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Functions of Mediator and the RNA Polymerase II C-terminal Domain in Transcription Initiation

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Summary

RNA polymerase II (RNAPII) has been identified almost 40 years ago, but the molecular details of its regulation and fine tuning during messenger RNA (mRNA) synthesis are still far from understood. Subsequently to RNAPII six general transcription factors (GTFs; TFIIA, TFIIB, TFIID, TFIIE, TFIIF, TFIIH) were discovered of which all except TFIIA are necessary and sufficient for promoter-dependent basal transcription initiation. In addition to the GTFs activator-dependent transcription requires the presence of a transcription cofactor, the Mediator complex. Mediator serves as a link between transcription activators, enhancers and the general transcription machinery. Initial studies revealed that Mediator stimulates the activity of the TFIIH associated kinase CDK7 and thereby facilitates RNAPII C-terminal domain (CTD) phosphorylation. Furthermore the Mediator complex interacts functionally with several signal transduction pathways and serves as an signal integration platform.

In order to dissect the process of transcription initiation, early studies made use of *in vitro* transcription systems reconstituted from recombinant or highly purified GTFs and RNAPII. In this system basal, activator-independent transcription does not require the presence of the Mediator complex. If however a more physiological nuclear extract transcription system is used, our laboratory and others have established previously that basal transcription becomes critically dependent on Mediator. Another difference between both transcription systems is that the first is insensitive to the kinase inhibitor H8 whereas in the second transcription can be inhibited by H8. This suggests that only the second transcription system is regulated by RNAPII CTD phosphorylation. In this thesis the interplay between Mediator, RNAPII, GTFs and transcription cofactors was studied using immobilized promoter template assays in combination with various immunodepleted nuclear extracts and recombinant factors.

Negative cofactor 2 (NC2) is an evolutionary conserved general cofactor that binds to many active genes *in vivo*. Previous studies in our laboratory had shown with recombinant proteins that NC2 competes with TFIIA and TFIIB for binding to TATA-binding protein (TBP) at a promoter *in vitro*. Genetic studies in yeast provided evidence that Mediator acts antagonistically to NC2. Here I have studied the role of NC2 on

preinitiation complex (PIC) formation and transcription in nuclear extracts. I observed rapid association of TFIID with promoters whereas NC2 enters PICs with a slow kinetic which is similar to that of TFIIB recruitment. My data indirectly suggest that TBP binds to DNA in a yet to be defined inactive form (perhaps as a TFIID complex) which is then slowly converted into an active TBP-TATA complex that is rapidly recognized by GTFs or NC2. My data support the notion that NC2 and TFIIB compete for binding to a PIC also in immobilized promoter assays under physiological conditions. NC2 concentrations in nuclear extracts appears to be tightly controlled. Doubling the NC2 concentration in a nuclear extract by adding recombinant NC2 (rNC2) abolished functional PIC formation and transcription. However, the *in vitro* analysis also showed that upstream of NC2 PIC formation is fully dependent on Mediator. Hence, TFIID binds to a promoter in a nuclear extract *in vitro* transcription system but we have no indication that a transcription competent PIC is formed in the absence of Mediator.

In yeast studies it was reported that upon transcription initiation *in vitro* several GTFs dissociate from the promoter DNA template whereas the Mediator complex is retained in a reinitiation complex. In the human system I recapitulate this observation for TFIIB and CDK7. In addition I provide evidence that Mediator partially dissociated from the promoter template upon transcription initiation. Upon transcription initiation the middle module subunit MED7 was retained on a promoter template, whereas the tail module subunit MED15 and CDK8 did dissociate. This data suggest that upon transcription initiation a head/middle module Mediator subcomplex is retained at the promoter whereas the tail and CDK8 modules dissociate.

Previous studies have established that Mediator promotes CDK7-dependent phosphorylation of the RNAPII CTD at serine-5 (ser-5). Various studies found that CTD ser-5 phosphorylation does coincide with transcription initiation. Using new monoclonal antibodies I observed two functionally distinct modes of CTD ser-5 phosphorylation *in vitro*: Hypo- and hyperphosphorylation of the largest RNAPII subunit Rpb1. I observed that CTD ser-5 hypophosphorylation is established already before complex opening by TFIIH. I found CTD ser-5 hypophosphorylation to be critically dependent on TBP, Mediator, TFIIB and CDK7. In addition I noted that CTD ser-5 hypophosphorylation correlates with the transcription potential of a PIC. CTD ser-5 hyperphosphorylation was

established in a Mediator-dependent fashion but independent of productive transcription. Immunodepletion of CDK7 did not led to a reduction in CTD ser-5 hyperphosphorylation. However, immunodepletion of CDK8 caused a reduction but not a loss of CTD ser-5 hyperphosphorylation upon transcription initiation indicating that another yet to be identified kinase might be involved in this process. These data suggest that CTD ser-5 hypophosphorylation is established only in the PIC context on RNAPII located at *bona fide* promoter regions but not on RNAPII complexes bound to DNA outside of promoter regions, e.g. in an open reading frame.

Recently phosphorylation of the RNAPII CTD at serine-7 (ser-7) was reported. In that study the entire coding region of the TCRβ locus was found to be associated with RNAPII CTD phosphorylated at ser-7. Starting from there I found that establishment of CTD ser-7 phosphorylation in the process of transcription initiation can be recapitulated in an immobilized template assay system *in vitro*. I confirmed the *in vitro* finding that establishment of CTD ser-7 phosphorylation correlates with transcription initiation with chromatin immunoprecipitation experiments on an inducible model gene system *in vivo*. Similar to CTD ser-5 phosphorylation, I observed two modes of CTD ser-7 phosphorylation: CTD ser-7 hypo- and hyperphosphorylation. In contrast to CTD ser-5 hypophosphorylation, which was established before complex opening, I observed establishment of CTD ser-7 hypophosphorylation predominantly after complex opening by TFIIH. Both, CTD ser-7 hypo- and hyperphosphorylation were found to be Mediator-dependent. A mass spectrometric screen for PIC associated kinases (in collaboration with the laboratory of Gerhard Mittler) yielded 13 kinases. Seven of the identified kinases were further tested for their potential to phosphorylate the RNAPII at ser-7 in an immobilized template assay.

Publications

Boeing, S.; Heidemann, M.; Eick, D.; Meisterernst, M.; Regulation of several RNA polymerase II CTD-phosphorylation states by Mediator upon transcription initiation. In preparation.

Albert, T.; Grote, K.; **Boeing, S.;** Stelzer, G.; Schepers, A.; Meisterernst, M.; Global distribution of negative cofactor 2 subunit-alpha on human promoters. Proc. Natl. Acad. Sci. U.S.A. 24 (2007), 10000-10005.

Uhlmann, T.*; **Boeing, S.***; Lehmbacher, M.; Meisterernst, M.; The VP16 activation domain establishes an active mediator lacking CDK8 *in vivo*. J.Biol. Chem. 282 (2007), 2163-2173. * equal contribution

Boeing, S. and Meisterernst, M.; Kontrolle der Genexpression durch Transkriptions-cofaktoren. Biospektrum 12 (2006), 311-312. Review. German.

A. Introduction

A.1 Transcription Overview

More than 30 years ago Robert Roeder and William Rutter purified RNA polymerase I, II and III (Roeder and Rutter, 1969). Since then six general transcription factors and several positive and negative cofactors have been identified but still the exact molecular mechanism of transcription initiation remains elusive. Messenger RNA biogenesis is a complex and highly interconnected process in which large protein complexes devoted to RNA synthesis, elongation, capping, splicing, polyadenylation and RNA export act simultaneously in the context of chromatin. Phosphorylation of the RNAPII CTD has been described in great detail but several mechanistic explanations are missing in particular with respect to CTD ser-5 phosphorylation. Till the present day it could not be conclusively resolved whether RNAPII moves along a DNA template during transcription, or whether the DNA is pulled through a large protein assembly called the transcription factory. Another mystery represents the mechanism by which RNAPII manages to transcribe through chromatin. (Hirose and Manley, 2000; Maniatis and Reed, 2002; Orphanides and Reinberg, 2002; Proudfoot et al., 2002). For the development and maintenance of an organism it is of paramount importance to regulate and coordinate all of the above processes properly. Gene expression can be controlled at several levels: The access of transcription activators to promoters is regulated by chromatin, transcription initiation by an complex interplay of transcription factors being recruited to promoters and enhancers. Before RNAPII switches to productive mRNA synthesis, abortive transcripts are produced. In addition transcription at many promoters appears to be regulated at the elongation step, as many genes are found to be associated with initiated but paused polymerases (Guenther et al., 2007). Furthermore is the stability of a particular mRNA subject to regulation by various mechanisms within the cell.

