from *Drosophila* by P-element-mediated gene tagging (Searles et al. 1982; Greenleaf 1983; Jokerst et al. 1989). *RPB1* and *RPB2* genes were subsequently isolated from yeast (Young and Davies 1983) and used for site-specific mutagenesis of the largest subunit genes of RNAPII (Ingles et al. 1984). The *Drosophila* and yeast genes served as probes for cloning the largest subunit genes of RNAPII from *Trypanosoma*, *Crithidia*, *Plasmodium*, *S. pombe*, *Caenorhabditis*, mouse, hamster, man, as well as from higher plants, such as soybean and *Arabidopsis* (Cho et al. 1985; Ahearn et al. 1987; Allison et al. 1988; Bird and Riddle 1989; Evers et al. 1989a,b; Li et al. 1989; Smith et al. 1989; Dietrich et al. 1990; Nawrath et al. 1990; Azuma et al. 1991).

Nucleotide sequence comparison of the largest subunit genes, *RPB1* and *RBP2*, revealed a phylogenetic conservation of 8–9 domains with homology to the β′ and β subunits of prokaryotic RNAPs, and to the largest subunits of RNAPI and III (for review, see Sawadogo and Sentenac 1990; Young 1991). The function of these domains has been deduced by confrontation of genetic and nucleotide sequence data (Allison et al. 1985; Himmelfarb et al. 1987; Nonet et al. 1987a; Sweetser et al. 1987; Jokerst et al. 1989; Nawrath et al. 1990; Scafe et al. 1990). The N-terminal domain of the largest subunit of RNAPII contains a conserved zinc-binding motif $CX_2CX_6CX_2HX_{11}HX_{12}CVCX_2C$. Analogous zinc-binding motifs, essential for interaction between the two largest subunits, also occur in the C-terminal domain I of the RPB2 subunit, and in the largest subunits of RNAPI and III (Yano and Nomura 1991; Young 1991). Several second-site mutations reverting the defects of the RPB1 subunit have been mapped in the C-terminal domain I, close to the nucleotide binding domain H of the RPB2 subunit. A number of mutations correcting the defects of the RPB2 subunit have been located in the domain H of RPB1 (Martin et al. 1990), and in the RPB6 common subunit which participates in DNA binding together with the two largest subunits (Mortin 1990; Archambault et al. 1992). A two-helix motif, known to contact the major groove of DNA, is located in a second N-terminal conserved domain of the RPB1 subunit (Ahearn et al. 1987; Jokerst et al. 1989; Nawrath et al. 1990).

About 120 amino acid residues identical in all eukaryotes define the central region of the largest subunit of RNAPII. A mutation causing α-amanitin resistance in mouse is localized in this region, and results in an asparagine to aspartate substitution in a conserved motif, VGQQ*N*VEG

(Bartolomei and Corden 1987). In yeast the Asn residue is replaced by serine, possibly explaining the insensitivity of yeast RNAPII to amatoxins. The largest subunits of plant RNAPII enzymes also contain a VGQQ*N*VEG motif. Nevertheless, plants can tolerate high concentrations of α-amanitin, possibly because they are capable of metabolically inactivating the toxin (Pitto et al. 1985). However, it cannot be excluded that the lower amatoxin sensitivity of plant RNAPIIs (see Sentenac 1985) results from a different amino acid exchange because the positions of other Ama^R mutations are not yet known. Certain Ama^R mutations cause pleiotropic developmental defects in a gene dosage-dependent fashion. In *Drosophila* the *C4* Ama^R mutation confers an ultrabithorax-like phenotype (*Ubl* locus, Greenleaf et al. 1980), whereas another Ama^R mutation in rat myoblasts prevents muscle differentiation (Crerar et al. 1983). These pleiotropic effects of Ama^R mutations correlate with a decreased rate in RNA elongation by α-amanitin-resistant RNAPII enzymes (Coulter and Greenleaf 1985; Shermoen and O'Farell 1991).

Electron crystallography of a yeast RNAPII lacking the RPB4/RPB7 subcomplex revealed a 25-Å-wide groove formed by the RPB1 and RPB2 subunits capable of accomodating the template DNA (Darst et al. 1991). The three-dimensional crystallographic image supports the genetic data, indicating that functional complementation between structurally altered RPB1 and RPB2 subunits is possible by conformational correction. The overall structure of yeast RNAPII is very similar to that of *E. coli* RNA polymerase, with the exception of a finger-like structure that protrudes from the molecule in the vicinity of the 25-Å groove. This structure corresponds to the hydrophylic C-terminal domain (CTD) of the largest subunit of RNAPII. This domain is composed of YSPTSPS heptameric repeats which are absent from the prokaryotic and archaebacterial RNAPs, as well as from the largest subunits of eukaryotic RNAPI and III enzymes. The number of heptapeptide repeats increases in correlation with the genomic complexity, but the degree of divergence from the consensus YSPTSPS motif varies among the CTD repeats in diverse organisms. In the CTD of *Plasmodium* the consensus heptamer is YSPTSPK and occurs nine times, whereas the number of consensus/variant YSPTSPS repeats is 18/8 in *S. cerevisiae*, 24/5 in *S. pombe*, 11/30 in *Caenorhabditis*, 15/26 in *Arabidopsis*, 2/42 in *Drosophila* and 21/31 in mouse and hamster (for review, see Allison et al. 1985, 1988; Corden et al. 1985; Bird and Riddle 1989; Nawrath et al. 1990; Azuma et al. 1991).

4 Regulation of RNAPII Activity by Phosphorylation of the C-Terminal Domain (CTD) of the Largest Subunit

The repeated pattern of proline residues in the CTD results in consecutive helical β-turns which add up to a tail-like, flexible secondary structure

(Matsushima et al. 1990). This structure is strongly antigenic and particularly sensitive to protease digestion during enzyme purification and when synthesized as part of CTD-fusion proteins in *E. coli* (Christmann and Dahmus 1981; Guilfoyle et al. 1984; Kim and Dahmus 1986; Lee and Greenleaf 1989; Peterson et al. 1992). Removal of the CTD by protease treatments converts the IIa and IIo forms of the largest subunit to form IIb (Dahmus 1983; Guilfoyle et al. 1984; Corden et al. 1985; Guilfoyle and Dietrich 1987). RNAPII enzymes purified from mouse and HeLa cells contain predominantly the IIo form (240 kDa) which can be converted to form IIa by phosphatase treatment (Cadena and Dahmus 1987). Casein kinase II phosphorylates the largest subunit at a single Ser residue of the C-terminal end in vitro (Dahmus 1981), but does not appear to be responsible for the phosphorylation of the CTD in vivo (Lu et al. 1991, 1992). Protease treatment of the in vitro or in vivo ^{32}P-labelled IIo form results in quantitative removal of the label, indicating that the CTD within the IIa form is a unique target for phosphorylation.

The effect of CTD phosphorylation was tested in various in vitro transcription systems (for review, see Sawadogo and Sentenac 1990). Initial studies suggested that all three forms of RNAPII can accurately initiate transcription from the adenovirus core Ad-2 MLP promoter. In these in vitro transcription assays RNAPII forms IIA and IIB were found to be more active than RNAP IIO, suggesting that the CTD or its phosphorylation may not be required for the initiation of transcription (Sentenac 1985; Kim and Dahmus 1989). In contrast, other data demonstrated that CTD-specific antibodies, as well as synthetic CTD heptapeptide repeats, can inhibit the initiation of transcription by RNAPII, but do not affect the transcript elongation (Dahmus and Kedinger 1983; Moyle et al. 1989; Thompson et al. 1989). These experiments also revealed that the CTD is required for transcription initiation from promoters lacking the TATA box, whereas the CTD-less RNA polymerase IIB can accurately initiate transcription from TATA-box containing promoters in vitro. However, when injected into *Xenopus* oocytes, CTD-specific antibodies inhibited the transcription from both the TATA-containing human histone H2b promoter and the TATA-less promoter of U1 small nuclear RNA gene (Thompson et al. 1989). These data showed that the CTD is absolutely required for the stabilization of the initiation complex in the absence of the TATA box, whereas general transcription factors can mediate the binding of RNAPII to TATA-box containing promoters even in the absence of the CTD.

