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General Transcription Factors Play Dual Roles In Initiation And Termination

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GENERAL TRANSCRIPTION FACTORS PLAY DUAL ROLES IN INITIATION AND TERMINATION

by

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INTRODUCTION

1.1 CENTRAL DOGMA

The survival of a cell depends on its ability to regulate its genetic program, which is crucial for growth, proliferation and homeostasis. Understanding how these programs operate and are regulated is the focus of a wide range of biological disciplines including evolution, genetics, biochemistry and developmental biology. The central dogma of molecular biology describes the process of gene expression. The first step in gene expression is the conversion of genetic programs coded in the bases of DNA to RNA by a process called transcription. In the next step, RNA is translated into proteins, which perform nearly all the vital functions of a cell. The basic unit of the genetic program is a 'gene', which is a region of the DNA that includes the promoter, the coding region and the terminator. Although, gene expression is regulated at multiple levels, transcription is the first major step, and a primary target of regulatory processes. In order to have an insight into the regulation of gene expression, therefore, it is critical to understand how transcription is regulated.

1.2 RNA POLYMERASES

In eukaryotes, the process of transcription is performed by at least three different RNA polymerases (RNAP) (253, 254). RNAP I and III transcribe ribosomal rRNA, tRNA, and small nuclear RNAs (snRNA), while RNAP II is the enzyme that transcribes protein coding genes as well as some snRNAs and non-coding RNAs (300). The overall process of transcription can be broken down into three basic steps: initiation, elongation and termination. Transcription often begins in response to a signal, which is transduced

to a protein inside the cell called the ‘activator’. This triggers the activator-dependent recruitment of the general transcriptional machinery onto the 5' end of a gene called the ‘promoter’ to form a preinitiation complex (PIC). The initiation of transcription takes place while polymerase is still bound to the promoter as a part of the initiation complex.

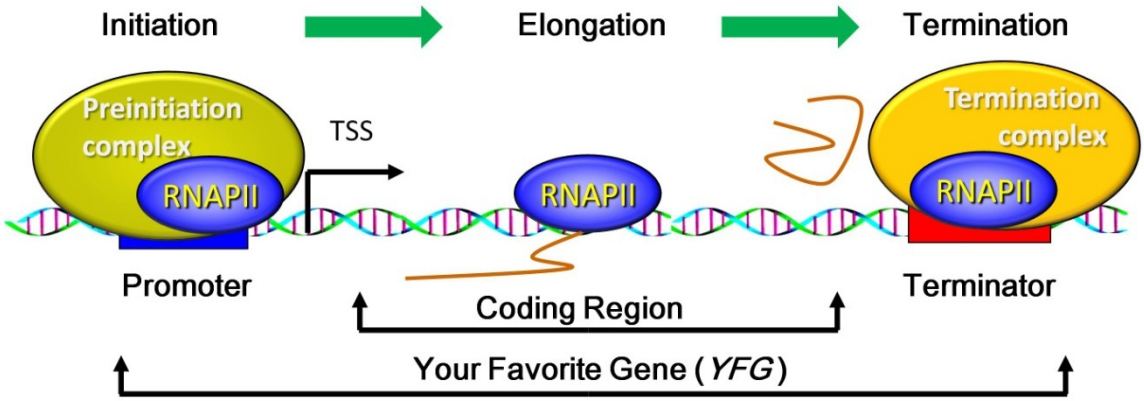

Figure 1. Schematic diagram of the transcription cycle for a gene

Soon after initiation, polymerase is released from the initiation complex leaving behind most of the components of initiation complex on the promoter as a ‘scaffold’ (324). This transition from initiation to elongation is called promoter clearance, which is accompanied by the recruitment of elongation factors. As RNA polymerase reaches the 3' end of the gene, the termination factors are recruited by the elongating polymerase. The termination factors facilitate 3' end processing of mRNA and the release of polymerase from the DNA template (266). A schematic of the basic steps in the transcription cycle for a protein coding gene is shown in figure 1.

All three forms of RNA polymerases exhibit similarity in structure and function over a wide range of taxa (174, 175). Eukaryotic RNAP II is comprised of 12 subunits (312). These subunits have been named Rpb1 to Rpb12. Of these 12 subunits, only Rpb4 and Rpb9 are not essential for survival of yeast cells (311, 313). Ten subunits

form the core enzyme, while Rpb4 and Rpb7 form a heterodimer that associates with the core to form the 12 subunit structure (39). The core enzyme can perform RNA synthesis, but is incapable of initiating transcription from a promoter, and often displays termination defect (256). Rpb4 and Rpb7 were found important for promoter-based initiation in vitro, while Rpb4 has also been implicated in the recruitment of 3' end processing/termination factors (11, 68, 161, 256). The subunits Rpb5, Rpb6, Rpb8, Rpb10 and Rpb12 are shared by all three RNA polymerases (291). With the exception of Rpb1, the remaining subunits also exhibit some degree of similarity among the three types of polymerases (116). Years of work from the Roger Kornberg and Patrick Cramer laboratories have provided key structural analysis of RNAP II in a complex with other transcription factors and DNA (11, 12, 38-40, 51, 69, 104, 148, 153, 154, 162, 195, 304, 305). These structural studies have provided key insight into the functional aspects of the transcription machinery. The two largest subunits of RNAP II together form the active site of the enzyme, and the binding sites for DNA and RNA. Rpb3, Rpb6 and Rpb11 are important for stabilization of the structure of the complex, while the remaining subunits are thought to provide interaction surfaces for regulatory factors (301). The largest subunit of RNAP II, Rpb1, contains a unique carboxyl terminal domain (CTD) consisting of an array of heptapeptide repeats that serve as a loading dock for the transcription factors and RNA processing factors during the transcription cycle (35).

The promoter and terminator regions mark the distal ends of a gene and are composed of specific sequence elements that bind transcription factors. Upstream activating or repressing sequences (UASs or URSs) are located upstream of the core promoter and bind activator and repressor proteins respectively. In yeast, the vast

majority of UASs and URSs are located upstream of the core promoter element, usually within a few hundred bp of the transcription start site (TSS, Fig. 2) (59, 60, 87, 91, 107, 214, 273, 319). The combinatorial input from these upstream sequences converge onto the promoter near the TSS through the action of coactivators and the general transcription factors (GTFs). A core promoter was first discovered in humans (Fig. 2), which is the minimal set of sequences required to initiate transcription (271). It is a sequence of 80 bp centered around the TSS that contains several conserved elements: TATA-box, initiator element (INR), TFIIB recognition element (BRE),

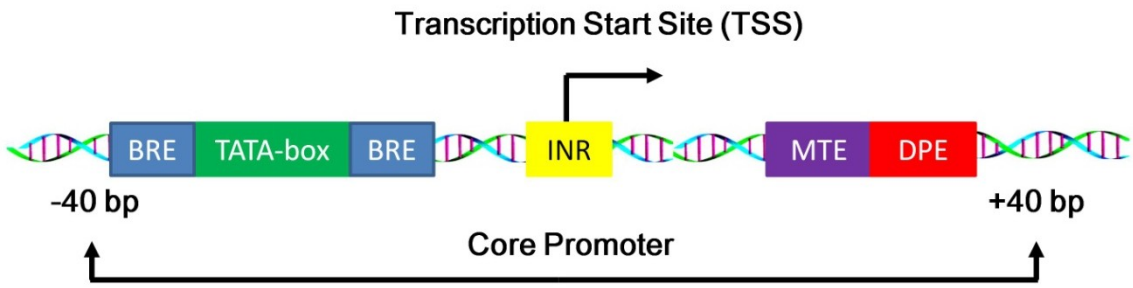

Figure 2. Schematic diagram of the core promoter

downstream positioning element (DPE) and the motif ten element (MTE). The TATA-box, INR, DPE and MTE are all sequences recognized by the general transcription factor IID (TFIID), while the BRE is an element recognized by the general transcription factor IIB (TFIIB) (146). In yeast, only the TATA box and INR like motifs have been found, the others are presumably present but too degenerate to be identified positively (116).

1.3 INITIATION

The initiation is the most well understood step of transcription owing to a large body of *in vitro* studies being performed using a promoter-containing DNA template and

the transcription competent cellular or nuclear extract. Purified fractions of cell or nuclear lysate were combined in order to find the various protein factors that could perform transcription on these templates. Through further purification of these fractions, a minimal set of transcription factors called the 'general transcription factors' (GTF) was identified which could support initiation of transcription from a generic promoter on a naked DNA template (259). These are TBP (TATA-binding protein), TFIIA, TFIIB, TFIIF, TFIIE and TFIIH (299). Inside the cell, however, the DNA is organized into a chromatin structure, which necessitates the requirement of a number of additional factors to overcome the nucleosomal barrier.

Initiation of transcription begins with the activator-dependent formation of the PIC on the promoter (Fig. 3). The PIC is formed by the ordered recruitment of the GTFs and Mediator complex (201). The chromatin remodelers and histone modifiers allow access

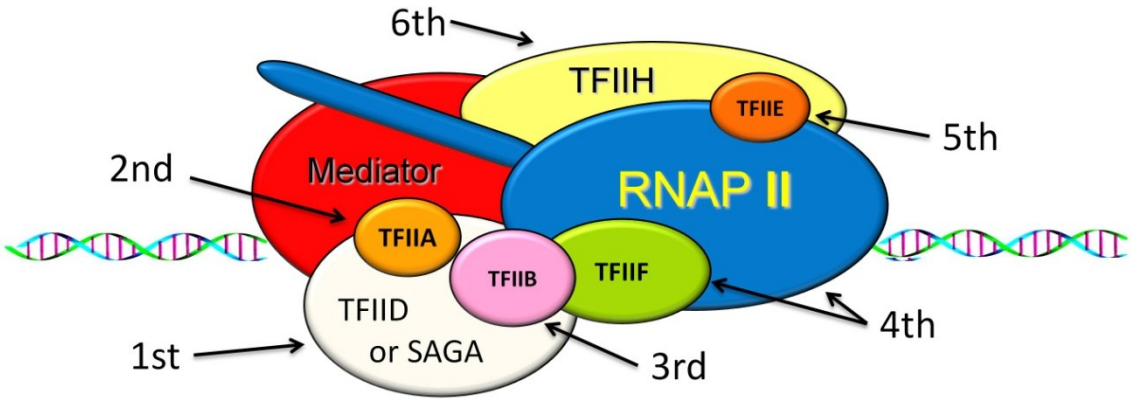

Figure 3. Schematic diagram of the pre-initiation complex (PIC). The numbering Indicates the order of recruitment of that factor during PIC assembly.

of GTFs and RNA polymerase II to the promoter sequence to form PIC. Activator-mediated recruitment of general transcription factors on the promoter is facilitated by Mediator complex. The general transcription factors are recruited in the following order: SAGA/TFIID, TFIIA, TFIIB, TFIIF along with RNAP II, TFIIE and then TFIIH (36). The

first factor to be recruited onto the core promoter is either TFIID or SAGA complex. In general, TATA-less promoters recruit TFIID, and TATA-containing promoters are dependent on SAGA complex for PIC formation (21, 24, 178, 184, 298). TFIID is complex composed of TBP (TATA-binding protein) and 14 TBP-associated factors (TAFs) (108). In yeast, SAGA was originally discovered for its histone H3 acetyltransferase activity (106). It is composed of 6 essential subunits and another 15 non-essential subunits. Five of the six essential subunits, TAF5, TAF6, TAF9, TAF10 and TAF12, are common with TFIID (24, 275, 315). In yeast, TBP binds to the promoter region at approximately 40-120 bp upstream of the TSS, and creates a bend in the DNA (Fig. 4) (118). Contrary to the general perception, roughly half of the genes in yeast contain

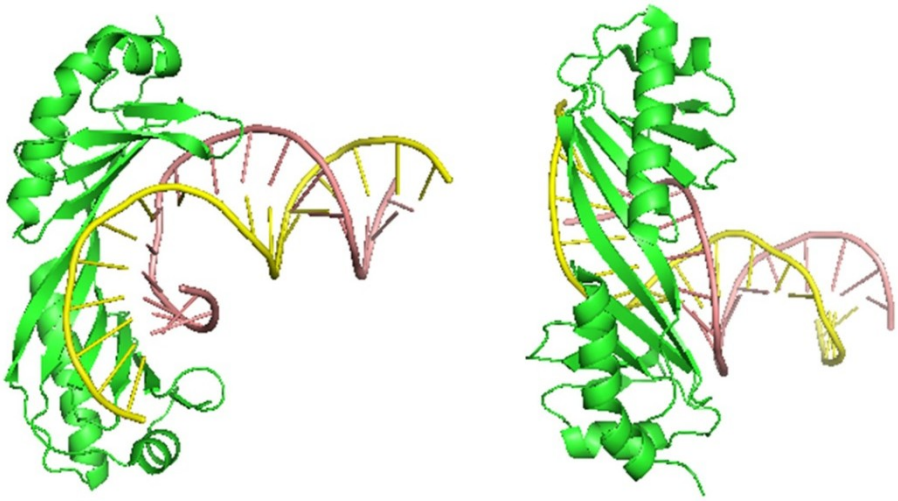

Figure 4. PyMol representation of structural data for TBP (green) binding and bending the DNA at the TATA-box. Created using 1RM1.pdb file.

TATA-less promoters (18). Current genomewide studies have revealed two mechanisms involved in transcription activation based on the recruitment of either SAGA or TFIID to the core promoter (138, 170, 181, 184, 185). The two mechanisms also differ in their requirement for the GTFs necessary to recruit TBP. Promoters

utilizing TFIID do not depend on Mediator or TFIIB for the recruitment of TBP to the promoter region, while SAGA-dependent promoters require them to recruit TBP (24-26, 185). TFIIA is a two subunit protein which binds next and stabilizes the TFIID interaction with the promoter. TFIIA has also been reported to inhibit repressor interaction with TFIID, thereby promoting transcription activation (213, 218). TFIIB is a single subunit factor that interacts with TFIID and short regions of DNA flanking the TATA box called the TFIIB recognition elements (BRE), which function to help position the polymerase at the correct start site (115, 285). TFIIF is a three subunit factor which is recruited to the promoter along with RNAP II, and stabilizes its interactions with TFIID and TFIIB (115, 129, 285). Some studies have also implicated TFIIF function in elongation step of transcription (285). TFIIE is a two subunit factor, which facilitates the recruitment of TFIIH to the promoter (36). TFIIH is an 11 subunit complex that functions to unwind the downstream DNA and phosphorylates the CTD of RNAP II (182).

One of the factors that has been found vital for transcription on a wide variety of promoters is Mediator complex. Mediator has been found to play a critical role in both activation and repression of transcription (30). Mediator is composed of 25 subunits and has been found to interact with various gene specific activators (27, 30, 31). It acts as a bridge between activators and the general transcription machinery (27). Mediator is therefore often described as a ‘coactivator’ that bridges the link between activator and the GTFs. Recent studies, however, have found Mediator crosslinking to almost every RNAP II-transcribed promoter, thereby giving rise to the speculation that Mediator is indeed a general transcription factor that gets dissociated form the core promoter immediately after initiation of transcription (308). Mediator complex is divided into 4

modules: the head, the middle, the tail and the kinase module. The head and kinase modules interact extensively with RNAP II, TFIID and TFIIH (88, 176, 180). The kinase module has been shown to inhibit transcription by different methods. First, the Srb10 subunit of the kinase module phosphorylates the CTD of RNAP II before the initiation of transcription causing a premature release of RNAP II from the PIC (127). Second, Srb10 phosphorylates TFIIH subunit Ccl1 that is a part of TFIIH-kinase submodule ultimately resulting in inhibition of kin28 CTD-kinase activity (4). Third, it sterically interferes with the recruitment of RNAP II to the PIC by making the head module inaccessible to interact with the polymerase (84, 287). The tail module has been shown to interact with activators and repressors and regulates recruitment of the transcription machinery (226). Each of the three subunits in the tail module, when fused to DNA binding domains, were able to activate transcription even in the absence of an activator (322). Recent evidence indicates that Mediator is the first transcription factor to respond to a signal from the activator and is instrumental in initiating the assembly of the PIC at least on a subset of promoters (308).

Structural studies and biochemical analysis of the PIC have given an insight into the mechanism of transcription by RNAP II. Following assembly of PIC, unwinding of about 10 bp surrounding the TSS results in formation of the ‘transcription bubble’ (116). With the unwinding of DNA, the PIC makes a transition from ‘closed’ to the ‘open’ conformation. The interaction of polymerase subunits with TFIIIF and TFIIB stabilizes the bubble. TFIIB contains a domain called the B-finger that inserts into the RNA exit channel of RNAP II (Fig. 7) (200). The B-finger contacts the active site in the enzyme and helps to correctly position RNAP II at the TSS (Fig. 7). After the synthesis of first 5-

7 nucleotides, the growing RNA chain begins competing for space with the B-finger in RNA exit channel and pushes the B-finger out, thereby dissociating TFIIB from the polymerase (162, 195, 257). Ssl2 subunit of TFIIH now contacts the DNA at about 30 bp downstream of the TATA-box and unwinds the DNA by a wrench like action (157). Ssl2 helicase activity then extends the bubble downstream. The forward movement of RNAP II on the template coincides with the collapse of the initiation bubble (238). Simultaneously, the Kin28 kinase activity of TFIIH phosphorylates RNAP II carboxy-terminal domain (CTD) at serine-5 (196). The phosphorylation severs the connection of polymerase with Mediator, resulting in the release of the complex from the core promoter (272). This step called ‘promoter escape’ or ‘promoter clearance’ marks the transition of RNAP II into productive elongation. Following initiation of transcription, a subset of factors consisting of SAGA/TFIID, TFIIA, TFIIE and TFIIH is left behind on the promoter forming a ‘scaffold’ that facilitates reinitiation of subsequent rounds of transcription (325).

1.4 ELONGATION

Transcription elongation begins immediately after promoter clearance, and in most eukaryotes is accompanied by DSIF (DRB-sensitive inducing factor)-mediated pausing of RNAP II just downstream of the promoter element (320). Such promoter proximal pausing of polymerase, however, has not been observed in budding yeast. The elongation of transcripts on a naked DNA template under in vitro conditions requires only TFIIF and TFIIS (140). The elongation in yeast is stimulated by the Ctk1/Bur1, which phosphorylates the CTD of RNAP II at serine-2, and facilitates recruitment of RNA processing factors (309). A similar role of serine-2 phosphorylation

has been demonstrated in higher eukaryotes (81). Apart from Mediator, TFIIF is the only GTF that also plays a role in elongation of transcription by RNAP II (120, 219, 261, 290). The AT-rich sequences in the body of a gene present a barrier to elongation as RNAP II frequently backtracks on such sequences leading to the misalignment of growing 3' end of RNA with the active site. TFIIS, a factor that possesses 3' to 5' exoribonuclease activity, promotes the release of backtracked RNAP II by realigning the active site with the 3' end of mRNA (168, 307). Under *in vivo* conditions, elongating polymerase has to overcome the nucleosomal barrier. There are three types of factors that help polymerase move through the chromatin template. These are histone chaperone, ATP-dependent remodeling factors and histone modifying enzymes. FACT and Spt6 are histone chaperones for H2A-H2B and H3-H4 respectively (32, 237). They are essential for smooth passage of polymerase through the chromatin template during elongation (9, 121, 149). The ATP-dependent chromatin remodelers like RSC and SWI-SNF complexes have also been shown to overcome nucleosomal barrier in yeast (42, 132). The histone modifying enzymes that are crucial for elongation step are COMPASS (Complex of Proteins Associated with Set1) and HAT complexes like NuA4 complex (75, 76, 215). As RNAP II progresses through the body of a gene, the COMPASS functions to methylate histones at H3K4 near the promoter, which helps recruit other histone modifying enzymes that acetylate histones in front of the progressing RNAP II. In turn, this allows for the ATP-dependent chromatin remodeling complexes RSC and Chd1 along with the histone chaperone FACT to function and allow passage of RNAP II around the nucleosomal barriers during elongation (183).

1.5 TERMINATION

Termination is one of the least understood steps of eukaryotic transcription cycle. Recent studies, however, have begun to shed light on the mechanism of termination of transcription by RNAP II. Termination requires both *cis*-acting elements and the *trans*-acting termination factors (73, 112, 205, 327). The termination by RNAP II involves cleavage and polyadenylation of the precursor mRNA, followed by the release of RNAP II from the template (29). Termination serves a variety of critical functions in the cell. First, it allows recycling of RNAP II to drive subsequent rounds of transcription. Second, the addition of a poly(A) tail provides protection to mRNA from 3' exonucleolytic cleavage (303). In addition, polyadenylation also improves the translatability of mRNA (206). Third, termination ensures that RNAP II doesn't progress further downstream wasting cellular energy and possibly interfering with the transcription of neighboring genes (100, 255). Last, proper termination also results in the recruitment of RNA export factors, which bind the polyadenylated RNA and export it to the cytoplasm for translation (206).

Termination is coupled to 3' end processing of precursor mRNA. The same set of factors is required for both the cleavage and polyadenylation of mRNA as well as dissociation of polymerase from the template (167). In yeast, the termination factors are organized into three complexes with a combined size of over a megadalton (Fig. 5.) (216). These are cleavage factor I (CF1) complex, cleavage and polyadenylation factor (CPF) complex, and the Rat1 complex. The CF1 complex is composed of 5 subunits: Rna15, Rna14, Pcf11, Hrp1, and Clp1 (109, 152). The CPF complex is composed of 15 subunits: Fip1, Yth1, Pfs2, Pta1, Yhh1, Ydh1, Ysh1, Pap1, Pti1, Ssu72, Glc7, Syc1, Swd2, Cft1, and Mpe1 (28, 150). The Rat1 complex is composed of 3 subunits: Rat1,

Rtt103, and Rai1 (198). The names of the CF1 and CPF complexes may be misleading based on in vitro studies that have shown a requirement for the entire CF1 complex and

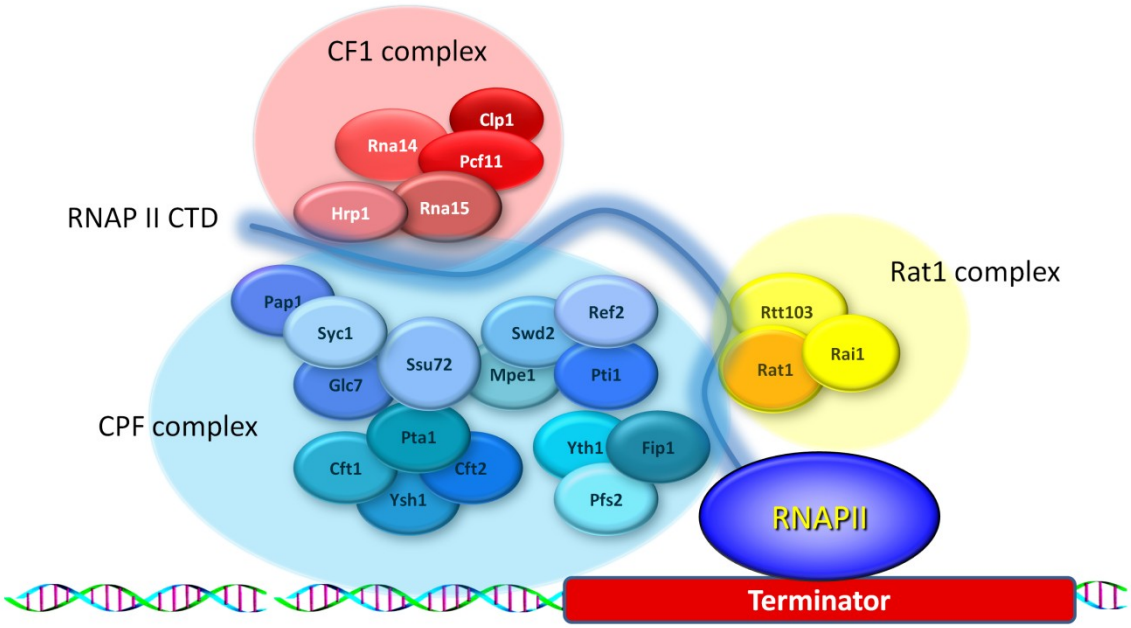

Figure 5. Schematic diagram of the 3 termination factor complexes and recruitment to RNAP II CTD during termination.

the majority of CPF subunits for cleavage and polyadenylation to occur. The Rat1 complex requires the exoribonuclease activity of the Rat1 protein in order to function in termination (198). Interestingly, export of the polyadenylated mRNA into the cytoplasm is compromised in mutants of the Rat1 complex (8).

There are two prevailing models explaining the mechanism of termination by RNAP II: (1) allosteric model, and (2) torpedo model. According to the allosteric model, there is a change in conformation of the transcription complex as RNAP II transcribes over the terminator (241). This change causes the release of elongation factors and the recruitment of termination factors which then bring about termination of transcription. According to torpedo model, termination is facilitated by the Rat1 exonucleolytic

complex, which begins degrading the cleaved RNA that is still attached to the transcribing polymerase at the 3' end of a gene. The Rat1 complex degrades the RNA until it physically 'bumps' into RNAP II (155). This causes a conformational change in RNAP II and subsequent release from the template. Evidence supports both models, and the actual mechanism of termination is widely accepted as a combination of both (198, 249).

1.6 TFIIB: STRUCTURE AND FUNCTION

Transcription factor TFIIB is a single subunit protein of about 38 kDa size. It is required for initiation of transcription as well as start-site selection. The gene, *SUA7*, codes for TFIIB in budding yeast. It was originally discovered in a genetic screen as a suppressor of a mutation that led to a downward shift in the transcription initiation site (245). *SUA7* is an essential gene in yeast that encodes a protein of 345 amino acids. Interestingly, the human TFIIB can recruit both human and yeast RNAP II to the promoter region, while the yeast TFIIB can only recruit the yeast RNAP II, indicating that an important change has occurred in the structure of protein during evolution (289). Genetic, biochemical and structural evidence have implicated TFIIB in multiple aspects of the RNAP II transcription cycle. It has been shown to play a role in activation, PIC formation, start-site selection as well as the termination of transcription (71, 101, 123, 133).

TFIIB consists of two basic domains, an N-terminal zinc-finger domain, and a C-terminal core domain (Fig 6). The zinc finger domain is known to interact with RNAP II and TFIIF, while the core domain interacts primarily with TBP as well as the BRE sequences flanking the TATA-box (37, 114, 316). The core domain of TFIIB, spanning

residues 120 to 345, is composed of two direct repeats made up of 5 α-helices that are 22% identical and 42% similar, and are separated by an amphipathic helix extending

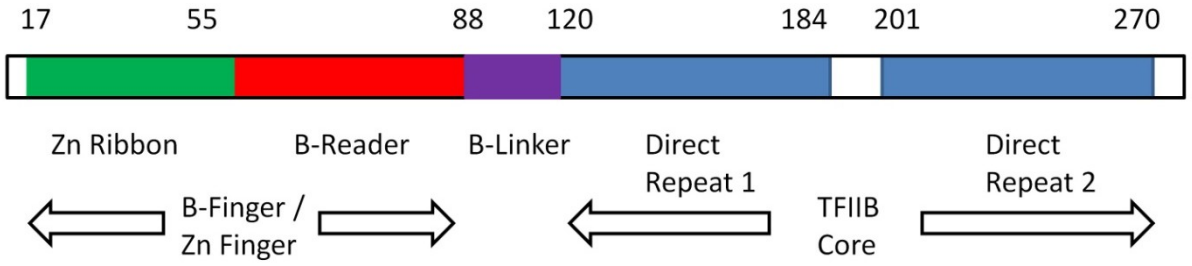

Figure 6. Schematic diagram of the functional domains of TFIIB.

from 184 to 201 residues (71). The species specific region accounting for the human and yeast differences has been mapped to 14 residues in the first direct repeat (268). In vitro, the TFIIB core domain can bind the promoter independently in the presence of TBP, but is incapable of recruiting RNAP II (14). The C-terminal core domain plays a crucial role in the initiation of transcription, possibly by helping orient DNA in the promoter-proximal region for unwinding.

Once the TBP binds the DNA, it bends the DNA by almost 90 degrees (230). The bend allows interaction of core TFIIB with the BREs upstream and downstream of the TATA box. The DNA downstream of the TATA-box unwinds in the preinitiation complex forming the transcription bubble, above the TBP saddle, and the template DNA strand is positioned at the RNAP II active site (145, 229). TFIIB forms the bridge between the TBP-TATA box complex and the polymerase surface, where the C-terminal core interacts with TBP and the N-terminal zing finger makes extensive contacts with Rpb1 and Rbp2 subunits of RNAP II (48, 85, 162, 195, 332).

Mutational and structural analysis of TFIIB has provided further insight into the importance of its N-terminal domain. The zinc finger domain can be broken down into three discrete functional units; the B-ribbon comprising residues 17-55, the B-reader with residues 56-88, and the B-linker from 89-120 (39, 50, 124, 333). The B-reader is immediately distal to the N terminal B-ribbon and is the most conserved TFIIB sequence known (71). It plays a crucial role in start-site selection (123, 187, 239, 246, 330). Two of the key alleles of *SUA7*, *sua7-1* and *sua7-3*, which exhibit altered start site selection, were the consequence of mutations E62K and R78C respectively, mapped to the B-reader (246). In addition to start site selection, these mutations also give rise to a cold-sensitive phenotype for which suppressors have been isolated. Suppressors of mutations in this domain were found in TFIIF and RNAP II subunits(277, 278). The structural studies further confirmed the interaction of B-finger with TFIIF and polymerase subunits (126). Surprisingly, an enhancer of a mutation in the B-reader was found in Ssu72, a 3' end processing/termination factor (278).

Structural studies have revealed that TFIIB makes contact with the active site of RNAP II in the initiation complex (40). The zinc ribbon domain first contacts RNAP II at the dock domain (40). The residues 20-54 of the zinc ribbon form three antiparallel β-strands, which surround a zinc ion (Fig. 7). The zinc ribbon domain contacts the RNAP II at Rpb1 residues 409-419, near the RNA exit channel of the polymerase (16, 37, 114, 133, 321). The TFIIB reader domain passes through the saddle region of RNAP II, between the clamp and the wall, and inserts into the active center of the enzyme (40). From there, it extends down to the base of the cleft, and then comes up and exits. In

addition, the B-finger and core also make multiple contacts with TFIIF, which is located near the RNA exit channel (41, 94, 126, 200).

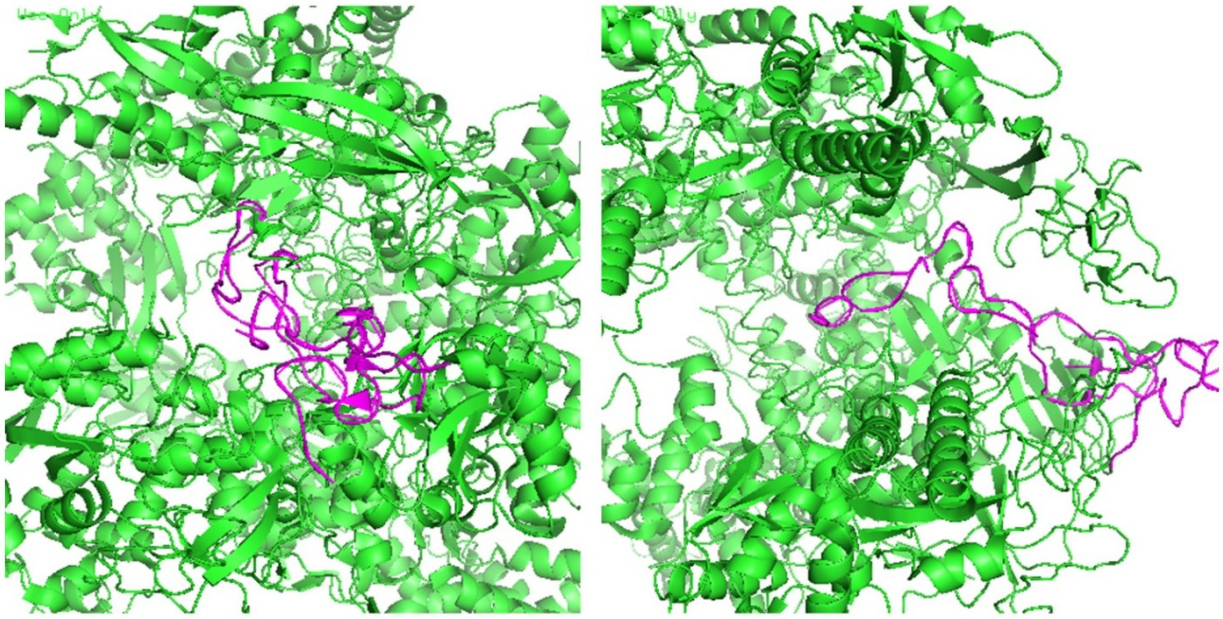

Figure 7. PyMol representation of the partial N-terminal TFIIB (red), Rpb1 and Rpb2 (green). The left view shown is looking down into the exit channel with TFIIB positioned above, the right view is a 90° rotation of the left showing the TFIIB reader in the active site of the enzyme. Created using 15RU.pdb file.

TFIIB is essential for initiation of transcription, but immediately after initiation, its zinc-finger becomes inhibitory for elongation of transcript as it competes with the growing RNA chain for occupancy of the polymerase saddle region and the exit channel (238). To allow transcription to proceed further, the zinc finger is ejected from the exit channel after RNAP II has transcribed first 10-11 nucleotides. This results in the separation of TFIIB from RNAP II and possibly from the promoter region. In vitro studies have clearly demonstrated the release of TFIIB from the initiation scaffold soon after initiation of transcription (325).

In keeping with its vital role in transcription initiation, TFIIB has been found to interact both physically and genetically with other components of PIC such as TFIID, TFIIH and Mediator complex as well as the transcription regulators such as activators and repressors. TFIIB has been shown to physically interact with TFIID subunits TAF1, TAF9, TAF10 and TAF12 in the TFIID-TFIIA-TFIIB complex formed on the promoter during PIC formation (114, 117, 258). TFIIB exhibits physical as well as genetic interaction with the TFIIH subunits Tfb4 and Ssl2 (105, 295). Mediator subunits Srb2, Srb5, Srb6 and Rgr1 have been found co-fractionating with TFIIB during chromatographic purification (197, 325), while interaction with Med15 subunit was shown in a split-ubiquitin screen (190).

Reconstituted transcription using purified GTFs, RNAP II and Gal4-VP16 fusion protein led to the discovery of a novel role of TFIIB in activation of transcription (191, 192). Work from multiple labs has since confirmed the transcription activation function of TFIIB. In keeping with its role in activation, interaction of TFIIB with a number of acidic activator proteins has been demonstrated in vitro (71). Most activators have been shown to interact with the amphipathic helix connecting the two direct repeats in the TFIIB core domain (1, 45, 172, 186, 316). However, there are some such as VP16 that interact with the direct repeats (192). A direct physical interaction of TFIIB with the Gal4-VP16 activator protein has also been demonstrated in vivo using the crosslinking approach (117). This is the only known case of a direct physical interaction of TFIIB with an activator under physiological conditions. In humans, thyroid hormone receptor beta (THRβ) can function as an activator or repressor depending on whether or not thyroid hormone is present. It was found that THRβ physically interacts with the N-terminal of

TFIIB without its hormone ligand and represses transcription. Upon binding of hormone, THRβ interacted with the TFIIB core domain and activated transcription (15).

TFIIB exists in two conformations, an 'open form' where the N and C-termini are free to interact with other proteins, and a 'closed form' where the N-terminal physically interacts with the second repeat of the C-terminal core (Fig. 8) (14, 101, 122, 124, 250, 317, 331). It has been reported that activators binding to TFIIB induces a change in its conformation (1, 124, 250, 316). In addition, TFIIB binding to BREs cause a conformational change that is important for start-site selection (90). Less well known is the fact that TFIIB can acetylate itself in the presence of acetyl-CoA on Lys238. The acetylation is important for stabilizing TFIIB-TFIIF interaction, and for transcription in vitro (54).

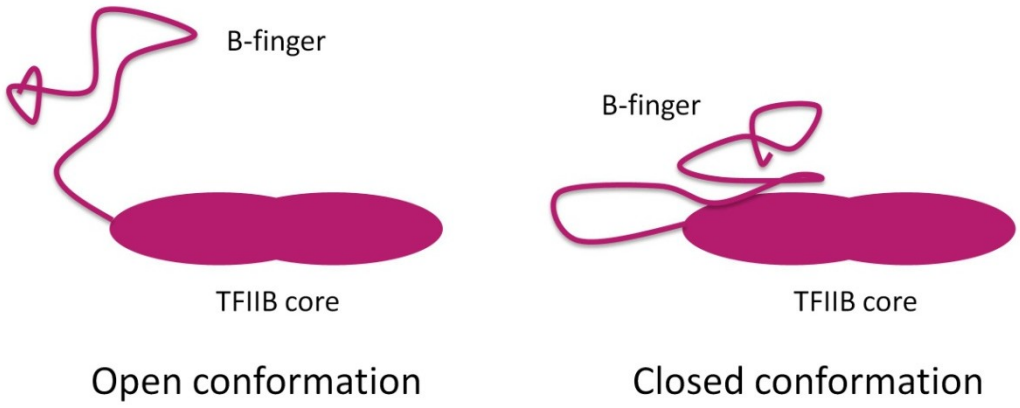

Figure 8. Schematic representation of the open and closed TFIIB conformations.