A.1.1 The Transcription Cycle

The transcription cycle of RNAPII can be divided into six subsequent steps: (1) Pre-initiation complex (PIC) formation, (2) transcription initiation and (3) promoter clearance, (4) elongation, (5) 3'end formation and finally (6) termination of transcription (Fig. 1). The first step in transcription initiation complex formation is the recruitment of

the transcription machinery to the promoter to form a preinitiation complex (PIC). The efficiency of this step is determined by the accessibility of the chromatin-embedded promoter, promoter specific regulatory factors and the core promoter elements (TATA box, initiator (INR), TFIIB recognition element (BRE) and downstream promoter element (DPE)). Two models were proposed: A stepwise assembly pathway and a holoenzyme model. In the stepwise assembly model the GTFs and RNAPII are recruited sequentially. This model describes the situation well if purified or recombinant proteins are used in an *in vitro* transcription assay (Orphanides et al., 1996). The holoenzyme model states that GTFs and RNAPII are recruited together with coactivators like Mediator in a preassembled complex to the promoter. This model is based on *in vitro* data in which nuclear extracts instead of purified or recombinant factors were used (Chang and Jaehning, 1997; Myer and Young, 1998). The yeast holoenzyme complex contains the GTFs TFIIB and TFIIH (Wilson et al., 1996). TFIID and TFIIA are not found in holoenzyme complexes. Upon PIC assembly components are recruited the ATP-dependent DNA helicase activity XPB, which is part of the TFIIH complex, catalyses the formation of the open complex. During open complex formation 11-15 bp of double-strand DNA close to the transcription start site are melted and the template strand is positioned into the active site of RNAPII (Wang et al., 1992). Formation of the open complex can be inhibited by ATPγS, which inhibits the TFIIH associated helicase (Stelzer et al., 1994). After formation of a 4 nt RNA, transcription is no longer sensitive to ATPγS, indicating that the TFIIH helicase is only required for the initial DNA melting (Stelzer et al., 1994). In several transcription systems multiple short RNAs (3-10 nt in length) are generated as so called abortive transcripts before RNAPII productively initiates synthesis of full length transcripts (Holstege et al., 1996; Luse and Jacob, 1987). During promoter clearance RNAPII is released from the PIC. Based on the finding that the Mediator complex does interact with unphosphorylated but not with hyperphosphorylated RNAPII CTD (Svejstrup et al., 1997) it is likely that CTD hyperphosphorylation plays a crucial role in promoter clearance. During PIC formation or transcription initiation CTD ser-5 phosphorylation is established but it has not been resolved in the literature at which point it is established. Furthermore it is not definitely solved to which degree the promoter associated CTD ser-5 kinases CDK7 and CDK8

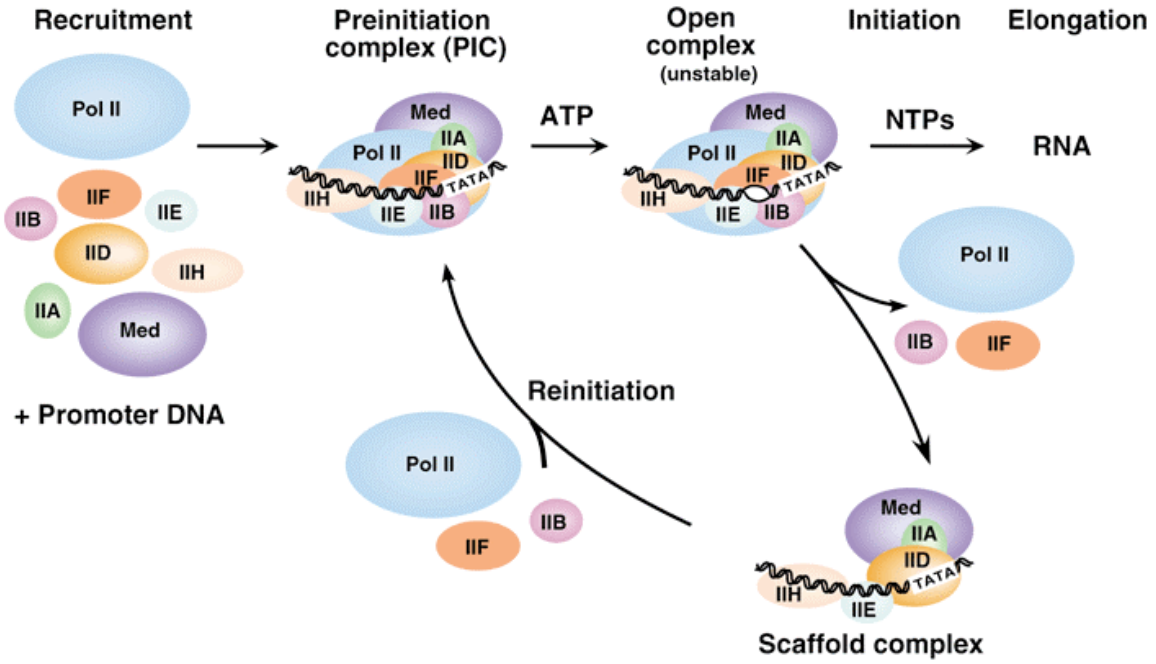

Figure 1: The pathway of transcription initiation and reinitiation for RNAPII. Taken from a review by Steven Hahn (Hahn, 2004).

contribute to CTD ser-5 phosphorylation of RNAPII (Lee and Young, 2000; Liu et al., 2004). Upon transcription initiation *in vitro* the PIC does partially dissociate. In yeast TFIIA, TFIID, TFIIE and p62 of TFIIH and several Mediator subunits remain associated with the promoter template whereas RNAPII, TFIIB and TFIIF do not. Notably the tail Mediator subunit MED15 (Gal11) appears to dissociate whereas the head middle subunits MED17 (Srb4), MED20 (Srb2) and MED6 remain attached to the promoter DNA template upon transcription initiation (Yudkovsky et al., 2000). RNAPII that managed to escape the promoter will finally switch to the elongation mode, which is associated with phosphorylation of the RNAPII CTD at ser-2, which in turn is mediated by CDK9 (Price, 2000). Upon reinitiation of transcription at the promoter RNAPII, TFIIB and TFIIF are recruited again to the promoter, and the transcription cycle starts over again with PIC formation (Hahn, 2004).

A.2. Regulation of RNA Polymerase II

Timing and location of RNAPII transcription initiation is tightly regulated by a variety of accessory factors such as general transcription factors, positive and negative cofactor complexes and chromatin modifying enzymes. These factors guide RNAPII to *bona fide* transcription start sites and facilitate transcription initiation. A particular regulatory surface is the C-terminal domain (CTD) of the largest subunit of RNAPII. It acts as a binding surface for transcription related protein complexes. Post-translational modifications of the CTD ensure recruitment of these complexes in the correct order (Meinhart et al., 2005). Furthermore transcription regulation by CTD post-translational modifications is proposed to occur in a combinatorial fashion, as stated in the CTD-code hypothesis (Buratowski, 2003). A common view on RNAPII function is that it travels along the DNA template during transcription. Given the fact that the assembled RNAPII transcription machinery represents a complex of several megadalton, it is also possible that the DNA is moved though a stationary transcription factory. Evidence for this scenario comes from a combination of three-dimensional fluorescence in situ hybridization, immunofluorescence and chromosome conformation capture (3C) experiments. They revealed that active genes are clustered in the nucleus and that these genes are recruited to sites of active transcription, and not *vice versa* (Cook, 1999; Faro-Trindade and Cook, 2006).

A.2.1. RNA Polymerase II

RNAPII is a 500 kDa multiprotein complex composed of 12 subunits. The subunits can be classified into three groups: (i) subunits that are shared between RNAPI, II and III (Rpb5, 6, 8, 10, 12); (ii) RNAPII specific core subunits (Rpb1, 2, 3, 11) and (iii) the RNAPII specific subunits Rpb 4 and 7 that are not essential for transcription elongation *in vitro* (Cramer et al., 2000). A unique feature of RNAPII is the presence of several repeats of the heptapeptide YSPTSPS in the C-terminal region of Rpb1. All amino acids of the heptarepeats have the potential to be post-translationally modified. The RNAPII CTD is part of the largest subunit of RNAPII, Rpb1, and attached to the body of RNAPII by a flexible linker region. In x-ray structures of RNAPII the CTD is not visible and

therefore may exhibit an unstructured conformation. Binding of transcription cofactors may induce distinct CTD structures (Meinhart and Cramer, 2004; Noble et al., 2005).

A.2.1.1. CTD

The C-terminal domain of RNAPII can be seen as regulatory switchboard for RNAPII transcription activity. It interacts with components of the preinitiation complex, e.g. the Mediator complex (Naar et al., 2002), as well as with factors required for RNA processing, e.g. the capping enzyme complex (Komarnitsky et al., 2000). By now it is established that RNAPII can be phosphorylated at ser-2, -5 and -7 of a heptarepeat. During the transcription cycle, RNAPII is incorporated into the PIC in the unphosphorylated form, and then sequentially phosphorylated at ser-5 and, as the polymerase switches to the elongation mode, at ser-2. The physiological significance of CTD ser-7 phosphorylation has not been elucidated in detail (Chapman et al., 2007; Phatnani and Greenleaf, 2006). Interestingly, *in vitro* transcription reactions involving highly purified or recombinantly expressed factors are not dependent on CTD phosphorylation, whereas *in vitro* transcription in the nuclear extract system is dependent on CTD phosphorylation and sensitive to the kinase inhibitor H8 (Serizawa et al., 1993).

A.2.1.2. CTD kinases

Several kinases have the potential to phosphorylate the CTD of RNAPII at least *in vitro*. Most CTD kinases belong to the class of cell-cycle dependent kinases (CDKs) and require a cyclin as cofactor for full activation. The largest subgroup within the CTD kinases represent the CDK1-related CDC2-like kinases CDK1, CDK9, CDK11, CrkRS and CDK13. The Mediator complex can be associated either with CDK8 or the CDK8-like kinase CDK11. Another PIC associated CTD kinase is CDK7. DNA-PK and ERK have been identified as CTD kinases, however little is know about the physiological significance of their particular CTD phosphorylation activities (Table 1).