UV cross-linking studies demonstrated that elongating transcripts are associated with the largest subunit of RNAP IIO carrying a phosphorylated CTD (Bartholomew et al. 1986; Cadena and Dahmus 1987). Reconstitution of transcription initiation and elongation complexes using purified transcription factors revealed, on the other hand, that the unphosphorylated form of RNA polymerase (IIA) is required for promoter binding and the formation of stable preinitiation complexes. During the transition from initiation to elongation, inactive RNAP IIA is specifically phosphorylated at

the CTD to yield an active enzyme, RNAP IIO (Laybourn and Dahmus 1989; Payne et al. 1989; Lu et al. 1991, 1992; Chesnut et al. 1992). Due to the apparent contradiction between in vitro and in vivo transcription data, the role of CTD in the regulation of transcription has been extensively discussed (for review, see Corden 1990; Sawadogo and Sentenac 1990; Chao and Young 1991; Young 1991).

5 Genetic Analysis of Interactions Between RNAPII and Transcription Regulatory Proteins

Genetic analysis of CTD mutants in yeast, *Drosophila* and mouse provided the first indication that the CTD is essential for the regulation of RNAPII transcription in vivo (Nonet et al. 1987b; Allison et al. 1988; Bartolomei et al. 1988; Zehring et al. 1988). The maintenance of at least 11 CTD heptapeptide repeats is required for viability in yeast. Mutants with 11–12 repeats are conditionally viable, but display heat and cold sensitivity and inositol auxotrophy (Nonet et al. 1987b). In *Drosophila* about 50% of the repeats is essential in vivo (Zehring et al. 1988). In mouse a reduction in the number of repeats to 29–31 reduces the cell growth and division rate and 25 or fewer repeats cause cell lethality (Bartolomei et al. 1988). Replacement of yeast RNAPII CTD with the first 26 repeats of hamster CTD did not affect viability, whereas an exchange for the less conserved *Drosophila* CTD caused lethality in yeast (Allison et al. 1988). Repeats differing in two or three amino acids from the consensus heptapeptide could not substitute for the conserved heptapeptide repeats in mouse cells (Bartolomei et al. 1988).

These observations indicated that both the position and amino acid composition of heptapeptide repeats can effect the CTD function in vivo. Combinations of yeast *INO1* and *GAL10* upstream activator sequences (UAS) with a "core" TATA-box promoter-*lacZ* reporter gene construct revealed a reduction in *INO1*, and *GAL10* mRNAs in a yeast mutant, *rpb1Δ104* that carried only 11 CTD repeats. The deficiency of transcriptional activation by the *GAL10* UAS was shown to result from a defective interaction between the truncated form of RNAPII CTD and the *GAL10*-specific transcription activator protein, GAL4 (Allison and Ingles 1989; Scafe et al. 1990). Using GCN4 and RNAPII affininity columns, Brandl and Struhl (1989) demonstrated that GCN4 and RNAPII can interact directly in vitro. This interaction required the basic DNA-binding domain of GCN4. Studies demonstrating that GAL4 can mediate transcriptional activation in *Drosophila* and mammalian cells underscore the importance of these results and suggest that analogous interactions between the C-terminal repeats of RNA polymerase II and promoter-specific activator proteins generally occur in all eukaryotes (Fischer et al. 1988; Kakidani and Ptashne 1988; Webster et al. 1988).

Isolation of second-site mutations suppressing the defects of CTD deletions in yeast led to the identification of intragenic and extragenic revertants. Intragenic revertants carried either point mutations in the conserved domains of the largest subunits of RNAPII, or duplications of the shortened CTDs. A dominant extragenic suppressor correcting the effect of CTD truncations was mapped to the yeast gene *SRB2*, which encodes a transcription factor that can apparently replace the function of the CTD (Nonet and Young 1989; Koleske et al. 1992). Data indicating that acidic activators, the CTD of RNAPII and transcription factors located at the TATA box can interact with each other opened the way to novel genetic approaches. Deletions removing the TATA box and/or UAS activator binding sites have been exploited for isolation of second-site revertants in yeast. These second-site mutations reverted either the inducibility of transcription by a UAS-specific activator, or reconstructed the basic level of uninduced transcription of diverse genes (i.e., *His3*, *His4*, *Suc2*, etc.). In a second system mutations in genes *SWI1–6*, which are positive regulators of the mating-type endonuclease gene *HO*, were exploited to isolate revertants expressing *HO*.

Isolation of suppressors reverting the defects (i.e., auxotrophy and cell cycle arrest in G1 phase) caused by a deletion of *HIS4* UAS elements yielded four classes of *sit* mutations. *sit1* and *sit2* proved to be mutations in the largest subunit genes of RNAPII. SIT3 is a transcription factor which probably interacts with the GCN4 and TATA-binding proteins. SIT4 is a protein phosphatase required for cell cycle-dependent regulation of gene *SWI4*. Since *sit1–sit4* and *sit2–sit4* double mutants were inviable, it was likely that SIT4 would be required for the dephosphorylation of the largest subunit of RNAPII (Arndt et al. 1989). Combinations of *sit4* alleles with *cdc28* and *bcy1* mutations caused lethality. This indicated that the SIT4 function is connected to regulatory pathways including the CDC28 cell cycle kinase and cAMP-dependent protein kinases harboring a BCY1 regulatory subunit (Hoekstra et al. 1991; Fernandez-Sabaria et al. 1992; Johnston and Lowndes 1992; see below).

CTD truncations of RNAPII and mutations in the *swi1*, *swi2*, and *swi3* genes were also found to prevent the GAL4-mediated activation of genes *GAL1* and *GAL10*, and to negatively affect the UAS-mediated transcriptional control of a large set of genes. In addition to SWI1, SWI2 and SWI3, UAS-dependent transcription from the *HO*, *GAL1*, *INO1*, *SUC2* and other promoters requires further factors, SNF5 and SNF6, which form a functional complex with the SWI1, SWI2 and SWI3 proteins (Laurent and Carlson 1992; Peterson and Herskowitz 1992; Winston and Carlson 1992). These data show that the CTD of RNAPII has a complementary and synergistic function with a transcription factor complex (including proteins SWI1, SWI2, SWI3, SNF5 and SNF6) which "tethers" the UAS-specific activators to other elements of the TATA-box bound initiation complex. Such complementary functions may explain why RNA polymerases lacking a CTD can respond to activation by certain UAS-specific transcription factors (e.g.,

Sp1 in mammals), and why CTD is required for transcription of another set of genes, e.g., those lacking a TATA box (Zhou et al. 1992). In fact, a mutation in the *SIN1* gene was found to suppress the effects of CTD truncation, as well as of *swi1*, *swi2*, and *swi3* gene mutations. *SIN1* encodes a HMG1-type protein which is a chromatin-associated general repressor (Kruger and Herskowitz 1991; Peterson et al. 1991). As the CTD truncations, *swi* and *sit* mutations block the cell cycle progression at the G1 phase.

6 Promoter Recognition and Transcription Initiation by RNAPII: A Phylogenetically Conserved Mechanism

Genetic and biochemical studies in recent years have produced a general concept of transcription initiation (for review, see Gabrielsen and Sentenac 1991; Greenblatt 1991a,b; Roeder 1991; Guarante and Bermingham-McDonogh 1992; Sharp 1992; White and Jackson 1992a,b; Rigby 1993). A brief discussion of this concept helps to understand the function of RNAPII CTD in the regulation of transcription.

Studies of the preinitiation complex (PIC) assembly on class II promoters, such as the adenovirus core promoter (Ad-2 MLP), showed that an ordered binding of RNAPII and several general transcription factors (TFs) to the TATA box and to downstream initiation sequences precedes the initiation of RNA synthesis. Transition of PIC to an active initiation complex (i.e., open complex) requires ATP, indicating the involvement of protein kinase(s) and/or ATPase(s). The rate-limiting step of PIC assembly involves TFs and activator proteins (Buratowski et al. 1991; Wang et al. 1992a). The most extensively characterized factors are TFIIA, -B, -D, -E, -F, -G/J, -H, -I, -S, and -X from HeLa cells which are related to factors α, β, γ, δ, ε and τ from rat liver, and to factors a, b, c, d, e and g from yeast (for review, see Roeder 1991).