1.7 TFIIH: STRUCTURE AND FUNCTION

Transcription Factor TFIIH is a complex of 11 subunits with a molecular mass of over 500 kDa(224). Apart from Mediator, it is the only GTF with two separate enzymatic activities: a DNA helicase and a kinase activity (110). TFIIH was discovered in 1989 in Conaway laboratory when they purified a factor from rat liver that was required for

accurate transcription initiation and was characterized by an ATPase activity that was stimulated by the TATA-box (62-64). Structurally, TFIIH can be divided into two modules: the core module (subunits Ssl1, Ssl2, Rad3, Tfb1, Tfb2, Tfb4, Tfb5 and Tfb6) and kinase module also known as TFIIK in yeast (subunits Tfb3, Ccl1 and Kin28) (61, 224). Sequencing and structural analysis strongly suggest the evolutionarily conserved nature of the factor. Electron microscopy (EM) and crystal structure found TFIIH a ring like structure where TFIIK is linked to the core through Rad3 (Fig. 9) (92, 99, 247, 280). The conserved enzymatic activities reside in the Ssl2 and Rad3,

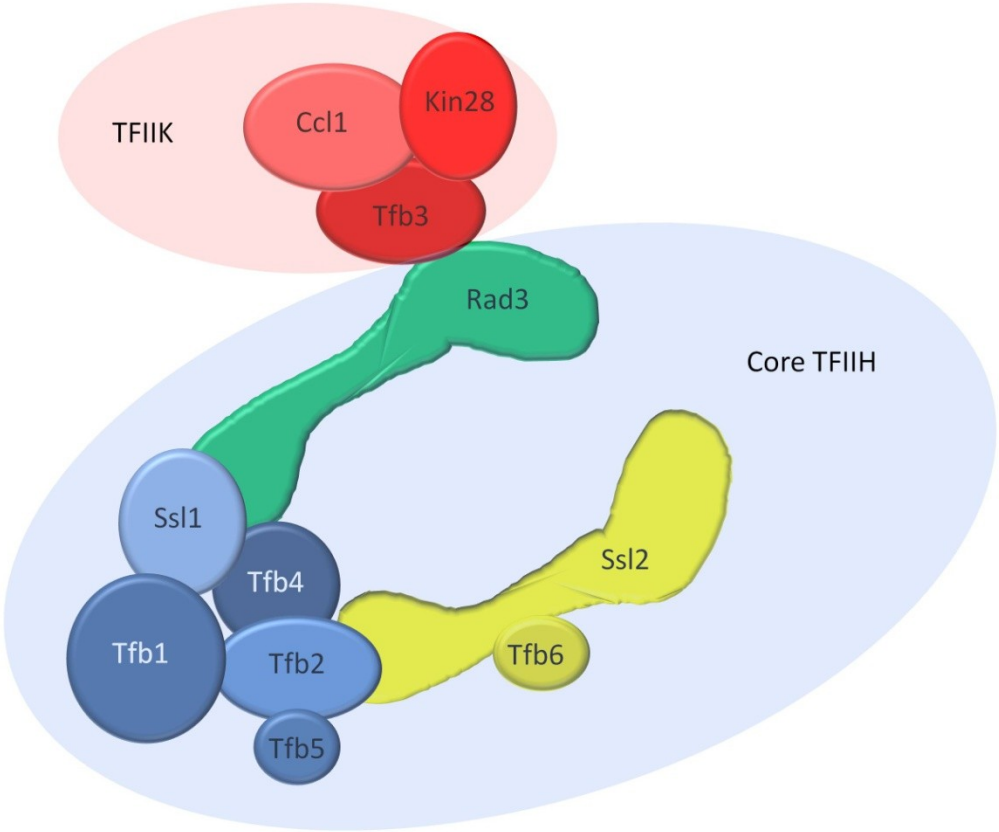

Figure 9. Schematic representation of TFIIH

which are 3' to 5' and 5' to 3' ATP-dependent helicases respectively; and Kin28 which is a serine kinase that phosphorylates CTD of Rpb1(61). In yeast, the Ssl1 subunit has also been found to contain an E3 ubiquitin ligase activity that functions in the

transcriptional regulation of genes involved in DNA repair (281). Apart from its essential function in transcription, TFIIH has also been found to play a crucial role in the repair of DNA by nucleotide excision repair (NER) pathway. A few studies have also implicated TFIIH in RNAP I transcription (13, 33, 77, 139, 260).

During transcription, the enzymatic activities of TFIIH play key roles in stabilization of PIC and in promoter escape (308). TFIIH is the last GTF to be recruited onto the promoter during PIC formation. It is not necessary for transcription in an in vitro transcription system when the template DNA is either supercoiled or partially melted near the TATA-box (135, 242). The core module forms a ring like structure that positions the Ssl2 helicase at the leading edge in front of the PIC and RNAP II, and the Rad3 helicase behind RNAP II. It is through this positioning that both helicases could function where Ssl2 will unwind the DNA in 3' to 5' direction from the leading edge toward RNAP II while Rad3 may unwind from the back in 5' to 3' orientation, melting the DNA and extending the transcription bubble with RNAP II (99). However, it is only the Ssl2 helicase activity that is required for formation of the open complex where 10 bp of DNA is melted just downstream of the TATA box (56, 57). Recent work indicates that Ssl2 may not function in the PIC as a 3' to 5' helicase and instead works as a DNA translocase. It was found that Ssl2 binds downstream of the bubble and may insert 15 bp of unwound DNA into the RNAP II active site (57, 111, 157).The requirement of Ssl2 helicase continues during initiation until the bubble reaches 17-18 bp and collapses back down to 10 which is the size during elongation (238).

The exact contacts that different subunits of TFIIH make during PIC formation are not well understood as the structural studies on TFIIH are not of high enough

resolution to map them into the RNAP II EM densities. Recently, Tfb6 was identified as the 11th subunit of TFIIH. It was found that Tfb6 interacts with Ssl2 and this interaction dissociates Ssl2 from TFIIH holoenzyme upon Tfb6 phosphorylation. Tfb6 is not an essential protein and purification of TFIIH from a Tfb6 deleted strain resulted in a 20 fold increase in yield of holo-TFIIH complex (224).

TFIIH kinase activity is stimulated by interactions with TFIIE, TFIIF and the Mediator along with the U1 snRNA (22, 88, 171, 233, 236). TFIIK phosphorylates RNAP II CTD at both serine-5 and serine-7 in the open complex, although in yeast it is unclear exactly where this happens in vivo as RNAP II scans downstream for about 20- 200 bp in order to find a TSS, which is well past the size of the bubble (3, 103, 199). This is in contrast to mammalian cells, where TSS is generally located 23-28 bp downstream of the TATA-box (86). The CTD phosphorlyation signals promoter escape and also breaks the contacts of Mediator head module with RNAP II CTD. The serine-5 phosphorylation mark then signals the recruitment of the capping machinery (53, 89). After the first round of initiation, TFIIH remains on the scaffold with TFIID, TFIIA and TFIIE in the presence of an activator in order to drive further rounds of reinitiation (325).

1.8 CTD PHOSPHORYLATION

The largest subunit of RNAP II, Rpb1, contains a region towards the C-terminus called the carboxy-terminal domain (CTD), which consists of multiple repeats of the heptapeptide sequence $Y_1S_2P_3T_4S_5P_6S_7$. Since its discovery in 1985, it has been a focal point of research (65, 66). The CTD repeats are highly conserved in sequence yet vary in number from organism to organism. There are 26 repeats in yeast, 46 in flies and 52 in human (35, 65). Deletion of the CTD is lethal in vivo, but not required for in vitro

transcription (231, 232, 328). The CTD is the target for many post translational modifications. Five of its seven residues, tyrosine-1, serine-2, threonine 4, serine-5 and serine-7, can be phosphorylated (Fig. 10). In addition, the proline residues at 3 and 6 can be isomerized into stable *cis* and *trans* configurations (81). In order to better understand the possible syntax of the CTD, research done in the Stiller and Shuman laboratories placed additional residues at key places throughout the CTD (193, 263, 276).

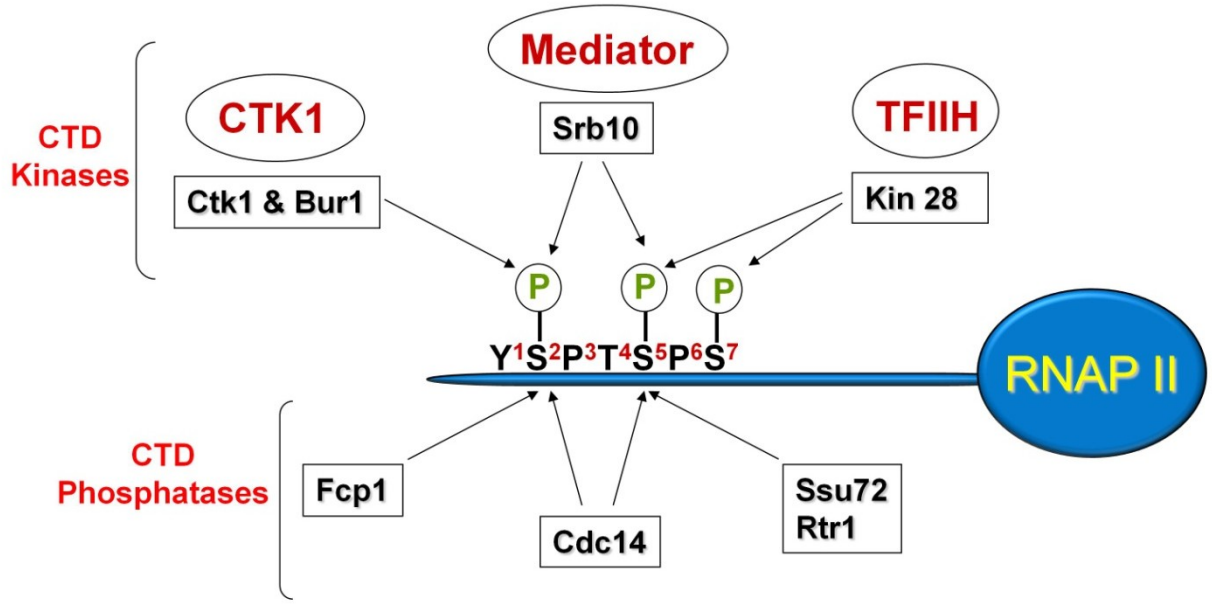

Figure 10. Schematic of the RNAP II CTD. Enzymes that add or remove phosphate at specific serine residues are indicated.

The results indicate that the CTD is actually composed of 11 residues long functional units, which comprises the first heptad and the next four residues in the chain (81, 194, 262, 276). Taken together, it would appear that the overall length of the CTD is important, along with keeping functional units intact with respect to tyrosine and serine-proline-serine spacing. In yeast, with 26 repeats, the overall length of an unordered CTD tail could stretch up to 900 A^{o}, which is nearly 6 times the diameter of RNAP II;

making it an ideal scaffold to recruit factors that need to physically interact with RNAP II over a variety of its surface during transcription and cotranscriptional RNA processing (81).

The enzymes responsible for most of the CTD posttranslational modifications have been discovered. In yeast, there are four known enzymes for phosphorylating the CTD: Kin28, Srb10, Ctk1 and Bur1. Kin28 is a subunit of TFIIH that performs serine-5 and ser7 CTD phosphorylation (103, 156). Srb10 is a subunit of Mediator and has been shown to phosphorylate serine-2 and serine-5 in vitro (127, 188). Ctk1 is a subunit of the elongation complex and is thought to perform the majority of serine-2 phosphorylation (52). Bur1 functions during elongation as part of the Bur1-Bur2 cyclin-dependent kinase complex and phosphorylates serine-2 and serine-5 in addition to phosphorylating the elongation factor Spt5 (309). In budding yeast, three more kinases that could possibly target the CTD have been identified on the basis of their phosphorylation target similarity with known CTD kinases. These are Brd4, Erk1 and Erk2 (81). It is thought that Brd4 may be a yet another kinase that may target serine-2. The CTD phosphorylation at tyrosine-1 and threonine-4 residues was recently demonstrated in yeast. However, identification of the kinases responsible for these modifications remains elusive (47, 130, 137). The prolyl-isomerase, Ess1, preferentially targets the proline-6 residue when serine-5 is phosphorylated and has been shown to be involved in initiation and termination of transcription (119, 163).

The serine phosphatases that remove CTD mark are well characterized. Ssu72, which is a component of the cleavage and polyadenylation factor (CPF) complex, removes serine-5 and serine-7 marks (19, 95, 165). Ssu72 is recruited at the promoter

and the terminator regions of a gene and is believed to function at both the ends of a gene (10). Cdc14 removes both serine-2 and serine-5 marks and functions during mitosis where it is required for mitotic exit (55). Rtr1 is a serine-5 phophatase that has been shown to function during the transition from initiation to elongation (220). Fcp1 is a serine-2 phosphatase which is also recruited at the promoter and terminator regions, but is only thought to function at the terminator (160).

Early ChIP experiments using some of the first antibodies to recognize CTD

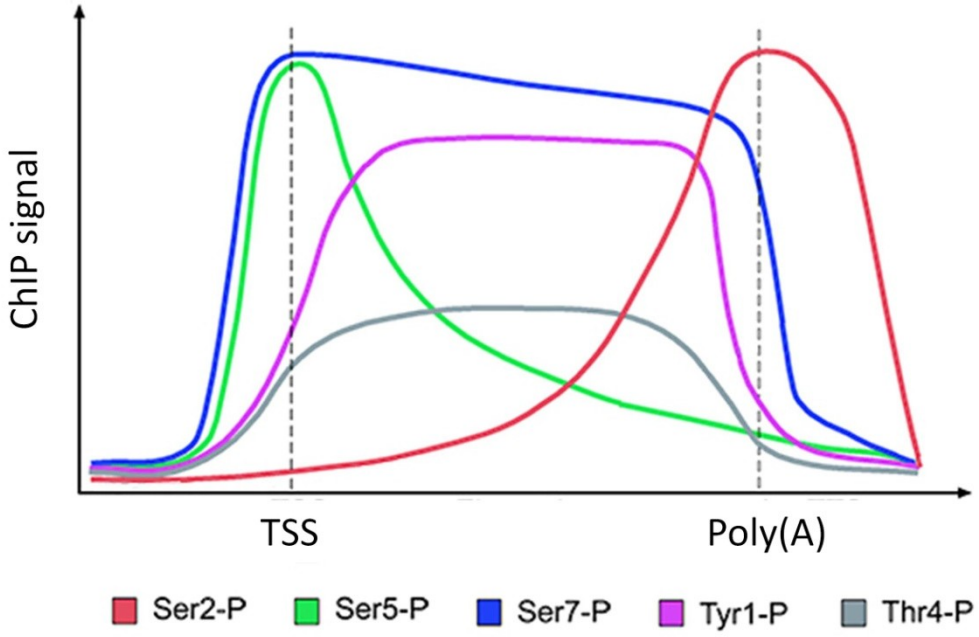

Figure 11. Model of the RNAP II CTD phosphorylation status on a gene

phosphorylation began to paint the picture of a CTD code that could be used to tell the position of RNAP II along a gene. In this now widely accepted model for protein coding genes, serine-5 phosphorylation peaks over the TSS and fades along a gene in a fashion opposite to that of serine-2 phosphorylation which starts downstream of the promoter and peaks over the poly(A)-site (Fig. 11) (35). Tyrosine-1 and threonine-4 phosphorylation remains at high levels throughout the coding region between the

promoter and the terminator. Serine-7 phosphorylation exhibits a similar pattern, except that it extends a little bit more into the promoter and terminator regions (81). The CTD phosphorylation pattern over a gene signals the stage of transcription of a gene. The initiation factors, such as Mediator and TFIIH, involved in PIC assembly, recognize hypophosphorylated CTD (158, 308). A notable exception is that some of the termination factors, such as Pcf11 and Ssu72, thought to be recruited through serine-2 phosphorylation at the terminator, are also recruited onto the promoters (6, 266).

More recently, numerous genomewide data sets have attempted to analyze this problem with new monoclonal antibodies that can better detect specific CTD phosphorylation patterns. These studies have revealed that different genes exhibit different patterns of CTD phosphorylation during transcription. A recent study found that the pattern of CTD phosphorylation differed on genes depending on their transcriptional activity and their isolation within the genome (286). Some genes displayed a more or less uniform serine-2, serine-5, and serine-7 phosphorylation pattern throughout the gene body. In contrast, some have only 5’ or 3’ phosphorylation peaks, while others have both 5’ and 3’ peaks of a particular modification. For example, some genes display a uniform serine-7 phosphorylation pattern throughout the gene, while a number of genes exhibit only the 5' enrichment (286). For the serine-5 phosphorylation mark, most genes displayed only a 5' peak, while some showed a peak at both the 5' and 3' ends. Regarding the serine-2 phosphorylation mark, while most genes conformed to the usual model with a 3’ peak, they did find those that either had uniform or 5’ peak distributions (286).

1.9 PROMOTER-TERMINATOR CROSSTALK

The transcription cycle can be divided into three basic steps: initiation, elongation and termination. Each of these steps requires a unique set of factors for its proper execution. The generally accepted view is that the transcription factors have exclusive roles dedicated to a particular step in the transcription cycle. The current body of evidence suggests that the different steps of the transcription cycle do not operate in isolation, but rather integrate into each other (167, 203, 212, 266, 297). This may seem intuitive for sequential steps where some initiation factors also participate in elongation, as the subsequent elongation step relies on completion of initiation. However, evidence suggests that the initiation factors may function in termination, and termination factors similarly help in initiation of transcription (6, 115, 207, 212, 221, 329). This promoter-terminator crosstalk is poorly understood, and it is not known how these functional relationships can influence the transcription cycle.

There are a number of factors operating at the promoter region of eukaryotic genes that physically or functionally communicate with the terminator-bound factors (Table 1). TFIIB, for example, interacts with a number of termination factors. These include both genetic and physical interactions. The first evidence regarding this

TABLE 1 Promoter bound factors which interact genetically or physically with terminator bound factos

Promoter bound Factor	Protein / subunit	Terminator bound Factor	Interacting partner/subunit	Type of interaction	Reference
TFIID	Taf2	CPF	Ssu72, Fip1, Cft2, Cft1, Mpe1, Ref2, Ysh1, Pta1, Pap1	Affinity-MS	Sanders et al., 2002
	Taf5	CPF	Cft1	Affinity-MS	Lee et al., 2011

	Taf6	3'-end RNA-processing complex	Pab1	Affinity-MS	Sanders et al., 2002
	Taf6	CF1	Rna14	Affinity-MS	Gavin et al., 2002
	Taf8, Taf12	Rat1 complex	Rtt103	(-) Genetic	Constanzo et al., 2010
	Taf9	CPF	Ref2	Synthetic lethal	Milgrom et al., 2005
	Taf14	Isw1b	Ioc2	(+) Genetic	Collins et al., 2007
TFIIB	TFIIB	CPF	Ssu72	Phenotypic enhancement, Synthetic rescue	Sun and Hampsey 1996
	TFIIB	CPF	Ssu72	Dosage rescue, Synthetic lethal	Wu et al., 1999
	TFIIB	CPF	Ssu72	Reconstituted complex	Wu et al., 1999
	TFIIB	CPF	Ssu72	Affinity-Western	Ganem et al., 2003
TFIIH	Rad3	CF1	Rna14	Synthetic rescue	Jensen et al., 2004
	Ssl1	Rat1 complex	Rtt103	(-) Genetic	Constanzo et al., 2010
	Kin28	CPF	Ssu72	Affinity-Western, Synthetic lethal	Ganem et al., 2003
Mediator	Med8	Rat1 complex	Rtt103	(-) Genetic	Constanzo et al., 2010
	Rox3	(3'-end RNA-processing)	Yra1	Affinity-MS	Krogan et al., 2006
	Srb2	CPF	Ssu72	(-) Genetic	Constanzo et al., 2010
	Med1	CPF	Syc1	(-) Genetic	Wilmes et al., 2008
	Soh1	CPF	Ssu72	(-) Genetic	Collins et al., 2007, Fielder et al., 2009
	Soh1	CPF	Syc1	(-) Genetic	Wilmes et al., 2008
	Soh1	Rat1 complex	Rtt103	(-) Genetic	Wilmes et al., 2008
	Gal11	CPF	Swd2	(-) Genetic	Collins et al., 2007
	Gal11	Rat1 complex	Rtt103	(-) Genetic	Collins et al., 2007
	Pgd1	Rat1 complex	Rtt103	(-) Genetic	Collins et al., 2007
	Pgd1	Isw1b	Ioc4	(+) Genetic	Collins et al., 2007
	Cse2	CPF	Syc1	(-) Genetic	Wilmes et al., 2008
	Sin4	Rat1 complex	Rtt103	(+) Genetic	Costanzo et al., 2010
	Sin4	Isw1b	Ioc2	(-) Genetic	Costanzo et

					al., 2010
	Srb5	CF1	Rna15	Affinity-Western	Mukundun et al., 2013
SAGA	Gcn5	CPF	Ysh1	Affinity-MS	Graumann et al., 2004
	Ada2	CF1	Pcf11	2 Hybrid	Uetz et al., 2000
	Ahc1	Isw1b	Ioc4	(-) Genetic	Costanzo et al., 2010
	Spt8	CF1	Rna14	Synthetic growth defect	Holbien et al., 2009
	Spt8	CPF	Pti1	(-) Genetic	Collins et al., 2007
	Spt8	CPF	Ssu72	(-) Genetic	Collins et al., 2007
	Spt8	CPF	Pta1	(-) Genetic	Collins et al., 2007
	Ubp8	Rat1 complex	Rtt103	Synthetic rescue	Hang et al., 2011
	Spt3	CPF	Pta1	(-) Genetic	Collins et al., 2007
	Spt3	CPF	Pti1	(-) Genetic	Collins et al., 2007
	Spt3	Rat1 complex	Rtt103	(-) Genetic	Collins et al., 2007
	Chd1	Isw1b	Ioc2	(-) Genetic	Collins et al., 2007
	Chd1	Isw1b	Ioc4	(-) Genetic	Costanzo et al., 2010
	Ngg1	3'-end RNA-processing complex	Pab1	Affinity-Western	Drysdale et al., 1998
	Sgf29	CPF	Pta1	(-) Genetic	Costanzo et al., 2010
	Hfi1	Isw1b	Ioc2	(-) Genetic	Collins et al., 2007
	Spt7	CPF	Cft1	Affinity-MS	Lee et al., 2011
	Sus1	(3'-end RNA-processing)	Yra1	Affinity-Western	Pascual-Garcia et al., 2008
	Sus1	(3'-end RNA-processing)	Yra1	Synthetic lethal	Rodriguez-Navarro et al., 2004
	Sgf73	CF1	Pcf11	(+) Genetic	Costanzo et al., 2010

came from a genetic screen for factors important for initiation. A point mutation (E62K) in the B-finger region of yeast TFIIB (*sua7-1*) altered transcription start site selection and conferred a cold-sensitive growth phenotype (246). A mutation in Ssu72 (*ssu72-1*)

enhanced this defect by shifting the start site further downstream and conferring a heat-sensitive phenotype (278). Yeast proteomic analysis identified Ssu72 as a subunit of the CPF complex (72, 97, 125, 274). In addition to the genetic interaction, TFIIB and Ssu72 also exhibit a physical interaction *in vitro* (317). These observations raised the intriguing possibility that TFIIB could be making additional contacts with the components of the 3' end processing machinery during transcription. These studies strongly suggested a role for TFIIB at the 3′ end of genes, and will be the focus of investigation in Aim# 1 of my thesis.

In addition to TFIIB, there are also reports of TFIIH interaction with termination factors (Table 1). Several subunits of TFIIH have been found physically and functionally interacting with the 3′ end associated factors. Kin28, which is the kinase subunit of TFIIH, interacts physically as well as genetically with Ssu72 (67, 97, 115, 165). The TFIIH-Ssu72 interaction as well as phosphorylation-dephosphorylation of serine-5 and serine-7 contribute to successful execution of the transcription cycle and will be discussed later in this chapter in the context of Ssu72. In addition to the Kin28-Ssu72 interaction, Ssl1 subunit was found to exhibit a genetic interaction with Rtt103 subunit of Rat1 complex, which is involved in termination of transcription (67, 155). The role of these interactions and TFIIH kinase activity at the 3' end of genes will be the focus of investigation in Aim# 2.

Mediator is another promoter-associated factor that exhibits interaction with the terminator-bound factors. The first evidence that Mediator could be contacting the termination factors came from a large scale yeast proteomic analysis (58). This study identified interaction of one of the head subunits, Rox3, with Yra1, a protein involved in

3′ end processing and mRNA export (Table 1). All other Mediator-termination factor interactions that have been reported so far are from the widely used ‘Epistatic Miniarray Profile’ (E-MAP) technology, which measures genetic interactions based on the pair-wise deletion of genes that have been linked to specific biological processes. This synthetic genetic approach established interaction of the head subunit, Med8, with Rtt103 (58), and identified further interaction of middle and tail submodules with the CPF subunits (Table 1) (58, 67, 306). Recently, Srb5 subunit of Mediator head module was also found interacting with the Rna15 subunit of CF1A complex (221)(Mukundan and Ansari, 2013).

The overwhelming evidence of the interaction of the promoter and terminator-bound factors in the cell raises an important question. What is the significance of such interactions in the context of the transcription cycle? A clue came from crosslinking studies of the factors on transcriptionally active genes. The chromatin immunoprecipitation (ChIP) analysis of formaldehyde-crosslinked cells revealed that the initiation and termination factors are not merely interacting with each other, but they are also interacting with the distal ends of genes (Fig. 12). TFIIB, for example, has been found occupying both the 5′ and 3′ ends of genes during transcription (83, 209, 210, 212, 225, 270, 293). A genomewide analysis revealed that about 80% of a selected group of 1140 transcriptionally active genes in yeast have TFIIB localized at both ends (225). The TFIIB signal at the 3′ end, however, was 2-times less than that on the 5′ end. The crosslinking of TFIIB to the extremities of genes during transcription is an evolutionarily conserved feature, being observed in humans as well (207, 323). A similar gene occupancy profile has been observed for TBP, both in yeast and humans (207,

225). Mediator subunit Srb5, TFIIF subunit Tfg1, as well as TFIIH subunits; Ssl2

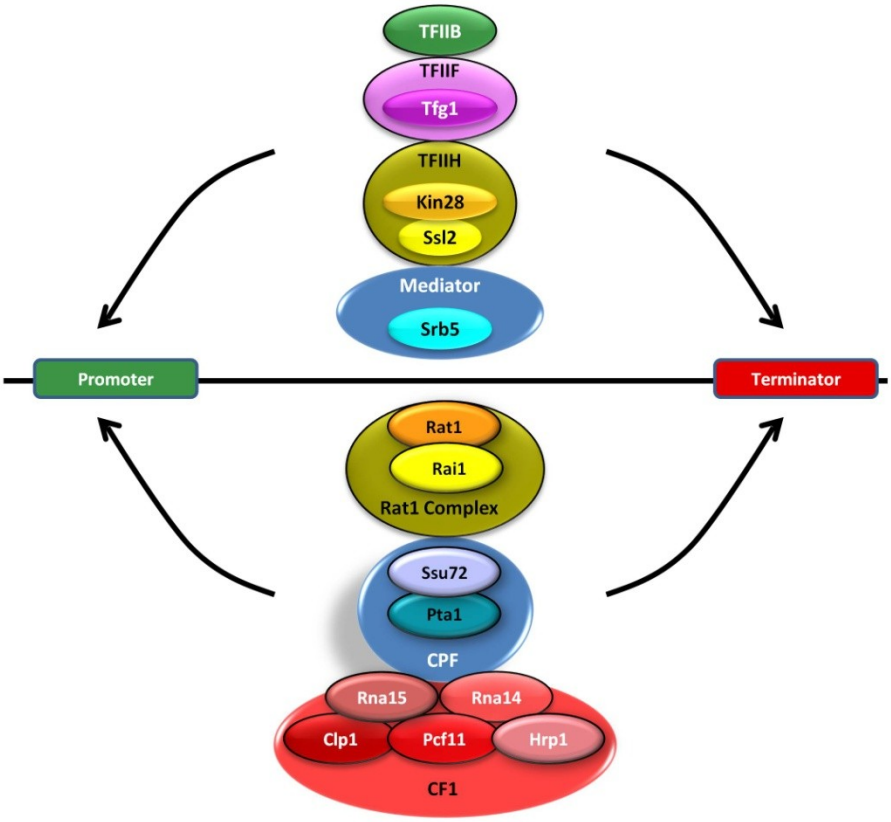

Figure 12. Initiation and termination factors that ChIP on both ends of a gene.

and Kin28, also occupy the distal ends of a gene in a transcription-dependent fashion (105, 210, 221, 248, 329). The transcription-dependent interaction of so many initiation factors with the 3′ end strongly suggests a biological role for these factors at the terminator end of genes.

Analogous studies with the termination factors found them localized to the 3′ end as expected, but a number of them were also found occupying the 5′ end of genes (Fig. 12). The subunits of both CF1 and CPF complexes in yeast exhibited the transcription-dependent crosslinking to gene boundaries (10, 43, 155, 212, 227). A similar localization of CPSF and CstF subunits at the ends of genes was observed in mammalian systems (102, 297). Recently, crosslinking of two termination factors, TTF2

and Xrn2, to the 5′ end of genes was reported in mammalian cells (34). The homologue of Xrn2 in yeast is Rat1. Both Rat1 and Xrn2 are believed to facilitate termination of transcription by the torpedo mechanism (155, 302). The Rat1 and its associated factor Rai1 also occupy both ends of genes during transcription (155).

One possible explanation for termination factors occupying the promoter region arose from the recent discovery of wide spread promoter driven upstream anti-sense transcription (240, 264, 265). Many of these short anti-sense transcripts were found to be cleaved and polyadenylated poly(A) signals (7). Interestingly, the U1 signals were found to be enriched in the sense direction relative to the poly(A); and the disruption of U1 snRNP activity resulted in the premature termination in the sense direction (7). Thus, the interaction of the initiation and termination factors is not merely the coincidental co-recruitment to the distal ends of a gene, but indicates a functional relationship that confers directionality to the promoter-bound polymerase and also influences overall transcription of a gene. Another possible interpretation of this evolutionarily conserved transcription factor occupancy pattern on gene extremities is that the initiation and termination factors may physically interact with each other on the chromatin template during transcription to form a gene loop that enhances efficiency of transcription.

1.10 GENE LOOPING

The ChIP data, along with the physical interaction data and the genetic analysis, have provided unequivocal evidence in support of the interaction of a number of initiation and termination factors with both the ends of a gene. A critical issue is how the initiation and termination factors that are expected to occupy the 5′ and 3′ ends respectively of a gene, are able to contact both ends of a gene. There are two possible

explanations for this rather unexpected distribution of transcription factors. One possibility is that these factors are recruited independently to both ends of a gene. The other possibility is that the presence of a factor at the two ends of a gene is the result of gene looping (102) (Fig. 13). When a gene is in looped

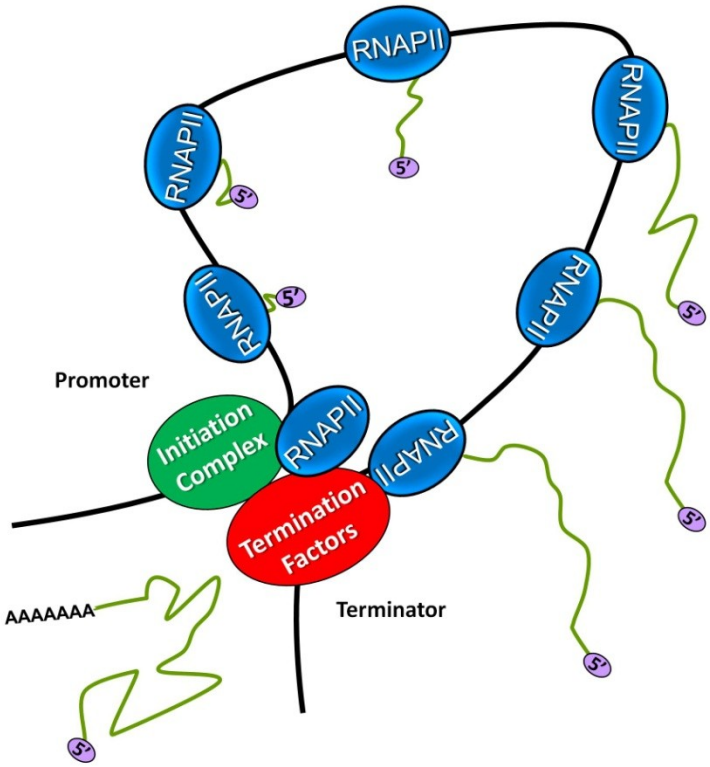

Figure 13. Schematic diagram of a gene loop

conformation, the close proximity of the promoter and terminator regions may facilitate the interaction of a promoter-bound factor with the terminator region, and that of a terminator-associated factor with the promoter. There is a high probability that the same molecule simultaneously contacts both ends of a looped gene (270). In such a scenario, the molecules occupying the promoters and terminators are not separate entities (Fig. 13). There is experimental evidence to support both possibilities (23, 102, 173, 207, 221, 270, 293).

A number of genes in yeast undergo looping in a transcription-dependent manner (6, 10, 23, 83, 102, 141, 217, 270, 283, 284). Chromatin analysis using the chromosome conformation capture (CCC) approach revealed that gene looping is due to the juxtaposition of the promoter and terminator regions of a gene (Fig. 13). When a gene is in looped conformation, TFIIB localizes to both ends of the gene (83, 270). Gene looping is completely abolished in the *sua7-1* mutant (E62K) of TFIIB in budding yeast. This is the same mutant that led to the discovery of one of the first known interactions of a promoter-bound factor with a terminator-linked factor, that of TFIIB with Ssu72 (102, 278). In this mutant, the recruitment of TFIIB at the promoter region remains unaffected, but its crosslinking to the 3′ end of a gene is almost completely abolished (270). Accordingly, TFIIB-interacting termination factors were observed at the 5′ end of a gene in wild type cells, but not in the looping defective *sua7-1* cells (6). Although the concept of gene looping is not so well established in higher eukaryotes, analogous studies have found evidence of genes assuming a looped architecture during transcription in mammalian systems as well (141, 179, 234, 243, 282, 326). There are also reports of transcription dependent gene looping in Drosophila and plants (70, 128). Gene looping explains localization of at least some transcription factors at the distal ends of a gene. The crosslinking of TFIIB, Mediator subunit Srb5 as well as TFIIH subunits; Ssl2 and Kin28 to both the ends of a gene in yeast occurs in a looping dependent manner, and therefore can be attributed to gene looping (83, 105, 210, 221, 248, 270, 329). Similarly, a number of CF1 and CPF subunits in yeast also crosslink to the promoter region in a looping-dependent manner (6, 10, 43, 155, 212, 227).

Gene looping, however, may not account for the interaction of all initiation and termination factors with the ends of genes. A substantial number of yeast genes exhibit anti-sense transcription initiating from their 3′ end (49, 228, 318). The genes with detectable 3' end initiated anti-sense transcripts display a promoter-like architecture at their 3′ end (225). Genomewide analysis revealed that a majority of such genes have TFIIB and TBP present at their 3′ end (225). Whether these genes assume looped conformation during anti-sense transcription remains to be elucidated, but at least some of them display anti-sense transcription as well as the TFIIB-TBP occupancy at the 3′ end in the absence of gene looping (225). The interaction of TFIIB and TBP with the 3′ end of genes in linear conformation, therefore, cannot be attributed to gene looping, but to the independent recruitment of these factors at the 3′ end of genes.

The physiological significance of gene looping has been recently demonstrated in a few key studies. First, gene looping was found to play a role in transcriptional memory, which is the process where the transcriptional machinery ‘remembers’ a previously activated state via gene loop formation, and thus the cell can respond much faster during reinduction (173, 283). Second, gene looping was linked to promoter directionality, where promoter driven anti-sense transcription decreased during gene loop formation (44, 284). Third, the intron-mediated enhancement of transcription (IME) has been found to require gene loop formation, and the addition of an intron to the 5’ proximal coding region can activate a gene and force a looped conformation (217). Fourth, gene looping helps in activator dependent enhancement of transcription by facilitating reinitiation (6, 83). Last, gene looping has also been shown to facilitate termination of transcription, possibly through the poly(A) site selection (6).