CDK	CTD-P specificity	General function	Cyclins/ Cofactors	Reference
CDK1	ser-2, ser-5	mitosis	A, B	(Cisek and Corden, 1989; Gebara et al., 1997)
CDK2	ser-2, ser-5	mitosis	E	(Deng et al., 2002)
CDK7	ser-5	mitosis/ transcription	H	(Trigon et al., 1998)
CDK8	ser-5	transcription	C	(Hengartner et al., 1998)
CDK8-like (aka CDK11)		transcription	C	(Tsutsui et al., 2008)
CDK9	ser-2, ser-5	transcription	T, K	(Price, 2000)
CDK11		alternative splicing	L	(Hu et al., 2003)
CDK12/CrKRS/ CDC2L7		transcription/ splicing	L	(Chen et al., 2006)
CDK13/CDC5L		splicing		(Chen et al., 2007)
CKII		ubiquitous		(Trembley et al., 2003)
DNA-PK	ser-2, ser-7	transcription reinitiation	Ku80, Ku86	(Trigon et al., 1998)
ERK1/2	ser-5	signal transduction		(Trigon et al., 1998)

Table 1: CTD kinases and their CTD phosphorylation specificity as reported in the literature

CDK1

In yeast the CDK1 homologue CDC2 (*S. pompbe*) or CDC28 (*S.cerevisiae*) are required for the G1-S and the G2-M transition. CDC2 is involved in the coordination of cell-cycle dependent gene transcription (Wittenberg and Reed, 2005). CDK1 is responsible for cell cycle-dependent CTD hyperphosphorylation during M-phase. As a consequence RNAPII-dependent transcription is shut down. During mitosis the hypophosphorylated IIa form of RNAPII virtually disappears and the hyperphosphorylated, inactivated form predominates (reviewed in (Bregman et al., 2000)). Purified CDC2/cyclin B kinase is sufficient to inhibit transcription in reconstituted transcription reactions with biochemical purified and recombinant basal transcription factors (Long et al., 1998). Yu and colleagues described CDK1 in *S.cerevisiae* as essential for GAL1 transcription. However, they find that the kinase activity of CDK1 is not essential for transcription initiation (Yu et al., 2005). CDK1 is activated by T-loop phosphorylation and a substrate of CDK7 within the CAK complex (Larochelle et al., 2007). Based on *in vitro* kinase assays with CTD peptides as substrates, CDK1 phosphorylates ser-2 and ser-5 within the CTD of RNAPII (Gebara et al., 1997).

CDK9 (CDC2L4)

CDK9 acts in conjunction with T-type cyclins and is the catalytic subunit of the positive transcription elongation factor (pTEFb) (Price, 2000). P-TEFb acts at the level of elongation and is regulated by reversible association of the regulator 7SK RNA and an RNA binding protein, HEXIM1 or HEXIM2 (Byers et al., 2005). The p-TEFb activity is required to overcome a transcription arrest during early elongation which is imposed by the DSIF and NELF complexes. In this process the RNAPII CTD is critical (Marshall et al., 1996; Marshall and Price, 1995) and phosphorylated predominantly by pTEFb associated CDK9 at ser-2. Another substrate of pTEFb is the hSpt5 subunit of DSIF (Price, 2008). CTD ser-2 phosphorylation increases towards the 3'end of a transcribed gene *in vivo* (Chapman et al., 2007).

CDK11 (CDC2L1/CDC2L2)

CDK11, formerly known as PITSLRE, is a member of the CDK1-related kinases. It is encoded by two different, but highly similar, genes CDC2L1 (cell devision control like 1) and CDC2L2 (cell devision control like 2) (Kahle et al., 2005). Three variants of CDK11 are found in a cell, CDK11(p110), CDK11(p58) and CDK11(p46). All isoforms are associated with L-type cyclins. A yeast two-hybrid screen found CDK11(p46) to interact with the CCR4-NOT complex component NOT2 (Shi and Nelson, 2005). The CDK11(p110) isoform has been implicated in splicing, as immunodepletion of CDK11 from a splicing extract reduces the appearance of spliced products in an *in vitro* system (Hu et al., 2003; Loyer et al., 2008). CDK11 co-purifies and is tightly associated with casein kinase II (CKII). CDK11 associated CKII phosphorylates the CTD of RNAPII as well as CDK11(p110) (Trembley et al., 2003). CDK11(p110) was found to be a substrate of CDK7 *in vitro* (Larochelle et al., 2006).

CrkRS (CDK12/CDC2L7)

CrkRS, also named CDK12 or CDC2L7 (cell devision control like 7), is a CDK1-related kinase that contains an arginine- and serine-rich domain, a feature that is found in several SR protein family splicing factors. CrkRS interacts with L-type cyclins and has been shown to regulate alternative splicing of an E1A minigene. (Chen et al., 2006). Anti-

CrkRS antibodies stain nuclei in a pattern that overlaps with that of splicosomal components and hyperphosphorylated RNAPII. Anti-CrkRS immunoprecipitations phosphorylate the RNAPII CTD *in vitro* (Ko et al., 2001). Human CDK9 is orthologous to *S.cerevisiae* BUR1, whereas the CTD kinase CTDK-I is more closely related to human CDK13 and CrkRS (Guo and Stiller, 2004).

CDK13 (CDC2L5)

CDK13, also known as CDC2L5 (cell devision control like 5), is like CrkRS involved in the regulation of alternative splicing. It interacts with L-type cyclins (Chen et al., 2007).

CDK7

CDK7 is a kinase with a dual function, depending on the complex it is associated with. The trimetric CAK complex is required for cell cycle progression (Larochelle et al., 2007). Genetic studies in yeast indicated that the *S.cerevisiae* CDK7 homologue Kin28 is essential for RNAPII gene expression (Cismowski et al., 1995; Valay et al., 1995, Holstege et al., 1998). A recent study using a chemical inhibitor raised specifically against an engineered Kin28 protein found that the kinase activity of Kin28 is dispensable for transcription (Kanin et al., 2007). CDK7 is part of the TFIIH complex and has been shown to phosphorylate RNAPII CTD at ser-5. Furthermore CDK7 has been shown to be required for the assembly of CDK1/cyclin B complexes as well as for activation of CDK2 (Larochelle et al., 2007).

CDK8/CDK11

CDK8 is component of the Mediator complex. It is a RNAPII CTD kinase that phosphorylates the CTD at ser-5 (Hengartner et al., 1998). A close homologue of CDK8 is the CDK8-like (CDK8L) kinase (Sato et al., 2003). Like CDK8 it is associated with the Mediator complex. In some publications this kinase is referred to as CDK11, however it is not identical to the CDK1-related CDK11 kinase (Tsutsui et al., 2008).

DNA Protein Kinase (DNA-PK)

DNA-PK is associated with PICs formed on immobilized promoter templates *in vitro*. In this context DNA-PK acts as a CTD kinase (Peterson et al., 1995). Inhibition of DNA-PK with wortmannin does not have an effect on *in vitro* transcription (Woodard et al., 1999).

Casein Kinase II (CKII)

Casein kinase II is a ubiquitous kinase involved in various processes. It was found to be the CTD kinase activity present in a CDK11 immunoprecipitation (Trembley et al., 2003). Furthermore it is required for downstream-promoter element (DPE) dependent transcription *in vitro* (Lewis et al., 2005).

A.2.1.3. CTD phosphatases

CTD phosphorylation is counteracted by at least two particular CTD phosphatases, Fcp1 and Ssu72. Fcp1 has been described as CTD ser-2 and ser-5 phosphatase and is associated with the general transcription factor TFIIF (Lin et al., 2002). The CTD also can be dephosphorylated at ser-5 by the Ssu72 phosphatase, which is associated with the cleavage and polyadenylation factor complex and also does interact with the general transcription factor TFIIB (Dichtl et al., 2002; Krishnamurthy et al., 2004). A model is proposed in which the Ssu72 phosphatase catalyzes ser-5 dephosphorylation after pre-mRNA capping in a manner that facilitates the transition of RNAPII from the initiation to the elongation stage of the transcription cycle (Reyes-Reyes and Hampsey, 2007).

A.2.3. General Transcription Factors

In *in vitro* transcription systems composed of only recombinant or highly purified proteins, a strict order of incorporation of general transcription factors into the PIC could be determined. The first factor to bind the TATA box of DNA in such a system is TFIID, then TFIIB is recruited to the DNA-IID complex. After that RNAPII is recruited together with TFIIF, and finally TFIIE and TFIIH are recruited into the PIC. TFIIA is not absolutely required for transcription initiation and therefore not considered a general transcription factor. In yeast, TFIIA acts at an early step of PIC assembly by enhancing the stable recruitment of TFIID. TFIIA was found to increase the rate of PIC formation,

but has no influence on the stability of a PIC (Ranish et al., 1999). If a more complex *in vitro* transcription system is used, e.g. the nuclear extract transcription system it is not clear whether PIC assembly follows the scheme above.