The PIC assembly begins with binding of the TFIID to the TATA element and continues with subsequent binding of TFIIA and TFIIB to class II promoters. TFIIA binds upstream of TFIID, whereas the binding site of TFIIB is located 3′ downstream of the TATA box. Genetic analyses of yeast mutations suppressing defects of the TATA box, GAL4 activation domain and RNAPII CTD truncations (see above) support biochemical data, indicating that TFIID is a complex of a TATA-binding protein (TBP) and multiple TBP-associated factors (TAFs). TAFs are thought to act as co-activators mediating the interactions between the TBP, the CTD of RNAPII, TFIIB and acidic activator proteins (Kelleher et al. 1990; Pugh and Tijan 1990; Dynlacht et al. 1991; Flanagan et al. 1991; Meisterernst et al. 1991; Lai et al. 1992; Laurent and Carlson 1992; Takada et al. 1992; Wang et al. 1992b; see below). TATA-binding proteins encoded by HeLa, rat, yeast, *Drosophila* and plant *TBP/TFIIDτ* genes were found to be interchangeable

in vitro. Comparison of TBP sequences revealed a phylogenetically conserved C-terminal domain which was shown to bind to the consensus TATA sequence (for review, see Gasch et al. 1990; Cormack et al. 1991; Gill and Tijan 1991; Greenblatt 1991b; Haaß and Feix 1992).

Measurement of RNA polymerase I, II and III activities in a TBP-deficient yeast mutant, *spt15*, led to the discovery that the TATA-binding protein is required for specific promoter recognition by all three eukaryotic RNA polymerases (Cormack and Struhl 1992; Schultz et al. 1992). Biochemical and genetic data so far indicate that general transcription factors (SL1, TFIID and TFIIIB/BRF1, see below) mediating RNAPI, II and III transcription in yeast, *Drosophila* and mammals contain only a single type of TATA-binding protein which is a functional equivalent of TBPs identified in plants. Studies of the crystal structure of *Arabidopsis* TBP protein correlate with the genetic data, and show association of two TBP molecules in a "concave" DNA-fold in which the basic repeat sits astride the DNA. Mutations destroying the DNA-binding map to this region, whereas others influencing RNA polymerase or species specificity cause alterations in the convex surface of the TBP dimer (Nikolov et al. 1992). Lysine residues on the convex surface of the TBF basic repeat are involved in the interaction with TFIIA, which stabilizes the TBP-TATA complex during PIC formation on TATA-box containing promoters (Lee et al. 1992; Nikolov et al. 1992).

Interaction between TBP and TFIIA is not essential for in vitro PIC assembly on core promoters. However, TFIIA is absolutely required for PIC formation when TBP is associated with TAFs in the TFIID complex, and for activator-dependent transcription (for review, see Roeder 1991; Conaway et al. 1992; Sayre et al. 1992a,b; Zhou et al. 1992). TFIIA, consisting of three proteins in HeLa, relieves the effect of inhibitors, such as Nc1, Nc2 and Dr1, which abolish class II gene transcription by binding to the TBP (Roeder 1991; Inostroza et al. 1992). Whereas TFIIA is required for basal transcription, a number of TAF proteins which associate with the TATA-binding protein in the TFIID complex may compete with TFIIA or enhance the interaction of the TFIID-TFIIA complex with gene-specific activators. Recently, using an epitope-labelled TBP, a holo-TFIID was purified from HeLa cells. This holo-TFIID supports transcriptional stimulation by different activation domains provided by transcription activators Sp1, E1a, Zta and GAL4-AH in the presence or absence of the TATA box. It cannot be excluded that on diverse promoters this holo-TFIID is associated with additional TAFs (i.e., transcription factors acting by protein-protein interaction; for discussion, see Pugh and Tijan 1990; Sharp 1992; Zhou et al. 1992). Different regulatory factors may therefore mediate diverse PIC assembly pathways. Thus, PIC assembly on promoters lacking the TATA box is not promoted by TFIIA, but requires TFII-I. TFII-I specifically binds to initiator sequences and acts cooperatively with TFIID (Roeder 1991).

The TFIIA-D-I complex on TATA-box containing promoters and the TFIID-I complex on TATA-less promoters interact with TFIIB. TFIIB-

TFIID interaction enhances the binding of RNAPII to the preinitiation complex. TFIIB has been cloned from yeast (gene *SUA7*) and HeLa cells, and identified as a protein that can associate with RNAPII in solution (Ha et al. 1991; Malik et al. 1991; Tschochner et al. 1992). TFIIB not only forms a bridge between TFIID and RNAPII, but also regulates the start-site selection by interaction with proteins located at the initiator sequences. TFIIB mutations were identified in yeast as suppressors of aberrant initiation (Pinto et al. 1992). Isolation of suppressors correcting a promoter A-block mutation in a tRNA gene and a mutation in the conserved basic domain of the TATA-binding protein led to the discovery of factor BRF, a TFIIB homolog, which is a subunit of the RNAPIII general transcription factor TFIIIB in yeast (Buratowski and Zhou 1992; López-De-León et al. 1992). TFIIB can specifically bind to the acidic activating region of sequence-specific transcription factors, such as GAL4 and HSV-1 VP16. By interacting with TFIIB, which can recruit RNAPII to promoters carrying TFIIA-D-I and TFII-D-I, acidic activators can dramatically increase the rate of PIC formation (Lin and Green 1991; see below).

6.1 The Role of TBP, and TFIIB Homologs in the Formation of Preinitiation Complexes with RNAPI, II and III

Early events leading to the entry of RNA polymerase into the preinitiation complex are very similar for RNAPI, II and III transcribed genes. PIC formation on RNAPI promoters requires two types of transcription factors: a species-specific promoter selectivity factor, SL1, and an upstream binding factor, UBF (for review, see Reeder 1990; Sollner-Webb and Mougey 1991). SL1 does not bind to DNA alone, but forms PIC by interacting with UBF and RNAPI. SL1 consists of the TATA-binding protein TBP, and three associated factors (TAFs). It is yet unknown whether any of the SL1-specific TAFs would be similar to TFIIB or BRF. General properties of UBFs are similar to those of sequence-specific activator proteins of RNAPII promoters (Bell et al. 1990; Jantzen et al. 1990, 1992). Activation of the transcription by the UBF-SL1-RNAPI complex is analogous to the process by which the TFIID-B-I complex and various sequence-specific activators mediate the formation of PIC with RNAPII on TATA-less promoters (Comai et al. 1992).

RNAPIII binds to PICs formed on TATA-less and TATA-containing promoters (Geiduschek and Tocchini-Valentini 1988; Mitchell and Tijan 1989; Reeder 1990; Gabrielsen and Sentenac 1991). TFIII general transcription factors recognize conserved sequence boxes A, B and C in RNAPIII transcribed promoters. In the promoter of 5S RNA genes the binding of TFIIIA (a protein with nine zinc fingers; see Theunissen et al. 1992) to box C is required for subsequent binding of TFIIIC and TFIIIB. In tRNA promoters TFIIIC binds to the A and B boxes and recruits TFIIIB alone. TFIIIA and TFIIIC function as enhancer and proximal element-binding factors which assemble with and position TFIIIB upstream of the transcription start site (Braun et al. 1992; Bartholomew et al. 1993). Transcription initiation on TATA-containing RNAPIII promoters requires TFIIIB and sequence-specific upstream activators (e.g., Oct-1). TFIIIB is a functional analogue of SL1 and TFIID complexes. In association with the TATA-binding protein TBP, TFIIIB carries two subunits, B′/BRF and B″ in yeast, and TAF-172 and TAF-L components in HeLa cells. In yeast the basic domain of TATA-binding protein interacts with the TFIIB-like TFIIIB′/BRF1 subunit and facilitates the assembly with the B″ subunit during binding to TFIIIC (Colbert and Hahn 1992; Kassavetis et al. 1992). Through the B″ subunit TFIIIB interacts with the C34 subunit of RNAPIII which thus enters into the TFIII(A)-C-B preinitiation complex (Bartholomew et al. 1993). The TAF-172 subunit of TFIIIB in HeLa cells functions similarly to BRF. By association with the TBP protein, TAF-172 prevents the TBP from interacting with TATA-box sequences of RNAPII promoters (Lobo et al. 1992; Taggart et al. 1992; White and Jackson 1992a,b; Rigby 1993). Thus, TFIIB, BRF, and probably an analogous subunit of SL1, together with other associated TAFs, can regulate the promoter and RNA polymerase specificity of the TATA-binding factor TBP.