1.11 RESEARCH FOCUS

While the prevalence and physiological significance of gene looping has been demonstrated over the last decade; the mechanism that physically links the promoter and terminator regions in order to form a gene loop is poorly understood. It is hypothesized that gene loops form due to the interaction of promoter and terminator bound factors. Namely, the general transcription factors (Mediator, TFIID, TFIIA, TFIIB, TFIIF and TFIIH) would physically and functionally interact with the termination factors (CF1 and CPF). In order to investigate if that is the case, my research focused on two of the general transcription factors: TFIIB and TFIIH.

TFIIB was an excellent candidate based on its localization on the terminator during transcription, its genetic interaction with the termination factor Ssu72 and the loss of gene looping in the mutant of TFIIB, *sua7-1.* In chapter II, my research focuses on investigating the molecular basis of gene loop formation through the isolation of a gene looping complex with TFIIB. Genome wide studies have also demonstrated that in addition to TFIIB, TFIIH localizes on the terminator (248, 292, 294). In chapter II, my research investigates a role for TFIIH in the termination of transcription and gene loop formation. In addition, I further demonstrate that these roles are dependent on the kinase function of TFIIH subunit, Kin28.

CHAPTER II

EVIDENCE FOR A HOLO-TFIIB COMPLEX CONTAINING TERMINATION FACTORS THAT FACILITATE GENE LOOP FORMATION

Most of this chapter has been published:

Medler et al., (2011) Evidence for a Complex of Transcription Factor IIB (TFIIB) with Poly(A) Polymerase and Cleavage Factor 1 Subunits Required for Gene Looping. J Biol. Chem. 286:33709-18. DOI: 10.1074/jbc.M110.193870

II.1. ABSTRACT

Gene looping is emerging as an important gene regulatory mechanism in eukaryotes. The presence of general transcription factors at the promoter region of a gene during transcription is well established. However, recent studies have revealed the localization of the general transcription factor TFIIB to the 3' end of a gene as well during transcription. Here we show that TFIIB localization at the terminator end of a gene requires a functional CF1 complex. TFIIB physically interacts with the all subunits of the CF1 complex. Affinity chromatography and sedimentation analysis revealed the existence of a holo-TFIIB complex consisting of Pap1 and CF1 subunits. This complex was resistant to MNase digestion suggesting that the interaction of TFIIB with termination factors was not mediated by RNA or DNA. The complex was also stable upon brief exposure to high salt. The sedimentation coefficient of the holo-TFIIB complex was similar to that of large ribosomal subunit, and was intermediate between that of TFIIH and TFIID. The general transcription factors TFIID and TFIIH as well as subunits of RNAP II could not be detected in the affinity-purified holo-TFIIB preparation. The holo-TFIIB complex was observed only in the looping competent strains, but not in

the looping defective *sua7-1* strain. We further show that in *sua7-1* cells, where a holo-TFIIB complex is not formed, the kinetics of activated transcription are altered. These results strongly suggest a role for the holo-TFIIB complex in gene looping, and a possible role of gene looping in activator-dependent transcription.

II.2. INTRODUCTION

Transcription of protein encoding genes by RNA polymerase II (RNAP II) involves several distinct steps that include the assembly of preinitiation complex, initiation, elongation, termination, and reinitiation (115, 310). Transcription starts with the recruitment of RNAP II and the general transcription factors TFIID, TFIIB, TFIIA, TFIIF, TFIIE, and TFIIH onto the promoter to form a preinitiation complex (PIC) in response to a signal. Gene specific activators respond to the signal by facilitating the assembly of PIC. RNAP II and general transcription factors are sufficient for accurate basal level transcription at least under in vitro conditions (252, 314). The response to activators requires additional cofactors that include chromatin modifiers and Mediator complex. Once the gene is activated, the amount of transcripts produced is determined primarily by the number of reinitiation events (74). Despite the remarkable progress made in understanding the molecular mechanisms that govern initiation of transcription in eukaryotes, relatively little is known about the processes that mediate reinitiation. It was hypothesized that efficient transfer of polymerase from the terminator to the promoter is facilitated by a DNA loop between distal ends of the transcribed gene (74, 169). The existence of such gene loops has been recently reported for RNAP II-transcribed genes in yeast, plants and mammalian cells (10, 234, 235, 243, 282). It has been shown that RNAP II-dependent gene looping is the consequence of the physical

interaction of the terminator with the promoter of the same gene during transcription. Gene looping has been shown to require both the *cis* acting poly(A) termination signal and the *trans* acting 3' end processing/termination factors (10, 243). The general transcription factor TFIIB, which exhibits a genetic interaction with the CPF subunit Ssu72, was also found essential for gene looping in yeast (270). Whether termination factors and TFIIB facilitate transfer of RNAP II from the terminator to the juxtaposed promoter is not known.

The emerging ubiquity of gene looping and its potential as an important transcription regulatory mechanism necessitates understanding the mechanism of gene loop formation. Our hypothesis is that the looped architecture is formed by the interaction of promotre-bound factors with the factors occupying the 3' end of the gene. The preliminary results produced in our laboratory and the published reports have identified TFIIB is an important determinant of gene looping. We therefore searched for the TFIIB interacting termination factors in this investigation. We believe that a macromolecular complex containing TFIIB and the termination factors may serve as a bridge between the promoter and the terminator regions during gene loop formation.

II.3. RESULTS

II.3.1. ACTIVATORS INTERACT WITH TFIIB DURING ACTIVATED TRANSCRIPTION

Activators bring about enhancement of transcription. Recent evidence from our laboratory suggests that the activator-dependent stimulation of transcription is dependent on gene looping. However, activators do not interact with the 3′ end of the gene during loop formation (83). These results imply that the activators may be facilitating gene looping by recruiting other factors that interact with both the 5' and the

3' ends of a gene. We have also shown that TFIIB is an important determinant of gene looping. These observations suggest that activator may be enhancing transcription through TFIIB-mediated gene looping.

A number of transcription activators in yeast, mammalian systems and plants have been shown to physically interact with TFIIB (71). In many of these studies, an activator-TFIIB interaction was demonstrated in the absence of transcription. None of the studies in yeast demonstrated physical contact of a native activator with TFIIB under in vivo conditions. The vast majority of interactions were performed using in vitro protein binding assays which demonstrated that the activators were interacting with the two direct repeats in the C terminus of TFIIB which also is shown to interact with RNAP II. The only in vivo evidence of a physical interaction is the crosslinking of a Gal4-VP16 fusion construct with TFIIB during induced transcription (117). If the activators bound to their UAS site are mediating gene looping through their interactions with TFIIB, we expect activator-TFIIB interaction to occur only during activated transcription when the gene is in a looped configuration.

To investigate interaction of TFIIB with activators, we chose Met28, Ino2 and Gal4, which are the gene specific activators of *MET16*, *INO1*, and *GAL1p-BUD3*, respectively. Transcription of *MET16* is regulated by methionine. In the presence of methionine, *MET16* is transcribed at a very low level. However, upon methionine depletion, transcription of *MET16* is stimulated by about 5-fold (83). Similarly, transcription of *INO1*, a gene involved in inositol metabolism, is enhanced by about 50-fold in the absence of inositol in the medium (83). *GAL1p-BUD3*, as we have shown earlier, is almost completely repressed in the presence of dextrose as a carbon source

(83). The addition of galactose brings about a 50-fold stimulation of transcription of *GAL1p-BUD3* (83). All these three genes are in a looped conformation during their induced transcriptional state. In the absence of activators, both gene looping and enhanced transcription of these genes is severely compromised.

We therefore performed coimmunoprecipitation with anti-HA antibodies in strains with HA-tagged Met28, Ino2, and Gal4. Coimmunoprecipitation was done during induced and non-induced states of *MET16*, *INO1*, and *GAL1p-BUD3* in formaldehyde crosslinked cells. There was no interaction of TFIIB with Met28, Ino2, and Gal4 under non-induced conditions (Fig. 14, lanes 3, 8, and 13). TFIIB was coimmunoprecipitated with the activators only under transcriptionally inductive conditions (Fig. 14, lanes 5, 10,

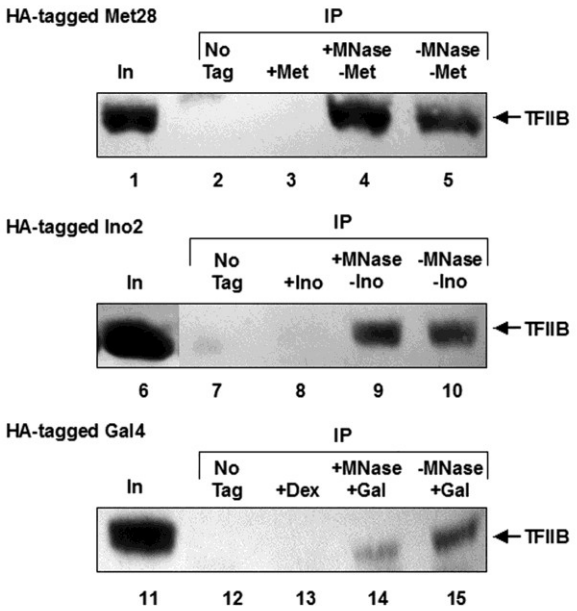

Figure 14. TFIIB cross-links to the activators during activated transcription.
Immunoprecipitations (IP) were performed during repressed and activated transcriptional states of *MET16, INO1, and GAL1p-BUD3* as described in appendix C. Immunoprecipitated samples were subjected to SDS-PAGE analysis, and Western blot was performed with anti-TFIIB polyclonal antibodies. Immunoprecipitation from the cell lysate without HA-tagged activators served as a control. *Dex,* dextrose; *Gal,* galactose*; Met,* methionine; *Ino,* inositol.

and 15). Activator-TFIIB interaction was not mediated by DNA because micrococcal

nuclease digestion of DNA did not abolish the interaction (Fig. 14, lanes 4, 9, and 14). The complete digestion of chromatin by micrococcal nuclease was routinely checked before performing the coimmunoprecipitation (Figure 15). A moderate decrease in Gal4-

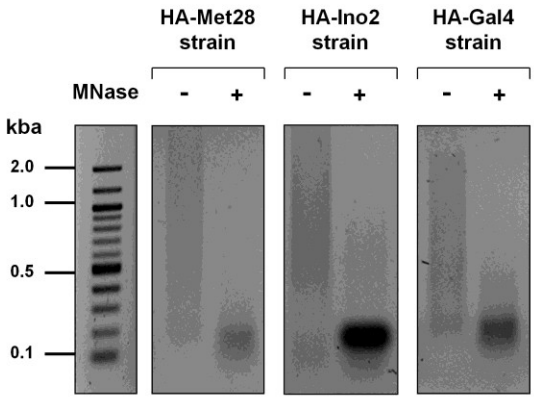

Figure 15. MNase digestion of chromatin in the cell lysate prepared for co-immunoprecipitation of TFIIB with HA-tagged activators. Following treatment of 10 ml of cell lysate containing HA-tagged Met28 or Ino2 or Gal4 with 10 units of MNase at 37°C for 30 min (+MNase), a 1 ml aliquot was treated as described in appendix C and incubated overnight at 65°C for reversal of crosslinks. After phenol-chloroform extraction and ethanol precipitation, DNA was electrophoresed on 1.5% agarose gel. The control samples (-MNase) were treated similarly except that no MNase was added.

TFIIB interaction was often observed in the absence of DNA. This suggested that Gal4 may be interacting with TFIIB through DNA under certain conditions. However, a reproducible Gal4-TFIIB interaction was always observed in the absence of DNA (Fig. 14, lane 14). These results demonstrate that an activator-TFIIB interaction occurs specifically during activated transcription in vivo.

II.3.2. TFIIB localization on the terminator region requires Pap1, Rna14 and Pcf11, but not Hrp1

Having shown the activator-TFIIB interaction during gene loop formation, we next investigated how TFIIB is facilitating gene looping. We reasoned that the promoter-bound TFIIB may be interacting with the factors present at the 3' end of the gene, and this interaction will bring the terminator and the promoter in close physical proximity. Our reasoning was based on three published results. First, TFIIB is known to exhibit genetic interaction with Ssu72, a component of CPF 3' end processing complex (278). Second, it has been recently demonstrated that TFIIB occupies both the promoter and terminator regions of *PMA1* and *BLM10* in an Ssu72-dependent manner (270). Third, looping of several yeast genes was abolished in *sua7-1*, a mutant of TFIIB that is defective in gene looping (270). Since TFIIB physically interacts with several gene specific activators in a transcription dependent manner, it was therefore a strong candidate for the factor mediating activator-dependent gene looping.

We therefore asked whether TFIIB association with the 3' end of genes is also dependent on termination factors such as Rna15, Rna14, Pcf11, Hrp1 and Pap1. TFIIB ChIP was therefore performed for *MET16* and *INO1* genes in the temperature-sensitive mutants of Rna15 (*rna15-2*), Rna14 (*rna14-1*), Pcf11 (*pcf11-2*), Hrp1 (*hrp1-5*), Pap1 (*pap1-1*) and isogenic wild type strains. TFIIB-ChIP was performed under induced transcriptional state of a gene at the permissive (25°C) and non-permissive (37°C) temperatures of the mutants. TFIIB crosslinked to both the ends of INO1, in the wild type strain at 25°C and 37°C during induced transcription (Fig. 16). In contrast, TFIIB crosslinking to the terminator was abolished in *rna15-2*, *rna14-1*, *pcf11-2* and *pap1-1* strains at restrictive temperature (37°C) (Fig. 16), while the crosslinking to the promoter remained intact (Fig. 16). Remarkably, TFIIB occupancy of the terminator region of

INO1 remained unaffected in *hrp1-5* strain following a temperature shift to 37°C (Fig. 16).

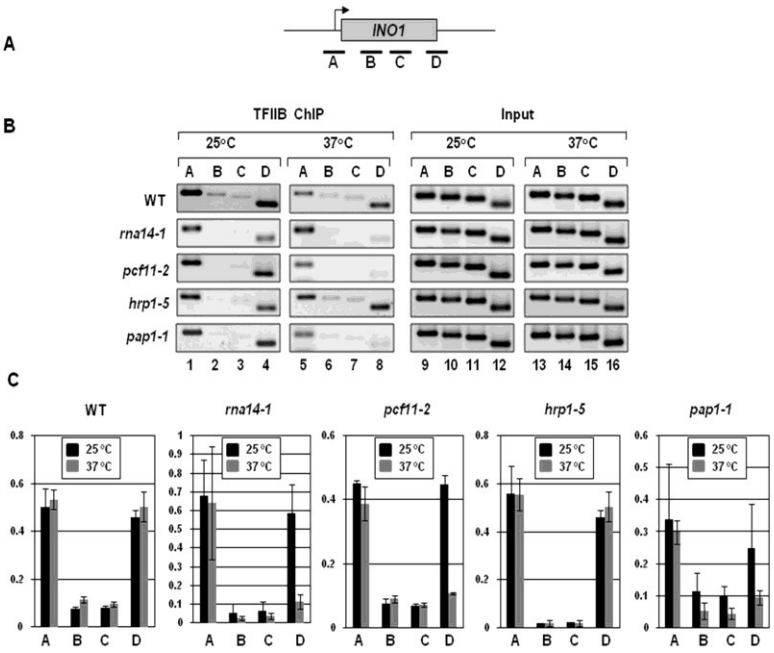

Figure 16. TFIIB crosslinking to the terminator region is dependent upon a functional CF1 complex and poly (A) polymerase. A, Schematic depiction of *INO1* indicating the position of ChIP primer pairs A, B, C and D. B, ChIP analysis showing crosslinking of TFIIB to different regions of *INO1* in wild type and mutant strains of Rna14 (*rna14-1*), Pcf11 (*pcf11-2*), Hrp1 (*hrp1-5*) and poly(A) polymerase (*pap1-1*) following 120 min. of induction andincubation at either permissive (25°C) or nonpermissive (37°C) temperatures. The Input signal represents DNA prior to immunoprecipitation. C, Quantification of the data shown in B, representing ChIP signal/Input signal.

Identical results were obtained with *MET16* (Fig. 17). Thus, Rna15, Rna14, Pcf11 and Pap1 are required for interaction of TFIIB with the 3' end of *MET16* and *INO1*. Hrp1 may not be required for TFIIB localization to the terminator regions of genes.

II.3.3. TFIIB forms a complex with CF1 subunits and Pap1

TFIIB is an essential general transcription factor (71). Recombinant TFIIB, with a molecular weight in the range of 32 to 38 kDa, could complement all functions of native, biochemically purified TFIIB in an in vitro transcription assay (113, 204, 244, 288). These results suggested that TFIIB is a single polypeptide protein that exists as a monomer in solution. There was no evidence of TFIIB being a part of a macromolecular

complex containing initiation factors or termination factors or any other protein. To find proteins associated with TFIIB under physiological conditions, the proteomic analysis was performed employing the TAP-approach (96, 97, 166). Neither a promoter nor a

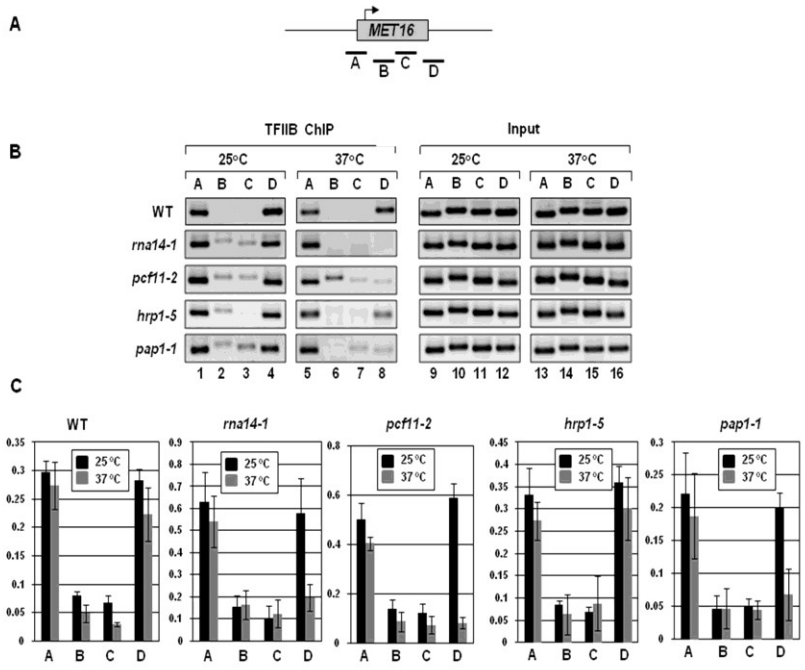

Figure 17. TFIIB crosslinking to the terminator region is dependent upon a functional CF1 complex and poly (A) polymerase. A, Schematic depiction of *MET16* indicating the position of ChIP primer pairs A, B, C and D. B, ChIP analysis showing crosslinking of TFIIB to different regions of *MET16* in wild type and mutant strains of Rna14 (*rna14-1*), Pcf11 (*pcf11-2*), Hrp1 (*hrp1-5*) and poly(A) polymerase (*pap1-1*) following 120 min. of induction andincubation at either permissive (25°C) or nonpermissive (37°C) temperatures. The Input signal represents DNA prior to immunoprecipitation. C, Quantification of the data shown in B, representing ChIP signal/Input signal.

terminator-bound factor was detected in the affinity purified TFIIB preparation in the first proteomic analysis carried out by Gavin et al., (97). However, in the second analysis, poly(A)-binding protein (Pab1) which interacts with the poly(A) tail of mRNA was identified as the only 3' end processing factor interacting with TFIIB (96). The study carried out by Krogan et al., found RNAP II subunits and two terminator-bound factors, CPF subunit Fip1 and Pab1 interacting factor Pan2, co-purifying with TFIIB (166). The absolute requirement of TFIIB in gene looping, crosslinking of TFIIB to both the promoter and the terminator regions of a looped gene and its functional interaction with

several 3' end processing/termination factors including CF1 subunit Rna15 (83, 270) suggested that a complex of TFIIB and termination factors exist in the cell.

We therefore investigated if TFIIB forms a macromolecular complex with CF1 subunits and Pap1 in yeast cells. Our experimental approach involved affinity purification of TFIIB followed by detection of CF1 subunits and Pap1 in the purified preparation by Western blot. To perform affinity purification of TFIIB, a triple hemagglutinin (3XHA) tag was inserted at the carboxy-terminus of TFIIB. Insertion of HA-tag did not interfere with the biological activity of TFIIB as both the transcription and gene looping of *MET16* and *INO1* remained unaffected in the tagged strain (data not presented). Additionally, a Myc-tag was integrated at the carboxy-terminus of each of the five subunits of CF1 complex and Pap1 for their detection by Western blot. Thus, six strains were constructed each carrying HA-tagged TFIIB and Myc-tagged version of one of the subunits of CF1 complex and Pap1.

Cell lysates from each of the six strains described above were purified over anti-HA-agarose beads. Proteins bound to the beads were eluted with HA oligopeptides. Western blot analysis of eluates revealed the presence of Rna14 (Fig. 18, lane 3), Rna15 (Fig. 18, lane 4), Pcf11 (Fig. 18, lane 5), Hrp1 (Fig. 18, lane 6), Clp1 (Fig. 18, lane 7) and Pap1 (Fig. 18, lane 8) in the affinity purified TFIIB preparation. As a control, purification was performed from a strain carrying untagged TFIIB. No signal for Rna14, Rna15, Pcf11, Clp1, Hrp1 and Pap1 was observed in the absence of HA-tagged TFIIB (Fig. 19), thereby confirming that the observed signals were due to the association of these factors with TFIIB. MNase digestion of cell lysate prior to affinity purification did not disrupt the association of CF1 subunits and Pap1 with TFIIB (Fig. 20, lanes 3 - 8).

These results indicate that the interaction of terminator-bound factors with TFIIB is not mediated by DNA or RNA. To rule out the possibility that copurification of Myc-tagged

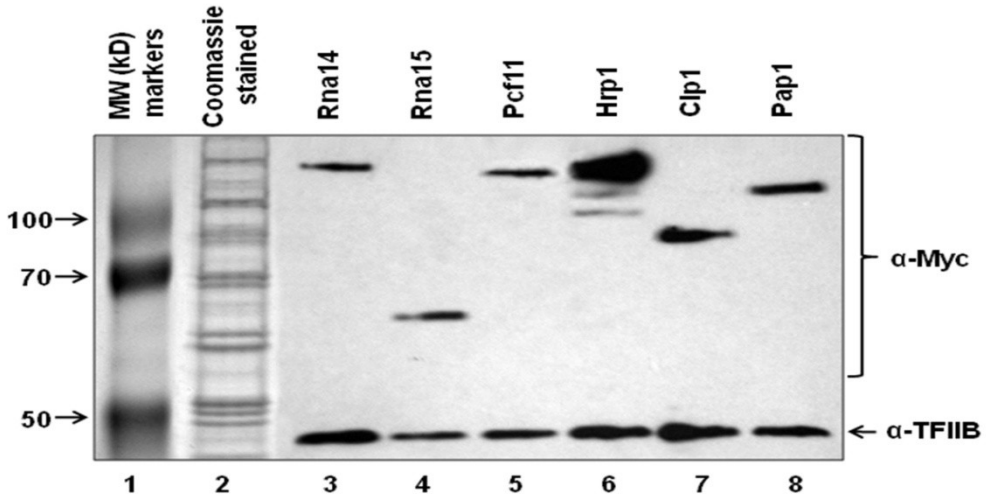

Figure 18. CF1 subunits and poly(A) polymerase copurify with TFIIB on an anti-HA affinity column. HA-tagged TFIIB was affinity purified from cells harboring Myc-tagged CF1 subunits or poly(A) polymerase. Purified samples were subjected to SDS-PAGE followed by Western blot analysis using anti-HA and anti-Myc antibodies. Lane 1 displays molecular weight marker proteins and lane 2 represents imperial Coomassie staining of the eluate from an anti-HA affinity column.

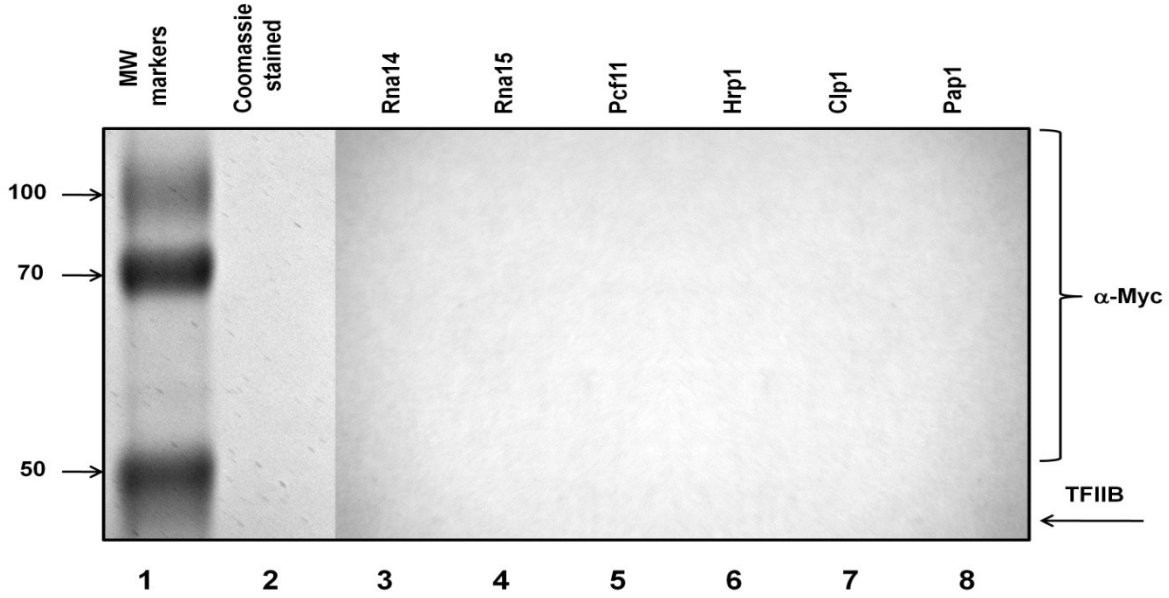

Figure 19. Association of CF1 subunits and poly(A) polymerase with TFIIB is not mediated by anti-HA beads. Affinity purification of untagged TFIIB was performed on anti-HA beads from a strain harboring Myc-tagged CF1 subunits and poly(A) polymerase as described in appendix C. Purified samples were subjected to SDS-PAGE followed by Western blot analysis as above. Lane 1 displays molecular weight marker proteins and lane 2 represents imperial Coomassie staining the eluate from an anti-HA affinity column.

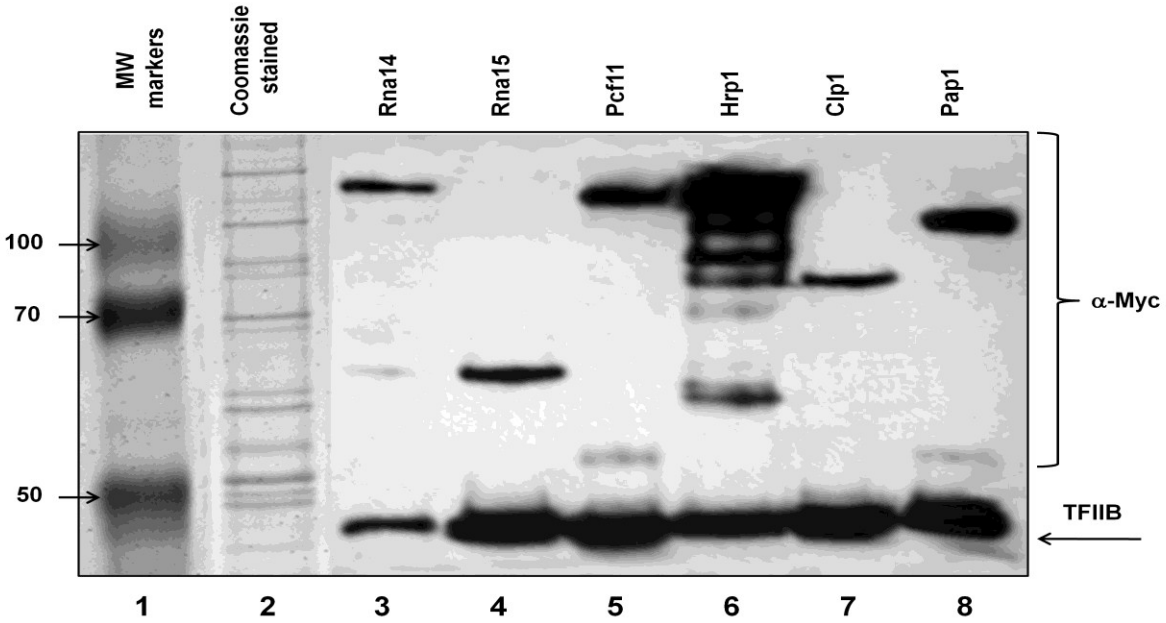

Figure 20. CF1 subunits and poly(A) polymerase copurification with TFIIB is MNase resisitant. Prior to affinity purification, samples were digested MNase as described in appendix C. Affinity purifications were performed for HA-tagged TFIIB from a strain harboring Myc-tagged CF1 subunits and poly(A) polymerase as above. Purified samples were subjected to SDS-PAGE analysis and followed by Western blot analysis as above. Lane 1 displays molecular weight marker proteins and lane 2 represents imperial Coomassie staining the eluate from an anti-HA affinity column.

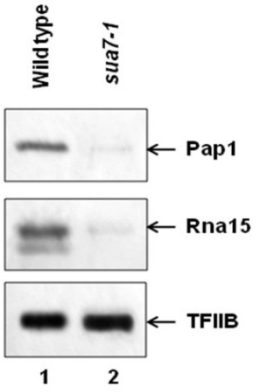

Figure 21. TFIIB interaction with Pap1 or Rna15. Affinity purifications were performed for HA-tagged TFIIB in the wild type and the looping defective mutant *sua7-1* strain. The eluates from the affinity columns were subjected to SDS-PAGE and Western blotting performed with antibodies against TFIIB, Rna15 and poly(A) polymerase.

CF1 subunits and Pap1 with TFIIB is not due to the interaction of the Myc-tag with TFIIB, the affinity purification was performed in a strain without a Myc-tag on any of the CF1 subunits or Pap1. Western blot analysis of affinity purified TFIIB, in this case using antibodies specifically directed against Pap1 and CF1 subunit Rna15, revealed that the interaction of these factors with TFIIB is not dependent on the Myc-tag (Fig. 21, lane 1). The affinity purification of a holo-TFIIB complex described above was performed at KCl concentration of 150 mM. To check the stability of the complex, we repeated the

purification at 500 mM KCl in the lysis buffer. High ionic strength did not affect the association of TFIIB with CF1 subunits and Pap1 during affinity purification (Fig. 22).

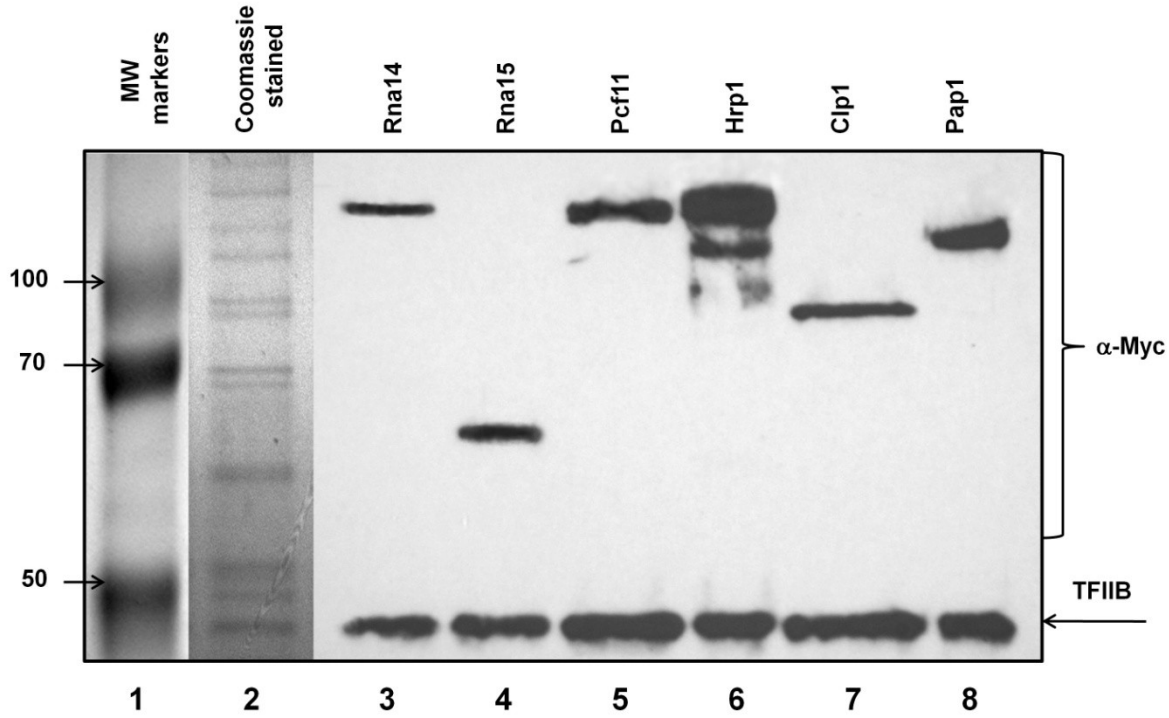

Figure 22. TFIIB interaction with CF1 subunits and poly(A) polymerase is stable at 500 mM KCl during affinity purification. Affinity purifications were performed for HA-tagged TFIIB in a strain harboring Myc-tagged CF1 subunits and poly(A) polymerase as described in appendix C in the presence of 500 mM KCl. Purified samples were subjected to SDS-PAGE analysis followed by Western blot analysis as above. Lane 1 displays molecular weight marker proteins and lane 2 represents imperial Coomassie staining the eluate from an anti-HA affinity column.

II.3.4. HOLO-TFIIB COMPLEX DOES NOT CONTAIN GENERAL TRANSCRIPTION FACTORS

TFIIB has been shown to interact, both genetically as well as physically, with TBP and RNAP II (71). We therefore checked for the presence of these proteins in the affinity purified TFIIB preparation using antibodies directed against TBP and Rpb1 subunit of polymerase. No signal for either TBP (Fig. 1B, lane 2) or Rpb1 (Fig. 1B, lane 4) was detected in the TFIIB preparation. We also did not find any evidence for the presence of another general transcription factor TFIIH in the affinity purified TFIIB

preparation. Western blot analysis using antibodies against TFIIH subunit Kin28 confirmed the absence of the factor in the TFIIB preparation (Fig. 1B, lane 6). These

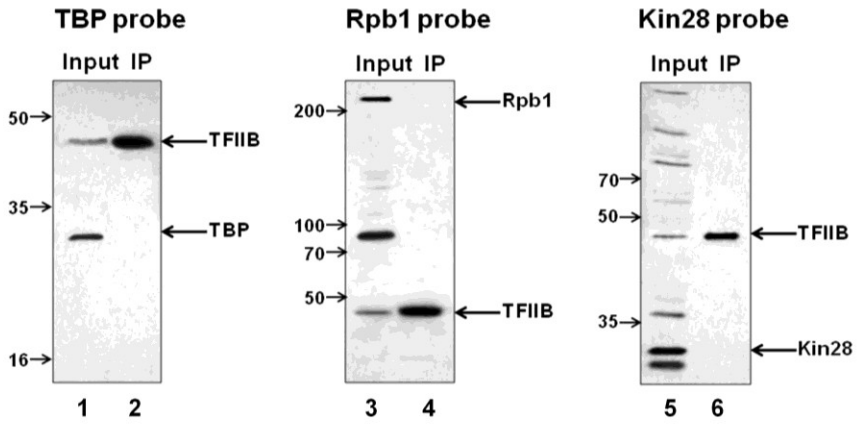

Figure 23. TFIIB only transiently interacts with PIC components. Affinity purified HA-tagged TFIIB was subjected to SDS-PAGE followed by Western blot analysis using anti-HA, anti-TBP, anti-Kin28 and anti-Rpb1 antibodies.

results suggest that the holo-TFIIB complex does not contain the factors that transiently interact with it during the transcription cycle.

II.3.5. HOLO-TFIIB COMPLEX IS NOT OBSERVED IN LOOPING DEFECTIVE CELLS

To determine the physiological significance of TFIIB-CF1-Pap1 complex in the context of gene looping, affinity purification of TFIIB was performed in a looping deficient mutant strain of TFIIB called *sua7-1*. Affinity purified TFIIB preparation from *sua7-1* cells was subjected to SDS-PAGE analysis followed by Western blotting using antibodies against Pap1 and CF1 subunit Rna15. Our results show that neither Pap1 nor Rna15 were found associated with TFIIB in *sua7-1* strain (Fig. 21, lane 2). Thus, TFIIB association with the terminator-bound factors occurred in a looping-dependent manner. These results argue in favor of a TFIIB-CF1-Pap1 complex playing a crucial role in loop formation.