Several laboratories purified a RNAPII holoenzyme complex, a pre-assembled complex composed of RNAPII, Mediator and the general transcription factors TFIIB, TFIIE, TFIIF and TFIIH. TFIID was not found in the holoenzyme complex (Chang and Jaehning, 1997; Maldonado et al., 1996; Ossipow et al., 1995; Wilson et al., 1996). In addition components of the hnRNP and SWI/SNF complexes were described as constituent of the holoenzyme complex (Kim and Nikodem, 1999; Wilson et al., 1996). Which PIC assembly model holds true *in vivo*, the stepwise GTF assembly model or the holoenzyme model is not conclusively determined till the present day.

A.2.3.1. TFIID

TBP, the TATA-box binding protein is a universal transcription factor required by all three eukaryotic RNA polymerases (Hernandez, 1993). The RNAPII-associated TFIID complex is composed of 14 TBP-associated proteins (TAFs) and about 700 kDa in size (Albright and Tjian, 2000; Tora, 2002). X-ray studies revealed that the core domain of TBP binds to a TATA box in a saddle-like conformation (Burley and Roeder, 1996). TBP binds the 8 bp TATA box unwinds it about a third of a helical turn and thereby bends the DNA in an 80 degree angle (Kim et al., 1993a; Kim et al., 1993b). The TBP molecule is not symmetric in itself and does not bind to TATA elements with high orientation specificity. TBP requires other promoter elements and initiation factors in order to assemble the transcription machinery in the right orientation (Cox et al., 1997).

A.2.3.2. TFIIB

TFIIB is recruited to the PIC after TBP. It interacts with DNA and can recognize the BRE-core promoter element (Imbalzano et al., 1994). TFIIB is at least in part responsible for transcription start site selection by RNAPII (Hawkes and Roberts, 1999). There are reports suggesting that TFIIB might be recruited by transcriptional activators such as VP16 (Roberts et al., 1993). Recently it was demonstrated that TFIIB helps to form a loop between the promoter and the polyA region of a gene (Singh and Hampsey, 2007).

A.2.3.3. TFIIF

TFIIF consists of two subunits Rap30 and Rap74. TFIIF binds tightly to RNAPII, suppresses unspecific DNA binding of RNAPII and stabilizes the PIC (Conaway and Conaway, 1993). TFIIF also has a function during elongation. It prevents pausing of RNAPII (Bengal et al., 1991). TFIIF has been shown to be associated with a serine/threonine kinase activity which may modulate TFIIF function (Rossignol et al., 1999).

A.2.3.4. TFIIE

TFIIE consists of two subunits, α and β, which form an $\alpha_2\beta_2$heterotetramer (Ohkuma et al., 1991). TFIIE interacts directly with the unphosphorylated form of RNAPII, TFIIF and TFIIH (Flores et al., 1989). TFIIE stimulates TFIIH recruitment as well as its helicase activities (Ohkuma, 1997).

A.2.3.5. TFIIH

TFIIH is a complex composed of nine polypeptides with four enzymatic activities: A DNA-dependent ATPase (Roy et al., 1994) two ATP dependent helicases (Schaeffer et al., 1993) and a kinase activity (Lu et al., 1992). The TFIIH complex consist of two distinct domains: a six subunit core complex, which contains the two DNA helicase activities and the CAK (CDK activated kinase) domain, consisting of the CDK7 kinase, cyclin H and MAT1 (Coin and Egly, 1998). The XPB DNA-helicase activity is essential for the formation of an open promoter complex (Holstege et al., 1996; Moreland et al., 1999; Wang et al., 1992). The CDK7 kinase activity is important for promoter clearance, the transition from transcription initiation to elongation (Dvir et al., 1997). Recently it has been demonstrated that the kinase module of TFIIH is recruited in a Mediator-dependent fashion to a promoter (Esnault et al., 2008). The CDK7 kinase activity is stimulated by the Mediator complex (Kim et al., 1994). The TFIIH complex is not only important in transcription initiation, but also part of the nucleotide excision repair pathway (Svejstrup et al., 1995).

A.2.4. Positive transcription cofactors

Most transcription cofactors were purified from the upstream stimulatory activity (USA) fraction. This fraction represses transcription in the absence of activators and stimulates it in the presence of activators (Meisterernst et al., 1991). The USA fraction contains six positive cofactors (PC1: PARP-1 (Meisterernst et al., 1997), PC2: Mediator complex (Kretzschmar et al., 1994; Malik et al., 2000), PC3: Topoisomerase I (Kretzschmar et al., 1993; Merino et al., 1993), PC4 (Kaiser et al., 1995), PC5 and PC6. PC1, PC3, PC4 and HMG2 are all non-sequence specific DNA binding proteins suggesting that these cofactors function by affecting the accessibility of RNAPII to promoters in the chromatin context (Kaiser and Meisterernst, 1996). PC3/Topoisomerase I has been reported to comprise an intrinsic kinase activity that phosphorylates splicing factors (Rossi et al., 1996). In addition two negative cofactors (NC) were identified in the USA fraction – NC1 and NC2 (see chapter A.2.5.). In addition the Integrator complex was described as a RNAPII cofactor which is essential for transcription of small nuclear RNAs (Baillat et al., 2005).

A.2.4.1. Mediator complex

Initially the Mediator complex was purified as the activity that is required for activator-dependent *in vitro* transcription in a system reconstituted from recombinant and highly purified factors. To do so, the Mediator complex functionally connects promoter- and enhancer-bound transcription activators to the general transcription machinery. Subsequently it was discovered that the Mediator has functions in transcription initiation beyond that. *In vitro*, transcription can be observed from a DNA template even in the absence of an activator, and termed basal transcription (Blazek et al., 2005). In a transcription system reconstituted from recombinant and purified factors no Mediator complex is required basal transcription. If however the more physiological nuclear extract

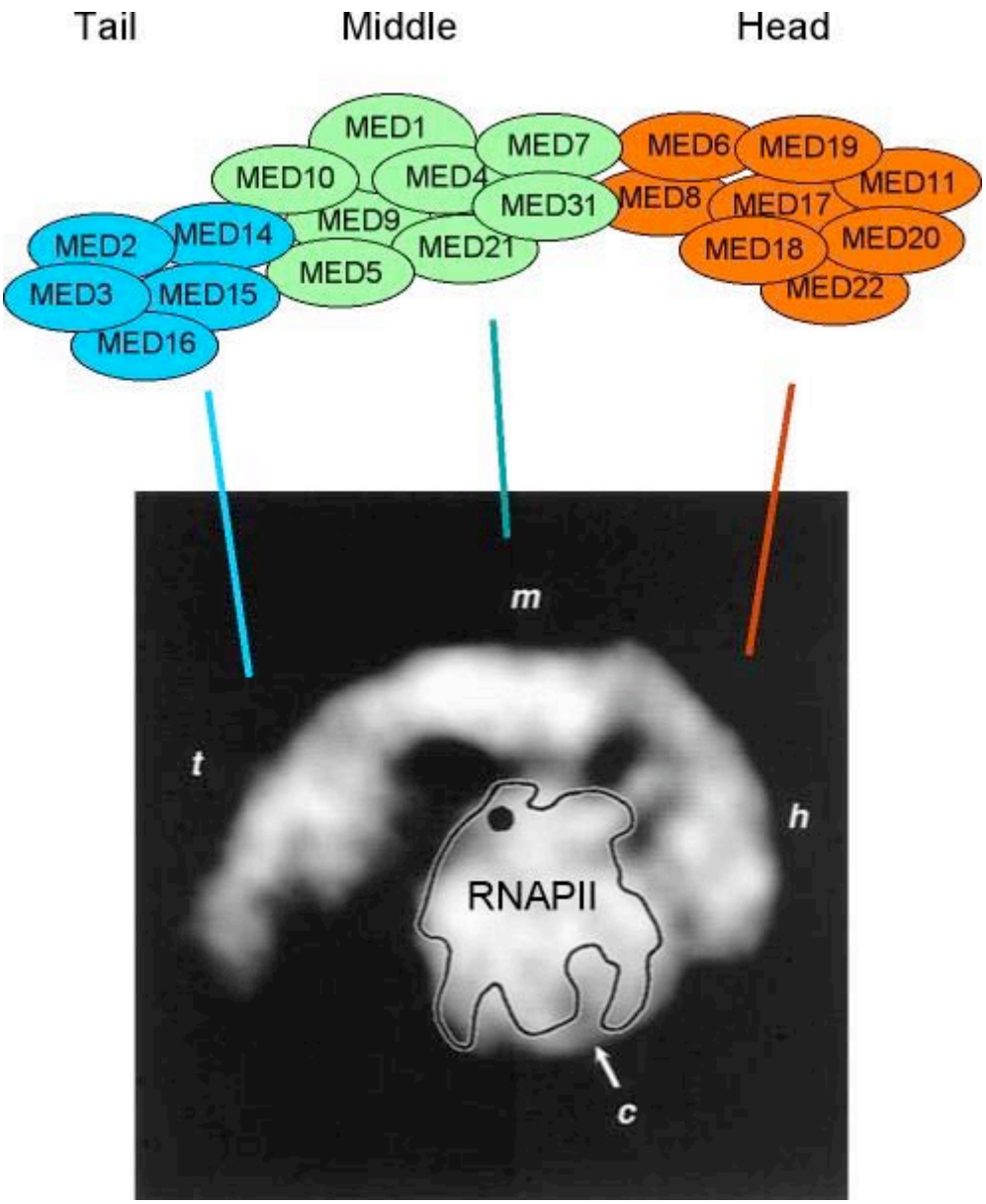

Figure 2: Schematic representation of the yeast Mediator (without the CDK8 module) and an EM micrograph (taken from (Asturias et al., 1999)) of Mediator attached to RNAPII. The black spot indicates the CTD attachment point; c indicates the location of the DNA binding channel.