6.2 Transcription Initiation: The Role of TFIIE, F, J/G, H, S and X

The association of RNA polymerase II with promoter-bound TFII(A)DB(I) complexes is aided by RNAPII-associated proteins (RAPs; for review, see Greenblatt 1991a). RAP30 and RAP70 associate to form a tetrameric ($\alpha_2\beta_2$) general transcription factor, TFIIF, which binds to RNAPII through the RAP30 subunit. TFIIF can abolish the aspecific DNA binding activity of RNAPII, and together with TBP and TFIIB, target the RNAPII to the TATA box in vitro (Flores et al. 1991; Finkelstein et al. 1992; Killeen and Greenblatt 1992; Killeen et al. 1992). A second class of RAP factors, TFIIE, binds to PIC together with TFIIF and RNAPII. TFIIE is also a heterotetrameric factor carrying two α (57 kDa) and two β (34 kDa) subunits. β is a modulator of the activity of the RNAPII-binding α-subunit. After binding of the TFII(A)DB(I)-RNAPII-TFIIE/F complex, TFIIE and -F are positioned downstream of the transcription initiation site and induce a dramatic conformational change in the PIC by clearing the start position (Buratowski et al. 1989, 1991; Ohkuma et al. 1990). A further RNAPII associated protein, RAP38 (also known as elongation factor TFIIS), acts as antiattenuator during elongation by preventing the pausing of RNAPII (Izban and Luse 1992). TFIIF, E and S probably remain associated with the elongation complex, and together with TFIIX, increase the rate of mRNA synthesis (Bengal et al. 1991). To convert the initiation complex to an active elongation complex, the binding of two additional factors, TFIIH and J/G, is required. These factors catalyze the phosphorylation of the C-terminal

domain of RNA polymerase II which is concomitant with a striking increase in ATP hydrolysis (see below; Sumimoto et al. 1990; Lu et al. 1991, 1992). This general pathway of PIC formation and transcription elongation appears to be phylogenetically conserved, although certain differences exist between the properties of the general transcription factors in diverse organisms (e.g., TFIIE and yeast factor a; for review, see Buratowski et al. 1989; Conaway et al. 1992; Sayre et al. 1992a,b).

6.3 Regulatory Interplay Between TFIID, TFIIB, Sequence-Specific Activators and the C-Terminal Domain of RNA Polymerase II

The assembly of PIC is accelerated by interactions between upstream sequence binding factors, TFIID, TFIIB and the CTD of RNAPII (Greenblatt 1991b; Roeder 1991; Guarante and Bermingham-McDonogh 1992). TFIIB and TBP/TFIID have been shown to specifically interact with activation domains of diverse sequence-specific transcriptional factors (Stringer et al. 1990; Lin and Green 1991; Nikolov et al. 1992). Certain transcriptional activators, such as the USF factor of the Ad-2 MLP promoter, Sp1, the plant zipper TGA1a and yeast GAL4 and GCN4, can function in heterologous transcription systems in vitro and in vivo (Horikoshi et al. 1988a; Kakidani and Ptashne 1988; Webster et al. 1988; Mitchell and Tijan 1989; Katagiri et al. 1990; Meisterernst et al. 1990; Ptashne and Gann 1990). However, in contrast to the TFIID complex, the TBP protein alone fails to promote activator-dependent transcription in vitro. An extensive search for "coactivators, adaptors and mediators" which can "tether or squelch" the effect of transcription activators on the TBP-TFIIB-RNAPII-TFIIE/F complex led to the identification of TAFs and other activator or suppressor proteins (Berger et al. 1990; Kelleher et al. 1990; Pugh and Tijan 1990; Smale et al. 1990; Dynlacht et al. 1991). USA is an interesting representative of such a class of factors. USA can repress basal transcription mediated by general transcription factors, but stimulates transcription in response to activators Sp1, USF, GAL4 or GCN4, particularly on chromatin substrates. USA appears to carry negative (NC1, NC2) and positive (PC1) elements which are probably able to bind TBP (Meisterernst et al. 1991; Roeder 1991).

Genetic and biochemical data demonstrate that a number of transcription factors which bind to TFIID/TBP and TFIIB can also interact with the CTD domain of the largest subunit of RNAPII (Allison and Ingles 1989; Brandl and Struhl 1989; Stringer et al. 1990; Liao et al. 1991; Peterson et al. 1991; Koleske et al. 1992). Thus, the CTD may be considered a major mediator/coactivator of gene-specific transcription factors and of the TATA-binding protein (Liao et al. 1991). The consensus CTD heptameric peptide repeat, when used as an affinity matrix, can quantitatively remove TFIID or TBP

from nuclear extracts, indicating that a tight interaction exists between the CTD and TBP. This interaction is regulated by phosphorylation. The phosphorylated form of CTD cannot bind to TBP, which supports the observation that binding to the preinitiation complex requires an unphosphorylated form (IIa) of the largest subunit of RNAPII (Lu et al. 1991; Chesnut et al. 1992; Conaway et al. 1992; Usheva et al. 1992). It is thus possible that, by titrating out TBP, the CTD of RNAPII can regulate transcription by RNA polymerase I and III.

The interaction between CTD and TBP may be abolished by viral oncoproteins, such as the E1A protein of adenovirus, which can specifically bind to the TBP (Horikoshi et al. 1991). E1A has been shown to activate transcription through the action of several transcription factors, such as ATF-2 or E2F (Horikoshi et al. 1988b). Genetic experiments indicate that E1A can directly interact with ATF-2 by forming a bridge to the TATA-binding factor (Lee et al. 1991). In addition to viral genes, the synthesis of E1A protein in adenovirus-infected cells *trans*-activates the transcription of several cellular genes including a heat-shock gene, *hsp70*. Interestingly, the activator-dependent transcription from the Ad-2 MLP and *hsp70* promoters is not affected by the deletion of the carboxyl-terminal domain of the largest subunit of RNAPII in vitro (Buratowski and Sharp 1990; Zehring and Greenleaf 1990; see below).

The interaction between CTD, TFIID, TFIIB and transcription activators may serve as a "short-cut" in the PIC assembly. Once elongation is initiated, the TFIID-B complex remains bound to the TATA-box region thus providing a potential entry site for RNAPII in recurrent transcription cycles (Rice et al. 1991; Roeder 1991). Multiple rounds of transcription on class II promoters are not promoted by general transcription factors, but require a reinitiation factor (RTF; Szentirmay and Sawadogo 1991) and/or transcription activators in vitro. Activator proteins, such as Oct-2, ATF, GAL4 or the EBV Zta protein, promote PIC assembly by interaction with TFIID, TFIIB and possibly CTD, and are continuously required at the promoter for recurrent initiation (for review, see Liebermann and Berk 1991; Arnosti et al. 1993). The effect of remote activators is mediated to proximal activators, and further to CTD, TFIID and TFIIB, by protein-protein interactions, resulting in selective *trans*-activation of various promoters by SV40 T, herpes simplex virus VP16 and adenovirus E1A oncoproteins (Johnson and McKnight 1989; Lai et al. 1992; Seipel et al. 1992; Tanaka et al. 1992).

Transcription, translation, nuclear translocation, DNA- and protein-binding activity of *trans*-acting factors is regulated by phosphorylation. GAL4, which can interact with the CTD, TFIID and TFIIB, is for example active when phosphorylated (Sadowski et al. 1991). The CTD of RNAPII, GAL4, Oct-1, E2F and many other transcription factors is phosphorylated during transcription initiation and at defined stages of the cell cycle (Hunter and Karin 1992).

7 Cell Cycle Regulation of Transcription and DNA Replication

Transcription in dividing cells is overruled by a cell cycle control which, by many interlocked signalling pathways and feedback mechanisms, regulates DNA synthesis, cell growth and division. All these mechanisms directly or indirectly affect the activity, assembly and stability of cyclin-dependent protein kinase complexes which determine the timing of cell cycle progression.