II.3.6. GLYCEROL GRADIENT ANALYSIS OF HOLO-TFIIB COMPLEX

To further confirm that a holo-TFIIB complex exists in yeast cells, affinity purified TFIIB was subjected to sedimentation analysis on a linear 5-30% (v/v) glycerol gradient in the presence of 150 mM KCl. Western blot analysis revealed that TFIIB fractionated as a single peak spanning fractions 12 to 19 (Fig. 24). Pap1, as well as CF1 subunits: Rna14, Rna15, Pcf11, Hrp1, and Clp1, cosedimented with TFIIB (Fig. 24). The peak of

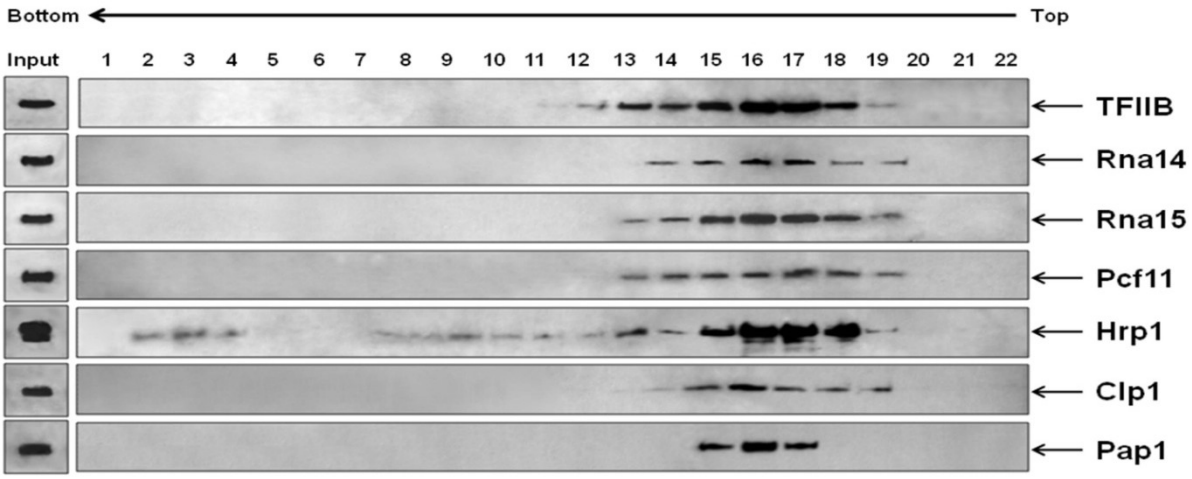

Figure 24. Sedimentation analysis reveals copurification of CF1 subunits and poly(A) polymerase with TFIIB. Affinity purified HA-tagged TFIIB was subjected to sedimentation analysis in 5 to 30% (v/v) glycerol gradient in 150 mM KCl. Fractions of 1.8 ml each were collected and subjected to SDS-PAGE analysis followed by Western blotting to visualize TFIIB, CF1 subunits and poly(A) polymerase. Input bands indicate affinity purified sample prior to sedimentation analysis.

CF1 subunits and Pap1 coincided with the TFIIB peak in fraction number 16 (Fig. 24). To conclusively prove that TFIIB cosedimenting with CF1 subunits and Pap1 is not free TFIIB, but TFIIB in a complex with 3' end processing/termination factors, it was important to determine the sedimentation behavior of free TFIIB. For this, we purified recombinant TFIIB from bacteria and carried out sedimentation analysis under identical conditions. Recombinant TFIIB sedimented in fractions 19 to 22 with the peak centered

on fraction number 20 (Fig. 25). These results suggest that almost all TFIIB in the affinity purified preparation is in complex with 3' end processing/termination factors.

However, when sedimentation analysis of affinity purified TFIIB was carried out in the gradient made in 500 mM KCl, TFIIB was separated from CF1 complex and

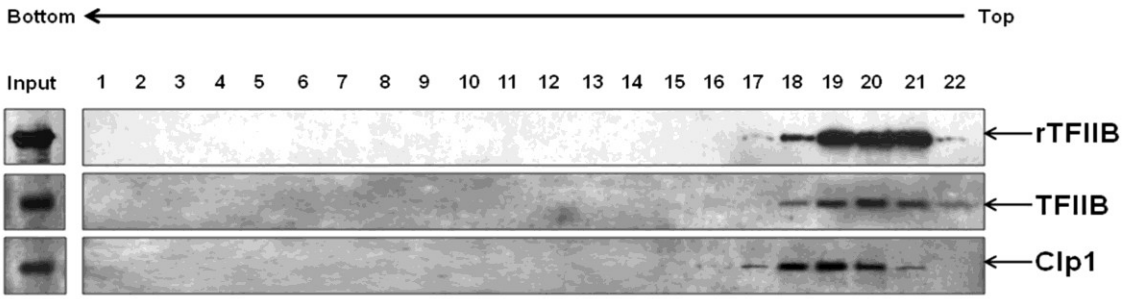

Figure 25. Free TFIIB has a higher sedimentation profile. Affinity purified His-tagged recombinant TFIIB (rTFIIB) was subjected to sedimentation analysis in 5 to 30% (v/v) glycerol gradient in 150 mM KCl. As a comparison for free TFIIB, affinity purified HA-tagged TFIIB in a stain also harboring a Clp1-Myc tag was subjected to identical sedimentation analysis in 5 to 30% (v/v) glycerol gradient in 500 mM KCl. Fractions were collected and processed as above.

sedimented at a lower rate in fractions 18 to 22 with the peak in fraction number 20 (Fig. 25). To further corroborate the position of free TFIIB in the glycerol gradient, sedimentation analysis of affinity purified TFIIB was also performed in a gradient which contained 0.1% SDS. TFIIB and two subunits of CF1 (Rna14 and Rna15) were all found in higher peak fractions near 19-20 (Fig. 26). These results correlate with the position of free TFIIB based on the recombinant TFIIB peak centered on fraction 20. Thus, holo-TFIIB complex is not stable upon prolonged exposure to high salt in a centrifugal field, though it is able to withstand high ionic strength for a short period of time during affinity purification.

We then compared the sedimentation profiles of the holo-TFIIB complex with the sedimentation profiles of RNAP II, TFIID and TFIIH complexes. The affinity purified

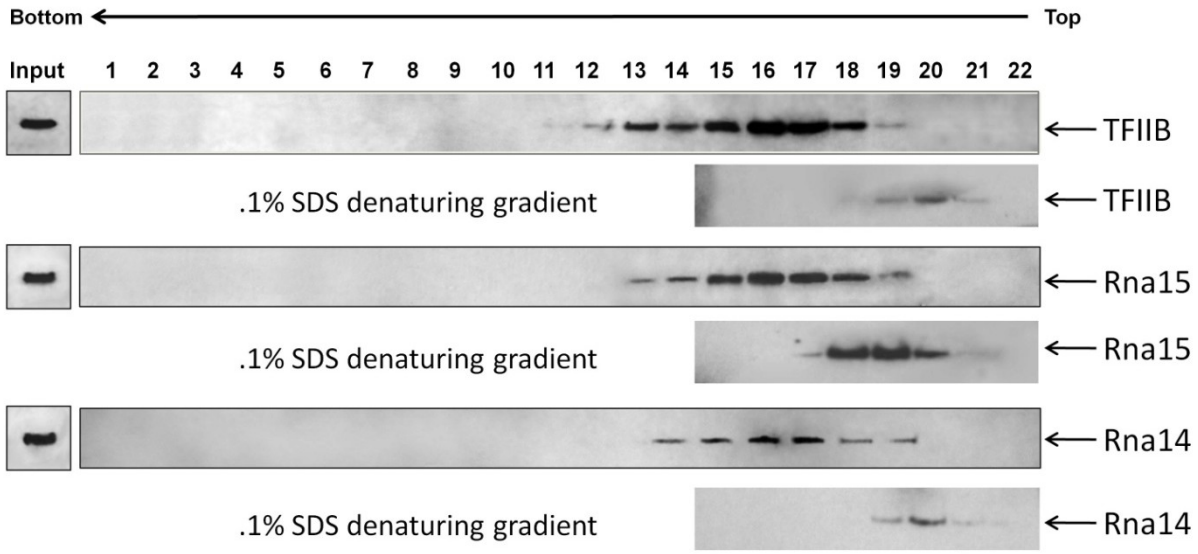

Figure 26. TFIIB migrates at a higher sedimentation profile under denaturing conditions. Affinity purified HA-tagged TFIIB in stains also harboring either a Rna14-Myc or Rna15-Myc tag were subjected to identical sedimentation analysis in 5 to 30% (v/v) glycerol gradient in 0.1% SDS. Fractions were collected and processed as above.

TFIIB, RNAP II, TFIID and TFIIH were subjected to sedimentation analysis under identical conditions (Fig. 27). The presence of RNAP II, TFIID and TFIIH in the gradient fractions was detected by Western blot analysis using antibodies against the Rpb1, TBP and Kin28 subunits of RNAP II, TFIID and TFIIH respectively. RNAP II sedimented in fractions 3 to 8 with the peak in fraction number 6 (Fig. 27). TFIID, which has a molecular weight of about 750 kDa (23), sedimented in fractions 7 to 15 with the peak in fraction number 11 (Fig. 27), while TFIIH with an approximate molecular weight of 500 kDa (80) sedimented in fractions 13 to 20 (Fig. 27). Thus, the sedimentation coefficient of the holo-TFIIB complex is intermediate between that of the TFIID and TFIIH complexes. We also looked for the presence of TFIIB in the gradient purified TFIID and TFIIH preparations. No signal for TFIIB was detected in the TFIID, TFIIH or RNAP II

glycerol gradient fractions (Fig. 27). This corroborated our earlier results that TFIIB is not in a complex with general transcription factors.

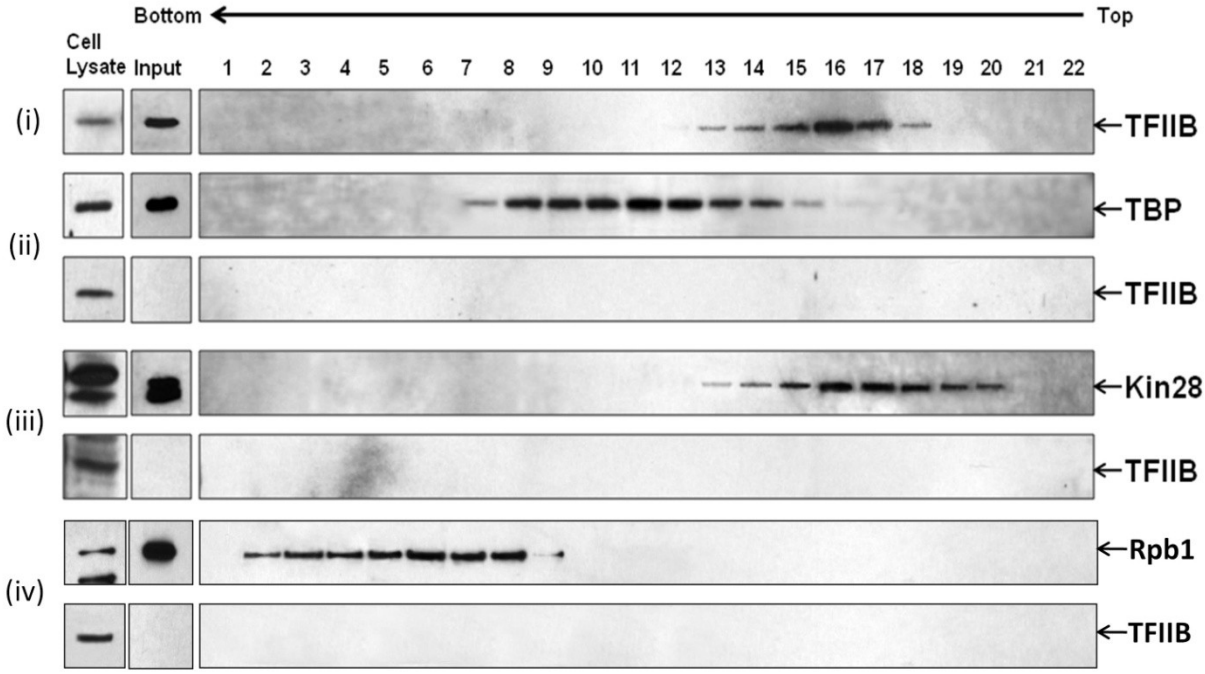

Figure 27. Holo-TFIIB complex sediments between TFIID and TFIIH complexes. Affinity purified preparations of TFIIB (i), TBP (ii), Kin28 (iii) and Rpb1 (iv) were subjected to sedimentation analysis in 5 to 30% (v/v) glycerol gradient in 150 mM KCl as described previously. Fractions were collected, processed as above and probed for TFIIB and TBP, Kin28 or Rpb1.

II.3.7. SIZING COLUMN CHROMATOGRAPHY OF THE HOLO-TFIIB COMPLEX

Sedimentation analysis cannot provide accurate information about the size of the complex because it measures the relative buoyancy in the gradient. In order to find out the size of the holo-TFIIB complex, size exclusion chromatography was performed using the HA-affinity purified TFIIB preparation on a superdex-200 column. The column was calibrated with known sizing column markers. Elution profiles for each size marker ranging in size from 669 kDa (Thyroglobin) to 158 kDa (Aldolase) was monitored for UV absorbance and plotted (Fig 28). The superdex-200 column can accurately determine the molecular sizes above 660 kDa. The elution profile of the affinity-purified TFIIB was

monitored by UV as well as western blotting to confirm the presence of TFIIB with CF1 subunits indicating the holo-TFIIB complex was still intact after sizing column

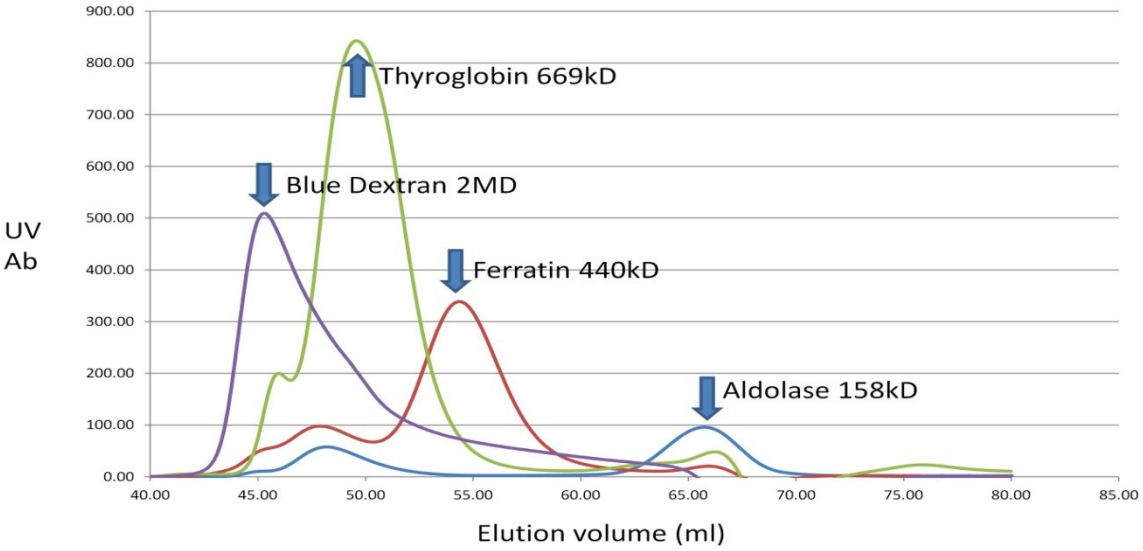

Figure 28. Elution profile for sizing markers after size exclusion chromatography with a Superdex 200 column.

chromatography (Fig 29). The peak fraction centered around an elution volume of 45 ml, which was near the 2 MDa blue dextran elution volume, indicating that the holo-

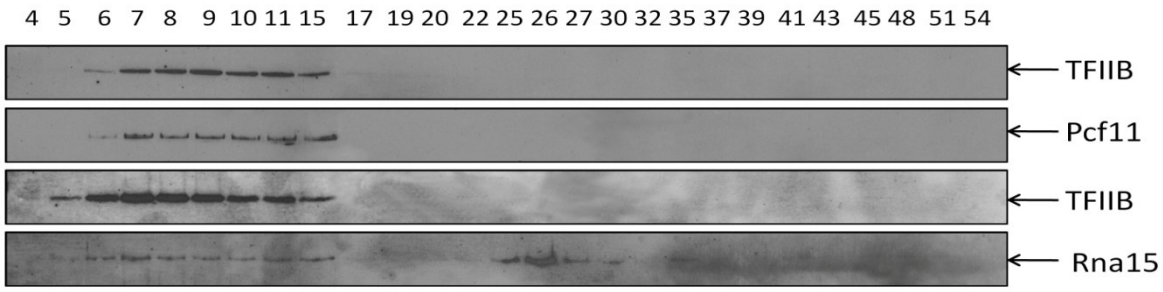

Figure 29. Holo-TFIIB complex is intact after size exclusion chromatography. Affinity purified preparations of HA-TFIIB strains harboring either Myc tagged Pcf11 or Rna15 were subjected to size exclusion chromatography using a Superdex 200 column as described in appendix C. 1 ml fractions were collected and subjected to SDS-PAGE analysis followed by Western blotting to visualize TFIIB and CF1 subunits.

TFIIB complex is less than 2 MDa (Fig 30). However, we could not accurately determine the size of holo-TFIIB complex from this column.

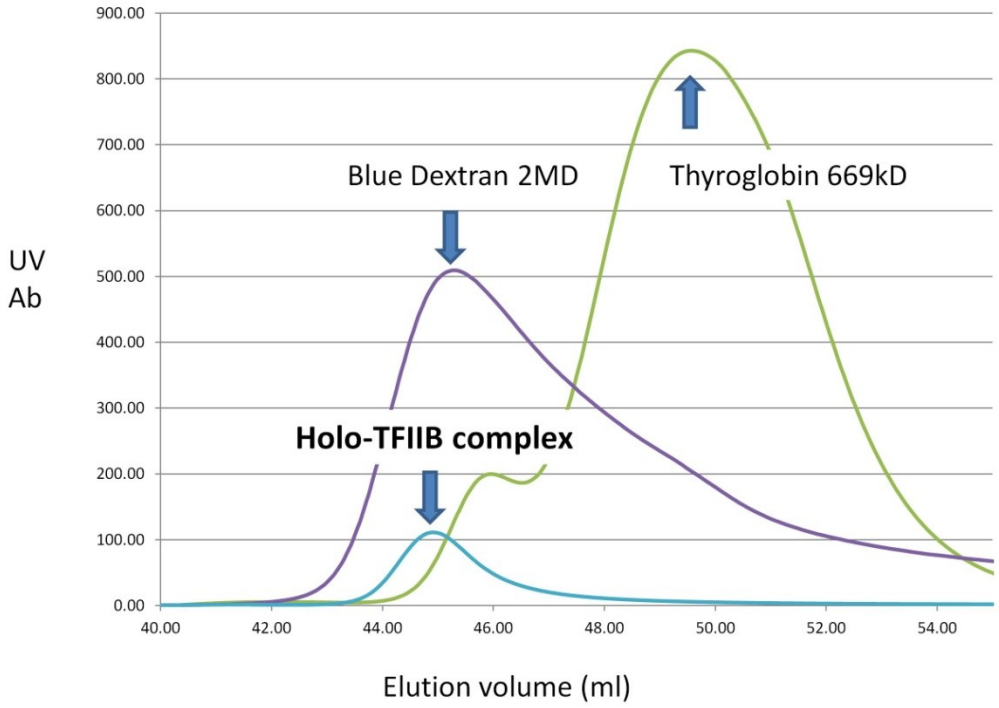

Figure 30. Holo-TFIIB complex is beyond the resolution for the Superdex 200 column. Affinity purified preparations of HA-TFIIB strains harboring either Myc tagged Pcf11 or Rna15 were analyzed as above. UV absorbance was plotted by fractions collected and the data was combined to identify the size of the Holo-TFIIB complex.

II.3.8. TFIIB AFFINITY PURIFICATION SPECIFICALLY ENRICHES FOR TFIIB IN A HOLO-TFIIB COMPLEX

Interestingly, there was no detectable signal for free TFIIB in either the glycerol gradient or the sizing column fractions. One possibility is that most of the TFIIB inside the cell is present in the holo-TFIIB complex. In order to address this issue, size exclusion chromatography was performed using a whole cell extract prepared from a strain that harbored the HA-tagged TFIIB. The purified fractions were then examined by Western blotting for TFIIB. A similar peak for the holo-TFIIB complex was observed around the elution volume of 45 ml along with three additional peaks near elution volumes of 52, 59 and 68 ml (Fig 31). A reasonable estimate based on the blotting intensities, would be that approximately 25-30% of the TFIIB in the cell forms a holo-

TFIIB complex. One possible explanation for this selective enrichment during affinity

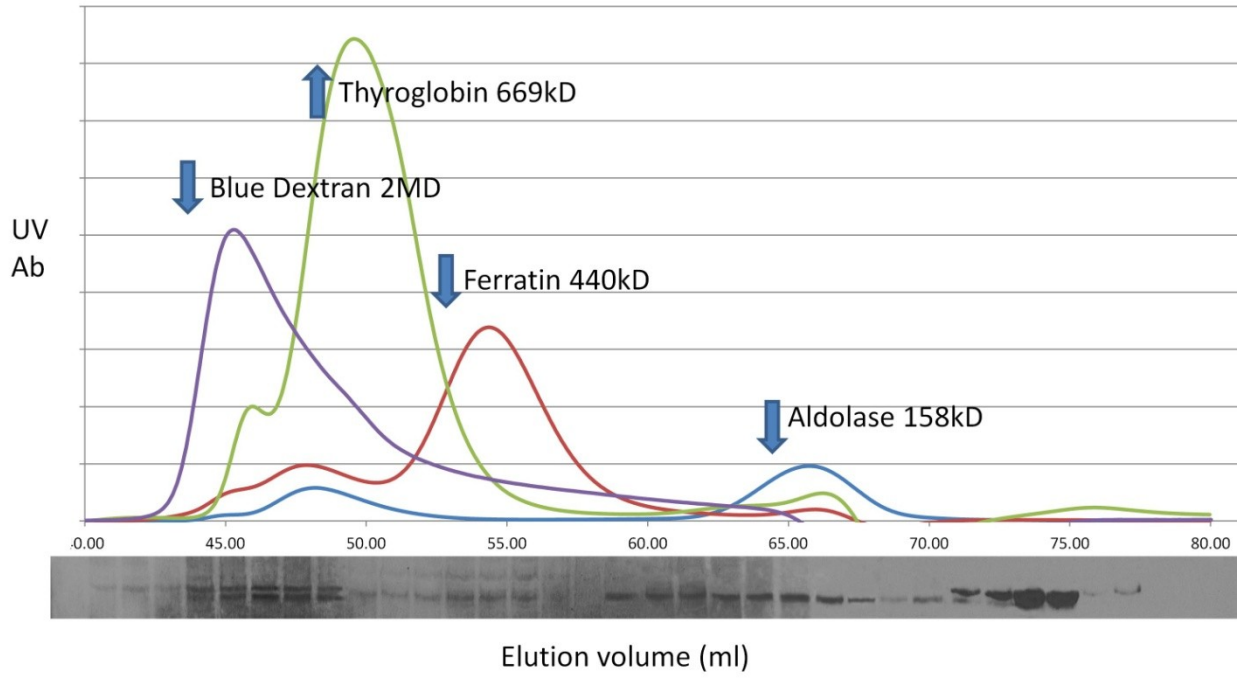

Figure 31. Affinity purification of TFIIB selectively enriches for the Holo-TFIIB complex. Whole cell extract was prepared from a strain harboring the HA tag on TFIIB and size exclusion chromatography was performed as in appendix C. Fractions were collected and analyzed as above for Western blot and UV absorbance.

purification could be due to the two different conformations of TFIIB. In the closed conformation, the N terminus and C terminus interact in a manner similar to a closed wallet which could obfuscate the HA tag on the C-terminus. In order for TFIIB to interact with activators and RNAP II, it switches to an open conformation where the N- and C-terminus are separated and free to physically interact with other proteins. TAP analysis of TFIIB were also attempted in an effort to increase the yield over the oligopeptide elutions. However, the addition of a TAP-tag to the C-terminal of TFIIB disrupted its physical interaction with the CF1 subunits, and this strategy wasn't pursued further.

II.3.9. KINETICS OF ACTIVATED TRANSCRIPTION IS COMPROMISED IN THE ABSENCE OF GENE LOOPING

We have earlier demonstrated that gene looping is conferred by the activator-dependent interaction of the promoter-bound proteins with the terminator-bound factors (83). Here we provide evidence for the existence of a complex of promoter-bound TFIIB with the terminator-associated factors in yeast cells. This complex could be the molecular basis of gene looping as it exists only in the looping competent strains, but not in the looping defective strain. Gene looping has been proposed to enhance transcription efficiency of a gene by coupling termination to reinitiation (235).

In such a scenario, efficiency of transcription is expected to decrease in the absence of gene looping. We therefore compared kinetics of activated transcription of *MET16* and *INO1* in wild type cells that harbor holo-TFIIB complex and in the looping defective *sua7-1* strain. Our results suggest that although both *MET16* and *INO1* exhibited induced transcription in *sua7-1* strain, activated transcription exhibited a kinetic lag in the looping defective strain (Fig. 32). The level of *MET16* RNA in *sua7-1*

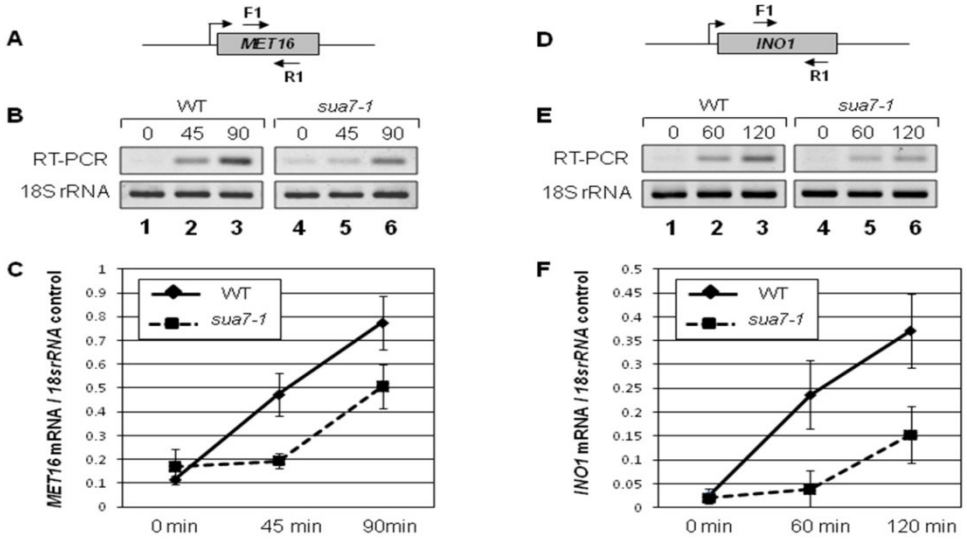

Figure 32. Induced transcription in the looping defective mutant of TFIIB exhibits a kinetic lag. *A* and *D*, Schematic depiction of *MET16* and *INO1* indicating the position of RT-PCR primer pairs. *B* and *E*, RT-PCR analysis of *MET16* and *INO1* in wild type (WT) and looping defective *sua7-1* strains following transfer of cells to transcription inducing conditions at indicated time points. *C* and *F*, Quantification of the data shown in *B* and *E*.

cells was about 1.5 times less than in isogenic wild type strain following 90 min after transfer of cells to inducing conditions (Fig. 32, lanes 3 and 6; Fig. 7C). Similarly, *INO1* RNA level in *sua7-1* cells was approximately 2.5 times less than in wild type cells at 120 min after induction of transcription (Fig. 32, lanes 3 and 6; Fig. 7F). A possible interpretation of these results is that a looped conformation helps a gene to achieve higher transcription efficiency within a short period of time following exposure of the cells to induction signal.

II.4. DISCUSSION

In this study, we have analyzed gene looping during transcriptional activation of protein encoding genes in budding yeast. Our results show that gene specific activators physically interact with TFIIB in a transcription dependent manner. An activator may function in association with TFIIB to keep a gene in the activated state through multiple rounds of transcription by facilitating reinitiation through gene looping. TFIIB plays a crucial role in gene looping. It has been proposed that the presence of TFIIB at the distal ends of a gene and a simultaneous absence of TBP from the terminator region are strong indicators of gene looping (83, 270). Our results with *MET16* and *INO1* corroborate this view. The first round of transcription requires the recruitment of all general transcription factors and RNAP II on the promoter to form a PIC. Following initiation of transcription, most of the general transcription factors are left behind on the promoter in the form of a scaffold (325). During scaffold-based reinitiation, very few components have to be recruited back to the promoter to form a preinitiation complex. Reinitiation is therefore faster than initiation at least under in vitro conditions (143). If RNAP II is transferred directly from the terminator to the scaffold, the rate of reinitiation

is expected to be augmented even further (74). A gene looping assisted transfer of polymerase from the terminator to the promoter, with a concomitant increase in transcription efficiency, has been demonstrated during mitochondrial transcription (208). Here we demonstrate that there is a kinetic lag in the looping defective TFIIB mutant, which strongly suggests that RNAP II transfer from the terminator may be leading to higher rates of reinitiation. Other work from our lab, which further corroborates this hypothesis, has shown that in the termination defective mutants, gene looping is abrogated and results in a 2 fold decrease of RNAP II recruitment on the promoter (6).

The localization of TFIIB on the terminator during active transcription and its genetic interaction with Ssu72 indicate that TFIIB could physically interact with the termination factors to mediate the formation of a gene looping complex. The results presented here show that TFIIB associates with the CF1 3' end processing complex and Pap1 in yeast cells. We provide several lines of evidence in support of the existence of a complex of TFIIB and termination factors. First, cross-linking of TFIIB to the 3' end of the gene, which is essential for loop formation, was abolished in looping-defective temperature-sensitive mutants of Pap1 and the CF1 subunits Rna14 and Pcf11 at non-permissive temperatures. Second, affinity purification of HA-tagged TFIIB yielded a complex composed of Pap1 as well as CF1 subunits Rna14, Rna15, Pcf11, Clp1, and Hrp1. Third, a holo-TFIIB complex is devoid of known TFIIB interacting proteins such as RNAP II and TFIID. Thus, a TFIIB complex is not formed by transiently interacting proteins. Fourth, a glycerol gradient sedimentation profile of affinity-purified TFIIB showed a TFIIB peak cosedimenting with CF1 subunits and Pap1. Fifth, the sedimentation rate of affinity purified TFIIB is more than that of free TFIIB, thereby

suggesting that it is in a complex. These results provide strong support in favor of the existence of a macromolecular complex composed of TFIIB and 3' end processing factors in yeast cells (Figure 33).

Our results indicate that the association of TFIIB with Pap1 and the CF1 complex

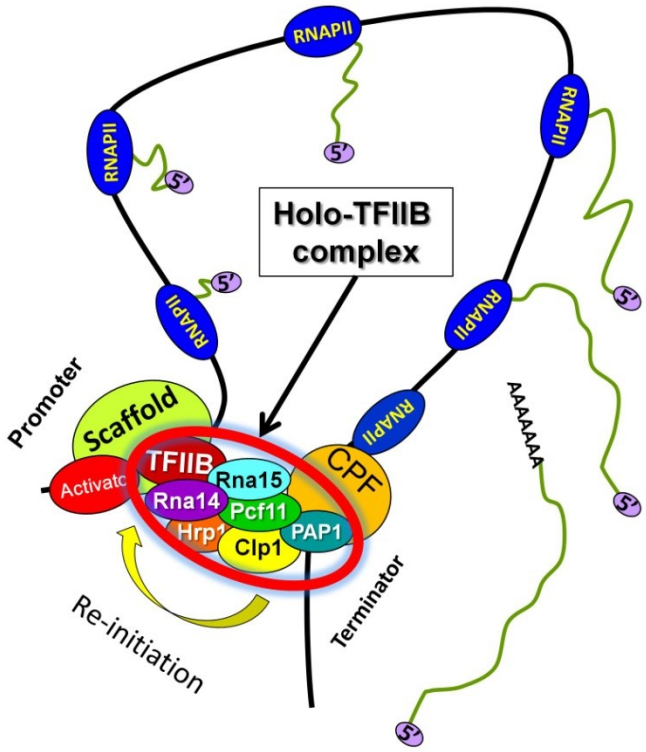

Figure 33. Schematic diagram of a gene loop where a Holo-TFIIB complex could facilitate transfer of RNAP II during re-initiation.

occurs only when the conditions are favorable for gene looping. First, Pap1 and CF1 subunit, Rna15, associate with TFIIB in a looping-competent strain. No such association was observed in looping-deficient *sua7-1* strain. Second, other work from our lab demonstrated CF1 subunits and Pap1 were found localized to the 5' end of a gene only when it was in a looped conformation (212). These results suggest that a complex of TFIIB, Pap1, and CF1 is formed at the promoter-terminator junction to facilitate loop formation.

We expected at least two populations of TFIIB in a cell: 1) free TFIIB that is not engaged in transcription; 2) TFIIB in association with the terminator-bound factors on genes that are in looped configuration. Contrary to our expectation, we did not find any low sedimentation coefficient peak of free TFIIB in the glycerol gradient nor was there any indication of free TFIIB in the fractions collected from the sizing column. One possible reason for this could be that our affinity purification step is selectively purifying the holo-TFIIB complex. Following the elution of TFIIB from affinity beads using oligopeptides, there was still a substantial amount of TFIIB bound to the beads that could be eluted with 0.5% SDS (data not presented). This tightly bound TFIIB could be free TFIIB that was not be eluted with the anti-HA oligopeptides. Accordingly, we demonstrated the presence of free TFIIB when the cell lysate was directly fractionated on the sizing column. The earlier attempt to purify native TFIIB from yeast did not observe a holo-TFIIB complex (288). A possible explanation for this is that the holo-TFIIB complex is not stable upon prolonged exposure to high ionic strength. When we performed sedimentation analysis of affinity-purified TFIIB at 500 mM KCl, TFIIB dissociated from the complex and sedimented at the position of free TFIIB. During the purification of native TFIIB from budding yeast by Tschochner et al. (288), at several steps in the purification protocol, ionic strength equivalent to or higher than 500 mM potassium acetate was used. This may have resulted in separation of TFIIB from the termination factors, and consequently, a holo-TFIIB complex was not observed. The holo-TFIIB complex may also include factors other than CF1 subunits and Pap1. It is likely that some components of CPF complex are present in the TFIIB macromolecular assembly. Ssu72, which is a subunit of CPF complex, exhibits a genetic as well as a

physical interaction with TFIIB, and is a strong constituent candidate of the TFIIB complex (72, 125, 278, 317). The presence of Pab1, Pab1-binding protein Pan1, and CPF subunit Fip1 in the tandem affinity-purified preparation of TFIIB makes them likely components of the TFIIB complex as well. Also, the presence of some promoter-bound factors in the TFIIB preparation cannot be ruled out.

A similar interaction of mammalian TFIIB with CPSF and CstF, which are homologues of yeast CPF and CF1 cleavage and polyadenylation complexes, has also been observed (296). TFIIB exhibited a physical interaction with CstF-64 and mSsu72 subunits of CstF and CPSF complexes, respectively. CstF-64 and mSsu72 were also found cross-linked to the distal ends of a gene in a manner analogous to their yeast counterparts. Furthermore, TFIIB phosphorylation was required for the recruitment of CstF-64 and mSsu72 to the promoter region of a gene. Whether association of TFIIB with CstF and CPSF complexes facilitates juxtaposition of the promoter and terminator regions to form a gene loop in higher eukaryotes remains to be elucidated.

TFIIB is absolutely required for initiation of transcription and its interactions with promoter-bound factors are well established. The essential role of TFIIB in gene looping, its interaction with the terminator-bound factors, and the kinetic delay in induced transcription suggests a novel role of TFIIB in looping-mediated transcriptional regulation. A genomewide search found TFIIB occupying both the promoter and the terminator regions of at least 120 genes in yeast (209). These results suggest that gene looping could be a general feature of transcriptionally activated genes in budding yeast. The role of gene looping may not be restricted to activation of transcription. We expect promoter-terminator interaction to have a wider implication in eukaryotic transcription. It

has been recently demonstrated that gene looping juxtaposes an inhibitory regulatory element located at the 3' end of BRCA1 gene near its promoter region, leading to transcriptional repression of the gene in breast tumor cell lines (282). In this case, gene looping represses rather than activates transcription. In human B- and T-lymphocytes, interaction of the promoter with the terminator region of CD68 had an effect on the splicing of its primary transcript (234). A role for gene looping in preventing transcription interference has also been proposed in budding yeast, where gene density is high with little intervening space between neighboring genes (235). Gene looping may have different consequences, but it is certainly emerging as a general, possibly ubiquitous, transcription regulatory mechanism among eukaryotes.