transcription system is used, also basal transcription becomes dependent on the Mediator complex. The fact that basal *in vitro* transcription can occur with recombinant or purified factors, but not in the nuclear extract system (which contains all factors present in the minimal transcription system) indicates that Mediator is required to inactivate a regulatory mechanism that acts negatively on transcription initiation. In the course of this study it was published that Mediator is required for the recruitment of the general

transcription factor TFIIB in the nuclear extract system (Baek et al., 2006). In addition Mediator was also discovered by means of yeast genetics. Mediator components were discovered in genetic screens for suppressors of CTD truncation mutants (Hengartner et al., 1995; Kim et al., 1994; Thompson et al., 1993). Five of these Srb-genes, are essential for yeast cell growth (Nonet and Young, 1989; Thompson et al., 1993). In addition Mediator was found to counteract the effects of the negative cofactors NC2 and the CCR4-NOT complex (Lee et al., 1998). In early studies Mediator was found to facilitate CTD phosphorylation by stimulating the TFIIH-associated kinase CDK7 (Kim et al., 1994). Due to its complexity, the Mediator complex also serves as an integration platform for intracellular signaling pathways (Blazek et al., 2005).

A.2.4.1.1. Head module

In *S.cerevisiae* the head module consist of the MED6, MED8, MED11, MED17 (Srb4), MED18 (Srb5), MED19, MED20 (Srb6) and the MED22 (Srb6) polypeptides and can be assembled from recombinant proteins as a distinct module (Takagi et al., 2006). Within the head module a MED8-18-20 submodule was identified to interact with TBP *in vitro* (Lariviere et al., 2006; Lariviere et al., 2008).

A.2.4.1.2 Midle module

The middle module comprises the MED1, MED4, MED5, MED7, MED9, MED10, MED21, and MED31 Mediator subunits. Like the head module it possesses several contact points with RNAPII (Davis et al., 2002).

A.2.4.1.3. Tail module

In yeast the tail module is composed of the MED2, MED3, MED14, MED15 and MED16 subunits. The Mediator tail can be deleted without loss of yeast cell viability (Li et al., 1995) or destruction of the overall Mediator structure as determined by electron microscopy (Dotson et al., 2000).

A.2.4.1.4. CDK8 module

The CDK8 module consist of the MED12 (Srb8), MED13 (Srb9), CDK8 (Srb10) and cyclin C (Srb11) subunits. It is found in large (e.g. ARC-L) but not in small Mediator complexes (e.g. CRSP). The CDK8 module containing Mediator has been shown to be inert in activator-dependent transcription *in vitro* (Taatjes et al 2002, Sune et al 1998). An electron microscopy (EM) study revealed that binding of RNAPII and the CDK8 module to Mediator is mutually exclusive (Elmlund et al., 2006). CDK8 has been shown to phosphorylate the CTD of RNAPII at ser-5 (Borggrefe et al., 2002) and in addition the regulatory subunit of CDK7, cyclin H. Phosphorylation of cyclin H leads to decreased amounts of transcript generated in *in vitro* transcription assays (Akoulitchev et al., 2000). At least for some genes transcription activation is dependent on the loss of the CDK8 module from the promoter *in vivo*. The loss of the CDK8 module, which coincides with transcription activation appears to be dependent on PARP-1 (Pavri et al., 2005). There is evidence that the CDK8 module may also contain the CDK8-like CDK11 kinase instead of CDK8 (Sato et al., 2003).

A.2.4.1.5. MED25-Mediator

The MED25 subunit of the Mediator complex is exclusively found in higher eukaryotes (Blazek et al., 2005; Bourbon, 2008). It was discovered as a subunit that strongly interacts with the activation domain of the viral activator VP16. However, the MED25 containing Mediator complex is dispensable for Sp1 activated *in vitro* transcription (Mittler et al., 2003). *In vivo*, MED25 is required for retinoic acid receptor (RAR)-activated transcription (Lee et al., 2007).

A.2.4.1.6. Mediator and RNAPII interactions

The Mediator complex has several physical interaction sites with RNAPII (Chadick and Asturias, 2005). Critical for the interaction of RNAPII and the Mediator complex appears to be the CTD of RNAPII. Nine Mediator subunits were initially discovered in genetic screens for suppressors of CTD truncation mutants (Hengartner et al., 1995; Thompson et al., 1993). Antibodies against the unphosphorylated RNAPII CTD displace Mediator from RNAPII (Svejstrup et al., 1997). Mediator is unable to stimulate transcription of a CTD-less RNAPII (Myers et al., 1998). Apparently CTD phosphorylation upon

transcription initiation leads to a break-up of the RNAPII-Mediator complex, allowing RNAPII to start transcription (Liu et al., 2004). The Tjian laboratory identified a particular Mediator dubbed CRSP by its ability to interact with GST-CTD in a pulldown assay. Addition of an excess of recombinant GST-CTD to an *in vitro* transcription assay inhibits CRSP-dependent transcription (Naar et al., 2002). In EM studies demonstrate that CRSP adopts a distinct structure upon GST-CTD binding which is different from the three dimensional structure of free CRSP complex. Furthermore its been shown that CRSP that is bound to the transcription activator VP16 does adopt the same structure as it does when bound to GST-CTD (Taatjes and Tjian, 2004). The CTD may bind to yeast Mediator between the head and middle modules, since recombinant head and middle modules independently interact with the CTD (Kang et al., 2001). EM studies with human Mediator complexes map the RNAPII CTD to a small region between the head and the middle module. This region is close to, but not identical to the VP16 binding site (Naar et al., 2002). The Gnatt laboratory described a particular Mediator responsive form of RNAPII which contains an additional thirteenth RNAPII subunit termed Gdown1. In *in vitro* transcription experiments Gdown1-containing RNAPII is selectively dependent on and responsive to Mediator (Hu et al., 2006).

A.2.5. Negative transcription cofactors

Over the past two decades a number of negative transcription cofactors have been described. Negative cofactor 2 (NC2) and the CCR4-NOT complex were found to be antagonists of Mediator complex function in a genetic screen (Lee et al., 1998). The ATPase BTAF-1 which is known in yeast as Mot1 acts like NC2 in a complex with TBP. NC2 and BTAF-1 act as negative transcription cofactors in *in vitro* transcription experiments, however they are recruited to transcriptional active genes *in vivo* (Albert et al., 2007; van Werven et al., 2008). DSIF and NELF act in conjunction after transcription initiation. These complexes pause RNAPII 20 to 50 nucleotides downstream of the transcription start site. This block appears to be another mode of RNAPII regulation and can be overcome by pTEFb which comprises the CDK9 kinase (Price, 2008). Furthermore DSIF has been reported to be involved in capping enzyme recruitment (Mandal et al., 2004).

A.2.5.1. NC2

NC2 is an evolutionary conserved heterodimer complex composed of the NC2α and NC2β subunits. It binds TBP-promoter complexes from the bent underside of the TATA-element. NC2 and TBP form a ring-like structure around the DNA which sterically occludes association of TFIIA and TFIIB with the PIC (Goppelt et al., 1996; Kamada et al., 2001; Mermelstein et al., 1996). The NC2 complex facilitates TBP interaction with promoters irrespective whether they contain a TATA box or not. Based on a ChIP on chip study NC2 is associated with a substantial fraction of active human promoters *in vivo* (Albert et al., 2007). Genetic studies have revealed both negative and positive effects of NC2 on gene expression. In *in vitro* transcription experiments only a negative function of NC2 could be detected. Recently it was found that the promoter bound DNA-TBP-NC2 complex exhibits a substantial mobility along the DNA template. This study suggested that by this mechanism NC2 may help to position TBP properly at the promoter (Schluesche et al., 2007). This represents the first molecular explanation for the apparent positive NC2 function *in vivo*. Furthermore NC2 is subject to post-translational modifications during Mitosis (E. Piaia and M. Meisterernst, unpublished).

A.2.5.2. BTAF-1

BTAF1 and its yeast orthologue Mot1 are general repressors of RNAPII-dependent transcription (Davis et al., 1992). BTAF-1 and Mot1 are able to dissociate TBP from DNA with their intrinsic ATPase activity (Chicca et al., 1998; Pereira et al., 2003). In mammalian cells/nuclear extract the majority of TBP is not found in TFIID but in a complex with BTAF1 dubbed B-TFIID (Timmers and Sharp, 1991). However recent genome wide gene expression profiling studies revealed that Mot1 affects gene expression negatively as well as positively (Andrau et al., 2002; Dasgupta et al., 2002; Geisberg et al., 2002). The proposed hypothesis for a BTAF-1 mechanism of action states that BTAF-1/Mot1 displaces TBP from non-promoter regions and deliver it thereby to promoter regions (Geisberg et al., 2002; Muldrow et al., 1999).