It is thought that in yeast the "cell cycle clock" contains a stable Thr/Ser-specific protein kinase, p34, which is the product of the *CDC28* and *cdc2* genes of *S. cerevisiae* and *S. pombe*, respectively. The p34 kinase is activated by binding to cyclins. The transcription, translation and stability of cyclins are regulated throughout the cell cycle. At the "Start", in late G1, the p34 kinase forms different complexes with "G1-specific" cyclins (CLN1–3) and activates DNA synthesis and replication of the microtubule organizing centre (MTOC). The "Start" is one of the checkpoints controlling cell proliferation by external and internal stimuli that influence the activity of both cyclins and CDC2/CDC28 kinases. After passing the "Start", B-type cyclins accumulate during the S-phase and bind to the CDC2/CDC28 kinase. The cyclin B-p34 complexes, also called pre-MPFs (i.e., mitosis promoting factors), are inactivated by Wee1/Mik1 kinase-mediated phosphorylation. A protein phosphatase, CDC25, converts pre-MPF in fission yeast to an active MPF which induces entry into mitosis at the G2/M boundary. Entry into mitosis is a second checkpoint at which the cell cycle is subjected to a complex physiological control involving feedback mechanisms sensing DNA replication and repair. MPF together with an array of interacting protein kinases and phosphatases is included in the activation of major mitotic events, such as chromosome condensation, destruction of the nuclear envelope and assembly of the mitotic spindle. The exit from mitosis is a third potential checkpoint for physiological control, such as regulation of MPF-activated pathways of protein degradation. Degradation of poly-ubiquitinated cyclins leads to inactivation of the MPF and release of CDC2 kinase for reinitiation of the cell cycle (for reviews, see Hartwell and Weinert 1989; Murray and Kirschner 1989; Draetta 1990; Nurse 1990; Enoch and Nurse 1991; Hoekstra et al. 1991; Reed 1991; Murray 1992).

Transcription, and the synthesis, repair, and mitotic and meiotic transmission of DNA, represent major downstream processes controlled by the cell cycle. Mutations and inhibitors affecting these processes are known to regulate the activity of CDC2-cyclin complexes at all three checkpoints of the cell cycle (for review and references, see Murray 1992). A simple model explaining coordinate control of transcription and DNA synthesis was derived from the genetic analysis of "Start" in yeast (Andrews and Herskowitz 1990). As described above, deletions of RNAPII CTD and suppressor mutations compensating the effect of CTD truncations cause a cell cycle arrest in G1 phase. Functions sensing the intactness and phosphorylation of RNAPII CTD could thus be involved in the regulation of DNA synthesis. To compensate for the truncation of the CTD, a mutation destroying the function of the SIT4 phosphatase is required in *S. cerevisiae*. This *sit4* mutation results in a deficiency of SWI4/SWI6 transcription factors, which in turn abolishes the expression of genes involved in DNA synthesis and decreases the level of G1/S-specific CLN cyclins. Due to the lack of cyclins, the formation of CDC28/CDC2-CLN cyclin complexes would be inhibited, preventing the phosphorylation and subsequent nuclear transport of SWI6 (and other transcription factors), and the G1/S phase transition in yeast (Lowndes et al. 1991, 1992; Nasmyth and Dirick 1991; Ogas et al. 1991; Fernandez-Sabaria et al. 1992; Johnston and Lowndes 1992; Merrill et al. 1992). The synthesis of CLN cyclins is coordinately regulated by multiple signalling pathways, the major elements of which are heterotrimeric G-proteins, membrane receptors, protein kinases (i.e., TPKs, STE11, STE7, FUS3, KSS1), and transcription factors (i.e., STE12, SWI1–6). In response to starvation or mating-type pheromones these pathways in yeast negatively regulate the levels of CLN cyclins, e.g., by FAR1, FUS3 and SWI6 functions, and cause a G1 cell cycle arrest. An important modulator of this cAMP-dependent pathway is BCY1, the regulatory subunit of A-type protein kinases. At low cAMP concentrations BCY1 inhibits the A kinases and cause a G1 cell cycle arrest, whereas in response to high cAMP levels BCY1 activates A kinases and cell division. The lethality of *sit4*/*bcy1* double mutants indicates the complementarity of cell cycle control and cAMP-dependent signalling pathways (Sprague 1991; Lew et al. 1992). How these pathways recognize the truncation and phosphorylation of the RNAPII CTD remains an intriguing question.

The situation in plants and other eukaryotes is somewhat more complex. Mammals encode at least nine different cyclins (A, B1–2, C, D1–3, E and F) which have been identified by complementation of yeast *CLN* cyclin mutations. These cyclins may associate with multiple cyclin-dependent kinases, CDC2/CDK1, CDK2, CDK3, CDK4 and CDK5. However, certain restrictions in cyclin-CDK combinations exist. Cyclin D in association with CDK2, CDK4 and CDK5 is now believed to regulate the "Start" in late G1, cyclin E-CDK2 acts in G1-S transition, whereas cyclin A-CDK2 is required for S-phase progression. In late S and G2 phases, cyclin A-CDC2 is a possible activator of cyclin B1-CDC2 and/or cyclin B2-CDC2, which are the regulators of G2-M phase transition (for review, see Clarke et al. 1992; Pagano et al. 1992a,b; Hunter 1993). Recent data indicate that the regulation of the yeast cell cycle may be even more complex as outlined above, and rather comparable in complexity to that of other eukaryotes (Courchesne et al. 1989; Elion et al. 1991; Surana et al. 1991; Yoon and Champbell 1991; Sorger and Murray 1992; Tsuchiya et al. 1992). Characterization of CDK-type kinase and cyclin genes in plants also supports the emerging view on the phylogenetic conservation of cell cycle control elements (John et al. 1989; Doonan 1991; Feiler and Jacobs 1991; Ferreira et al. 1991; Hata 1991;

Hata et al. 1991; Hirayama et al. 1991; Hirt et al. 1991; Hemerly et al. 1992; Nitschke et al. 1992).

7.1 Oncogenes and Tumor Suppressors: Conserved Functions in the Cell Cycle

G1 arrest of the cell cycle induced by starvation or mating-type pheromones in yeast is suppressed by dominant mutations which either cause an overproduction of CLN cyclins or prevent their ubiquitin-mediated degradation (see Cross and Tinkelenberg 1991; Reed 1991; Tyers et al. 1992). Similarly, in animal tumors chromosome translocations and DNA virus insertions were observed to increase the expression of D- or A-type G1/S cyclins. In mammals the passing of "Start or restriction point" at G1 activates the cell cycle by overcoming the G2/M control observed in *S. cerevisiae*. Overexpression of cyclin D is implicated in parathyroid and lymphoid neoplasias (for review, see Motokura and Arnold 1993). G1 progression in mammals, as in yeast, is controlled by limiting nutrients, and can be stimulated by several peptide growth factors. Some of these growth factors, such as CSF-1, activate the transcription of D-type cyclin genes (Matsushime et al. 1991). Cyclins D1, 2 and 3 form complexes with CDK2, CDK4 and CDK5 kinases, which are located in the nucleus during G1. Cyclin D1 was found in association with the proliferating cell nuclear antigen PCNA (Xiong et al. 1992). PCNA is the regulatory subunit of DNA polymerase δ which, together with DNA polymerase α, functions in DNA replication and repair (Yang et al. 1992). Similarly to cyclin D, cyclin A is located in the nucleus from mid-G1 to G2, until it is replaced by cyclin B. Cyclin A overexpression was observed in primary liver cancers, which in a few cases result from hepatitis B virus integration into cyclin A genes (see Bréchot 1993). Cyclin A is known to function at the G1/S transition and in S-phase when it is associated with CDK2, whereas in G2 phase it is bound to CDC2. Cyclin A plays a major role in viral oncogenesis. It has been identified in complex with the E1A protein of adenovirus type 5, as well as in complexes carrying the CDK2 kinase and an associated protein, p107 (for review, see Moran 1993).