CHAPTER III

A NOVEL ROLE OF TFIIH KINASE, KIN28, IN TERMINATION OF TRANSCRIPTION IN BUDDING YEAST

III.1. ABSTRACT

TFIIH is a general transcription factor with two different enzymatic activities, a kinase and a helicase activity. The kinase activity resides in the Kin28 subunit of TFIIH. There are conflicting reports regarding the role of Kin28 kinase in transcription cycle. Using an analog-sensitive mutant of Kin28, here we show that the Kin28 kinase is required for optimal transcription of both inducible and non-inducible genes in budding yeast. Transcription run-on analysis confirmed that the Kin28 kinase dependent enhancement of transcript level was the consequence of a direct affect of the kinase on transcription rather than on RNA stability. More importantly, RNAP II reads through the termination signal into the downstream regions of genes during kinase inhibition, thereby indicating a termination defect. The recruitment of Rna15 and Ssu72 subunits of CF1 and CPF termination complexes respectively near the 3′ end of genes was adversely affected in the kinase mutant. Both Rna15 and Ssu72 coimmunoprecipitated with Kin28 thereby suggesting a physical interaction of the kinase with the CPF and CF1 termination complexes. ChIP analysis revealed Kin28 crosslinking to both the 5′ and the 3′ ends of transcriptionally active genes. The localization of Kin28 towards the 3′ end of genes and its interaction with Rna15 and Ssu72, however, was compromised in the kinase-defective mutant. These results strongly suggest a novel role of Kin28 kinase in termination of transcription. CCC analysis revealed that gene looping, which is the physical interaction of the promoter and terminator regions of a gene during

transcription, was severely compromised in the Kin28 kinase mutant. We propose that Kin28 kinase dependent gene looping could be playing a crucial role in TFIIH-mediated termination of transcription in budding yeast.

III.2. INTRODUCTION

Although TFIIH is a general transcription factor, its fundamental role in cell is not limited to transcription. It has additional cellular functions in DNA repair, and cell cycle regulation (80). Mutations in TFIIH subunits Ssl2 and Rad3 (XPB and XPD in humans) adversely affect both transcription and DNA repair, and cause autosomal recessive disorders; xeroderma pigmentosum, Cockayne syndrome and trichothiodystrophy (61). These diseases are characterized by predisposition of the affected individuals to cancer, ageing, developmental and neurological defects. The multiplicity of functions has made TFIIH the focus of intense investigation.

TFIIH is a multisubunit factor with a molecular weight of more than 500 kDa. It has been remarkably conserved during evolution, and has essentially the same subunit structure in yeast and higher eukaryotes (20, 99). The holo-TFIIH complex is composed of 11 subunits organized into two subcomplexes (Fig. 13) (224). The subunit organization of TFIIH reflects its multiplicity of functions in the cell. The core subcomplex comprising of Tfb1, Tfb2, Ssl1, Tfb4, Tfb5 and Rad3 in budding yeast, is required for transcription as well as DNA repair (99). Another subunit Ssl2 is loosely associated with the core subcomplex, and is indispensable for both transcription and repair of damaged DNA (224). Recently, an additional subunit Tfb6 was identified that facilitates dissociation of Ssl2 from the core subcomplex following the initiation of transcription (224). The kinase subcomplex, also known as TFIIK, is composed of Tfb3,

the cyclin Ccl1, and the cyclin-dependent kinase Kin28 (93). TFIIK phosphorylates the carboxy-terminal domain (CTD) of RNAP II at serine-5 and serine-7 residues, and is required for transcription as well as cell cycle control (3, 156). The structural analysis revealed TFIIH as an open ring like structure with a hole in the center that contacts DNA. The three-dimensional reconstruction of TFIIH on the basis of electron microscopy and X-ray diffraction studies places critical catalytic subunits of TFIIH in proximity of their target substrates (46). Ssl2 helicase crosslinked to DNA about 30 bp downstream of the transcription start site, while Kin28 kinase was found positioned near its CTD substrate. Rad3 helicase was localized near the DNA upstream of the transcription initiation bubble (126).

TFIIH is the only GTF with two distinct enzymatic activities: the DNA-dependent helicase activity and the cyclin-dependent kinase activity (80, 99). The helicase activity resides in two subunits, Ssl2 and Rad3, while Kin28 is the cyclin-dependent kinase. Ssl2 contacts template downstream of the transcription start site, and act as a molecular wrench to unwind DNA beyond the transcription bubble (126). This causes collapse of the initiation bubble, and facilitates release of the polymerase from the promoter for elongation, a step often referred to as the promoter clearance (199). Kin28 kinase phosphorylates serine-5 of CTD in the initiation complex (35, 159). The CTD-serine-5 phosphorylation is believed to disrupt the interaction of the polymerase with the components of the initiation complex, thereby helping further in the promoter clearance (4, 196, 279, 308). The serine-5 phosphorylation also facilitates recruitment of the capping enzyme for 5' end processing of nascent mRNA (93, 251). Kin28 was recently found to additionaly phosphorylates serine-7 of CTD (3, 79, 156).

There are contradictory reports regarding the role of TFIIH kinase, Kin28, in transcription by RNAP II (20, 136, 147, 196). Transcription starts with the formation of PIC at the promoter. During PIC assembly, recruitment of GTFs and polymerase takes place in an ordered fashion, starting with TFIID followed by TFIIA, TFIIB, RNAP II-TFIIF, TFIIE and TFIIH in that order (Fig. 3) (111, 126, 248, 269, 285). RNAP II is recruited in a completely dephosphorylated form. The phosphorylation of CTD is not required either for PIC assembly or initiation of transcription (5, 35). The polymerase initiates transcription while sitting on the promoter, and still in contact with the general transcription factors (36, 164). The phosphorylation of serine-5 of CTD takes place immediately after initiation (159). The role of serine-5 phosphorylation has been found crucial for promoter clearance, which is the release of polymerase from the initiation complex for elongation (35, 142, 202, 308). The significance of serine-5 phosphorylation in the recruitment of capping enzyme for 5' end processing of mRNA has been unequivocally demonstrated (53, 89, 211, 251). Our initial understanding of the function of TFIIH kinase under physiological conditions has come from studies with the temperature-sensitive mutants of Kin28 in budding yeast. Kin28 is an essential gene in yeast, as the cells lacking Kin28 are not viable. The shifting of Kin28 mutants to the elevated temperature adversely affected the recruitment of TFIIH complex at the promoter resulting in a dramatic decrease in the CTD-serine-5 phosphorylation, and a concomitant decrease in the level of steady state mRNA level in the cells (134, 196). The temperature-sensitive mutation has been found to affect the catalytic activity of Kin28 kinase as well as its interaction with other subunits of TFIIH (144, 151). To determine the specific role of Kin28 kinase in transcription by RNAP II, the ATP-binding

pocket of the enzyme was engineered to make it respond to a specific inhibitor NA-PP1, which is an analog of ATP. In the presence of NA-PP1, the kinase activity of analog-sensitive Kin28 mutant (Kin28-as) is almost completely inhibited without affecting its interaction with the subunits of TFIIH complex (196). The studies using Kin28-as mutant revealed that the kinase activity is not required for recruitment of TFIIH at the promoter region. The startling finding was that Kin28 kinase is not essential for transcription (136, 147, 308). A drastic decline in global transcript level in the absence of Kin28 kinase activity, however, was observed (136). This decrease in mRNA level in the Kin28-as mutant was attributed to the effect of serine-5 phosphorylation on capping of mRNA at the 5′ end rather than a direct role of Kin28 kinase in transcription (136). This view has been challenged by recent studies, which have reaffirmed the role of Kin28 kinase in promoter clearance (142, 308). These studies also implicated serine-5 phosphorylation in release of Mediator complex from the promoter-proximal region following initiation of transcription. Thus, the role of Kin28 kinase in transcription cycle remains elusive.

To investigate the precise role of Kin28 kinase in transcription by RNAP II, we examined the transcription of a number of inducible and constitutively expressed genes in Kin28-as mutant in the presence and absence of NA-PP1. Our results suggest that Kin28 kinase is not the absolute requirement for transcription, but is necessary for optimal transcription of both inducible and non-inducible genes. The kinase crosslinked to both the 5′ and 3′ ends of transcriptionally engaged genes. In the presence of analog NA-PP1, Kin28-as localization to the 5′ end of genes remained unaffected, but crosslinking to the 3′ end exhibited a dramatic decline. The delocalization of Kin28 from the 3′ end coincided with the polymerase reading through the termination signal.

Furthermore, gene looping was severely compromised in the kinase defective mutant. These results suggest a novel role of Kin28 kinase in termination of transcription, possibly through gene looping.

III.3. RESULTS

III.3.1. KIN28 KINASE IS REQUIRED FOR OPTIMAL TRANSCRIPTION OF BOTH INDUCIBLE AND CONSTITUTIVELY EXPRESSED GENES

The gene coding for Kin28 is essential for survival of yeast cells. Apart from the CTD-kinase activity, Kin28 is also essential for the recruitment of the holo-TFIIH complex at the promoter region of transcriptionally active genes. The CTD-kinase activity of Kin28 is neither essential for transcription nor for the survival of yeast cells (147). The growth of cells, however, is severely inhibited in the kinase-defective mutant, thereby suggesting that either Kin28 kinase is affecting the transcription cycle in a subtle way, or it is playing a role in a yet unknown aspect of cellular dynamic (Fig. 34) (147). To further probe the function of Kin28 kinase in transcription by RNAP II, we used

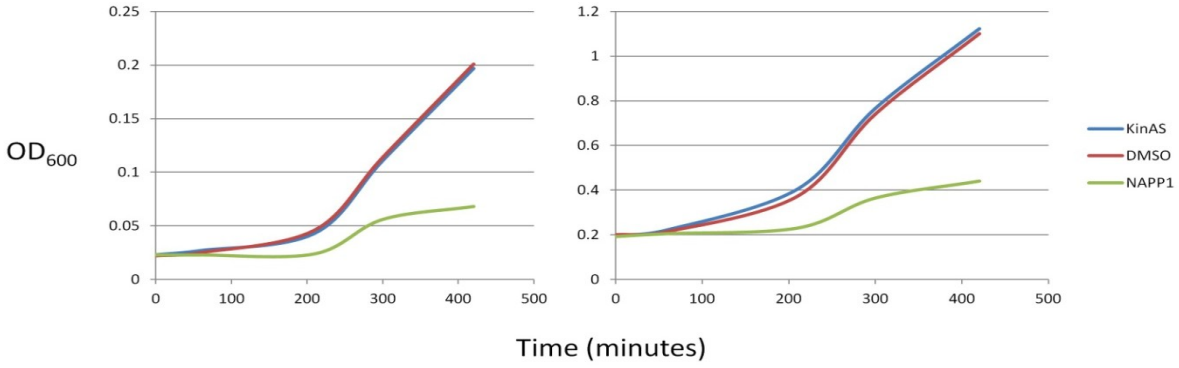

Figure 34. Kinase inhibition with NA-PP1 severely impacts growth during pre-log and early log phase. Cells harboring the kinase mutant (Kin28as) were equalized by cell number and then allowed to grow under the indicated conditions and OD was measured at 45 minute intervals. For DMSO and NA-PP1 conditions, 20 ul of DMSO or DMSO + NA-PP1 (6 uM final concentration) was added to 100 ml of culture at time 0.

an analog-sensitive strain of Kin28 called Kin28-as that has been used previously (196).

The Kin28-as strain is able to accommodate the analog NA-PP1. The analog is a highly specific competitive inhibitor of Kin28 kinase. In the presence of 5-10 μM NA-PP1 in the growth medium, the CTD kinase activity of Kin28-as mutant is almost completely inhibited within 60 minutes.

To investigate the precise function of Kin28 kinase in transcription cycle, we examined transcription of both inducible and constitutively expressed genes in Kin28-as mutant in the presence and absence of 7 μM NA-PP1 in the growth medium. Equal numbers of cells were harvested at 0, 30, 60 and 90 minutes following addition of NA-PP1 to the cultures in mid-log phase. Total RNA was isolated and steady state level of mRNA was determined by RT-PCR approach. We first checked the effect of Kin28 kinase on five inducible genes; *HXT1*, *MET16*, *CHA1*, *GAL10* and *INO1*. Our results

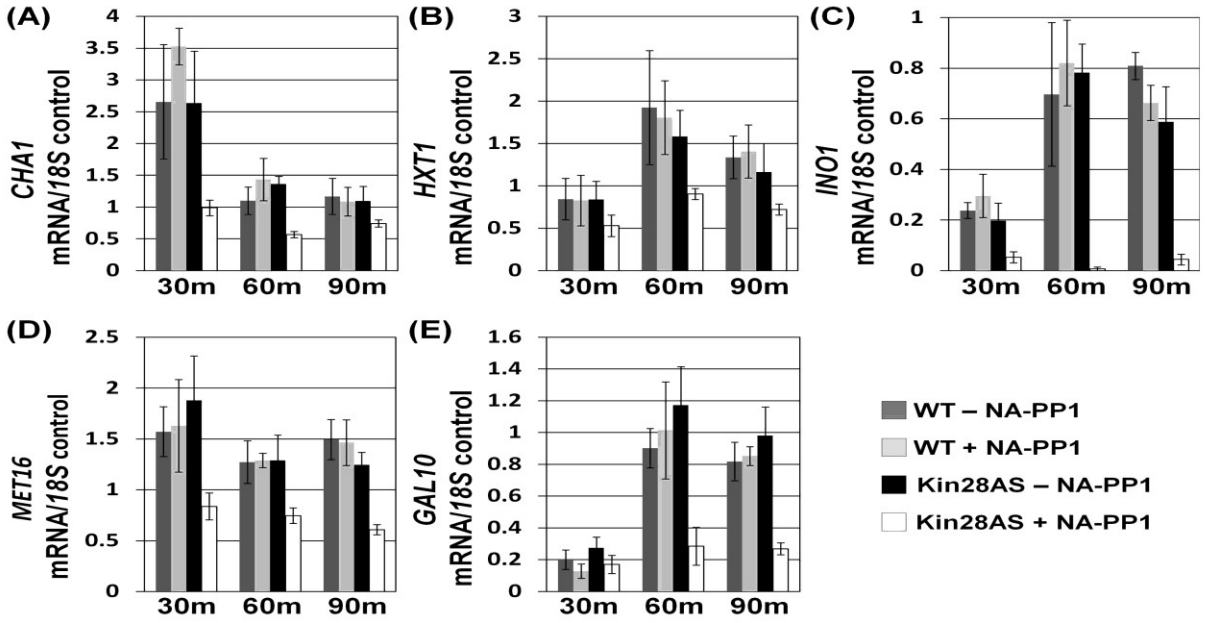

FIGURE 35. Kinase inhibition reduces mRNA steady state levels of inducible genes. A-E, quantification of transcript levels in wild type (grey bars) and the analog-sensitive Kin28 mutant (Kin28AS, black and white bars) for the inducible genes *CHA1*, *HXT1*, *INO1*, *MET16* and *GAL10* at the indicated time points. The cells were treated with either DMSO (solvent for NA-PP1, dark grey and black bars) or 6uM NA-PP1 (light grey and white bars) during the induction time frame. Error bars represent one standard deviation.

show that a defect in Kin28 kinase affected the mRNA level of different inducible genes

to different extent. The transcript level of *HXT1*, *MET16* and *CHA1* decreased by about 2-3 fold, of *GAL10* by 5 fold, and of *INO1* by about 10 fold in the Kin28-as strain in the presence of NA-PP1 (Fig. 35). No such decrease in RNA level was observed in the isogenic wild type strain in the presence of NA-PP1 or in the Kin28-as strain in the absence of NA-PP1 (Fig. 35). Next, we checked the role of Kin28 kinase on transcription of five constitutively expressed genes; *ACT1*, *ASC1*, *MSN5*, *SPC2* and *CMP2*. A similar decline in mRNA level was observed for all five non-inducible genes tested in our experiments. The steady state amount of transcripts of *ACT1*, *ASC1*, *MSN5*, *SPC2* and *CMP2* registered a more or less uniform moderate decline of 2-3 folds in the analog-sensitive mutant in the presence of NA-PP1 (Fig. 36).

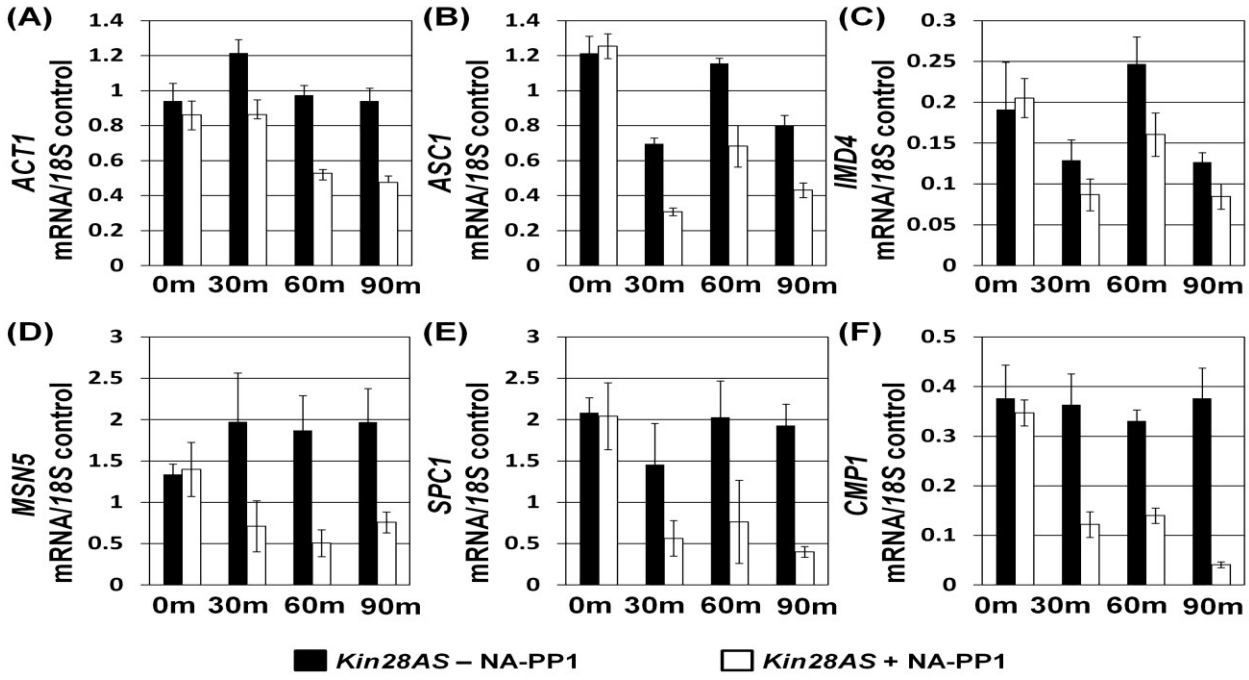

Figure 36. Kinase inhibition reduces mRNA steady state levels of constitutively expressed genes. A-F, quantification of transcript levels for the constitutive genes *ACT1*, *ASC1*, *IMD4*, *MSN5*, *SPC1* and *CMP1* in the analog-sensitive Kin28 mutant (Kin28AS, black and white bars) following treatment with either DMSO (solvent, black bars) or 6uM NA-PP1 (white bars) for 1 hour. Error bars represent one standard deviation.

The Kin28 kinase activity is essential for capping of mRNA at the 5′ end of genes.

Since capping of mRNA has been shown to affect the stability of mRNA, it was possible that the observed decrease in mRNA level of genes in the absence of kinase activity was not due to the effect of kinase on transcription, but on the stability of transcripts. To clarify the issue, we checked the nascent transcription of two inducible genes *CHA1* and *HXT1*, as well as two constitutively expressed genes *ACT1* and *ASC1*, by strand-specific transcription run-on (TRO) approach. TRO assay is a better indicator of transcriptional activity of a gene than RNAP II density ChIP as it measures the position of transcriptionally active polymerase on a gene (Fig. 37). The Kin28-as cells were grown to the mid-log phase, and transcription was induced in the presence and absence of NA-PP1 as described previously. The strand-specific TRO analysis was carried out using Br-dUTP as described in Al Husini et al., (2013). The results show that the nascent transcription of *CHA1* decreased by about 8 fold and of *HXT1* by more than 20 fold in the presence of NA-PP1 (Fig. 38). The nascent transcription of two constitutively

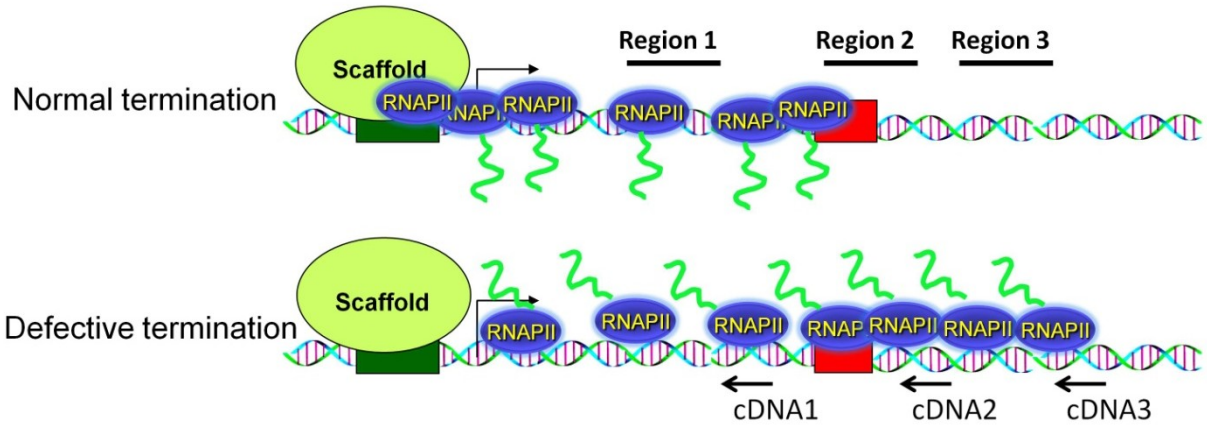

Figure 37. TRO assay detects the termination defect as a read through of RNAP II past the terminator region

expressed genes *ACT1* and *ASC1* also registered a steady 1.5-2 fold decline in the absence of Kin28 kinase activity (Fig. 38).

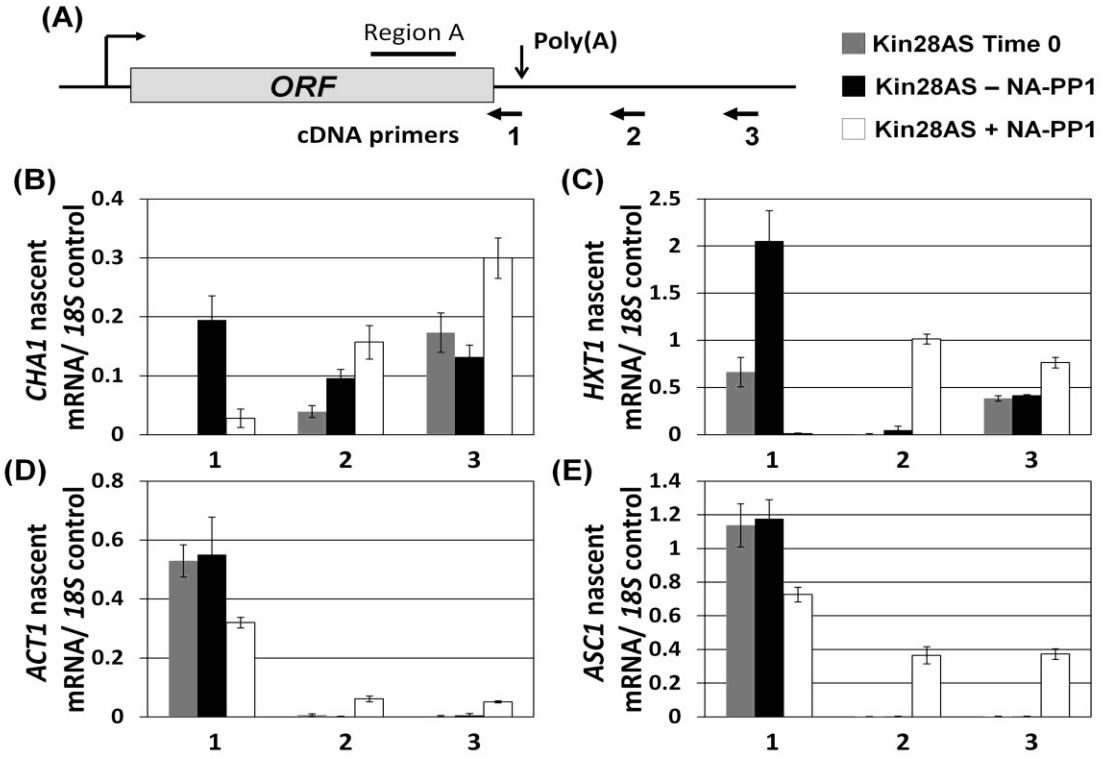

Figure 38. Kinase inhibition reduces nascent transcription levels and leads to transcription read through. The analog-sensitive Kin28 mutant cells were grown to an O.D. of 0.8 and then induced for 1 hour in appropriate media, with either DMSO or 6 μM NA-PP1. Time 0 is an equal aliquot of cells prior to induction and treatment. A, schematic indicating the 3 regions of a gene that were used for cDNA synthesis following TRO analysis. Region A represents the amplicon used for each cDNA. B-E, quantification of TRO results for region A. Error bars represent one standard deviation.

Thus, the decrease in the steady state mRNA level of both inducible and non-inducible genes observed by RT-PCR analysis was the consequence of a direct effect of kinase on transcription of genes. An additional indirect role of Kin28 kinase on stability of the transcripts has been shown, and cannot be ruled out here.

III.3.2. KIN28 KINASE IS REQUIRED FOR TERMINATION OF TRANSCRIPTION

The strand-specific TRO analysis of genes in the Kin28-as strain revealed a startling rather unexpected role of Kin28 in RNAP II transcription cycle. In all four genes that we tested, polymerase read through the termination signal in the absence of Kin28 kinase activity thereby signifying a defect in termination of transcription of these genes (Fig. 38). The TRO assay detects the presence of transcriptionally active RNAP II on a

gene. When termination is efficient, the active polymerase is restricted between the promoter and terminator regions of a gene. In the termination-defective mutants, however, polymerase reads through the terminator signal into the downstream region of the gene. TRO analysis revealed a weak polymerase signal in the downstream regions 2 and 3 of *CHA1*, *HXT1*, *ACT1* and *ASC1* in the mutant in the absence of NA-PP1 (Fig. 38). In the presence of NA-PP1, however, TRO signal in the downstream regions 2 and 3 of all four genes increased by about 2-10 fold (Fig. 38). Thus, RNAP II was not able to read

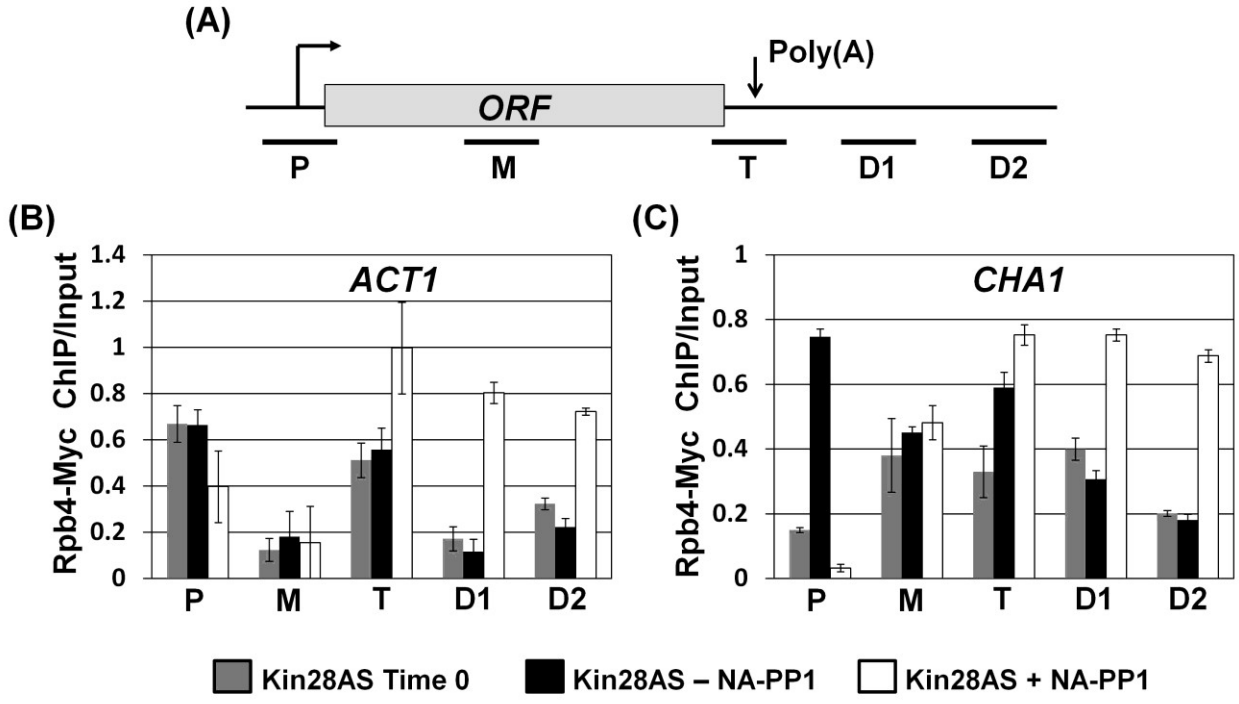

Figure 39. Kinase inhibition leads to increased RNAP II density downstream of genes. The analog-sensitive Kin28 mutant cells were grown to an O.D. of 0.6 and then induced for 1 hour with either DMSO or 6uM NA-PP1. Time 0 is an equal aliquot of cells prior to induction and treatment. A, schematic indicating the 5 regions of a gene that were subjected to ChIP analysis. B and C, quantification of ChIP results for the indicated regions. Input represents DNA prior to immunoprecipitation. Error bars represent one standard deviation.

the termination signal of *CHA1*, *HXT1*, *ACT1* and *ASC1* efficiently under kinase-defective condition and continued transcribing the downstream regions. These results suggest a role for Kin28 kinase in termination of transcription. The RNAP II density ChIP

assay corroborated readthrough of polymerase beyond the termination signal under kinase defective condition (Fig. 39).

To further probe the role of Kin28 kinase in termination, we checked the recruitment of CF1 and CPF termination complexes, which are required for both 3' end processing/termination near the terminator region of genes in the kinase defective mutant. We expected that if Kin28 kinase activity is required for termination of transcription, the recruitment of either CF1 or CPF or both complexes will be adversely affected under kinase defective condition. Both CF1 and CPF complexes are composed

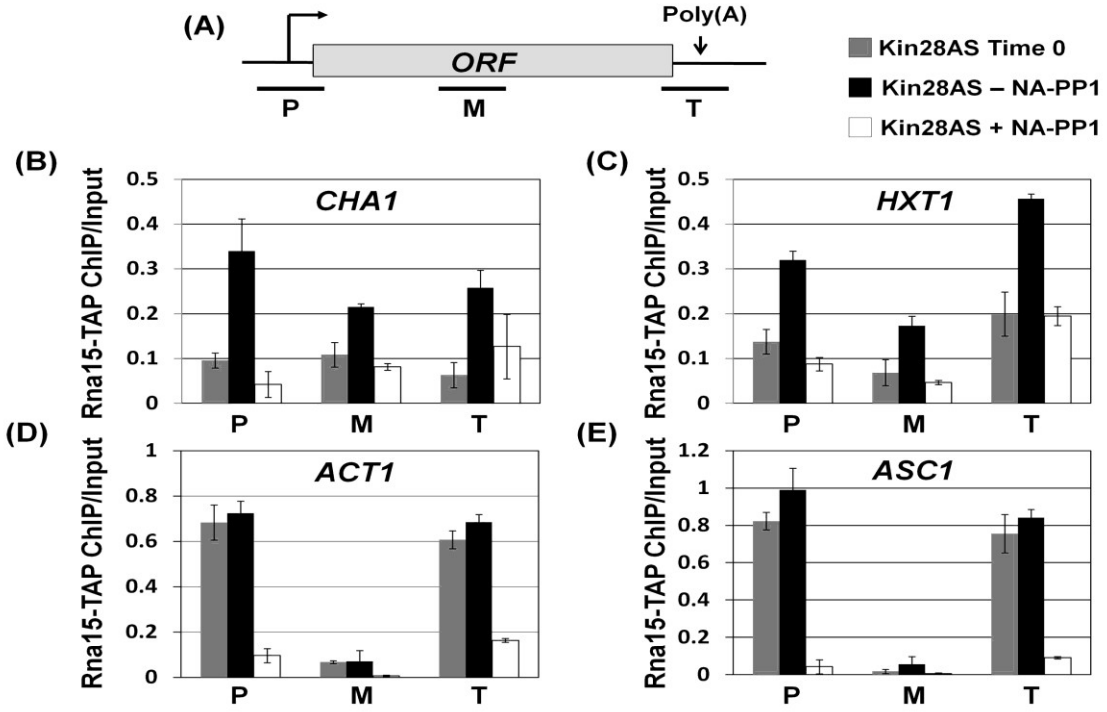

Figure 40. CF1 subunit Rna15 crosslinking to the promoter and terminator is dependent on Kin28 kinase function. The analog-sensitive Kin28 mutant cells were grown to an O.D. of 0.6 and then induced for 1 hour with either DMSO or 6uM NA-PP1. Time 0 is an equal aliquot of cells prior to induction and treatment. A, schematic indicating the 3 regions of a gene that were subjected to ChIP analysis. B-E, quantification of ChIP results for the indicated regions. Input represents DNA prior to immunoprecipitation. Error bars represent one standard deviation.

of multiple subunits (205, 249). We used a ChIP approach to monitor the recruitment of CF1 complex towards the 3' end of *CHA1*, *HXT1*, *ACT1* and *ASC1* genes using its Rna15 subunit, while SSu72 subunit was used to detect the recruitment of CPF

complex in the mutant strain. ChIP analysis revealed that both Rna15 and Ssu72 occupied the terminator region of all four genes in the mutant in the absence of NA-PP1 (Figs. 40 and 41). In the presence of NAPP1, however, crosslinking of Rna15 to the 3'

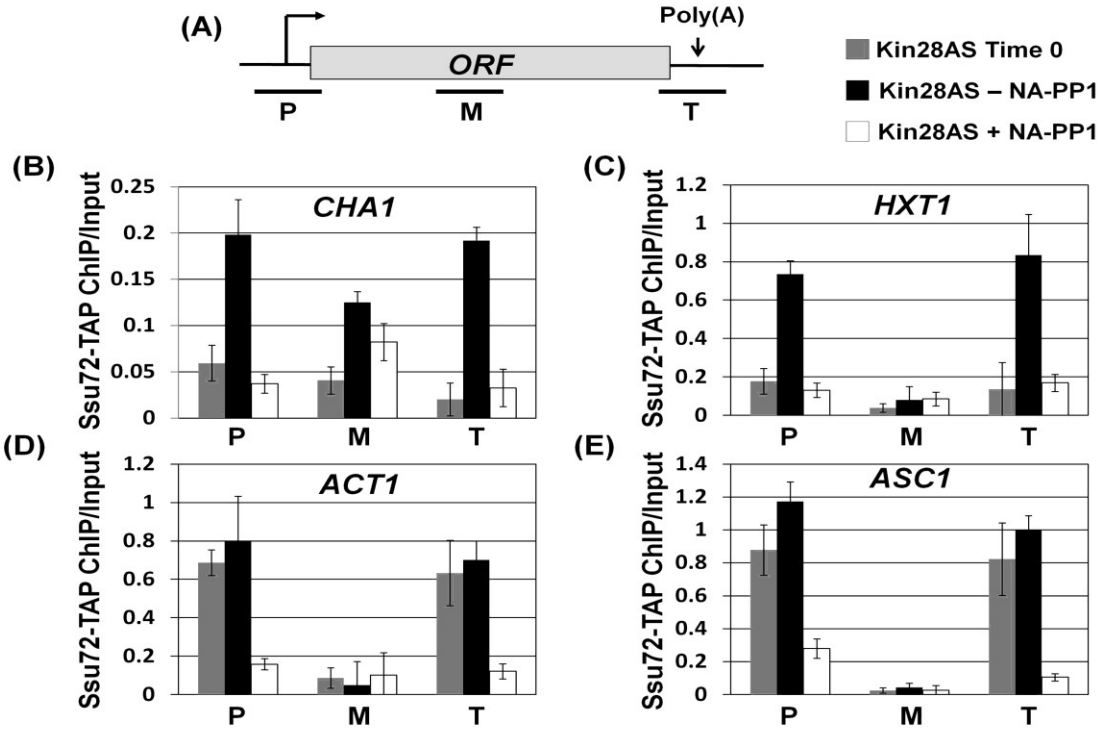

Figure 41. CPF subunit Ssu72 crosslinking to the promoter and terminator is dependent on Kin28 kinase function. The analog-sensitive Kin28 mutant cells were grown to an O.D. of 0.6 and then induced for 1 hour with either DMSO or 6uM NA-PP1. Time 0 is an equal aliquot of cells prior to induction and treatment. A, schematic indicating the 3 regions of a gene that were subjected to ChIP analysis. B-E, quantification of ChIP results for the indicated regions. Input represents DNA prior to immunoprecipitation. Error bars represent one standard deviation.

end decreased by about 50-80% (Fig. 40), while that of Ssu72 declined by more than 75% (Fig. 41). Thus, the recruitment of both the CF1 and the CPF complexes towards the terminator region of *CHA1*, *HXT1*, *ACT1* and *ASC1* was compromised under the condition of deficient Kin28 kinase activity. Taken together these results strongly suggest a novel role for Kin28 kinase in termination of transcription for at least a subset of genes in budding yeast.