A.2.5.3. DSIF

DRB-sensitivity inducing factor (DSIF) was initially discovered as a factor, that renders an partially reconstituted *in vitro* transcription system (comprising the 0.5 M and 0.85 M P11 fractions) sensitive to the inhibitors DRB or H8 (Wada et al., 1998a). DSIF is conserved from yeast to man and composed of two subunits hSpt4 and hSpt5 (Yamaguchi et al., 1999). Subsequently DSIF was found to be an essential factor in switching the RNAPII into the elongation mode. The mechanism involves binding of DSIF to RNAPII during promoter escape (Yamaguchi et al., 1999), which in conjunction with the NELF complex induces pausing. The inhibition can be overcome by the factor pTEFb and the kinase activity of CDK9. If a nuclear extract is used to examine DSIF effects, hexokinase-treated nuclear extracts are used (Wada et al., 1998b). Notably, DSIF is unable to mediate DRB-induced transcription inhibition if the extract is treated with ATP during PIC formation (Wada et al., 1998b). Recently a function of DSIF in early transcription initiation was revealed by Malik et al. (Malik et al., 2007). It was shown that DSIF confers Mediator-dependence to an *in vitro* transcription system consisting of recombinant and highly purified transcription factors. This transcription system did not contain pTEFb or CDK9. Another hint that DSIF may have a function in early transcription initiation steps comes from the Fisher laboratory. This laboratory discovered that CDK7 is able to phosphorylate DSIF *in vitro* (Larochelle et al., 2006). Presumably DSIF has a second function prior to promoter escape. Another DSIF function appears to be the recruitment of capping factors that involves a direct interaction of the large DSIF subunit hSpt5 with capping enzyme (CE) (Mandal et al., 2004).

A.3. Initiation and elongation control of transcription

Based on their RNAPII occupancy and distribution three classes of genes can be distinguished: In the first class RNAPII is not detected throughout the gene and also no transcript is produced. The second class comprise genes that are transcribed in their entire length. They show a fairly uniform RNAPII distribution with many genes exhibiting a slightly higher polymerase occupancy at the 5’ and 3’ ends of the gene. The third class of genes, which appears to be larger than previously anticipated, includes genes at which transcription is initiated but no full-length transcript is produced. At this genes abortive

short transcripts are produced but the proximal DSIF/NELF imposed block on processive transcription can not be overcome (Price, 2008). Apparently a significant portion of developmental control genes are regulated by elongation control rather than by initiation control (Guenther et al., 2007).

A.4. Chromatin and transcription regulation

Transcription *in vivo* occurs in a chromatin context. A first layer of regulation is provided by regulation of chromatin compactation. Transcription is controlled at this level by physical accessibility of promoters and enhancers for RNAPII and transcription factors. Once RNAPII has initiated transcription it still faces a physical barrier in from of histones on the DNA template. Several accessory factors are thought to assist RNAPII in making its way through DNA wrapped around nucleosomes. Histone post-translational modifications are associated with distinct transcription states of DNA. H3-K9 trimethylation, which provides a docking site for the heterochromatin protein HP-1, is found at transcriptionally inert heterochromatin. H3-K4 trimethylation is found as a distinct peak close to the transcription start site of active RNAPII genes. In addition to that mark transcriptionally active genes are decorated with H3-K36 and H3-K79 methylation (Guenther et al., 2007; Peters and Schubeler, 2005). Histone acetylation which is thought to make chromatin more accessible occurs predominantly on histone H4 (Sterner and Berger, 2000).

A.5. Aim and scope of this work

The main goal of this work was to further define Mediator as a general transcription factor. To further define the underlying molecular mechanisms of basal and activated *in vitro* transcription I used a twofold approach: *In vitro* assays such as immobilized promoter template and transcription assays to screen for molecular mechanisms and *in vivo* chromatin immunoprecipitation assays on an inducible model gene to confirm *in vitro* results.

B. Experimental Procedures

B.1. Biochemistry

B.1.1. Preparation of nuclear extract

The difference between NAM and Dignam protocol is the salt concentration, at which the extraction takes place. Apparently the NAM protocol (210 mM NaCl) extracts more hypo- and less hyperphosphorylated RNAPII than the Dignam protocol (420 mM NaCl). All HeLa nuclear extracts were prepared according to the Dignam protocol, Jurkat nuclear extracts according to the NAM protocol.

B.1.1.1. Dignam protocol for the preparation of HeLa nuclear extract

HeLa nuclear extracts were prepared according to a protocol published by Dignam and colleagues (Dignam et al., 1983). All steps were carried out on ice in the cold room. For the preparation of transcription competent nuclear extracts it is helpful to collect the cells during the logrhythmic growth phase at a density of about 5 x 10^5 cells per ml medium. HeLa cells for nuclear extracts were grown in spinner cultures. 10-15 liters of cell culture were processed in one extraction. HeLa cells were harvested by centrifugation in a Beckman J-6B centrifuge (10 min, 2500 rpm, 4ºC) and collected in 50 ml polypropylene tubes. About 5 ml packed cell volume (PCV) were aliquoted into each 50 ml tube. Cells were washed once with 45 ml ice-cold PBS and the cells pelleted by centrifugation in a Heraeus Multifuge 3L-R at 2500 rpm and 4ºC. Each 5 ml of PCV were resuspended in 45 ml HB buffer. This hypotonic buffer leads to swelling of the cells. The swollen cells were pelleted by centrifugation (5 min, 2500 rpm, 4ºC) and the swollen cell volume (SCV) was determined. The supernatant was removed and the pellet resuspended in 2 x PCV HB buffer and further swelling was allowed by incubation for 10 min on ice. After that the cell suspension was transferred to a chilled glass homogenizer equipped with a loose type B pestle (Wheaton). Cell membranes were disrupted by 15 hard pestle strokes. After completion it was checked in the microscope that cell membranes were broken up efficiently and that the solution contained mainly nuclei. Then the suspension was centrifuged (15 min, 3900 rpm, 4ºC) and the cytosolic supernatant collected.

The pellet consisting of nuclei was resuspended in half a nuclear pellet volume (NPV) low salt buffer and homogenized again in a glass homogenisator with a type B pestle (six hard strokes). The resulting suspension was transferred into a plastic beaker, a stir bar was added and over the period of 30 min half a NPV high salt buffer was added dropwise. After the final potassium chloride concentration of about 420 mM was reached, the suspension was stirred for another 30 min. After that the suspension was centrifuged to clear the nuclear extract from debris (Sorvall centrifuge equipped with a SS34 rotor, 14 000 rpm, 30 min, 4ºC). The supernatant containing the nuclear extract was removed from the debris and dialyzed against BC0 buffer (supplemented with PMSF to a final concentration of 0.2 mM and β-mercaptoethanol to a final concentration of 1 mM) until the conductivity had reached that of BC100 (conductivity measurements were carried out on a Knick Konduktometer 703). After a final centrifugation step (Sorvall centrifuge equipped with a SS34 rotor, 14 000 rpm, 15 min, 4ºC) the nuclear extract was aliquoted and snap-frozen in liquid nitrogen and stored at -80ºC.

Hypotonic buffer (HB):	10 mM Tris-Cl, pH 6.8 at RT
	1.5 mM $MgCl_2$
	10 mM KCl
Low salt buffer:	20 mM Tris-Cl, pH 6.8 at RT
	25 % (v/v) glycerol
	20 mM KCl
	1.5 mM $MgCl_2$
	0.2 mM EDTA
	1 mM β-mercapto-ethanol
	0.2 mM PMSF

High salt buffer:	20 mM Tris-Cl, pH 6.8 at RT
	25 % (v/v) glycerol
	1200 mM KCl
	1.5 mM $MgCl_2$
	0.2 mM EDTA
	1 mM β-mercapto-ethanol
	0.2 mM PMSF

B.1.1.2. NAM Protocol for Jurkat nuclear extract

All steps were carried out at 4ºC and on ice in the cold room. DTT and PMSF were added to the buffers freshly prior to each extraction. Jurkat extracts in this work were prepared from isolated Jurkat nuclei (prepared by Cellex Biosciences Inc.). Nuclei were thawed on ice and after that resuspended in half a nuclear pellet volume (NPV) ice-cold NAM-buffer A. The suspension was transferred to a chilled glass homogenizer equipped with a loose type B pestle (Wheaton) and the nuclei broken up by 10 soft pestle strokes. After that the suspension was poured into a plastic beaker and while steering 1 NPV NAM-buffer C was added dropwise over a period of 30 min (final potassium chloride concentration: 210 mM). After another 30 min incubation the suspension was cleared from debris by centrifugation (Sorvall centrifuge equipped with a SS34 rotor, 18 000 rpm, 20 min, 4ºC). The cleared nuclear extract was dialyzed against BC0 until the conductivity of BC100 was reached. After a final centrifugation step (Sorvall centrifuge equipped with a SS34 rotor, 18 000 rpm, 20 min, 4ºC) the nuclear extract was aliquoted and snap-frozen in liquid nitrogen and stored at -80ºC.