As described above, E1A can form a bridge between transcription factors (i.e., ATF-2/CRE-BP1 cAMP response factors) and the TATA-binding protein TBP, and thus mediate *trans*-activation of diverse cellular genes including *N/c-myc*, *c-myb*, *c-fos*, *c-jun* and *hsp70* (Horikoshi et al. 1988b, 1991; Hiebert et al. 1989; Mudryj et al. 1990; Simon et al. 1988; Lee et al. 1991; Shi et al. 1991). E1A and related DNA virus oncoproteins, such as the SV40 T antigen or the E7 protein of human papillomavirus (HPV), have a second major function in the initiation of G1 cell cycle transition and DNA replication. This function is based on their capability to distrupt protein complexes suppressing the cell cycle in differentiated cells. A target for the E1A protein is the retinoblastoma suppressor protein RB, which in

G1 forms a complex with the transcription factor E2F. Other E1A targets are the cyclin E-CDK2-p107 complex in G1 and the cyclin A-CDK2-p107 complex in S, which also carry an E2F subunit (see Devoto et al. 1992; Nevins 1992). E1A dissociates E2F from RB-E2F and cyclin A/E-CDK2-p107 complexes which results in the activation of E2F by phosphorylation (see Bagchi et al. 1989, 1990; Moran 1993).

The recognition sequence and regulatory spectrum of E2F are similar to those of transcription factors SWI4/SWI6, which in yeast activate the transcription of genes encoding cyclins CLN1 and 2, DNA polymerase α and other enzymes involved in DNA synthesis (Andrews and Herskowitz 1990; Gordon and Campbell 1991; Johnston and Lowndes 1992; Merrill et al. 1992). In mammals E2F belongs to a family of homologous factors, binding sites of which have been identified in the promoter region of genes required for G1-S transition and DNA synthesis (i.e., *Myc*, *myb*, dihydrofolate reductase, thymidilate synthase and ribonucleotide reductase). E2F binding sites are also present in the promoter of cyclin A gene (Bréchot 1993). E1A-mediated dissociation of an E2F factor from cyclin E-CDK2-p107 complexes therefore may induce the transcription of the cyclin A gene which in turn leads to the activation of the cyclin A-CDK2-p107-E2F complex in G1/S (Lees et al. 1992; Pagano et al. 1992b). Phosphorylation of E2F results in the transcription of human DNA polymerase α gene. As in yeast, DNA synthesis in mammals is coordinately regulated by signalling pathways responding to growth factor stimuli (Bagchi et al. 1989; Pearson et al. 1991). Whereas the transcription of DNA polymerase α is activated by E2F and CDK2 kinase in G1-S, the enzyme is inactivated by CDC2-cyclin B-mediated phosphorylation in G2/M (Nasheuer et al. 1991).

The adenovirus E1A oncoprotein binds to the retinoblastoma suppressor RB by splitting the RB-E2F complex. The released E2F in turn activates the cell cycle and DNA replication. Formation of RB-E2F complex in differentiated cells inhibits the cell cycle. Unlike its homologue p107, RB cannot bind cyclins and thus prevents the formation of E2F-cyclin-CDK complexes (Ewen et al. 1991, 1992; Goodrich et al. 1991; Cobrinik et al. 1992; Faha et al. 1992; Hamel et al. 1992). In contrast to RB, p107 may perform a positive regulatory function by recruiting E2F and cyclin E/A to activate the CDK2 kinase (Cao et al. 1992). RB inhibits the E2F-induced expression of *CDC2* kinase, cyclin A, *c-myc* and *c-myb* genes (Dalton 1992; Hiebert et al. 1992; Pagano et al. 1992a). c-Myb counteracts RB by activating the *cdc2* gene in response to growth factor stimuli (Ku et al. 1993). It is intriguing that the transcription of a *cdc2/cdk* gene was also observed to be under growth factor control in higher plants (Hirt et al. 1991). In mammals RB can be inactivated in somatic cells by protein kinases which induce RB phosphorylation in response to growth factor and mitogenic stimuli (Chen et al. 1989). During the cell cycle, RB is phosphorylated in at least three steps (in G1, S and G2/M) probably by the CDK4-cyclin D, CDK2-cyclin E and CDK2/CDC2-cyclin A kinases (Hinds et al. 1992; Matsushime et al. 1992; Shirodkar et al. 1992). During mitosis, RB binds to the catalytic subunit of phosphoprotein

phosphatase PP1 and is activated by dephosphorylation (for review, see Hollingsworth et al. 1993).

RB alone, or in complex with E2F and E1A, can act as transcriptional repressor or activator of E2F- and E1A-regulated genes, respectively. Nuclear localization of RB, p107, E2F, E1A, CDK2 complexes and collaborating phosphatases indicate that these proteins can interact with general and site-specific transcription factors and RNA polymerases to coordinately regulate DNA replication and transcription. In contrast to E2F, the RB-E2F complex was shown to inhibit the activation of adenovirus promoters (Weintraub et al. 1992). RB acts as a tissue-specific activator of several genes. Thus, RB expression in lung epithelial cells stimulates, whereas in mouse fibroblasts represses the *c-fos* promoter (Robbins et al. 1990). RB binds to similar sequence elements as the transcription factor Sp1 (RB control elements, RCEs). RB control elements occur in combination with E2F and Myc/Max binding sites in many promoters (Blackwood et al. 1992; see regulation of *hsp70* promoter above).

Overexpression of c-Myc in various tumors can override the RB-caused G1 arrest. The amino-terminal domain of c/N-Myc was observed to interact with RB in vitro, and plays a role in the downregulation of RB activity (Rustgi et al. 1991; Evan and Littlewood 1993). The N-terminal domain of the E1A oncoprotein can interact in an analogous way with RB and with other similar putative tumor suppressor proteins, such as p130 and p300. p300 is a ubiquitous nuclear protein conserved between mammals and is involved in the regulation of genes encoding members of AP transcription factor and Hsp70 heat-shock protein families (see Moran 1993).

Coregulation of transcription and DNA replication is further illustrated by the fact that RB can also interact with the SV40 T antigen. The SV40 T antigen and topoisomerase I can efficiently initiate DNA replication in the presence of a replication factor RF-S. RF-S contains a CDK-cyclin complex and a replication protein, RPA, which is activated by phosphorylation during G1 (D'Urso et al. 1990; Dutta and Stillman 1992; Fotedar and Roberts 1992; see above). The SV40 T antigen also binds to p53, a second class of major tumor suppressor proteins (see Perry and Levine 1993). In animals, p53 acts as a negative regulator of the cell cycle, which in response to DNA damage (i.e., γ-irradiation) causes a G1 arrest (Kastan et al. 1992). When expressed in yeast, p53 similarly induces a G1 arrest which is stimulated by coexpression of human CDC2Hs (Nigro et al. 1992). p53 has been found in association with cell cycle kinase complexes in transformed cells, and to be phosphorylated in vitro by CDK2/CDC2-cyclin A and CDC2-cyclin B complexes (Bischoff et al. 1990; Milner et al. 1990). CDC2/CDK2 has been found in association with a complex consisting of SV40 T antigen, cyclin A and RF-A single-stranded DNA-binding protein in replication forks, which provided an indication for an interaction between CDC2/CDK2-cyclin A and p53 in vivo (see above; Dutta et al. 1991). Although the role of p53 in cell cycle progression is not completely deciphered, it is likely that p53 is phosphorylated, as RB, during late S and G2. In contrast to RB, p53 is inactivated by dephosphorylation during mitosis.

Similarly to RB, p53 is also a transcription factor, which binds to specific sequences located upstream of rRNA genes and *c-fos* (Farmer et al. 1992; Zambetti et al. 1992). p53 may thus be implicated in the regulation of rRNA transcription. Moreover, p53 was found to bind to the *hsp70* promoter and to inhibit heat-shock gene expression by interacting with the CCAAT factor, a component of the differentiation switch (for review, see Umek et al. 1991; Rorth and Montell 1992; Agoff et al. 1993). p53 also binds to sequences adjacent to the SV40 replication origin and inhibits DNA replication through competition with the SV40 T antigen (Bargonetti et al. 1991, 1992). Conversely, the SV40 T antigen was observed to inhibit p53 in *trans*-activation or repression of diverse promoters (Mietz et al. 1992). As tumor suppressor, p53 performs a redundant function with RB, while as a transcription factor it counteracts RB.