III.3.3. MECHANISM OF TERMINATION OF TRANSCRIPTION BY KIN28

The termination of transcription by RNAP II is dependent on CTD-serine2 phosphorylation and requires CF1 and CPF 3′ end processing/termination complexes in yeast (2, 17, 29, 35, 72, 189, 249). The serine-2 phosphorylation starts during early elongation, continues throughout the coding region and drops sharply after the poly(A) site (78, 159). The phosphorylation of CTD at serine-2 facilitates recruitment of CF1 and CPF complexes near the 3′ end of a gene, which then brings about termination of transcription (131, 249). So far, there is no report of CTD-serine-5 or CTD-serine-7 playing any role in termination of transcription in yeast or higher eukaryotes.

The Kin28 kinase may affect termination indirectly by influencing CTD

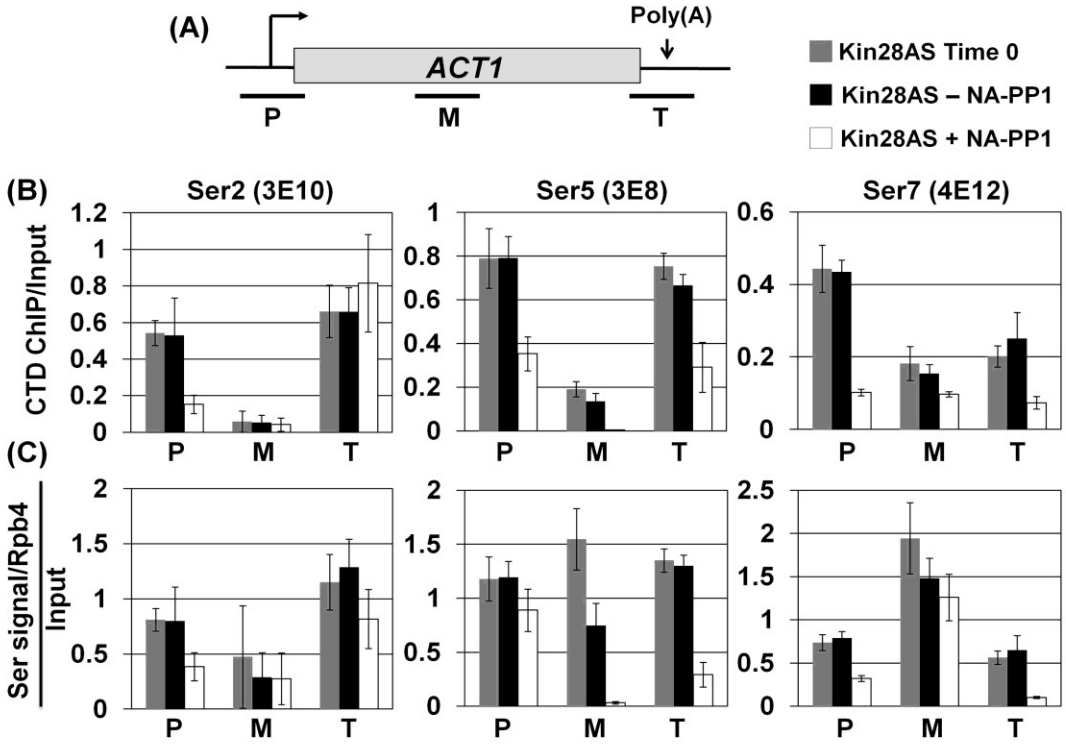

Figure 42. Kinase inhibition leads to an altered CTD phosphorlyation pattern along the *ACT1* gene. The analog-sensitive Kin28 mutant cells were grown to an O.D. of 0.6 and then induced for 1 hour with either DMSO or 6uM NA-PP1. Time 0 is an equal aliquot of cells prior to induction and treatment. A, schematic indicating the 3 regions of a gene that were subjected to ChIP analysis using the indicated antibodies for RNAP II CTD phosphorylation. B-E, quantification of ChIP results for the indicated regions. Input represents DNA prior to immunoprecipitation. Error bars represent one standard deviation.

phosphorylation towards the 3' end of genes, or directly by interacting with the

termination factors and facilitating their recruitment near the terminator region. To test the first scenario, we checked CTD phosphorylation status in different regions of *CHA1* and *ACT1* in the analog-sensitive Kin28 mutant in the presence and absence of NA-PP1. ChIP analysis revealed no change in the CTD-serine2 phosphorylation pattern near the 3′ end of any of the two genes in the presence of NA-PP1 (Fig. 42 and 43). We then examined the phosphorylation status of CTD-serine-5 and CTD-serine-7 near the 3′ end of *CHA1* and *ACT1* in the mutant strain. Although phosphorylation of serine5

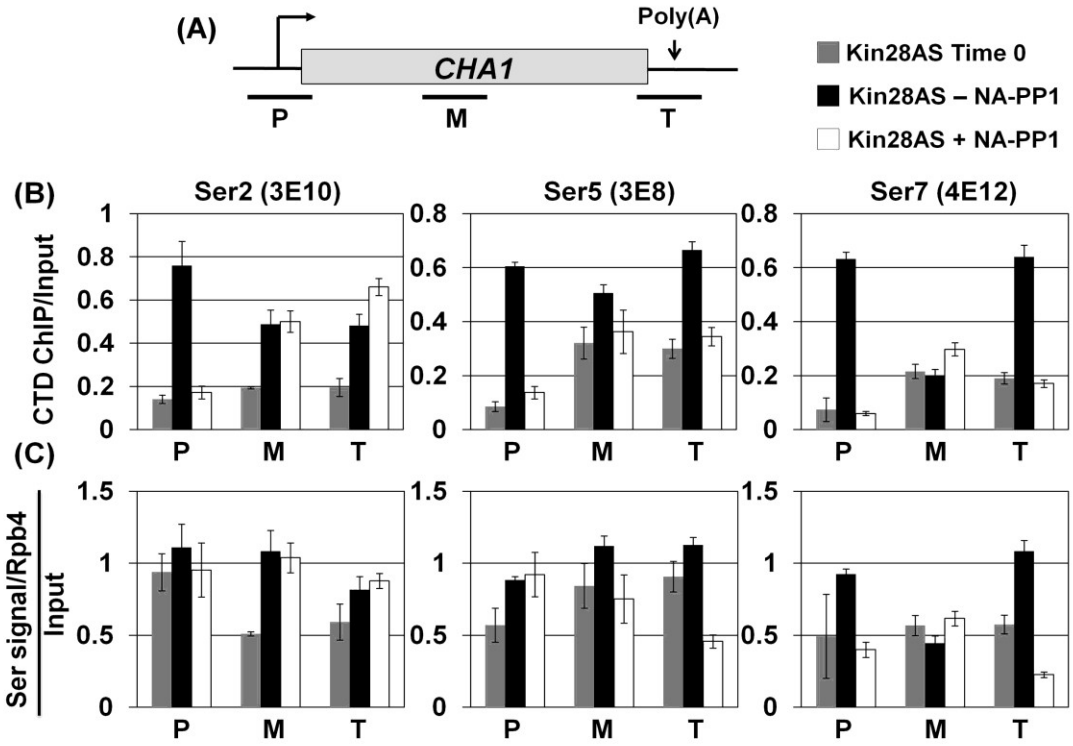

Figure 43. Kinase inhibition leads to an altered CTD phosphorlyation pattern along the *CHA1* gene. The analog-sensitive Kin28 mutant cells were grown to an O.D. of 0.6 and then induced for 1 hour with either DMSO or 6uM NA-PP1. Time 0 is an equal aliquot of cells prior to induction and treatment. A, schematic indicating the 3 regions of a gene that were subjected to ChIP analysis using the indicated antibodies for RNAP II CTD phosphorylation. B-E, quantification of ChIP results for the indicated regions. Input represents DNA prior to immunoprecipitation. Error bars represent one standard deviation.

near the promoter-proximal region is well established, there are conflicting reports regarding its phosphorylation near the 3′ end of genes (78, 286). A few recent studies demonstrated phosphorylation of serine-5 at the 3′ end in a subset of yeast genes (78,

286). Our results are in agreement with these reports. We found elevated levels of serine5 phosphorylation at the 3′ end of both *CHA1* and *ACT1* (Fig. 42 and 43). A similar elevated level of serine-7 phosphorylation was observed near the terminator region of both genes (Figs. 42 and 43). In the presence of NA-PP1, however, a 2-4 fold decrease in phosphorylation of both serine-5 as well as serine-7 was observed for two genes in the analog-sensitive mutant (Fig. 42 and 43). These results ruled out the possibility of Kin28 kinase playing an indirect role in termination of transcription by affecting CTD-serine-2 phosphorylation near the 3′ end of genes. Since inactivation of Kin28 kinase resulted in lowering of both CTD-serine-5 and serine-7 phosphorylation near the 3′ end of genes (Fig. 42 and 43), the possibility of serine-5 and serine-7 playing a role in termination of transcription cannot be ruled out.

III.3.4. KIN28 PHYSICAL INTERACTION WITH RNA15 AND SSU72 IS DEPENDANT ON ITS KINASE ACTIVITY

To check the possibility of a direct role of Kin28 in termination of transcription, we examined its interaction with the CF1 and CPF complexes using coimmunoprecipitation approach. The strains were constructed with the TAP-tagged version of Kin28-as allele, and either Myc-tagged Rna15 or Myc-tagged Ssu72. Kin28 was immunoprecipitated using IgG-Sepharose beads, and the presence of either Rna15 or Ssu72 was detected in the immunoprecipitated fraction in the presence and absence of NA-PP1. Our results show that Kin28 interacts with both CF1 subunit Rna15 and CPF subunit Ssu72 in the absence of NA-PP1 (Fig. 44). In the presence of NA-PP1, however, Kin28 interaction with both RNA15 and Ssu72 was completely abolished (Fig. 44). These results show

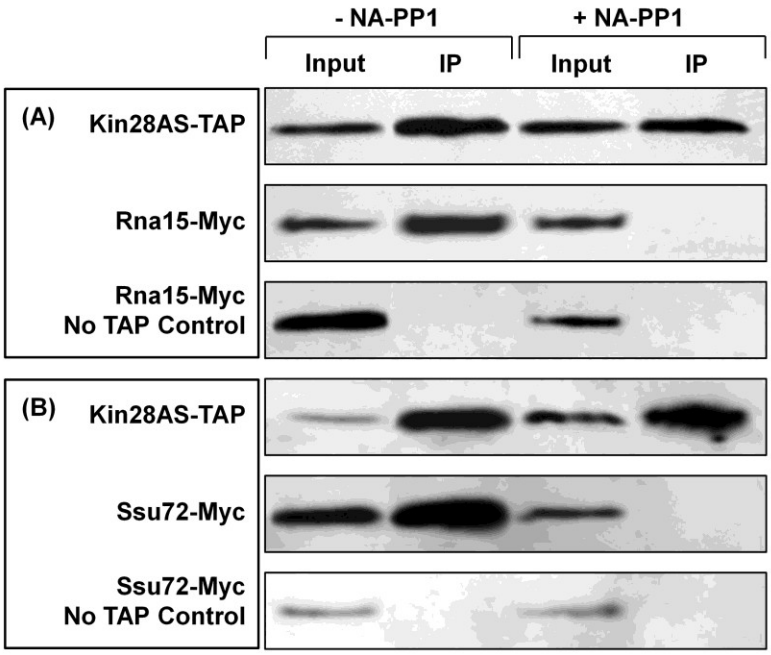

Figure 44. Kin28 physical interaction with Rna15 and Ssu72 is dependent on its kinase function. A-B, a C-terminal TAP tagged analog-sensitive Kin28 mutant was affinity purified on an IgG column from Kin28-as cells harboring either a C-terminal Myc tagged Rna15 (A) or Ssu72 (B). Purified samples were subjected to SDS-PAGE followed by Western blot analysis using anti-Myc antibodies. IP, immunoprecipitation.

that Kin28 interacts with both the CF1 and CPF complexes, and this interaction is completely dependent on its kinase activity. In order to rule out the possibility that this interaction is DNA dependent, an identical co-immunoprecipitation was performed with an additional MNase treatment prior to purification (Fig. 45). Whether Kin28-CF1 and Kin28-CPF interactions are facilitated by the Kin28-mediated phosphorylation of CTD or that of a subunit of CF1 or CPF complex needs further investigation. The possibility of Kin28 influencing termination both indirectly by affecting the CTD phosphorylation in the terminator region,

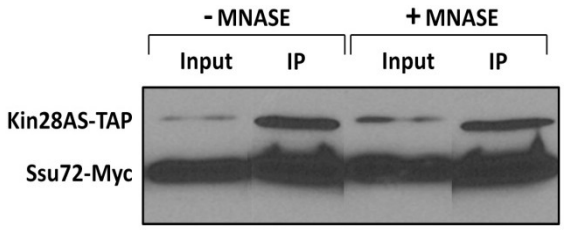

Figure 45. Kin28 physical interaction with Ssu72 is Mnase resistant. A C-terminal TAP tagged analog-sensitive Kin28 mutant was affinity purified on an IgG column from Kin28-as cells harboring either a C-terminal Myc tagged Ssu72. Prior to affinity purification, samples were digested with MNase as described in appendix C. Purified samples were subjected to SDS-PAGE followed by Western blot analysis using anti-Myc antibodies. IP, immunoprecipitation.

and directly by interacting with the termination factors near the 3′ of the gene also cannot be ruled out.

III.3.5. TFIIH LOCALIZATION TO THE DISTAL ENDS OF GENES DURING TRANSCRIPTION REQUIRES ITS KINASE ACTIVITY

The experiments described above firmly established the role of Kin28 kinase in termination of transcription of at least a subset of yeast genes. In order to have an insight into the role of Kin28 kinase in termination of transcription, we checked if Kin28 brings about termination by physically interacting with the 3′ end of genes and if the kinase activity is required for this interaction. The chromatin immunoprecipitation (ChIP) was performed in a strain bearing TAP-tagged version of Kin28-as allele. ChIP was performed in cells growing in the presence and absence of NA-PP1. As expected, Kin28

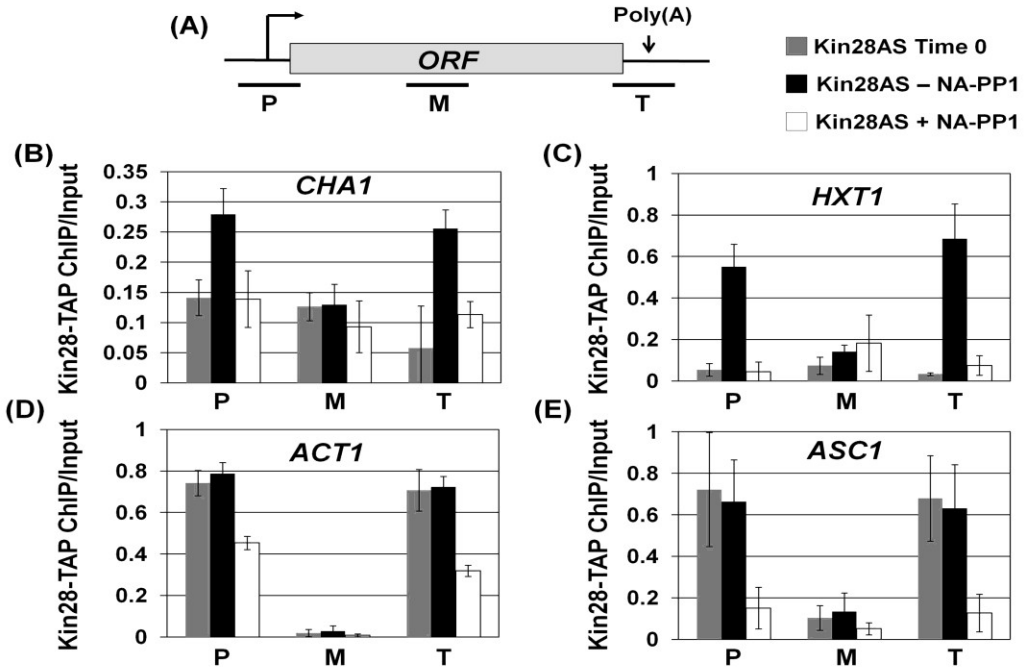

Figure 46. Kin28 cross-links on the terminator in a kinase dependent manner. The analog-sensitive Kin28 mutant cells were grown to an O.D. of 0.6 and then induced for 1 hour with either DMSO or 6 μM NA-PP1. Time 0 is an equal aliquot of cells prior to induction and treatment. A, schematic indicating the 3 regions of a gene that were subjected to ChIP analysis. B-E, quantification of ChIP results for the indicated regions. Input represents DNA prior to immunoprecipitation. Error bars represent one standard deviation.

was recruited towards the 5' end of *CHA1*, *HXT1*, *ACT1* and *ASC1* in the absence of

NA-PP1 (Fig. 46). In the presence of NA-PP1, however, crosslinking of Kin28 to the promoter region of both inducible and non-inducible genes registered a 50-80% decline (Fig. 46). Interestingly, Kin28 was also found localized near the 3' end of all four genes tested here (Fig. 46). A genomewide analysis has also found Kin28 crosslinked to the 3' end of a number of transcriptionally active genes in yeast (248). It was, however, not clear from this study if the recruitment of Kin28 near the terminator region required its kinase activity. We therefore repeated the ChIP experiment in Kin28-as mutant in the presence of inhibitory analog. Our results show that the crosslinking of Kin28 towards the terminator region of both genes was significantly reduced in the absence of its

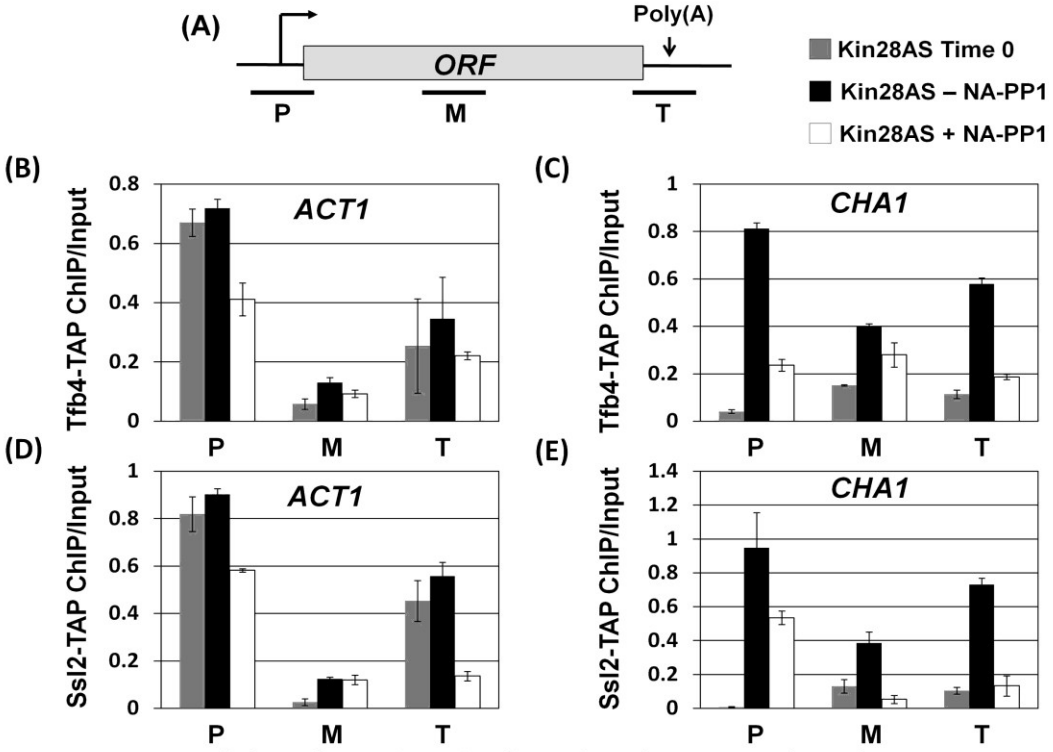

Figure 47. Core-TFIIH cross-links on the terminator in a kinase dependent manner. The analog-sensitive Kin28 mutant cells were grown to an O.D. of 0.6 and then induced for 1 hour with either DMSO or 6 μM NA-PP1. Time 0 is an equal aliquot of cells prior to induction and treatment. A, schematic indicating the 3 regions of a gene that were subjected to ChIP analysis. B-E, quantification of ChIP results for the indicated regions. Input represents DNA prior to immunoprecipitation. Error bars represent one standard deviation.

kinase activity (Fig. 46). The Kin28 ChIP signal at the 3' end of *CHA1* and *ACT1*

decreased by nearly 50%, while at *HXT1* and *ASC1* more than 75% decline was observed under kinase-defective condition (Fig. 46).

In order to distinguish if Kin28 is being recruited as part of the TFIIK submodule or the entire TFIIH, ChIP was performed in kinase sensitive strains harboring a C-terminal TAP-tagged Ssl2 or Tfb4. In both cases, the recruitment profile on the promoter and terminator regions were very similar to that observed for Kin28 in the presence and absence of the NA-PP1 inhibitor (Fig. 47). The overall conclusion of these results is that TFIIH, and not just TFIIK, is recruited at the promoter as well as terminator regions of a gene during transcription, and the kinase activity is required for the optimal recruitment of TFIIH to a gene.

III.3.6. KIN28 KINASE IS REQUIRED FOR GENE LOOPING

We have earlier demonstrated the role of Mediator complex in transcription termination (221). We showed that Mediator-facilitated termination of transcription was dependent on the gene assuming a looped conformation. We therefore asked if Kin28 kinase-mediated termination of transcription is also dependent on gene looping. CCC analysis of *CHA1*, *HXT1*, *ACT1* and *ASC1* was carried out in Kin28-as cells in the presence and absence of kinase activity. We have previously used this approach to demonstrate looping of genes in a transcription-dependent manner (83). In CCC assay, the physical interaction of the promoter and terminator regions of a gene is converted into a PCR product obtained using primers flanking the promoter (P1 primer) and the terminator (T1) regions as shown in Fig. 10 (82). CCC analysis revealed that *CHA1*, *HXT1*, *ACT1* and *ASC1* assume a looped gene conformation during transcription in wild type cells as indicated by a strong P1T1 PCR signal for all four genes (Fig. 48). In the

Kin28-as mutant also, a strong P1T1 looping signal was observed during induced

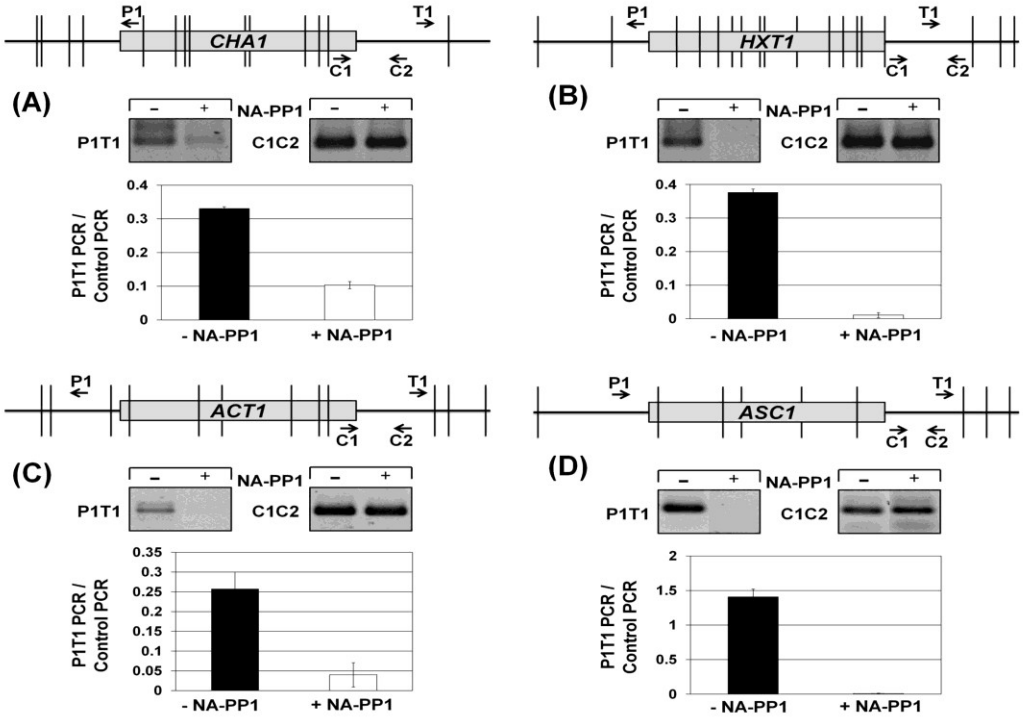

Figure 48. Gene looping is dependent on Kin28 kinase function. The analog-sensitive Kin28 mutant cells were grown to an O.D. of 0.6 and then induced for 1 hour with either DMSO or 6 μM NA-PP1. A-D, top panel represents a scaled schematic of a gene indicating the positions of AluI and NlaIV restriction sites (vertical lines) and the PCR primers (arrows) used for CCC analysis. Middle panels show the CCC analysis to detect gene looping. P1T1 PCR reflects the looping signal and the C1C2 PCR reflects the control indicating that an equal amount of template DNA was used in each reaction. Bottom panels show quantification of the CCC analysis. Error bars represent one standard deviation.

transcription of *CHA1*, *HXT1*, *ACT1* and *ASC1* in the absence of NA-PP1 (Fig. 48). In the presence of NA-PP1, however, the looped gene architecture of all four genes was almost completely abrogated as there was 3-40 folds decline in P1T1 PCR signal for different genes (Fig. 48). Thus, the kinase activity of Kin28 is essential for gene looping. Whether Kin28 kinase-mediated gene looping contributes to the termination of transcription, however, needs further investigation.

III.4. DISCUSSION

Since its discovery, the function of CTD kinase activity of TFIIH in transcription has been the focus of intense scrutiny. Using the analog-sensitive mutant of Kin28, it has been demonstrated that Kin28 kinase is neither essential for transcription nor for

survival of yeast cells (136, 147). These studies measured transcription in terms of steady state mRNA level, which is dependent on transcription as well as RNA degradation. The observed decrease in global transcript level in the absence of Kin28 kinase activity was attributed to its affect on RNA stability rather on transcription per se (136). We therefore measured nascent transcription of selected genes in the presence and absence of Kin28 kinase activity by a TRO assay. Our results suggest that Kin28 kinase is not an absolute requirement for transcription, but is required for optimal transcription of genes. Kin28 kinase may not be essential for transcription of all protein-coding genes. The prevailing view is that the general transcription factors TFIID, TFIIB, TFIIF, TFIIE and TFIIH are required for the transcription of a vast majority of RNAP II-dependent genes (201, 223, 292). This may not be entirely true. A recent study revealed that the TFIIB is required for transcription of only a subset of genes in humans (98). We propose that the Kin28 kinase activity of TFIIH may also not be necessary for transcription of all RNAP II-transcribed genes. If the TFIIH-kinase is required for transcription of a subset of non-essential genes, the cell may still be viable in the absence of the kinase activity, but the cell fitness may be adversely affected. This may explain why a defect in Kin28 kinase does not affect the cell viability and global poly(A)-mRNA level appreciably, but is still necessary for normal growth of yeast cells.

The Kin28 kinase occupies both the 5′ and the 3′ end of genes. Most studies have focused on the role of TFIIH kinase at the 5′ end of genes. We, however, were curious regarding a possible function of Kin28 kinase at the 3′ end of genes. Here we demonstrate a novel role of Kin28 kinase in termination of transcription. We provide several lines of evidence in support of our claim. First, localization of Kin28 at the 3′ end

of genes is dependent on the kinase activity of protein. Second, recruitment of CF1 and CPF termination complexes towards the terminator end of gene is compromised in the absence of kinase activity. Third, the interaction of Kin28 with the CF1 and CPF complexes is almost completely abolished in the Kin28 kinase-defective mutant. Fourth, RNAP II reads through the termination signal into the downstream region under the kinase-defective condition. TFIIH is not the only general transcription factor that has been implicated in termination of transcription. A similar termination function has been found for TFIIB as well. Just like Kin28, TFIIB crosslinks to the 3′ end of genes and facilitates recruitment of the termination factors there (212). The termination function of TFIIB has been remarkably conserved during evolution, as it has been observed in a wide range of organisms as yeast, mammals and flies (128, 222, 296). We recently demonstrated the role of another initiation factor, Mediator complex, in the termination of transcription (221, 222). The emerging view is that the initiation and termination factors do not have exclusive roles in the initiation and termination steps of transcription respectively. We have shown that at least some initiation factors participate in the termination of transcription, while additionally, some termination factors function in the initiation/reinitiation of transcription (6).

We have previously demonstrated that gene looping facilitates interaction of the promoter-bound factors with the 3′ end of genes, and of terminator-bound factors with the 5′ end of genes (6, 83, 212, 221). We hypothesize that it is gene looping that allows a transcription factor to function at both the ends of a gene. The termination function of TFIIB is completely dependent on its interaction with the 3′ end of a gene. We propose that Kin28 crosslinking to the 3′ end of a gene, and its consequent role in termination of

transcription is also dependent on gene looping. Accordingly, we show that there is no gene looping in the Kin28 kinase defective mutant. These results suggest that the Kin28 kinase activity is essential for promoter-terminator interaction. Loss of gene looping in Kin28-kinase deficient mutant coincides with the loss of Kin28 from the 3′ end of genes, and a defect in termination of transcription. A role of gene looping in termination of transcription by TFIIB has already been demonstrated. The possibility of Kin28-kinase-mediated gene looping contributing to its termination function, therefore, cannot be ruled out.

CHAPTER IV

CONCLUSIONS

IV. 1. SUMMARY

The physical and genetic interactions of the promoter and terminator bound factors were initially only a hint that the initation and termination steps in the transcription cycle are intimately linked. The results presented here demonstrate that initiation factors such as TFIIB and TFIIH are simultaneously present at both the promoter and terminator regions of a gene during active transcription. Other work from our lab has shown similar results with the 'promoter bound' Mediator complex and 'terminator bound' CF1 complex. Work using temperature sensitive mutants has revealed that these reciprocal localizations at the distal ends of a gene depended on the presence of functional complexes. The hypothesis that the physical contact of these complexes is the molecular basis of gene looping is strongly supported by the isolation of a holo-TFIIB gene looping complex containing CF1 subunits and the CPF subunit Pap1. Perhaps the most suprising result was the failure to detect TFIIH (Kin28) as part of the holo-TFIIB complex considering it also genetically interacts with Ssu72 and localizes on the terminator. This might be explainable given that in vitro evidence shows the release of TFIIB from the inititation scaffold that is left behind after the first round of transcription, composed at least partially of TFIID, TFIIA, and TFIIH. In vivo, it appears that TFIIB remains on the promoter, probably through gene looping interactions with the termination factors. Although at this point, the involvement of other promoter bound factors such as Mediator, in stabilizing TFIIB on the promoter, can not be ruled out.

TFIIH localization on the terminator region was interesting with regard to gene looping because of the kinase activity of subunit Kin28. Phosphorylation is perhaps the most well investigated post translational modification which can dictate an ‘on’ or ‘off’ state for many proteins. While TFIIH wasn’t detected in the holo-TFIIB gene looping complex, TFIIH could still be playing crucial roles in the formation of such a complex. The results presented here clearly show a role for TFIIH in the termination process. While in vitro studies have shown termination factor recruitment on a CTD peptide phosphorylated at serine-2, it is unclear how stable these complexes are in vivo. The CTD ChIP results hint that TFIIH could be indirectly affecting their stability based on phosphorylation of the CTD at serine-7. In addition, TFIIH could be playing a more direct role based on its physical interaction with termination factors and phosphorylating them in order to function properly. Although it is not definitively proven, TFIIH appears to be responsible for phosphorylating TFIIB at serine-65, which has been shown to be required for the recruitment of termination factors to the promoter and terminator (296). In addition, p53 has been shown to override a lack of TFIIB for the transcription of damage response genes and also recruit termination factors in a manner similar to TFIIB forming a gene loop (267). This indicates that there may be other mechanisms which can result in the formation of gene loops, and perhaps the only requirement for any promoter bound factor is that it also interacts with termination factors. This could possibly be tested by simply fusing an activator protein with one of the termination factors. Also, many viral activator proteins are thought to recruit TFIIB directly to promoters without the formation of a usual PIC, driving a ‘short circuit’ to transcription initiation and gene looping. Recently, it has been shown that in differentiated kidney

cells, TFIIB is not necessary for transcription of all genes as previously thought. It was however, required for expression of the herpes simplex virus-1 (98). In the future, a better understanding of how gene loops form could be important for disrupting viral transcription.

IV. 2. FUTURE DIRECTIONS

My work presented here is far from a complete story, and seems to create more questions than it has answered. The TFIIB work begs to ask at least a few questions regarding the holo-TFIIB complex. How big is it really? What are all of the composing factors? What is TFIIB interacting with directly where a single amino acid change prevents the formation of a gene looping looping complex? Furthermore, while not shown here, why does a C terminal TAP tag on TFIIB interfere with isolating a gene looping complex, but doesn't affect TFIIB localization on the terminator?

In order to determine how big it really is, a different column needs to be used. Then, large amounts of the holo-TFIIB complex could be purified and concentrated enough to get good mass spec data. The most interesting question is finding what directly interacts with TFIIB near the region containing the E62K that is critical for gene looping. I have begun the process of creating point mutants (2 so far) that are capable of incorporating a photo-crosslinkable amino acid (Bpa) at the site. The factor(s?) could then be discovered using a Western blot if they had an affinity tag. Otherwise, bands could be cut out of the gel where a crosslinked TFIIB was present and then subjected to mass spec to identify the interacting protein.

The work presented here on TFIIH opens up several lines of interesting research in order to understand how TFIIH is affecting termination and gene looping. Is TFIIH

phosphorylating termination factors directly? Is the kinase activity of TFIIH facilitating the recruitment at the promoter and terminator through the CTD?It is also possible that TFIIH could be working in a kinase cascade with other kinases involved in initiation and termination.

While a few of the targets of Kin28 kinase are known, it is not known if any of the termination factors are among them. If TFIIH is targeting termination factors this could explain the lack of recruitment during kinase inhibition due to the inability of that factor to stabilize the association of that termination complex. Alternatively, it could also be due to the inability to phosphorylate other promoter bound factors perhaps acting in a role similar to TFIIB, where TFIIB phosphorylation stabilizes CF1 recruitment by direct interaction with Rna15 (296). TFIID and SAGA are both relatively large complexes (over 15 subunits) and a few Kin28 targets have been found within them. In order to investigate this in an unbiased manner, inititiation and termination complexes would need to be purified and subjected to 2-dimensional PAGE to compare the spots with/out kinase inhibition. Those spots would then have to be analyzed with mass spec in order to identify the target proteins. Furthermore, those factors identified would then need to be mutated at the appropriate phosphorylation sites to ascertain if they are involved in gene loop formation.

While genomewide studies have provided key insights into patterns of CTD phosphorylation, recent studies indicate that creating an average pattern may not be reflective for all genes (286). Understanding the exact nature of how CTD phosphorylation patterns affect the stability and recruitment of the termination machinery will require the creation of several specific CTD mutants where each

phosphorylation event can be examined independently and in conjuction with each other. More specifically, does Kin28 physically associate with termination factors because both are binding to the CTD in close proximity? Given that the length of the yeast CTD can wrap entirely around RNAP II, it is not known if the specific phosphorylation marks have to be within the same 11 residue functional unit. It is necessary to create mutants where only one of the serines can be phosphorylated in isolation from the others to rule out that the proximity of marks on the CTD is a stabilizing factor.

IV. 3. SPECULATIONS

It is interesting to contemplate how gene loops are actually formed from the correct juxtaposition of a specific terminator with its own promoter. How does a terminator know which promoter to associate with? According to Peter Cook, RNAP II never really escapes the promoter in vivo and instead pulls the DNA through it as it transcribes (177). So, promoter DNA remains in contact with the PIC and so does the elongating polymerase as it progresses to the terminator. This implies that formation of a gene loop begins during the elongation step of transcription. Upon reaching the terminator, this would bring that region into juxtaposition with the promoter and a bonified gene loop could be stabilized during termination. Further rounds of reinitiation would then form ‘elongation loops’ within the promoter-termination gene loop. Perhaps the ‘background’ signal in CCC analysis within a gene reflects this concept, especially where it concerns the intron/exon borders which indicate there are regions within a gene that associate with the promoter and terminator regions during transcription (217).