NAM-buffer A:	10 mM Tris-Cl, pH 7.3 at RT
	1.5 mM $MgCl_2$
	10 mM KCl
	0.2 mM EDTA
	0.5 mM DTT
	0.2 mM PMSF

NAM-buffer C: 20 mM Tris-Cl, pH 7.3 at RT
25 % (v/v) glycerol
420 mM NaCl
1.5 mM $MgCl_2$
0.2 mM EDTA
0.5 mM DTT
0.2 mM PMSF

B.1.2. Expression and purification of recombinant proteins in *E.coli*

B.1.2.1. Expression of recombinant proteins

Recombinant proteins were expressed in the *E.coli* BL21 strain. The expression plasmid encoding the desired protein was transfected into the bacteria by heat-shock transfection. For protein expression, an overnight starter culture was diluted to an OD_{600} of 0.05 in LB medium to a total volume of 200 ml per shaker flask. Bacteria were grown at 37ºC to an OD_{600} of 0.6. Protein expression was induced by adding IPTG to a final concentration of 0.5 mM. In order to enhance the yield of full length protein, the bacteria cultures were shifted to an incubation temperature of 30ºC after induction. Protein expression was allowed for 3 to 6 hrs, depending on the construct expressed. Subsequently bacteria were harvested by centrifugation for 15 min at 3500 rpm and 4ºC. All following purification steps of the recombinant proteins were carried out at 4ºC.

B.1.2.2. Purification of recombinant GST-tagged proteins

The bacteria pellet of 200 ml expression culture was resuspended in 10 ml ice-cold lyses buffer supplemented with 10 mg lysozyme. After 10 min incubation on ice the suspension was sonicated with a microtip and the following settings on a Branson sonifier: Output amplitude 30%; total sonication time: 2 min; sonication cycles: 10 sec on, 50 sec off. During the sonication, the suspension was cooled with an ice-water bath. Then the lysate was cleared by centrifugation (10 min, 10 000 x g, 4ºC).

For the purification of the recombinant GST-tagged proteins 200 µl gluthathione-sepharose 4B (Amersham, cat. no.: 17-0756-01) were equilibrated by washing the beads

three times in 30 CV lysis buffer. The lysate was incubated together with the equilibrated beads for at least 2 hours in the cold room at 4ºC on a rotating wheel. After binding of the GST-fusion proteins to the glutathione beads the beads were washed 5 times with 50 CV BC2000 buffer. GST-VP16 constructs as well as the GST-CTD fusion protein were stored bound to the beads in BC2000 buffer containing 0.03% NaN_3. To determine the amount and purity of the GST-fusion proteins bound to the beads, a 10 µl aliquot of the beads was analyzed by SDS-PAGE and Coomassie staining of the gel. BSA protein solutions of known concentrations were used to determine the amount of protein bound to the beads.

Lysis Buffer:	20 mM HEPES-KOH pH 7.5
	100 mM KCl
	1 mM EDTA
	10% (v/v) glycerol
	0.1% (v/v) IGEPAL-CA-630 (NP-40)
	0.1% (v/v) protease inhibitor cocktail (Sigma)

B.1.3. Immunoprecipitations

All centrifugation steps in this section were carried out for 3 min at 700 x g and 4ºC, if not indicated otherwise.

B.1.3.1. Coupling of antibodies to sepharose beads

Protein A (Amersham Biosciences, cat. no.: 17-0618-03) or protein G sepharose beads (Amersham Biosciences, cat. no.: 17-5280-03) were washed 3 times in 30 to 50 column volumes (CV) PBS. Usage of protein A or protein G IP-beads depended on the species in which the antibody that was to be bound to the IP-beads, was raised (protein A beads for rabbit antibodies, protein G beads for goat, rat and mouse antibodies). IP beads were then blocked for at least 10 min in 10 CV of PBS containing 0.5% (w/v) BSA (Sigma, cat. no.: A3294). In case purified and concentrated antisera were to be bound to the IP-beads the antiserum was added directly to the blocking buffer such that 1 µg antibody is added for each µl of IP-bead volume. If hybridoma supernatants were to be bound to IP-

beads, the blocking buffer was removed and 10 µl hybridoma supernatant per 1 µl IP bead volume added. The beads were incubated together with the antibody for at least 4 hrs at 4ºC. After binding the antibody coated beads were either equilibrated in immunoprecipitation buffer (for an immunoprecipitation) without a covalent crosslink of antibodies and beads, or forwarded to the covalent crosslink procedure (B.1.3.2.).

B.1.3.2. Covalent crosslink of antibodies to sepharose beads

Antibody-loaded IP-beads (see section B.1.3.1.) were washed twice in 10 column volumes (CV) 0.2 M sodium borate, pH 9.0. Then the beads were resuspended in 10 CV 0.2M sodium borate, pH 9.0 containing 20 mM dimethyl pimelimidate (Sigma, cat. no.: D8388; the dimethyl pimelimidate degrades rapidly if dissolved, therefore it was added as powder immediately before the buffer was used). After 30 min incubation at RT on a roller, the IP beads were peletted by centrifugation and then washed once in 10 CV 0.2 M ethanolamine, pH 8.0. After that wash, the beads were resuspended in 10 CV 0.2 M ethanolamine, pH 8.0 and incubated for 2 hrs at RT on a roller. Finally the crosslinked IP beads were washed 3 times in 30 to 50 CV PBS. The beads were either used directly, or stored at 4ºC in PBS supplemented with 0.02% NaN_3.

B.1.3.3. Immunoprecipitation

HeLa or Jurkat nuclear extracts (see section B.1.1.) containing 5-15 mg/ml of protein were supplemented with KCl to a final chloride ion concentration of 150 mM, 0.1 % (v/v) IGEPAL-CA-630 (NP-40), 0.2 mM PMSF and then incubated with the indicated antibody coupled to protein A (rabbit antibodies) or protein G Sepharose (rat, goat and mouse antibodies) beads for at least 3 hrs at 4°C. As an isotype control for the rat monoclonal antibodies (MED15, MED25) the 83M (9A12) rat monoclonal antibody was used, as an isotype control for the goat polyclonal CDK8 antibody normal goat serum (Santa Cruz, cat. no.: sc2043) was used and for the rabbit polyclonal antibodies normal rabbit serum (Santa Cruz, cat. no.: sc-2027) was used. Antibody loaded beads were prepared as described in sections B.1.3.1. and B.1.3.2. and equilibrated in BC150 / 0.1% (v/v) IGEPAL-CA-630 (NP-40) / 0.2 mM PMSF before the incubation with nuclear extract. Then the beads were washed either three times in 50 column volumes (CV) IP wash buffer 150 or three times in 50 CV IP-wash buffer 800 and thereafter three times in

50 CV IP wash buffer 150. Protein-loaded IP beads were used to supplement immobilized template assays, *in vitro* transcription assays, *in vitro* kinase assays or were analyzed directly by immunoblot. In order to elute proteins from the beads for immunoblot analysis, three bead volumes 2 x SDS PAGE loading dye was added directly to the washed beads. Then the suspension was heated for 15 min at 60ºC in the heat block, the IP beads peletted by centrifugation and the supernatant loaded onto an SDS-PAGE gel.

IP-wash buffer 150: BC150 containing
0.1% (v/v) IGEPAL-CA-630 (NP-40)
1 mM DTT
0.2 mM PMSF

IP-wash buffer 800: BC150 containing
0.1% (v/v) IGEPAL-CA-630 (NP-40)
1 mM DTT
0.2 mM PMSF

B.1.4. Immunodepletion

Immunodepletions were carried out essentially according to the immunoprecipiation protocol described in the previous sections. IP-beads were loaded as described in section B.1.3.1., except for the CDK1 immunodepletion. In that case 1 µl of IP bead (α-CDK1 as well as α-isotype beads) volume was loaded with 2.2 µg α-CDK1 antibody (Santa Cruz, cat. no.: sc-954). In order to immunodeplete 500 µl nuclear extract, two sequential incubations with 100 µl antibody-loaded IP-beads for each incubation was carried out. The first incubation step was carried out for 3, the second step for 2 hrs. In case of the CDK1 depletion, 100 µl of nuclear extract were immunodepleted with two sequential incubations with 50 µl CDK1 or isotype loaded beads. After completion of the depletion, nuclear extracts were centrifuged at high speed (5 min, 13200 x g, 4ºC) in order to remove remaining IP-beads. Then immunodepleted nuclear extracts were snap-frozen in liquid nitrogen and stored at -80ºC.

B.1.5. GST-pulldown experiments

VP16 pulldown assays were performed essentially as described previously (Ikeda et al., 2002). HeLa or Jurkat extracts containing between 5 and 15 mg/ml of protein were adjusted to 25 mM Hepes-KOH pH 7.6, 100 mM KCl, 0.1% NP-40, 1 mM DTT, 0.2 mM EDTA, 10% glycerol, 0.2 mM phenylmethylsulfonyl fluoride (PMSF) and incubated in siliconized Eppendorf tubes with VP16 derivatives fused to GST and immobilized on glutathione–Sepharose at concentrations of 1 mg/ml for 16 hrs at 4°C. After 4 washes with 50 column volumes HEGN-100 buffer (25 mM Hepes-KOH pH 7.6, 100 mM KCl, 0.1% NP-40, 1 mM DTT, 0.2 mM EDTA, 10% glycerol, 0.2 mM PMSF) the bound proteins were eluted with 500 mM KCl in HEGN buffer (2CV) and analyzed by immunobloting.