7.2 Cell Cycle Regulation of RNAPII: CTD and CDC Kinases

From the data described above it is apparent that the transcriptional activity of RNA polymerases is regulated by many diverse pathways and cellular functions. Is there a major mechanism modulating the activity of the RNA polymerase enzymes themselves? So far one can logically argue both ways. No, because evidently the transcription of RNAP genes, the translation, secondary modification and assembly of RNAP subunits and their interaction with transcription factors provide endless combinations for regulation. Yes, because many connections between cell cycle regulation, transcription factors, *trans*-activating oncoproteins and tumor suppressors point to the role of RNA polymerase II CTD as a major mediator between transcription and diverse regulatory pathways. In the light of the complexity of these regulatory interactions it is not surprising that there is considerable confusion in explaining the role of the CTD (see Corden 1990; Sawadogo and Sentenac 1990; Woychik and Young 1990; Young 1991).

Once assembled, the activity of RNAPII is primarily regulated by phosphorylation of the C-terminal YSPTSPS repeated domains (CTD) of the largest subunit. As reviewed above, the CTD plays a major role in interactions with the TATA-binding factor TBP, transcription activators and repressors, and is also involved in the regulation of the "Start" during the cell cycle. These functions of the CTD are reminiscent of those of viral oncoproteins. Phosphorylation of the CTD provides a twofold regulation. CTD phosphorylation of "free" RNAPII inactivates the enzyme and prevents its binding to promoters. Once RNAPII is stably bound to preinitiation complexes in the chromatin, however, the phosphorylation of CTD is required for the activation of RNAPII and initiation of transcription. Data described above show that phosphorylation of the CTD in initiation complexes is concomitant with the phosphorylation of transcription activators and repressors which interact with the CTD. Consequently, protein kinases phosphorylating the CTD are integral components of transcriptionally active chromatin. It is logical to assume that multiple protein kinases involved in

the regulation of the cell cycle, transcription, DNA replication and repair control the phosphorylation of the CTD.

In fact, studies of CTD-specific protein kinases indicate that this assumption is correct. Thus far, several classes of CTD kinases have been isolated from yeast (Lee and Greenleaf 1989), *Aspergillus* (Stone and Reinberg 1992), plants (Guilfoyle 1989), mouse (Cisek and Corden 1989, 1991; Zhang and Corden 1991a,b), rat liver (Serizawa et al. 1992) and human cells (Dvir et al. 1992; Lu et al. 1992; Peterson et al. 1992; Gottlieb and Jackson 1993; Payne and Dahmus 1993). Because of the double effect of CTD phosphorylation, these kinases can be classified into two families: (a) template- or DNA-bound kinases which cannot phosphorylate histone H1 but are able to phosphorylate the RNAPII when bound to PIC or DNA, and (b) cell cycle kinases which display histone H1 kinase activity in vitro and can efficiently phosphorylate the RNAPII CTD in solution.

Template-dependent CTD kinases belong to two families. The first family includes general transcription factors, such as TFIIH from HeLa cells, δ factor from rat liver, and factor b from yeast which catalyze ATP hydrolysis and phosphorylation of the RNAPII CTD during the initiation of transcription (Arias et al. 1991; Feaver et al. 1991; Conaway et al. 1992; Flores et al. 1992; Lu et al. 1992; Serizawa et al. 1992; Payne and Dahmus 1993). TFIIH factors from HeLa cells were shown to contain two to five subunits (92/95, 62, 43, 40 and 35/33 kDa), whereas the most purified mouse δ factor carries eight subunits (94, 85, 68, 46, 43, 40, 38 and 35 kDa), and the yeast factor b consists of three subunits (85, 75 and 50 kDa). All three factors catalyze the ATP-dependent phosphorylation of the CTD after binding to PIC. TFIIE is a major stimulator of the TFIIH CTD kinase activity and is required for the entry of TFIIH into the preinitiation complex. TFIIJ binding to PIC further increases the activity of TFIIH. TFIIH alone or in complex with general transcription factors TFIIADBEF poorly phosphorylates the CTD in solution. The CTD kinase activity of TFIIH is greatly stimulated by DNA templates with TATA or initiator elements. The DNA-dependent ATPase activity of TFIIH-like factors accounts for a helicase function which was thought to be performed by TFIIE or F previously (Greenblatt 1991a). Properties of the less characterized *Aspergillus* KI and HeLa CTDK2 kinases resemble those of the TFIIH family. KI has been shown to phosphorylate Ser at the fifth position of YSPTS*PS repeats, thus other members of the TFIIH family are also expected to display a similar substrate specificity (Stone and Reinberg 1992). TFIIH and related kinases are required for PIC formation and fulfil a CTD kinase function required in both differentiated and dividing cells.

The second family of DNA-dependent kinases is represented by the human Ku antigen-associated DNA-PK CTD kinase (Dvir et al. 1992; Peterson et al. 1992; Gottlieb and Jackson 1993). DNA-PK is a well-characterized protein kinase of 300/350 kDa. DNA-PK-Ku phosphorylates the CTD at both Ser the Thr residues but only when RNAPII is bound to DNA or in the preinitiation complex. Moreover, DNA-PK specifically phosphorylates the Ku antigen, p53, the SV40 T antigen, and the transcription factors Sp1, Oct1, Oct2, c-Fos and c-Myc in DNA-bound forms (see Gottlieb and Jackson 1993). Ku is known to bind to double-stranded DNA ends, and is associated with chromatin in a cell cycle-dependent manner. Ku binds to DNA ends aspecifically in cooperative interaction with several transcription factors (see above substrates for DNA-PK) and slides to specific internal sequences which are similar to Sp1, Oct1, Oct2 and Ap1 (Jun, Fos, Myc) recognition sites. DNA-PK-Ku may have multiple functions. By phosphorylation of the CTD, DNA-PK can probably inhibit the aspecific binding of RNAPII to free DNA ends. Phosphorylation of Thr residues in the CTD suggests that DNA-PK may also phosphorylate RNAPII during elongation which may lead to irreversible inactivation of RNAPII in the absence of Thr-specific phosphatases. DNA-PK may thus arrest transcription in response to DNA damage. The p70 subunit of Ku is also referred to as transcription factor PSE1/TREF1/TREF2, which is an activator of the U1 snRNA promoter (Reeves and Sthoeger 1989; Gunderson et al. 1990; Knuth et al. 1990). Therefore, an alternative function for DNA-PK-Ku could be the inhibition of RNAPII activity in response to transcription by RNAPI or RNAPIII.

The second major class of CTD kinases is represented by the cyclin-dependent cell cycle kinases described above. Using CTD heptapeptide repeats as substrates Cisek and Corden (1989) have identified CTD kinase activities in mouse, human, hamster, and yeast cell extracts. Purification of CTD kinase activities from mouse Ehrlich ascites cells resulted in two major enzymes, E1 and E2, which phosphorylate the CTD at serines in the second and fifth positions of the YS*PTS*PS heptamer. E1 has proved to be a G2/M-specific cell cycle kinase complex consisting of a p34 kinase and a p62 cyclin B subunit. E2 carried the p34 CDC2 kinase subunit in combination with p58, a yet unidentified cyclin A-like subunit (Cisek and Corden 1991; Zhang and Corden 1991a,b). Lee and Greenleaf (1989) detected similar CTD kinase activities in yeast, *Drosophila* and HeLa cells, and purified an enzyme from *S. cerevisiae* which consists of 58, 38 and 32 kDa subunits (Lee and Greenleaf 1989). A related CTD kinase, KIII, displaying histone H1 kinase activity, was purified from *Aspergillus*, but did not cross-react with antibodies raised against the conserved PSTAIR motif of CDK2/CDC2 kinases (Stone and Reinberg 1992).