The role of TFIIH in gene looping can also be further highlighted with regard to its function in DNA repair. Work from other labs have shown that during transcription coupled DNA repair, the region of DNA being repaired is recruited to the nuclear pore. This recruitment also leads to the transcription of otherwise inactive genes during their repair. This coincides with work from the Proudfoot lab that demonstrated that gene loops are dependant on nuclear pore localization (283). Perhaps TFIIH is the key player, where once it is recruited to the promoter or a site of damage, this invokes a shuttle mechanism to the nuclear pore. Transcription could then stabilize the formation of an elongation loop inititating through TFIIB phosphorylation, which occurs immediately after CTD-serine-5 phosphorylation (297). This mechanism also implies the existence of various 'short circuits' to transcription initiation that can bypass the usual progression of PIC formation dictated by the mediator response to gene specific activators.

In support of this 'short circuit' hypothesis, is the phenomenon of intron mediated enhancement of transcription (IME). The insertion of an intron into a repressed gene causes it to switch to into a fully on state with regard to transcription. This could occur due to transcription initiating from the distal ends of a gene, which occurs during basal and anti-sense transcription. If RNAP II transcribes to the intron without being destabalized, this would recruit the splicing machinery, and then the gene switches to an 'elongation loop' mode that leads to gene loop formation and the full on state for that gene. This would also explain why IME functions based on the proximity of the intron to the distal ends of a gene.

APPENDIX A: EXPERIMENTAL PROCEDURES

A.1.1 CELL CULTURE (RT-PCR, 3C, ChIP or TRO)

Cell cultures were began from fresh plates (less than 3 weeks) in 5ml tubes in either YP-dextrose or synthetic Ura- drop out media. Cultures were grown at 30 °C for 6-7 hours until evening and diluted 1:100 into 100 ml flasks of similar media. 100 ml flasks were grown overnight and the optical density was measured the next morning on a spectrophotometer at wavelength 600 nm (OD_{600}). If cultures were overgrown, OD_{600} >0.6, cultures were diluted in similar media down to an OD_{600}~0.3 and grown to an OD_{600}~0.4 to 0.6 depending on the experiment and genes being examined. Cultures were then transferred to appropriate media for induction of 1-2 hours. For MET16, CHA1 or HXT1 cells were grown to an OD_{600}~0.5 and induced for 1 hour unless specified for a time course. For INO1 and GAL10 cells were grown to an OD_{600}~0.4 and induced for 2 hours unless specified for a time course. For induction using the kinase inhibitor NA-PP1, 6um final concentration in DMSO was used when the cells were transferred into induction media. For induction using strains that are temperature sensitive, cells were grown at the permissive temperature of 25 °C to an OD_{600}~0.4 to 0.6 and then transferred to the non-permissive temperature of 37 °C for 2 hours unless specified otherwise. After induction the cells were then processed accordingly for each experiment.

A.1.2 CELL CULTURE (CO-IP, Glycerol Gradient, FPLC)

Cell cultures were began from fresh plates (less than 3 weeks) in 5 ml tubes in either YP-dextrose or synthetic Ura- drop out media. Cultures were grown 6-7 hours until evening and diluted 1:200 into 1L flasks of similar media. 1L flasks were grown

overnight and optical density was measured the next morning on a spectrophotometer at wavelength 600nm (OD_{600}). If cultures were overgrown, OD_{600} >2.0, cultures were diluted in similar media down to an OD_{600}~0.6 and grown to an OD_{600}~1.2 to 1.5. If cells were being induced the cells were transferred at an OD_{600}~1.0 into 1L flasks of appropriate media and allowed to grow for an additional 1-2 hours before being processed for the experiment.

A.2 RT-PCR

Cells were grown as above. Cell pellets were washed with 5 ml DEPC treated water. Cells were transferred to 1.5 ml lock top tubes using 500 μl of Trizol. 250 μl of acid washed glass beads were added and cells were lysed by vortexing for 30 mintues at 4 °C. Tubes were then punctured using a 22 gauge needle and drained into 15 ml tubes by centrifugation for 2 minutes at 2k rpm at 4 °C. Lysate was transferred to a fresh 1.5 ml eppendorf and 500 ul of Trizol was added and incubated for 5 minutes at 25 °C. 200 μl of chloroform was added and mixed by vortexing, then centrifuged for 15 minutes at 14k at 25 °C. The supernatant was transferred to a another eppendorf and three RNA phenol chloroform extractions were performed. The RNA was precipitated by adding 0.1 volume of LiCl and 2.5 volumes of 100% EtOH and centrifuging for 20 minutes at 14k at 25 °C. The RNA pellet was resuspended in 51 μl of DEPC treated water and the quantity was measured using a nanodrop. The RNA concentration was adjusted to 100 ng/μl. Mulv reverse transcriptase was used to make cDNA using oligo-dT and 18S cDNA primers. Reactions were incubated using a thermocycler for the extension and deactivation steps. A negative control without reverse transcriptase was performed to ensure no DNA contamination contributed to any RT- PCR signal. cDNA was diluted

prior to PCR amplification by the addition of 180 μl of 1x TE. The gene specific PCR primers used are listed in appendix C. Each PCR was normalized against the 18S ribosomal RNA control.

A.3 STANDARD REVERSE TRANSCRIPTION REACTIONS (RT-PCR or TRO)

Reverse transcription reactions were all performed using Mulv reverse transcriptase (NEB). 10 μl of diluted RNA (as specified in either RT-PCR or TRO) was incubated with either 2 μl of oligo dT (25 [pM]) or gene specific primer at 65 °C for 5 minutes followed by 4 °C for at least 2 minutes. To each sample, 8 ul of a master mix (4 μl H_20, 2 μl of 10x Mulv buffer, 1 μl of dNTPs (NEB), 1 μl of Mulv RT enzyme) was added. Samples were then incubated in a thermocycler at 37 °C for 45 minutes followed by inactivation at 65 °C for 20 minutes and then held at 4 °C. cDNA for RT analysis was diluted with the addition of 180 μl 1x TE and cDNA for TRO analysis was diluted °C with the addition of 80 μl of 1x TE.

A.4 STANDARD PCR REACTIONS (RT-PCR, 3C, ChIP or TRO)

All PCR reactions were performed using Taq polymerase (NEB). Each PCR was performed as a 25 μl reaction using 16.5 μl water, 2.5 μl of 10x Taq polymerase buffer (NEB), 0.5 μl of dNTPs (NEB), 0.5 μl of Taq polymerase enzyme (NEB), 2 μl of a pre-mixed primer pair (5 μl of each primer from 250 [pM] stock and 190 μl water) and 3 μl of template (either DNA or cDNA). For a first round analysis, a 30 cycle reaction would be performed followed by gel electrophoresis. Based on the initial band strength determined using the Kodak 1D software, other reactions would be performed in order to get band strengths in the 3000 to 80000 linear range for quanfification. If one of the primer pairs was either extremely strong or weak, either a different pair was chosen or it

was run separately for the necessary cycles to adjust the intensity into same range with the rest. For input or 18S normalization controls 26-29 reaction cycles were used (usually on the lower end). For ChIP and RT PCR using gene specific primers 28-32 reaction cycles were used. For TRO and CCC the signals were often hard to see and required 33 to 36 reaction cycles to amplify quantifiable bands using 6 μl of template instead of the normal 3 μl. Quantification of band signals was performed as described below.

A.5 TRANSCRIPTION RUN-ON ASSAY (TRO)

Cells were grown as described above. The transcription run-on assay was performed as described in Al Husini et al., 2013. 50 ml of cells were harvested after induction at an OD_{600}~0.8. The cell pellet was washed with 10 ml of ice cold TMN buffer (10 mM Tris-HCl pH 7.5, 5 mM $MgCl_2$, 100 mM NaCl) and resuspended in 940 µl of DEPC (Diethylpyrocarbonate)-treated ice cold water. Cells were permeabilized by the addition of 60 μl 10% sarkosyl and incubated in an ice pack for 25 minutes while nutating at 4 °C. Cells were pelleted again using low speed centrifugation 1.2g for 6 minutes at 4 °C. In vivo transcription elongation was then performed by suspending the cells in 150 μl of reaction buffer (50 mM Tris-HCl pH 7.5, 100 mM KCl, 10 mM $MgCl_2$, 2 mM DTT, 0.75mM ATP, CTP, GTP and Brd-UTP, with 5 μl RNAse inhibitor cocktail NEB). To allow for proper elongation, the reactions were incubated at 30 °C for 5 minutes. The elongation reaction was immediately stopped with the addition of 500ul of ice cold Trizol reagent (Sigma). Cells were then lysed with 250 μl acid washed glass beads (Sigma) for 20 minutes using a vortex at 4 °C. The tubes were punctured using a 22 gauge needle and drained into 15 ml tubes by spinning 2k rpm at 4 °C. The lysate

was transferred to a 1.5 ml eppendorf and 500 μl of Trizol was added and incubated at 25 °C for 5 minutes. 200ul of chloroform (Sigma) was added and the tubes were vortexed briefly and centrifuged for 20 minutes at 14k rpm at 25 °C. Approximately 700 μl of supernatant was transferred to another 1.5 ml eppendorf tube and 700 μl of RNA phenol chloroform pH 4.2 was added and vortexed briefly. Tubes were then centrifuged for 15 minutes at 14k rpm at 25 °C. The RNA phenol chloroform extraction was repeated 2x more to purify the RNA. Total RNA was precipitated with 1/16th volume of 5M NaCl and 2.5 volumes of 100% ethanol and incubated overnight at -20 °C. RNA was pelleted by centrifugation for 20 minutes at 13.2k at 4 °C. The RNA pellet was washed once with ice cold 70% EtOH and resuspended in 100 μl of DEPC treated water.

A bed volume of 25 μl Anti-BrdU conjugated agarose beads (Sigma) were washed 3x using 500 ul of binding buffer (0.25x SSPE buffer, 1mM EDTA, 0.05% Tween20, 37.5 mM NaCl). Beads were blocked using 500 μl of blocking buffer (485 μl binding buffer, 5 μl of 10% polyvinylpyrolidone, 10 μl of Ultrapure BSA, Sigma) by nutating for 1 hour at 4 °C. RNA was further purified using the Qiagen RNA Easy kit and eluted twice with 50 μl of DEPC treated water. Beads were washed 2x using 500 μl of binding buffer and then 400 μl of binding buffer was added to the beads and placed on ice. The eluted RNA was incubated at 65 °C water bath for 5 minutes and immediately placed on ice for 2 minutes. The RNA was then added to the beads and bound by nutating for 2 hours at 4 °C. Beads were then washed sequenctially using 500 ul of binding buffer, 500 μl of low salt buffer (0.2x SSPE, 1 mM EDTA, 0.05% Tween20) 500 μl of high salt buffer (0.25x SSPE, 1 mM EDTA, 0.05% tween20, 100 mM NaCl) and 500 μl of TET buffer (1x TE buffer, 0.05% tween20). RNA was eluted 2x with 150 μl of

elution buffer (20 mM DTT, 150 mM NaCl, 50 mM Tris-HCl ph 7.5, 1 mM EDTA, 0.1% SDS) by incubating at 42 °C for 5 minutes followed by a final elution with 200 μl of elution buffer at 42 °C for 5 minutes. To the 500 μl of elution, 500 μl of RNA phenolchloroform ph 4.2 was added and vortexed and then centrifuged for 15 minutes at 14k rpm at 25 °C. Supernatent was transferred to fresh 1.5 ml eppendorf and RNA was precipitated with 0.1 volume LiCl and 2.5 100% EtOH by centrifugation for 20 minutes at 14k rpm at 25 °C. RNA was resuspended in 26 μl of DEPC treated water and the quantity was measured using a nanodrop. RNA concentration was adjusted to 50 ng/μl or the lowest concentration in the set. cDNA was made using strand specific primers listed in appendix C. The primers were designed to synthesize cDNA at a region just 3' of the open reading frame, the region near the poly (A) signal, and two more regions over 100 bp downstream from the poly (A) signal.

A.6. CHROMATIN IMMUNOPRECIPITATION (ChIP)

Cells were grown as described above. Cell cultures were crosslinked by adding 1% formaldehyde and shaking for 20 minutes at 25 °C. Crosslinking was quenched by the addition of 125 mM Glycine and shaking for 5 minutes at 25 °C. The cells were transferred to 50 ml conical tubes and pelleted by centrifugation at 3k rpm for 5 minutes at 4 °C. Cell pellets were washed with 10 ml of ice cold cell wash buffer (1x TBS, 1% Triton X100). Cells were transferred to 1.5 ml lock top tubes with 500 μl of FA lysis buffer (50 mM Hepes-KOH ph 7.9, 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% Na deoxycholate, 1 mM PMSF). 250 μl of acid washed glass beads were added and the cells were lysed by vortexing for 30 minutes at 4 °C. The tubes were punctured with a 22 gauge needle and drained into 15 ml tubes by centrifugation for 2 minutes at 2k rpm

at 4 °C. The lysate was transferred to a 1.5 ml eppendorf tube and centrifuged for 15 minutes at 13.2k rpm at 4 °C. The pellet was washed with 500 μl of FA lysis buffer and then transferred to a 15 ml tube with 4ml of FA lysis buffer. Sonication was performed with the Branson digital sonifier and a 2 mm probe. Lysates were sonicated while suspended in an ice water batch at 25% duty cycle using 20 second pulses followed by 20 second rest, for a total sonication time of 12 minutes. Sonicated lysate was then transferred into 1.5 ml eppendorf tubes and centrifuged for 15 minutes at 13.2k at 4 °C. Chromatin supernatents were then pooled per sample and aliquoted into 400 μl amounts to be used for Input or immunoprecipitation.

For ChIP experiments in strains harboring a C-terminal Myc tagged protein, anti-Myc conjugated agarose beads (Sigma) were used. For ChIP experiments in strains harboring a C-terminal HA tagged protein, anti-HA conjugated agarose beads (Pierce) were used. For ChIP experiments in strains harboring a C-terminal TAP tagged protein, IgG conjugated agarose beads (GE healthcare) were used. For ChIP experiments involving the use of antibodies against a selected protein, protein-A conjugated agarose beads (Sigma) were used. For CTD ChIP experiments, the antibodies for Ser2, Ser5 and Ser7 phosphorylation were 3E10, 3E8 and 4E12 respectively (Millipore). 800 μl of chromatin was incubated for 4 hours using 5 ul (or 20 μl for 4E12) of antibody with gentle shaking at 4 °C prior to incubation with the agarose beads.

A bed volume of 20 ul of beads was washed three times with 500 μl of FA lysis buffer. 800 μl of chromatin was added to the beads and incubated overnight with gentle shaking at 4 °C. Beads were then spun gently for one minute at 1.5k rpm at 25 °C. All of the subsequent washing steps were performed at 25 °C. Supernatent was removed and

the beads were washed 2x with 1 ml FA lysis buffer containing 0.1% SDS. The beads were then washed 2x with 1ml FA lysis buffer containing 500 mM NaCl and 0.1% SDS. The beads were then washed 2x with 1ml ChIP wash buffer (10 mM Tris-HCl pH 8.0, 250 mM $LiCl_2$, 0.5% Na deoxycholate, 1 mM EDTA, 0.5% tergitol, 0.1% SDS). The beads were then washed with 1 ml of 1x TE (10 mM Tris-HCl pH 7.5, 1 mM EDTA). Purified chromatin was eluted 2x with 200 ul of elution buffer (50 mM Tris-HCl pH 7.5, 10 mM EDTA, 1% SDS) by incubating at 65 °C for 10 minutes.

Eluted samples and input were then treated with 10 μg of RNAse A by incubation at 37 °C for 15 minutes. For input samples 0.1% SDS was added. Samples were treated with 20 μg proteinase K at 42 °C for 90 minutes. Reversal of crosslinking was done by incubating samples at 65 °C for overnight (or 6 hours minimum). DNA was purified by performing 2x DNA phenol chloroform pH 7.5 extractions. The DNA was precipitated by EtOH precipitation using 0.1 volume Na Acetate and 2.5 volumes EtOH and 2 μl of glycogen as a carrier and also to aid in visualizing the pellet. For every batch of sonicated chromatin, one set of input was resuspended in 20 μl of 1x TE and 3 μl of 10x loading dye to verify DNA fragment size was below 500 bp by gel electrophoresis. Input was resuspended in 101 μl 1x TE and immunoprecipitated (IP) samples were resuspended in 51 μl of 1x TE and the quantity was measured using a nanodrop. Inputs were routinely normalized to 100 ng/μl and IP samples were normalized to 50 ng/ul or to the lowest concentration in the set of IP samples. The primers used for PCR analysis were chosen based on the region of a gene to detect and to be of similar sized amplicons when possible. The primers used for ChIP PCR analysis are in appendix C.

A.7. CHROMOSOME CONFORMATION CAPTURE ANALYIS (3C)

Cell cultures were grown as described above (A.1). Crosslinking was performed by the addition of 1% formaldehyde and incubation for 30 minutes at 25 °C with gentle shaking. Crosslinking was quenched by the addition of 125 mM Glycine and shaking for 5 minutes at 25 °C. The cells were transferred to 50 ml conical tubes and pelleted by centrifugation at 3k rpm for 5 minutes at 4 °C. Cell pellets were washed with 10 ml of ice cold cell wash buffer (1x TBS, 1% Triton X100). Cells were then washed again with 10 ml of ice cold 1x TBS). Cells were transferred to 1.5 ml lock top tubes with 500 ul of FA lysis buffer (50 mM Hepes-KOH ph 7.9, 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% Na deoxycholate, 1 mM PMSF). 250 μl of acid washed glass beads were added and the cells were lysed by vortexing for 30 minutes at 4 °C. The tubes were punctured with a 22 gauge needle and drained into 15 ml tubes by centrifugation for 2 minutes at 2k rpm at 4 °C. The lysate was transferred to a 1.5 ml eppendorf tube and centrifuged for 15 minutes at 13.2k rpm at 4 °C. The pellet was resuspended in 1ml of ice cold FA lysis buffer with 0.1 % SDS and incubated at 4 °C with gentle shaking. Samples were then centrifuged for 15 minutes at 13.2k rpm at 4 °C and the pellet was resuspended in 1 ml FA lysis buffer and incubated for 10 minutes at 4 °C with gentle shaking. Samples were then centrifuged for 15 minutes at 13.2k rpm at 4 °C and the pellet was resuspended in 1 ml of ice cold water and centrifuged again similarly. Washed pellets were then resuspended in 500 μl of TM buffer (10 mM Tris-HCl pH 7.5, 5 mM $MgCl_2$) and aliquotted in 45 μl amounts in 1.5 ml flat eppendorf tubes. Samples were flash frozen in liquid nitrogen and stored at -80 °C until use.

5 μl of 10% SDS was added to each 45 μl sample and incubated for 30 minutes at 37 °C with gentle shaking, followed by the addition of 8 μl of 10% Triton X-100 and 12

ul of water and another incubation for 30 minutes at 37 °C with gentle shaking. 10 μl of 10x restriction enzyme buffer (3.1 or cutsmart, NEB) and 10 μl each of AluI and NlaIV (10 U/μl, NEB) were added to each sample and incubated at 37 °C for 6 hours with gentle shaking. An overnight double digestion was then performed by the addition of 70 μl water, 10 μl of 10x restriction enzyme buffer and another 10 μl of each enzyme followed by an overnight incubation at 4 °C with gentle shaking.

The next morning inactivation was performed by the addition of 5 μl of 10% SDS and incubation at 65 °C for 30 minutes. The SDS was then chelated by the addition of 75 μl of 10% Triton X-100 and 95 μl of water followed by incubation for 30 minutes at 37 °C with gentle shaking. Ligation was performed by the addition of 375 μl of 2x Quick Ligation Buffer (NEB) and 5 μl of Quick Ligase enzyme and incubation for 90 minutes at 25 °C with gentle shaking. Proteins were then digested by the addition of 7.5 μl of 10% SDS, 20 μl of 5M NaCl and 5 μl of 20 mg/ml proteinase K followed by incubation for 2 hours at 42 °C. 10 μl of additional proteinase K was then added to each sample and crosslinks were reversed by incubation overnight at 65 °C. The next morning DNA was purified by 3x DNA phenol chloroform pH 7.5 extractions. The supernatant was then transferred to a 2 ml microcentrifuge tube and the DNA was precipitated by EtOH precipitation using 0.1 volume 3 M Na acetate pH 5.2 and 2.5 volumes 100% EtOH and 2 μl of 20 mg/ml glycogen followed by centrifugation for 30 minutes at 14k rpm at 25 °C. The DNA pellet was then resuspended in 51 μl of 1x TE and quantity measured on a nanodrop. The concentration was then adjusted to either 100 ng/μl or the lowest concentration in the set of tubes. Gene specific primers were then chosen in order to identify promoter terminator interactions by a specific length ligation product. Control

primers spanning undigested regions were used to normalize 3C PCR signals against. The primers used for PCR analysis are listed in appendix C.

A.8. CO-IMMUNOPRECIPITATION (CO-IP)

Cells were grown as described above (A.1.2). Each liter of culture was split into 2 500 ml centrifuge tubes and centrifuged at 3k rpm for 5 minutes at 4 °C. The cell pellet obtained was washed once with 50 ml of ice-cold 1x TBS buffer containing 1% Triton X-100 and centrifuged again. The cell pellet was then washed with 50 ml of ice cold water and transferred to 50 ml conical tubes and centrifuged at 3k rpm for 5 minutes at 4 °C. The pellet (usually close to 7 ml) was resuspended in 9 ml of IP lysis buffer (20 mM Tris-HCl pH 8.0, 150 mM KCl, 0.5 mM EDTA, 1 mM $MgCl_2$, 0.1% Triton X-100, and 1 mM PMSF) and flash frozen drop by drop using an automated pipet in liquid nitrogen. The frozen cell pellet was homogenized to a very fine powder using a liquid nitrogen chilled mortar and pestle, transferred to a beaker, and allowed to thaw slowly on ice. The resulting cell lysate was centrifuged at 16,400 rpm for 20 min in a Sorvall SS-34 rotor, and the supernatant was transferred to a 15 ml tube. A 200 μl aliquot was transferred to a 1.5 ml eppendorf and 50 ul of 5x laemmli buffer (50% glycerol, 25% 2-mercaptoethanol, 10% SDS, 300 mM Tris-HCl pH 6.8, 0.01% bromophenol blue) was added to be used for input

For CO-IP experiments in strains harboring a C-terminal Myc tagged protein, anti-Myc conjugated agarose beads (Sigma) were used. For CO-IP experiments in strains harboring a C-terminal HA tagged protein, anti-HA conjugated agarose beads (Pierce) were used. For CO-IP experiments in strains harboring a C-terminal TAP tagged protein, IgG conjugated agarose beads (GE healthcare) were used. For CO-IP

experiments using anti-TFIIB antibodies, 50 μl of antiserum was bound with lysate for 1 hour at 4 °C with gentle agitation prior to incubation with protein A Sepharose beads (GE healthcare). A bed volume of 50 μl of affinity beads was washed 3x with IP lysis buffer prior to binding. For the MNase controls, the cell lysate was incubated with 300 units of MNase at 37 °C for 30 min prior to binding to the affinity beads. For the high salt controls, the IP lysis buffer was prepared as described above using a 500 mM KCl concentration.

Cell lysate was used to transfer the beads back into the 15 ml tube for binding. The binding was performed at 4 °C for 4 hours with gentle shaking. Following binding, the 15 ml tubes were centrifuged at 1k rpm for 2 minutes at 4 °C and another 200 ul aliquot was removed from the supernatant for flow through, the remaining supernatant was discarded. The beads were transferred to microcentrifuge tubes and washed five times with 1 ml each of IP wash buffer (20 mM Tris-HCl, pH 8.0, 50 mM KCl, 0.5 mM EDTA, 1 mM MgCl 2 , 1 mM PMSF, and 0.1% Triton X-100).

Elution was performed by one of three methods depending on the experiment and the affinity tag used for immunoprecipitation. For experiments that only required further SDS PAGE and Western blot analysis, elution was performed by using Laemmli buffer directly. For experiments that required further analysis by glycerol gradient sedimentation or FPLC analysis elution was performed with specific peptides for either cMyc or HA tags; or using proteolytic cleavage of the TAP tag.

For elution with Laemmli buffer, 200 μl of water and 50 μl of 5x Laemmli buffer was added to the beads. The samples were eluted by incubating for 15 minutes at 25

°C with gentle shaking. The samples were then centrifuged at 1.5k for 2 minutes at 25 °C and the supernatents were transferred to1.5 ml eppendorf tubes.

For elution with HA or c-Myc oligopeptides (Genscript), 200 μl of oligopeptide elution buffer (see below) was added to the beads and incubated for 30 minutes at 25 °C with gentle shaking. Due to the different number of peptide repeats in each C-terminal tag, 3x HA and 13x c-Myc, the concentrations for each oligopeptide were adjusted for elution. The HA-oligopeptide elution buffer used a concentration of 100 mg/ml in IP lysis buffer, and the c-Myc oligopeptide elution buffer used a concentration of 500 mg/ml in IP lysis buffer. After elution, samples were centrifuged at 1.5k rpm for 2 minutes at 25 °C and the supernatant was transferred to a 1.5 ml eppendorf tube and kept on ice to be used for either glycerol gradient sedimentation or FPLC analysis.

For elution against a TAP tagged protein using proteolytic cleavage, TurboTEV (MC Labs) was used against the TEV sequence in the TAP tag. The elution was performed by the addition of 195 μl of IP lysis buffer (worked better than the supplied buffer) with 2 mM DTT and 5 μl of TurboTEV enzyme followed by incubation either for 60 minutes at 25 °C, or overnight at 4 °C. After elution, samples were centrifuged at 1.5k rpm for 2 minutes at 25 °C and the supernatant was transferred to a 1.5 ml eppendorf tube and kept on ice for further use.

A.9 SDS PAGE and WESTERN BLOTTING

Eluted samples containing 1x Laemmli buffer were first heated for 20 minutes at 95 °C prior to loading. 10% gels were prepared using 1.5 mm premade cassettes (Invitrogen). The eluted proteins were separated by SDS-PAGE and transferred to a polyvinylidene difluoride (PVDF) membrane using an Amersham Biosciences TE70

semi-dry transfer apparatus. Following transfer, the membrane was additionally blocked by submerging in 100% methanol for a few seconds and then allowed to either dry overnight or in the 65 °C oven for 15 minutes. 14 ml of antibody solutions were prepared in 1x TBS with 0.25 g of nonfat dry milk according to manufacturer recommended dilutions for western blotting. For primary antibodies 140 μl of 1% sodium azide was added as a preservative and primary solutions were used for up to 3 months. Blotting was performed by incubating the membrane protein side down in the antibody solution for 1-2 hours, followed by two 5 minute washes in 1x TBS with gentle shaking. The protein bands were visualized using the Pierce Pico chemiluminescent reagents.

A.10 ACTIVATOR-TFIIB CO-IP

For coimmunoprecipitation using HA-tagged transcription activators, the cells were grown in 1 liter of appropriate medium to an A 600 of 1.0–1.2 and then induced for 2 h. Cross-linking was performed by incubating the cells in formaldehyde (final concentration, 0.5%) for 20 min, and the reaction was stopped by the addition of glycine (final concentration, 125 mM). The cell pellet was washed and lysed as described above. Cell lysate was subjected to sonication (30 pulses of 10s each at 25% duty cycle with 30s rest in between). The resulting cell lysate was centrifuged at 16,400 rpm for 20 min in a Sorvall SS-34 rotor. The supernatant was used for immunoprecipitation. One sample of the cell lysate, grown under induced conditions, was pre-treated with 10 units of micrococcal nuclease (WorthingtonLabs) for 30 min at 37 °C prior to binding. A 1-ml aliquot of the micrococcal nuclease-treated cell lysate was checked for complete digestion of chromatin by running on 1.5% agarose gel following deproteinization and crosslinking removal of samples.

A.11. GLYCEROL GRADIENT SEDIMENTATION

Affinity-purified samples were pipette mixed with an equal volume of ice-cold IP lysis buffer without glycerol and incubated for 20 min on ice. For each sample 20 ml of IP lysis buffer with 5% glycerol and 30% glycerol were prepared. Gradient analysis was performed in Seton 25 mm x 89 mm polyallomer tubes by first layering 19.5 ml of IP lysis buffer with 5% glycerol on the bottom using a syringe. Then carefully adding to the bottom, 19.5 ml of IP lysis buffer with 30% glycerol using a syringe placed in the center of the tube. The gradient tubes were then mixed on a Biocomp gradient master to create linear gradients using the settings: Time 1:30 seconds, Angle 75 degrees and Speed 25. The purified samples were loaded on the top of the gradient by pipetting slowly and touching the drops to the surface. The samples were then immediately centrifuged at 30,000 rpm for 18 h at 4 °C. Fractions of 1.8 ml each were collected manually using a Beckman fraction recovery system by puncturing the bottom, and 40 ul of each fraction was used for SDS PAGE and Western blotting.

A.12. FAST PRESSURE LIQUID CHROMATOGRAPHY (FPLC)

Affinity purified samples were combined from 4L of culture into a 1ml eppendorf tube. Sample was loaded into a 1 ml loop on the FPLC machine and a custom program was used to automate the collection of 1 ml fractions following passage of the void volume (39 ml). 54 fractions were collected after size exclusion chromatography using a superdex 200 column. Chromatography was performed using IP lysis buffer.

A.13 YEAST STRAIN CREATION

All yeast strains were created following a Lithium acetate transformation protocol. Cells were grown to an OD_{600}~0.6 and washed with 10 ml of ice cold Lithium acetate

buffer (100 mM LiOAc, 10 mM Tris-HCl pH 8.0, 1 mM EDTA). Cells were transferred with 100 μl of ice cold Lithium acetate buffer into eppendorf tubes containing the construct DNA and gently shaken for 5 minutes at 25 °C. The construct DNA was created from PCR using the high fidelity advantage enzyme and gene specific primers coupled with a tag or knock out plasmid listed in appendix C. Cells were then incubated with 280 μl of PEG solution (50% PEG 4000,100 mM LiOAc, 10 mM Tris-HCl pH 8.0 and 1 mM EDTA) for 45 minutes at 25 °C. Cells were then heat shocked with the addition of 43 μl DMSO for 5 minutes at 42 °C and immediately transferred to ice for at least 2 minutes. Cells were then centrifuged at 4k rpm for 30 seconds at 25 °C and the pellet was washed with 500 μl of either sterile water or media. For transforms using kanamyacin, cells were allowed an outgrowth period of two hours in non-selective media prior to plating. Cells were then resuspended and plated on selective plates. Colonies were isolated and positive transformants were screened by gene specific primers to verify the strain.

A.14 CLONING AND PURIFICATION OF RECOMBINANT TFIIB

The gene coding for yeast TFIIB, *SUA7*, was cloned into the NdeI-EcoRI sites of pET24a. Recombinant plasmid was transformed into the BL21 strain of *Escherichia coli*. Induction of recombinant TFIIB and preparation of cell lysate were performed as described by Ansari and Schwer (14). His-tagged TFIIB was affinity-purified on a Cobalt resin (Pierce Scientific) following the manufacturer's guidelines.

A.15. QUANTIFICATION

Initial quantification was performed using two independent replicas of each experiment followed by two rounds of PCR. For each PCR reaction, lanes were loaded

on duplicate gels in order to account for human pipetting error while loading. For each set of gels to be quantified following PCR, fresh agarose was prepared and all gels were poured at once in order to avoid differences in gel density when staining with ethidium bromide. Gels were stained for 20 minutes in a fresh solution of 200 ml 1x TAE with 10 ul of ethidium bromide (10 mg/ml). The signal strength of gel bands was analyzed using the Kodak Gel Logic 200 system after 2 second exposure. Pictures were analyzed using the Kodak 1D software to calculate the net intensity. The net intensities were then used to calculate a ratio of the signal of interest / control, and these ratios were averaged and the standard deviation was calculated. If standard deviations overlapped or displayed large variation, first another round of PCR and quantification was performed and averaged in. If there were still large standard deviations, a third replica experiment was performed and analyzed.

APPENDIX B: STRAINS

B.1. STRAINS USED IN CHAPTER II

Strain	genotype	Reference
FY23	MATa ura3-52 trp1∆63 leu2∆1	Madison and Winston, 1997 (1)
SSR2	MATa ura3-52 trp1∆63 leu2∆1, MET28-3xHA, KanMX	Kaderi et al, 2009
SAM6	MATa ura3-52 trp1Δ63 leu2Δ1, Δmet28::KanMX	Kaderi et al, 2009
BY4733	MATa his3∆200 trp1∆63 leu2∆0 met15∆0 ura3∆0	Hampsey lab
SRR3	MATa his3Δ200 trp1Δ63 leu2Δ0 met15Δ0 ura3Δ0, INO2-3xHA, KanMX	Kaderi et al, 2009
BEK3	MATa his3Δ200 trp1Δ63 leu2Δ0 met15Δ0 ura3Δ0 Δino2::KMX	Kaderi et al, 2009
YMH867	MATa leu2-3 can1-100 ura3-1 ade2-1 his3-11,15 trp1-1 GAL1 promoter upstream of BUD3, HIS3+	Ansari and Hampsey, 2005 (2)
SRR4	MATa leu2-3 can1-100 ura3-1 ade2-1 his3-11,15 trp1-1, Gal1p-BUD3, HIS+, GAL4-3xHA, KanMX	Kaderi at al, 2009
SAM4	MATa leu2-3 can1-100 ura3-1 ade2-1 his3-11,15 trp1-1,Gal1p:BUD3, HIS+, Δgal4::KMX	Kaderi at al, 2009
SRR1	MATa leu2-3 can1-100 ura3-1 ade2-1 his3-11,15 trp1-1,Gal1p-BUD3, HIS+, RNA15-TAP,TRP+	Kaderi at al, 2009
AA1	MATα ura3-1 trp1-1 ade2-1 leu2-3,112 his3-11,15 rna14-1 SUA7-TAP, TRP+	Medler et al, 2011
AA2	MATa ade1/ade2 lys2 ura3-52 pap1-1 SUA7-TAP, TRP+	Medler et al, 2011
SRR7	MATa his3Δ200 trp1Δ63 leu2Δ0 met15Δ0 ura3Δ0 RNA14-TAP, URA+	Medler et al, 2011
SRR8	MATa his3Δ200 trp1Δ63 leu2Δ0 met15Δ0 ura3Δ0 PCF11-TAP, URA+	Medler et al, 2011

SAM50	MATa his3Δ200 trp1Δ63 leu2Δ0 met15Δ0 ura3Δ0, RNA14-13xMyc, TRP+	Medler et al, 2011
SAM51	MATa his3Δ200 trp1Δ63 leu2Δ0 met15Δ0 ura3Δ0, RNA15-13xMyc, TRP+	Medler et al, 2011
SAM52	MATa his3Δ200 trp1Δ63 leu2Δ0 met15Δ0 ura3Δ0, PCF11-13xMyc ,TRP+	Medler et al, 2011
SAM53	MATa his3Δ200 trp1Δ63 leu2Δ0 met15Δ0 ura3Δ0, CLP1-13xMyc, TRP+	Medler et al, 2011
SAM54	MATa his3Δ200 trp1Δ63 leu2Δ0 met15Δ0 ura3Δ0, HRP1-13xMyc, TRP+	Medler et al, 2011
SAM55	MATa his3Δ200 trp1Δ63 leu2Δ0 met15Δ0 ura3Δ0, PAP1-13xMyc, TRP+	Medler et al, 2011
SAM56	MATa his3Δ200 trp1Δ63 leu2Δ0 met15Δ0 ura3Δ0, SUA7-3xHA, HIS+	Medler et al, 2011
SAM58	MATa his3Δ200 trp1Δ63 leu2Δ0 met15Δ0 ura3Δ0, SUA7-3xHA, HIS+, RNA14-13xMyc, TRP+	Medler et al, 2011
SAM59	MATa his3Δ200 trp1Δ63 leu2Δ0 met15Δ0 ura3Δ0, SUA7-3xHA, HIS+, RNA15-13xMyc, TRP+	Medler et al, 2011
SAM60	MATa his3Δ200 trp1Δ63 leu2Δ0 met15Δ0 ura3Δ0, SUA7-3xHA, HIS+, PCF11-13xMyc ,TRP+	Medler et al, 2011
SAM61	MATa his3Δ200 trp1Δ63 leu2Δ0 met15Δ0 ura3Δ0, SUA7-3xHA, HIS+, CLP1-13xMyc, TRP+	Medler et al, 2011
SAM62	MATa his3Δ200 trp1Δ63 leu2Δ0 met15Δ0 ura3Δ0, SUA7-3xHA, HIS+, HRP1-13xMyc, TRP+	Medler et al, 2011
SAM63	MATa his3Δ200 trp1Δ63 leu2Δ0 met15Δ0 ura3Δ0, SUA7-3xHA, HIS+, PAP1-13xMyc, TRP+	Medler et al, 2011
YMH14	MATα cyc1-5000 cyc7-67 ura3-52 leu2-3,112 cyh2	Pinto, 1994
YMH124	MATα cyc1-5000 cyc7-67 ura3-52 leu2-3,112 cyh2 sua7-1	Pinto, 1994
SAM64	MATα cyc1-5000 cyc7-67 ura3-52 leu2-3,112 cyh2 sua7-1, SUA7-3xHA,	Medler et al, 2011

	KANMX	
SFS1	MATα cyc1-5000 cyc7-67 ura3-52 leu2-3,112 cyh2 sua7-1, SUA7-3xHA, KANMX, PCF11-13xMyc, TRP+	Medler et al, 2011
SFS2	MATα cyc1-5000 cyc7-67 ura3-52 leu2-3,112 cyh2 sua7-1, SUA7-3xHA, KANMX, PAP1-13xMyc, TRP+	Medler et al, 2011
SFS3	MATα cyc1-5000 cyc7-67 ura3-52 leu2-3,112 cyh2 sua7-1, SUA7-3xHA, KANMX, RNA15-13xMyc, TRP+	Medler et al, 2011
SAM68	MATa his3Δ200 trp1Δ63 leu2Δ0 met15Δ0 ura3Δ0, TBP-3xHA, HIS+	Medler et al, 2011
YMH804	MATa ura3 leu2 trp1 his3 ade2 ade3 can1 kin28Δ::trp1 (pKIN28-HA)	Hampsey lab
SHY407B	MATα ade2Δ his3Δ200 leu2Δ0 met15Δ0 trp1Δ ura3Δ0 RPB9-Flag1-TAP::TRP1	Rani, 2004
rna14-1	MATα ura3-1 trp1-1 ade2-1 leu2-3,112 his3-11,15 rna14-1	Minveill-Sebastia, 1994
AA1	MATα ura3-1 trp1-1 ade2-1 leu2-3,112 his3-11,15 rna14-1 SUA7-TAP, TRP+	Medler et al, 2011
pcf11-2	MATa ura3-1 trp1Δ ade2-1 leu2-3,112 his3-11,15 pcf11Δ::TRP1/pEL36-pcf11-2	Amrani, 1997
NAH12	MATa ura3-1 trp1Δ ade2-1 leu2-3,112 his3-11,15 pcf11Δ::TRP1/pEL36-pcf11-2 SUA7-TAP, TRP+	Medler et al, 2011
hrp1-5	MATα cup1Δ ura3 his3 trp1 lys2 ade2 leu2 hrp1::HIS3[pRS315-hrp1-L205S (LEU2)]	Kessler, 1997
NAH13	MATα cup1Δ ura3 his3 trp1 lys2 ade2 leu2 hrp1::HIS3[pRS315-hrp1-L205S (LEU2)] SUA7-TAP, TRP+	Medler et al, 2011
pap1-1	MATa ade1/ade2 lys2 ura3-52 pap1-1	Patel, 1992
AA2	MATa ade1/ade2 lys2 ura3-52 pap1-1 SUA7-TAP, TRP+	Medler et al, 2011

B.2. STRAINS USED IN CHAPTER III

BY4733	MATa his3Δ200 trp1Δ63 leu2Δ0 met15Δ0 ura3Δ0	Hampsey lab
BPM5	MATa his3Δ200 trp1Δ63 leu2Δ0 met15Δ0 ura3Δ0, TFB4-TAP, URA+	This study
SAM89	MATa his3Δ200 trp1Δ63 leu2Δ0 met15Δ0 ura3Δ0, KIN28-TAP, URA+	This study
SAM90	MATa his3Δ200 trp1Δ63 leu2Δ0 met15Δ0 ura3Δ0, SSL2-TAP, URA+	This study
SAM93	MATa his3Δ200 trp1Δ63 leu2Δ0 met15Δ0 ura3Δ0, KIN28-TAP, URA+, RNA15-13xMyc, TRP+	This study
SAM51	MATa his3Δ200 trp1Δ63 leu2Δ0 met15Δ0 ura3Δ0, RNA15-13xMyc, TRP+	El Kaderi et al, 2009
SAM94	MATa his3Δ200 trp1Δ63 leu2Δ0 met15Δ0 ura3Δ0, KIN28-TAP, URA+, SSU72-13xMyc, TRP+	This study
SAM103	MATa his3Δ200 trp1Δ63 leu2Δ0 met15Δ0 ura3Δ0, SSU72-13xMyc, TRP+	This study
yFR763	MATα, ade::higG, his3Δ200, leu2Δ0, lys2Δ0,met15Δ0,trp1Δ63,ura3Δ0 kin28::kin28-L83G [pSH579, ARS CEN URA3 kin28-L83G]	Liu Y et al, 2004
SAM99	MATα, ade::higG, his3Δ200, leu2Δ0, lys2Δ0,met15Δ0,trp1Δ63,ura3Δ0 kin28::kin28-L83G [pSH579, ARS CEN URA3 kin28-L83G] Kin28as-TAP, TRP+	This study
SAM101	MATα, ade::higG, his3Δ200, leu2Δ0, lys2Δ0,met15Δ0,trp1Δ63,ura3Δ0 kin28::kin28-L83G [pSH579, ARS CEN URA3 kin28-L83G] Kin28as-TAP, TRP+,	This study

	RNA15-13xMyc, HIS+	
SAM102	MATα, ade::higG, his3Δ200, leu2Δ0, lys2Δ0,met15Δ0,trp1Δ63,ura3Δ0 kin28::kin28-L83G [pSH579, ARS CEN URA3 kin28-L83G] Kin28as-TAP, TRP+, SSU72-13xMyc, HIS+	Pinto, 1994
SAM104	MATα, ade::higG, his3Δ200, leu2Δ0, lys2Δ0,met15Δ0,trp1Δ63,ura3Δ0 kin28::kin28-L83G [pSH579, ARS CEN URA3 kin28-L83G] RNA15-13xMyc, HIS+	This study
SAM105	MATα, ade::higG, his3Δ200, leu2Δ0, lys2Δ0,met15Δ0,trp1Δ63,ura3Δ0 kin28::kin28-L83G [pSH579, ARS CEN URA3 kin28-L83G] SSU72-13xMyc, HIS+	This study
SAM106	MATα, ade::higG, his3Δ200, leu2Δ0, lys2Δ0,met15Δ0,trp1Δ63,ura3Δ0 kin28::kin28-L83G [pSH579, ARS CEN URA3 kin28-L83G] RPB4-13xMyc, HIS+	This study

APPENDIX C: PRIMERS

C.1. RT Primers

cDNA

Name	Sequence
Oligo dT	TTTTTTTTTTTTTTTTTTTTTTTT
18s	GACGGAGTTTCACAAGATTACC
CHA1 d1	GGAAAAAATCAATACTAGCAAAATA
CHA1 d2	GCTTTTCTTCACTTAGTAAGGATTAA
CHA1 d3	CTGGGGTCTTCATTTGTGTCA
ACT1 d1	GATAAAGTCAGTGCTTAAACACGTC
ACT1 d2	ATAAAACTGAAAAGCGATGAAGAG
ACT1 d3	TTTGCGTAACGTTTGGATGG

18s Control-PCR

Name	Sequence
18s F	GGAATAATAGAATAGGACGTTTGG
18s R	GTTAAGGTCTCGTTCGTTATCG

Gene Specific RT-PCR Primers

Name	Sequence
CHA1 F	AATTCAAAAGGACGGTAAAAGAT
CHA1 R	AAGGGATGAACATAAATGGGC
MET16 F	CATTTGGTTTGACTGGCTTGG
MET16 R	TCGTACTTGTCATCATCTTTCTCC
INO1 F	GATATCCAGAATTTCAAAGAAGAAAAC
INO1 R	TATTCTGCGGTGAACCATTAATATAG

ACT1 F	TACTCTTTCTCCACCACTGCTG
ACT1 R	GATTTCCTTTTGCATTCTTTCG
MSN5 F	CAATGCCAATCCAAACAGTG
MSN5 R	CGCACTATTACACAGCACATTTA
CMP1 F	AACCGCAGAATAATGAATAAAGTG
CMP1 R	GATATAAGGTTGGGTTCTTTGCT
SPC1 F	GTGCTCTCGCTACTTTTCTGG
SPC1 R	CATTGTGCTGTTCAGAGAACCA
IMD4 F	ATTGGTATGGGTTCTGGGTC
IMD4 R	GCCTTCAATCTCTTACCATCC
ASC1 F	CTTACGCTTTGTCTGCTTCTTG
ASC1 R	GATGGTCTTGTCACGGGAAC
GAL10 F	GATCTTCCATACAAAGTTACGGG
GAL10 R	CACAAATCTTGCGTCATAACG
HXT1 F	ATTTGGTATGAAGCACCACGA
HXT1 R	GGGCGACCTCAGATATTAGCA

C.2. C-Terminal Tagging Primers

Name	Sequence
Gal4-HA-tag-F2	ATAACTATCTATTCGATGATGAAGATACCCCACCAAACCCAAAAAAAGAG CGGATCCCCGGGTTAATTAA
Gal4-HA-tag-R1	ATGCACAGTTGAAGTGAACTTGCGGGGTTTTTCAGTATCTACGATTCATT GAATTCGAGCTCGTTTAAAC
5'Gal4-HA-tag-D	TGTGCGCCGTTTCTGTTATC

Ino2-HA-tag-F2	AGTCCATTAGAAGCGCAAATGAAGCACTACAGCACATACTGGATGATT CC CGGATCCCCGGGTTAATTAA
Ino2-HA-tag-R1	AAAATACATCCAACGGGAGGCCATTTTCATCACTAATAGCTTGTATGAG C GAATTCGAGCTCGTTTAAAC
5'Ino2-HA-tag-D	TTGTCTCCTTCCAGTTCGGG
Met28-HA-tag-F2	TTAAGTCTTTGAAATTGTTGAATGACATTAAGAGACGGAACATGGGCAG G CGGATCCCCGGGTTAATTAA
Met28-HA-tag-R1	ATCGAAGTTGGAGAGGAAAAACAAGACATCAGGCCCGCACGTTTCGC GGG GAATTCGAGCTCGTTTAAAC
5'Met28-HA-tag-D	GGTGGGAAAAGAAATCAACAAAC
F2-Myc-RNA14	TTTTAAATGATCAAGTAGAGATTCCAACAGTTGAGAGCA CCAAGTCAGGTCGGATCCCCGGGTTAATTAA
R1- Myc-RNA14	AGATGTGTTGGTATAAATATTCATATATACCTATTTATTA ACGTAATGTTAGAATTCGAGCTCGTTTAAAC
F2- Myc-RNA15	CTATTTGGGACTTAAAACAAAAAGCATTAAGGGGAGAA TTTGGTGCATTTCGGATCCCCGGGTTAATTAA
R1- Myc-RNA15	ATCATTGCGGAACCGCATTTTTTTTTTGTATTTTTGCCTCC CTAGTTTCAGAATTCGAGCTCGTTTAAAC
F2- Myc-PCF11	CTAATAGTGGCAAGGTCGGTTTGGATGACTTAAAGAAAT TGGTCACAAAACGGATCCCCGGGTTAATTAA
R1- Myc-PCF11	TAATATAATATATAGTTATTAAATTTAAATGTATATATGC AGTTCTGCTCGAATTCGAGCTCGTTTAAAC
F2- Myc-*HRP1*	GTCGCGGTGGATACAATAGACGTAATAATGGCTACCATC CATATAATAGGCGGATCCCCGGGTTAATTAA
R1- Myc-*HRP1*	TGAATTATACAAGAAAACTTTTCTCTAGTTTTCTACACTT TTCTTTTTTTGAATTCGAGCTCGTTTAAAC
F2- Myc-*CLP1*	GCCGACTTCCCAGCAAGGCGATGATTCTAACTTCATATA GATATTTAGAGCGGATCCCCGGGTTAATTAA
R2- Myc-	TACGATATTTGTATGGATTTGATATAAGGCTCTTGAACA

CLP1	GATAATTTTACGAATTCGAGCTCGTTTAAAC
F2- Myc-PAP1	CTGCTTCAGGTGACAACATCAATGGCACAACCGCAGCTG TTGACGTAAACCGGATCCCCGGGTTAATTAA
R1- Myc-PAP1	TGACTGATTAACCTATATTAATAAACTATTCAACTATAA ATAGGAATGTCGAATTCGAGCTCGTTTAAAC
F2-HA-TFIIB	TTGCTAATGGTGTAGTGTCTTTGGATAACTTACCGGGCG TTGAAAAGAAACGGATCCCCGGGTTAATTAA
R1-HA-TFIIB	CACGAGTACCCGTGCTTCTTGTTCCTATAATTTACTGTTT TATCACTTCAGAATTCGAGCTCGTTTAAAC
RNA15-Myc-Diag	TCCAGGCCGCAAGAAGAG
RNA14-Myc-Diag	AAGAGACTCAGAGCTTCCAACAG
PCF11-Myc-Diag	GTCCAATTTGTAAGGAAACCG
HRP1-Myc-Diag	AGCAAGATTCAAATGCCACTC
PAP1-Myc-Diag	GTAACAGATGAAAATAAAGAGGAAGAA
CLP1-Myc-Diag	TTGAGTCCTTATGCTATTGGTGTT
TFIIB-HA-Diag	CCGATGCAAGTCACTACTTCTG
Myc-tag-Diag	CAAGTCTTCCTCGGAGATTAGC
HA-tag-Diag	GGTAGAGGTGTGGTCAATAAGAGC
5’ KIN28-Myc/HA-F2	TCAAAGAATTACCACCACCAAGTGACCCGTCTTCAATAAAAATACGTAA CCGGATCCCCGGGTTAATTAA
3’ KIN28-Myc/HA-F2	GATACATCTAATGTCAATAACACAGATTCTACAAATTTTATAAAATCATA GAATTCGAGCTCGTTTAAAC

5’ KIN28-Tag-Diag	CGCCTTAGATTTTATGTGTGGA
5’ SSU72-Myc-F2	GGCAAAGCTCACATTCTCAACTACCGTCATTATACGCTCCTTCATATTACCGGATCCCCGGGTTAATTAA
3’ SSU72-Myc-R1	ATGAGGGCCGCTTAATGCTTATGCTTTTCTACAGTAATTGACCGTTTTGTGAATTCGAGCTCGTTTAAAC
5’ SSU72 –Tag diag	ATGATGATGAAAATGCTAAAATTG
5’ TFB4-C-TAP	GGAAACCAGTTGTTCCAAGGTTGAAAGCCAAAAAGAAGGTGACGAAACCATCCATGGAAAAGAGAAG
3’ TFB4-C-TAP	ATTGTGACGAAGGTTACCTGCTTGAAACGGATAATGTTCATTCCTTTCGTTACGACTCACTATAGGG
TFB4-TAP-DIAG	AGGACATCATGCTATTTAACAGGG
5’ C-TAP Ssl2	AGGAACATCATCCATTAATCAGAAAGATGTATTATAAGAATTTGAAGAAGTCCATGGAAAAGAGAAG
3’C-TAP Ssl2	TATGACTGAATAGATTCAAAATAGGAAGGTGACAATGAAACCAAGCCTATTACGACTCACTATAGGG
5’C-TAP SSL2-Diag	TTACACATTTACACGGAATGGAG
5’C-TAP kin28	TCAAAGAATTACCACCACCAAGTGACCCGTCTTCAATAAAAATACGTAACTCCATGGAAAAGAGAAG
3’C-TAP kin28	GATACATCTAATGTCAATAACACAGATTCTACAAATTTTATAAAATCATATACGACTCACTATAGGG
5’C-TAP kin28-Diag	CGAAGTTTCTTCCTTTATGACG
5’RNA15-TAP-C	CTATTTGGGACTTAAAACAAAAAGCATTAAGGGGAGAATTTGGTGCATTTTCCATGGAAAAGAGAAG
3’RNA15 –TAP-C	ATCATTGCGGAACCGCATTTTTTTTTTGTATTTTTGCCTCCCTAGTTTCATACGACTCACTATAGGG
5’TAP-Rna15-D	TCCAGGCCGCAAGAAGAG

5'Ssu72-C-TAP	GGCAAAGCTCACATTCTCAACTACCGTCATTATACGCTCCTTCATATTACTCCATGGAAAAGAGAAG
3'Ssu72-C-TAP	ATGAGGGCCGCTTAATGCTTATGCTTTTCTACAGTAATTGACCGTTTTGTTACGACTCACTATAGGG
5'Ssu72-C-TAP-Diag	GTGAAGATTTGATGAATAGAGG
3' ANY TAP Diag	GTTGAATTTGTTGTCTACTTTCGG

C.3. ChIP Primers

***INO1*-ChIP**

Name	***Sequence***
CHA1 A	GATAGCCTCTTGCGACCTTATT
	CATTCATATTTCAAGAAAAATTGTG
CHA1 B	AATTCAAAAGGACGGTAAAAGAT
	AAGGGATGAACATAAATGGGC
CHA1 C	GGTGGAAACGAATGGATGTC
	TCTTAGTGTTGTAACCCAAATGC
CHA1 D	GGAAGAAGCGTTGGATAGCAT
	CCCCTTTATACAAATTCTGTGC

***MET16* ChIP**

Name	**Sequence**
INO1 A	GCTTGTTCTGTTGTCGGGTTC
	GGAGGTGATTGGAGCAATATTATC
INO1 B	GATATCCAGAATTTCAAAGAAGAAAAC
	TATTCTGCGGTGAACCATTAATATAG
INO1 C	GTATTAAACCGGTCTCCATTGC

	CCGACGGGCTTCATATATTTG
INO1 D	CTCATTTCAACGACTCTCTTTTTC
	GCACTTTCTCGCATCTACCTCA

***HXT1* ChIP**

Name	Sequence
HXT1 P	GCGATGAGATAAGATAAAAGGGA
	GATTACCGATTCCTCTACTTTTGA
HXT1 M	ATTTGGTATGAAGCACCACGA
	GGGCGACCTCAGATATTAGCA
HXT1 T	GGTGGAAACGAATGGATGTC
	TCTTAGTGTTGTAACCCAAATGC

***CHA1* ChIP**

Name	Sequence
CHA1 P	GCCCCAGCGGAAATGTAA
	CATTCATATTTCAAGAAAAATTGTG
CHA1 M	GCCCAGGTTATCGTGAGTG
	CACCTCCACCAACGCTGC
CHA1 T	GGAAGAAGCGTTGGATAGCAT
	CAGTAGTTTATGCTTTATGCTCG
CHA1 D1	GCACAGAATTTGTATAAAGGGG
	GCTTTTCTTCACTTAGTAAGGATTAA
CHA1 D2	GTTCCGTAATAATCTTCCCAGC
	CTGGGGTCTTCATTTGTGTCA

***ASC1* ChIP**

Name	Sequence
ASC1 P	GACTGCTCCTTTGGTTTTCC
	GGTTGACCAGCAGAAGTAGCC
ASC1 M	CGAAAAAGCTGATGATGACTCTG
	TTGATGTTGGAGTTGTGACCG
ASC1 T	TGGCAAGTTATGACTGCTAACTAAG
	GCCAAGGAGACTGAATTTAATG

***ACT1* ChIP**

Name	Sequence
ACT1 P	CAAACTCGCCTCTCTCTCTCC
	GCAAGCGCTAGAACATACCAG
ACT1 M	CATACCTTCTACAACGAATTGAGAG
	CTTCATCAAGTAGTCAGTCAAATCTC
ACT1 T	TGGTCCATCTATCGTTCACCA
	ATAAAACTGAAAAGCGATGAAGAG
ACT1 D1	GTTTTGTCTCTCCCTTTTCTACG
	GGTATCAAAACGCCGGACTC
ACT1 D2	CCGCCATTAGAATTTGAGTCC
	TTTGCGTAACGTTTGGATGG

C.4. CCC Primers

HXT1 CCC

Name	Sequence

HXT1 P1	TCGGGTGTTAAGAAATATTTTGC
HXT1 T1	TTAACAGATAACCGAGTCGATCTC
HXT1 C1	AATACCACATAGGCGCTATACATAG
HXT1 C2	CGTCTTTTCTTTACTGCTTCACC

CHA1 CCC

Name	Sequence
CHA1 P1	GGAAAATGTTTATACAGTTTTCTCTT
CHA1 T1	GGAAAATGTTTATACAGTTTTCTCTT
CHA1 C1	GGAAGAAGCGTTGGATAGCAT
CHA1 C2	CGTTTTGGATATGTTGATGCTTAC

ACT1 CCC

Name	Sequence
ACT1 P1	CGAGTTTGGTTTCAAAACGG
ACT1 T1	CCGCCATTAGAATTTGAGTCC
ACT1 C1	TGGTCCATCTATCGTTCACCA
ACT1 C2	AATTTTCGTAGAAAAGGGAGAGAC

ASC1 CCC

Name	Sequence
ASC1 P1	GACTGCTCCTTTGGTTTTCC
ASC1 T1	CTTTATTTCCTTTATTGTGGTATTAG
ASC1 C1	ATGCTGTTTCTTTGGCTTGG
ASC1 C2	TGTACATATGTATTTTCGCAGCA

APPENDIX D: MEDIA

YEAST EXTRACT-PEPTON-DEXTROSE (YPD) medium (1 liter)

Component	Quantity	Notes
Yeast extract	10 g	
Peptone	20 g	
Dextrose	20 g	100 ml of 20% stock-add after autoclaving
Agar	20 g	For plates only
NaOH	1 pellet	For plates only

INOSITOL DROP-OUT MEDIUM (1 liter)

Component	Quantity	Notes
Ammonium Sulfate	5 g	
Vitamin Stock	1 ml	Of 1000X stock solution
Trace Elements Stock	1 ml	Of 1000X stock solution
Salt Mix	1.7 g	
Inositol drop-out amino acid Mix	230 mg	
Dextrose	20 g	100 ml of 20% stock-add after autoclaving

TRACE ELEMNTS STOCK (1000X; 100 ml)-FOR INOSITOL DROP-OUT MEDIA

Component	Quantity	Notes
Boric acid	50 mg	Autoclave Store in a dark bottle at 4^{o}C
Copper sulfate	4 mg	
Potassium iodide	10 mg	
Ferric chloride	20 mg	
Manganese sulfate	40 mg	
Sodium molybdate	20 mg	
Zinc sulfate	40 mg	

VITAMIN STOCK (1000X; 100 ml)- FOR INOSITOL DROP-OUT MEDIA

Component	Quantity	Notes
Biotin	2 mg	Autoclave Store in a dark bottle at 4^{o}C
Calcium pantothenate	200 mg	
Folic acid	0.2 mg	
Niacin	40 mg	
β-Aminobenzoic acid	20 mg	
Pyridoxine hydrochloride	40 mg	
Riboflavin	20 mg	
Thiamin hydrochloride	40 mg	

SALT MIX- FOR INOSITOL DROP-OUT MEDIA

Component	Quantity	Notes
Potassium phosphate monobasic	85 g	
Potassium phosphate dibasic	15 g	
Magnesium sulfate	50 g	
Sodium chloride	10 g	
Calcium chloride	10 g	

AMINO ACID MIX- FOR INOSITOL DROP-OUT MEDIA

Component	Quantity	Notes
Adenine hemisulfate	40 mg	
Histidine	20 mg	
Leucine	60 mg	
Lysine	30 mg	

Methionine	20 mg	
Tryptophan	40 mg	
Uracil	20 mg	

INOSITOL STOCK (100 X; 100 ml)

Component	Quantity	Notes
Inositol (for plus inositol medium)	1 g	1 ml/ liter of inositol drop-out medium

METHIONINE DROP-OUT MEDIUM (1 liter)

Component	Quantity	Notes
Yeast nitrogenous base	6.7 g	without amino acids
methionine drop-out mix	1 g	
agar	20 g	For plates only
NaOH	1 pellet	For plates only
Dextrose	20 g	100 ml of 20% stock-add after autoclaving

METHIONINE DROP-OUT MIX- FOR METHIONINE DROP-OUT MEDIA

Component	Quantity	Notes
Adenine	2.5 g	
L-arginine	1.2 g	
L- asparatic acid	6.0 g	
L- glutamic acid	6.0 g	
L-Histidine	1.2 g	
L-leucine	3.6 g	
L-lysine	1.8 g	
L-phenylalanine	3.0 g	

L-tryptophan	2.4 g	
L-tyrosine	1.8 g	
L-valine	9.0 g	
Uracil	1.2 g	

AMMONIUM SULFATE MEDIUM (1 liter)-FOR *CHA1* REPRESSION

Component	Quantity	Notes
Yeast nitrogenous base	1.7 g	Without amino acids Without ammonium sulfate
Ammonium sulfate	5 g	
Amino acid mix	230 mg	
Dextrose	20 g	100 ml of 20% stock-add after autoclaving

SERINE-THREONINE MEDIUM (1 liter)-FOR *CHA1* ACTIVATION

Component	Quantity	Notes
Yeast nitrogenous base	1.7g	Without amino acids Without ammonium sulfate
L-serine	1 g	
L-threonine	1 g	
Amino acid mix	230 mg	
Dextrose	20 g	100 ml of 20% stock-add after autoclaving

AMINO ACID MIX FOR *CHA1* MEDIA

Component	Quantity	Notes
Adenine hemisulfate	40 mg	
Histidine	20 mg	
Leucine	60 mg	
Lysine	30 mg	

Methionine	20 mg	
Tryptophan	40 mg	
Uracil	20 mg	

TRYPTON DROP-OUT MEDIUM (1 liter)

Component	Quantity	Notes
Yeast nitrogenous base	6.7 g	without amino acids
Trypton drop-out amino acid mix	1 g	
agar	20 g	
NaOH	1 pellet	
Dextrose	20 g	100 ml of 20% stock-add after autoclaving

TRYPTON DROP-OUT MIX

Component	Quatity	Notes
Adenine	2.5 g	
L-arginine	1.2 g	
L- asparatic acid	6.0 g	
L- glutamic acid	6.0 g	
L-Histidine	1.2 g	
L-leucine	3.6 g	
L-lysine	1.8 g	
L-methionine	1.2 g	
L-phenylalanine	3.0 g	
L-tyrosine	1.8 g	
L-valine	9.0 g	

Uracil	1.2 g	

G418 PLATES (KMX-MEDIUM) -1 liter

Component	**Quantity**	**Notes**
Yeast nitrogenous base	10.0 g	without amino acids
peptone	20.0 g	
agar	20.0 g	
Dextrose	20 g	100 ml of 20% stock-add after autoclaving
G418	1.0 ml	Of 400 mg/ml

2XYT MEDIUM-1 liter

Component	**Quantity**	**Notes**
Yeast extract	10.0 g	without amino acids
Tryptone	16.0 g	
NaCl	5.0 g	
Agar	20.0 g	For plates only

APPENDIX E: BUFFERS AND SOLUTIONS

STOCK SOLUTIONS

Reagent	Molarity/ concentration/ percentage	Notes
Tris-HCl- pH 8.0	1.0 M	• Adjust pH using HCl
EDTA pH 7.0 to 8.0	0.5 M	• Adjust pH using NaOH
NaCl	5.0 M	• Autoclave
KCl	2.0M	• Autoclave
SDS	10%	• Filter sterilize
$CaCl_2$	1.0 M	• Autoclave
$MgCl_2$	1.0 M	• Autoclave
PEG (Mw 4000)	50 %	• Filter sterilize
LiOAc	1.0 M	• Filter sterilize
Glycine	2.5 M	• Autoclave
Ammounium acetate	7.5 M	• Autoclave
NaOAc pH 5.2	3.0 M	• Adjust pH using glacial acetic acid
Glycerol	50 %	• Autoclave
Tergitol	10 %	• Autoclave
Triton X-100	10 %	• Filter sterilize
LiCl	5.0 M	• Autoclave
HEPES pH 7.9	1.0 M	• Adjust the pH using KOH • Filter sterilize
Sodium deoxycholate	10%	• Filter sterilize
KOH	10.0 M	• Autoclave

Dextrose	20 %	• Autoclave
PMSF	100 mM	• Don't autoclave • Keep at 4^{o}C
Glycogen	20 mg/ ml	• Filter sterilize
DTT	1.0 M	• Filter sterilize
Ethedium bromide	10.0 mg / ml	• Don't autoclave • Keep at 4^{o}C
Ammonium acetate	7.5 M	• Autoclave
TE	10X	• 100 mM Tris.HCl pH 8.0 • 10 mM EDTA
TAE	50 X	• 2.0 M Tris-acetate • 50 mM EDTA
TBS	10X	• 100 mM Tris-HCl pH 8 • 2M NaCl

AGAROSE GEL ELECTROPHORESIS BUFFER (1X TAE)

Component	Concentration	Notes
Tris-acetate	40 mM	• Autoclave
0.5 M EDTA pH 8.0	1 mM EDTA	• Autoclave

SOLUTIONS FOR YEAST GENOMIC DNA EXTRACTION

Reagent	Composition	Notes
Lysis buffer	2% Triton X-100 100 mM NaCl 10 mM Tris-HCl pH 8.0 10 mM EDTA 1% SDS	

SOLUTIONS FOR LiOAc/DMSO YEAST TRANSFORMATION

Reagent	Composition	Notes
LiAOAc buffer	0.1 M LiAOAc 10 mM Tris-HCl(pH=8.0) 1 mM EDTA	
PEG solution	50 % w/v PEG (M.W. = 4000)	Filter sterilize

	0.1 LiAOAc 10 mM Tris-HCl (pH=8.0) 1 mM EDTA	
DMSO	100 %	

SOLUTIONS FOR PLASMID MINIPREP

Solution	Composition	Notes
Solution I	50 mM Dex 10 mM EDTA 25 mM Tris-HCl pH 8.0	
Solution II	0.1 N NaOH 1% SDS	
Solution III	30 ml 5M KOAc 5.75 ml glacial HOAc 14.25 ml H2O	• Store at – 20 ^{o}C

YEAST CELL WASH

component	Concentration	Notes
Wash buffer I	1X TBS	• Autoclave
Wash buffer II	1XTBS 1% Triton X-100	• Autoclave

CHROMATIN IMMUNOPRECIPITATION (ChIP) BUFFERS AND SOLUTIONS

FA-LYSIS BUFFER

Reagent	Concentration	Notes
HEPES-KOH pH 7.9	50 mM	• Store at -20 °C
NaCl	140 mM	
EDTA	1 mM	
Triton X-100	1 %	
Sodium Deoxycholate	0.1 %	
PMSF	1 mM	
SDS	0.07 %	

FA-LYSIS BUFFER + 500 mM NaCl

Reagent	Stock Concentration	Volume added
HEPES-KOH pH 7.9- 8.0	50 mM	• Store at –20 °C
NaCl	500 mM	
EDTA pH 8.0	1 mM	
Triton X-100	1 %	
Sodium Deoxycholate	0.1 %	
PMSF	1 mM	
SDS	0.07 %	

ChIP WASH BUFFER

Reagent	Concentration	Notes
Tris-HCl pH 7.5 to 8	10 mM	• Store at -20 °C
LiCl	250 mM	
Triton X-100	0.5 %	
EDTA pH 8.0	1 mM	
Sodium Deoxycholate	0.5 %	
SDS	0.1 %	

ChIP ELUTION BUFFER

Reagent	Concentration	Notes
Tris-HCl pH 7.5 to 8.0	50 mM	• Store at room temperature
SDS	1 %	
EDTA pH 8.0	10 mM	

REVRESE TRANSCRIPTION PCR (RT-PCR) BUFFERS AND SOLUTIONS

HIGH TE BUFFER

Reagent	Concentration	Notes
Tris-HCl pH 7.5	50 mM	• Store at RT
EDTA	20 mM	

RNA-LYSIS BUFFER

Reagent	Concentration	Notes
Tris-HCl pH 8.0	80 mM	
$CaCl_2$	10 mM	
β-mercatoethanol	10 mM	
VCR (Shake well)	10 mM	

CHROMOSOME CONFORMATION CAPTURE SOLUTION

TM BUFFER

Component	Concentration	Notes
Tris HCl pH 7.5- 8.0	10 mM	
$MgCl_2$	5 mM	

TRANSCRIPTION RUN-ON ASSAY SOLUTIONS AND BUFFERS

Reagent	Composition	Notes
20X SSC	3 M NaCL 300mM Na3CitrateX2H2O	• Adjust pH to 7.0 using HCl
Sarkosyl	10%	
Boiling solution	0.4N NaOH 1 mM EDTA	
Hybridization solution	0.5M potassium phosphate pH 7.2 7% SDS	
Membrane wash I	0.1% SDS 1% SSC	
Membrane wash II	0.1% SDS 0.1% SSC	

2.5 XRun-on buffer	50 mM Tris-HCl pH 7.5 500 mM KCl 80 mM $MgCl_2$ 5 mM DTT	
NTPs/RNase inhibitor mix	10 mM each of CTP, ATP, and GTP 300 units of RNase Inhibitor 7 µl of [α-^{32}P]-UTP (3000 Ci/mmol, 10 µCi/µl	
TMN buffer	10 mM Tris-HCl pH 7.5 5 mM $MgCl_2$ 100 mM NaCl	
NaOAc/HOAc mix	0.3 M sodium acetate pH 5.2 0.5 µl of glacial acetic acid	
LETS buffer	0.1M LiCl 0.2% SDS 10 mM EDTA 10 mM Tris-HCl pH 7.5	

IMMUNOPRECIPITATION ASSAY BUFFERS AND SOLUTIONS

Component	Concentration	Notes
IP lysis buffer	10% glycerol 20 mM Tris-HCl pH 8.0 50 mM KCl 0.5mM EDTA 1 mM MgCl2 0.1% TritonX-100 1 mM PMSF (add directly before use)	• Autoclave • Keep at 4°C
30%Acrylamide:Bis Solution	1 % Bisacrylamide 29% Acrylamide	
4% stacking gel	125 mM Tris-Cl, pH 6.8 0.1% SDS 5% Acrylamid mix 0.1 Ammonium persulfate	• Keep at 4°C
Electrode buffer	25 mM tris 250 mM glycine 0.1 SDS	
5X laemeli buffer	250 mM Tris HCl pH 6.8 50 % Glycerol 10% SDS 2.8 M β-mercaptoethanol 0.1% Bromophenol blue	
Transfer Buffer	20% Methanol	

	24 mM Tris-base 192 mM Glycine	

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ABSTRACT

GENERAL TRANSCRIPTION FACTORS PLAY DUAL ROLES IN INITIATION AND TERMINATION

by

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Gene looping, defined as the interaction of the promoter and the terminator regions of a gene during transcription, is emerging as an important gene regulatory mechanism in eukaryotes. The role of promoter bound general transcription factors during initiation is well established. However, recent studies have revealed that some initiation factors also interact with the 3' end of a gene. The biological role of initiation factors at the 3' end of a gene is unknown. The general transcription factors TFIIB and TFIIH have been found to interact genetically with Ssu72, a component of CPF 3' end processing complex. Accordingly, we found that TFIIB and TFIIH localize to the distal ends of genes in a transcription dependent manner. TFIIB localization at the terminator region during transcription requires a functional CF1 complex. TFIIB physically interacts with the all subunits of the CF1 complex in an activator dependent manner. TFIIH also interacts with the CF1 and CPF 3' end processing complexes in a manner depending on its kinase activity. Employing affinity chromatography and glycerol gradient centrifugation, we show that TFIIB associates with poly(A) polymerase and the CF1 complex in yeast cells to form a holo-TFIIB complex. This complex was resistant to

MNase digestion and brief exposure to high salt. The sedimentation coefficient of the holo-TFIIB complex was intermediate between that of TFIIH and TFIID. Initiation factors which remain bound on a promoter scaffold in vitro, were not found in a holo-TFIIB complex with termination factors. The holo-TFIIB complex was observed only in the looping competent strains, but not in the looping defective sua7-1 strain. We further show that in sua7-1 cells, where a holo-TFIIB complex is not formed, the kinetics of activated transcription is altered. These results strongly suggest a role for TFIIB in termination of transcription. Similarly, the kinase dependent presence of TFIIH at the 3' end of genes suggested a role for the factor in termination. Accordingly, we show that RNAP II read through the termination signal in the absence of Kin28 kinase activity. Furthermore, the recruitment of CF1 and CPF subunits at the 3' end of a gene is impaired in the TFIIH kinase defective mutant. We propose that initiation factors are in contact with the terminator during gene looping and play an active role in transcription termination.

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Scott Medler, Nadra Al Husini, Sarita Raghunayakula, Banupriya Mukundan and Athar Ansari. (2011) Evidence for a complex of TFIIB with poly(A) polymerase and cleavage factor 1 subunits required for gene looping. *J.Biol.Chem*. **286**, 33709-33718.

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AWARDS

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