7.3 A Plant CTD Kinase Provides a Link Between Regulation of RNAPII Transcription, DNA Replication and Cell Cycle

Recently, we have initiated an extensive purification and characterization of cell cycle kinase complexes from alfalfa cell cultures, and analyzed their CTD kinase activity using either CTD fusion proteins or RNAPII holoenzyme as substrates. CDC2/CDK2 kinases were identified by antibodies raised against their conserved PSTAIR motif and purified by means

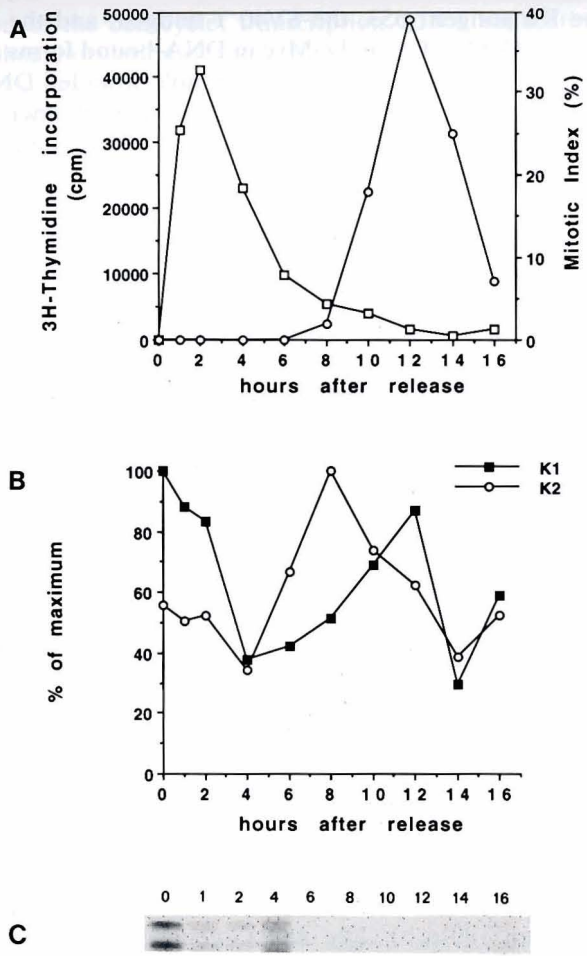

Fig. 1. Oscillation of CTD kinase activity and transition of diverse cell cycle kinase complexes throughout the cell cycle in plants. **A** DNA synthesis and mitotic division in an alfalfa cell suspension following the release of aphidicolin caused G1/S phase arrest. *Squares* show [^{3}H]thymidine incorporation in pulse-labelled cells after removing the DNA synthesis inhibitor; *circles indicate* the mitotic index (%) determined by counting an average of 1000 nuclei. **B** Cell extracts, prepared at defined time intervals after the release of the aphidicolin block, were resolved by DEAE-Sepharose chromatography to *K1* and *K2* cell cycle kinase fractions, which were characterized previously. Equivalent amounts of proteins from K1 and K2 fractions were bound to p13-Sepharose beads, and after extensive washes, the matrix-bound CDC2/CDK2 activities were determined using histone H1, as substrate. **C** Oscillation of CTD kinase activities during the cell cycle. K1 kinase fractions described above were incubated with a CTD fusion protein carrying the CTD domain of *Arabidopsis* RNAPII fused to *Staphylococcus* protein A, and separated by SDS-PAGE. The autoradiography shows typical gel shifts of CTD-fusion proteins which are due to phosphorylation by CTD kinase(s) associated with the K1 enzyme fraction

of their ability to interact with p13, the product of yeast *Suc1* gene (Brizuela et al. 1987; Ducommun et al. 1991). Higher plants, as other eukaryotes, accumulate diverse CDK-cyclin complexes during the cell cycle. Alfalfa cells arrested at G1/S phase by aphidicolin (an inhibitor of DNA polymerase α) accumulate a mixture of CDK kinases, p31 and p32, which bind to p13, phosphorylate histone H1, display CTD kinase activity and occur in different complexes of about 300 kDa (Fig. 1, K1). After releasing the cells from the G1/S block, the CTD kinase activity decreases throughout the S-phase, but a second peak of CTD-kinase activity rises during the onset of G2/M and mitosis. A histone kinase activity appearing in G2/M is associated with complexes of 75 kDa which contain only the p32 kinase subunit, a homologue of yeast CDC28 kinase (Fig. 1, K2; Hirt et al. 1991). This G2/M phase-specific kinase, K2, however, does not phosphorylate the CTD, indicating either that in our experiment some cells have escaped the aphidicolin block and contaminated the G2-M samples, or that the CTD kinase activity is associated with another type of CDC/CDK kinase complex in G2/M. Preliminary data indicate that the p31 kinase subunit of K1 complexes, which mediate CTD phosphorylation, is probably a homologue of animal CDK2 kinases. As CDK2, the p31 subunit of K1 complexes can bind p13 and is recognized by PSTAIR antibodies. In addition to K1 and K2 complexes, PSTAIR-reactive proteins have also been detected in association with DNA, but failed to phosphorylate the RNAPII in solution (Bakó unpubl.).

The overall analogy of substrate specificity, timely distribution and complexity between plant and animal cell cycle kinase complexes prompted us to address the specific question whether plant cell cycle kinases would carry a subunit with E2F binding specificity. A screening for specific binding to the consensus E2F recognition sequence revealed that, similarly to animals, the G1/S specific plant cell cycle kinase fraction, K1, carries an E2F binding activity. Since the same kinase fraction was implicated in specific phosphorylation of the RNAPII CTD, it appeared logical to assay whether RNAPII binding to these cell cycle kinase complexes can regulate the E2F binding activity. As illustrated by Fig. 2, RNAPII binding to the G1/S kinase fraction K1 dramatically increases the E2F binding activity, probably by dissociation of an E2F-like factor from the K1 cell cycle kinase complex(es).

8 Conclusion

The role of RNAPII in interaction with cell cycle kinase complexes is probably multiple. In analogy to viral oncogenes, this process may induce DNA synthesis by E2F and inactivate the free RNAPII pool in G1/S phase. By phosphorylation of RNAPII the cell cycle kinase could also differentially regulate transcription, since phosphorylation abolishes the ability of the CTD to titrate out the TATA-binding protein, which in turn would enhance

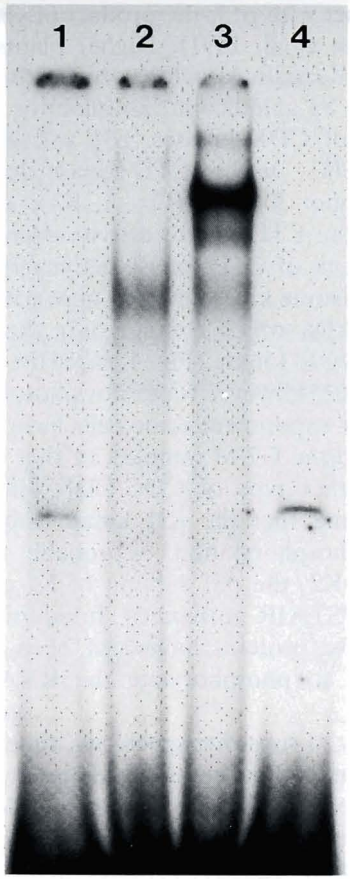

Fig. 2. E2F binding activity is associated with the K1 CTD kinase fraction and inducible by RNAPII. An oligonucleotide carrying the consensus TTTGGCGGGAA E2F binding site was end-labelled by T4 polynucleotide kinase and incubated in the presence of a 1000-fold excess of poly(dI/dC) competitor (*1–4*), with a 300-kDa fraction of purified K1 CTD kinase (*2*), with the K1 CTD kinase and tomato RNAPII holoenzyme (*3*), or with tomato RNAPII alone (*4*). A similar increase in E2F binding and formation of novel oligonucleotide-protein complexes were observed using RNAPII enzymes from alfalfa and wheat. These complexes could be specifically competed with unlabelled E2F oligonucleotides, but not with increasing amounts of poly(dI/dC) (data not shown)

the formation of SL1 and TFIIIB complexes required for RNAPI and RNAPIII transcription. As outlined above, certain genes would be able to escape this regulation, because transcription from their promoter may not require the CTD function. Alternatively, the binding of RNAPII to CDK-cyclin complexes could perform a *trans*-activating function by recruiting to promoters cell cycle kinases which would facilitate recurrent transcription by phosphorylating the RNAPII and interacting with transcription regulatory

proteins. In animals a competition of RNAPII with viral oncoproteins, such as adenovirus E1A or SV40 T antigen, may ultimately result in the accumulation of free phosphorylated RNAPII and E2F, which would trigger the replication of viral DNAs and deregulate transcription. The data described above suggest that many additional regulatory pathways exist, the uncovering of which will contribute to further understanding of the connections between transcription, DNA replication and cell cycle control.

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