B.1.6. *In vitro* transcription assays

In vitro transcription experiments were carried out essentially as described previously (Gilfillan et al., 2003; Malik and Roeder, 2003). *In vitro* transcription reactions were carried out either with an SmaI linearized major-late (ML) promoter fragment, that was purified by CsCl-gradient centrifugation, or on a isolated PIC that was formed prior to the *in vitro* transcription experiment on an immobilized template (as described in section B.1.6.). In reactions in which SmaI linearized ML promoter templates were used, 50 ng template were added to each 25 µl reaction. In cases there VP16-activated transcription was studied, 10 ng of GAL4-VP16 was added to each 25 µl reaction on a SmaI linearized major-late promoter template. If nuclear extracts were used in *in vitro* transcription experiments directly, 75 µg HeLa or 25 µg Jurkat nuclear extract were used per 25 µl reaction. The reaction was composed such that the criteria defined in table 2 were matched. On an immobilized promoter template formed PICs were washed, and then resuspended in a buffer that fulfilled the criteria defined in table 2.

Reaction volume	25 µl
Chloride ion concentration	60 mM
DTT	10 mM
Total BC-buffer content	40-50%
$MgCl_2$	5 mM
IGEPAL-CA-630 (NP-40)	Max. 0.03%
PEG-8000	0.1 mg/ml
HEPES-pH8.2	20 mM
RNase Inhibitor (Promega, cat. no.: N211A)	10 units
BSA (Roche, cat. no.: 711454)	0.5 mg/ml

Table 2: *In vitro* transcription parameters

For typical *in vitro* transcription experiments, the components were mixed, and a PIC was formed in the presence of 1 µM ATP, if not stated otherwise. PIC formation was carried out for 45 min (if not indicated otherwise) at 30ºC in a reaction volume of 23.5 µl. After that 1.5 µl NTP mix was added, and the transcription reaction was allowed to proceed for 45 min at 30ºC. The NTP mix consisted of 1 µl α-^{32}P-UTP (3000 Ci/mmol, Amersham, cat. no.: PB10203) and 0.5 µl of 50 x NTP-low UTP mix. In case a template with a G-less cassette was used (e.g. the ML-promoter template) an 50 x NTP-low UTP OMG mix was used instead of the 50 x NTP-low UTP mix.

In vitro transcription reactions were stopped by addition of 400 µl transcription stop buffer. The RNA was isolated by phenol-chloroform extraction and subsequent isopropanol precipitation at -20ºC. The RNA was pelleted by centrifugation (45min, 4ºC, 13200 x g), washed once with 80% ethanol, air dried and resuspended in 10 µl RNA-PAGE loading dye. RNA samples were incubated for 20 min at 60ºC and then loaded onto a 5% polyacrylamide-urea gel. The gel was run at a constant voltage of 250 V. The RNA was fixed in the gel by submerging it in 10% acetic acid for 10 min. Next the gel was washed three times in water, and dried on the vacuum gel dryer. Finally, the dried gel was exposed to either a phosphor-imager plate or to BioMax MR film (Kodak, cat. no.: 8736936).

50 x NTP-low UTP Mix:	5 mM ATP (Amersham, cat. no. 272056) 5 mM CTP (Amersham, cat. no. 272066) 5 mM GTP (Amersham, cat. no. 272076) 0.25 mM UTP (Amersham, cat. no. 272086)
50 x NTP-low UTP OMG-Mix:	5 mM ATP (Amersham, cat. no. 272056) 5 mM CTP (Amersham, cat. no. 272066) 0.25 mM OMG (3'-O-methyl-guanosine triphosphat; Amersham, cat. no. 274675) 0.25 mM UTP (Amersham, cat. no. 272086)
Transcription stop buffer:	7 M urea 10 mM Tris-HCl, pH 7.8 10 mM EDTA, pH 8.0 300 mM sodium acetate 0.5 % (w/v) SDS 100 mM lithium chloride 0.4 mg/ml yeast tRNA
RNA-PAGE loading dye:	97% (v/v) formamide 20 mM Tris-Cl, pH 7.3 10 mM EDTA, pH 8.0 0.1% (w/v) Bromophenolblue 0.1% (w/v) Xylenecyanol
5 % Urea-PAGE Gel:	1 x TBE 8 M urea 5 % (v/v) acrylamide:bisacrylamide (19:1) 0.1 % (v/v) APS 0.01 % (v/v) TEMED

B.1.7. Immobilized template assays

Immobilized template assays were performed on either a biotinylated MRG5 promoter construct or on a biotinylated major-late (ML) promoter construct. Early PIC formation studies were performed on the MRG5 promoter construct, later studies were carried out on the ML promoter template due to the fact that a more robust *in vitro* transcription results could be obtained by using this template. This immobilized template assay procedure is based on protocols published by the Hahn, Chiang and Carey laboratories (Black et al., 2006; Ranish et al., 1999; Wu et al., 2003).

B.1.7.1. Promoter template preparation

Biotinylated promoter templates were generated by PCR. The MRG5 promoter templates were amplified from the vector pGL2MRG5, that in turn stems from pMRG5 plasmid, which contains a Luciferase expression cassette in place of the G-free cassette of pMRG5. The promoter comprises 5 GAL4 bindings sites immediately upstream of a synthetic HIV/ML core promoter as described previously (Xie et al., 2000). The primers were: 5'- GCA TTC TAG TTG TGG TTT GTC CAA (biotinylated) and 5'- GCC GGG CCT TTC TTT ATG TT. To produce promoter templates with a mutated TATA box the same primer were used on plasmid pPHS99 in which the TATA box was mutated. The resulting 412 bp templates contain 91 bp of the luciferase coding region which in theory would generate a run-off transcript of that length. Biotinylated ML promoter templates were amplified from a 5xGAL4 site/major-late promoter/G-less cassete containing plasmid (pG5MLT) using the primers 5'- CGA TTC ATT AAT GCA GCT GG (biotinylated) and 5'- AAC TCG ACT GCA GCA TAT GTA TCA TAC ACA TAC G. The 700 bp template comprises five GAL4 binding sites upstream of the major late promoter and a 380 bp G-less transcription cassette downstream of the promoter region. The templates were purified on 0.7% agarose gels and recovered using a gel band extraction kit (Quiagen, cat. no.: 28704).

B.1.7.2. Coupling of promoter templates to paramagnetic beads

Biotinylated DNA templates were coupled to paramagnetic streptavidin beads (Promega, cat. no.: Z548C) as follows: paramagnetic beads were washed three times in binding and washing (B&W) buffer. Beads were resuspended in B&W buffer, and 8.5 ng biotinylated MRG5 promoter template or 15 ng biotinylated ML promoter template (in TE supplemented with 1 M NaCl) was added for each µg magnetic beads. After shaking for 30 min at room temperature, beads were washed once in B&W buffer containing 0.5 mg/ml BSA (Sigma, cat. no.: A3294). For blocking, beads were resuspended at a concentration of 1 mg/ml in blocking buffer. After that the paramagnetic beads were washed three times in TXN wash buffer and finally resuspended in TXN wash buffer at a concentration of 10 µg paramagnetic beads per microliter TXN wash buffer.

B&W buffer:
5 mM Tris-HCl, pH7.5
1 mM EDTA
1 M NaCl
0.003% (v/v) IGEPAL-CA-630 (NP-40)

Blocking buffer:
20 mM Hepes-KOH, pH 8.2
5 mM $MgCl_2$
50 % (v/v) BC120
0.1 mg/ml PEG-8000
1 mM DTT
0.025% (v/v) IGEPAL-CA-630 (NP-40)
5 mg/ml BSA (Sigma, cat. no.: A3294)
5 mg/ml polyvinylpyrolidone (Sigma, cat. no.: 81420)

TXN Wash buffer: 20 mM Hepes-KOH, pH 8.2
5 mM $MgCl_2$
50% (v/v) BC120
0.1 mg/ml PEG-8000
0.025% (v/v) IGEPAL-CA-630 (NP-40)
1 mM DTT
0.2 mM PMSF

B.1.7.3. PIC formation reaction

PIC formation reactions were conducted such that the conditions resembled those of a classical *in vitro* transcription experiment and carried out in lubricated tubes. A typical PIC formation reaction was conducted in a total volume of 200 µl and adjusted to meet the following criteria:

Basic components	70 µg paramagnetic beads coupled to 2.4 pmol promoter template 10-20% (v/v) nuclear extract, recombinant proteins 40-50% (v/v) total BC-buffer content 60 mM total chloride ion concentration 20 mM Hepes-KOH, pH 8.2 5 mM $MgCl_2$ 0.5 mg/ml BSA (Roche, ca. no.: 711454) 0.025 % (v/v) IGEPAL-CA-630 (NP-40) 0.1 mg/ml PEG-8000 40 units RNase inhibitor (Promega, cat. no.: N211A) 25 ng/µl poly(dG:dC) (Amersham, cat. no.: 27-7890-02) 10 mM DTT 0.2 mM PMSF
Optional components	1 µM ATP or 1 µM ATPγS or 500 µM H8 200 ng GAL4-VP16 or GAL4-Sp1 or GAL4-TA1

Table 2: PIC formation conditions for immobilized template assays

Typically, the reaction mixture was prepared without adding nuclear extract, recombinant proteins or IP-beads, then activator proteins (GAL4-VP16 or GAL4-Sp1 or GAL4-TA1) were added, and finally extracts, recombinant proteins and IP-beads were added as indicated in the figures. ATP, ATPgS or H8 were added to the PIC formation reaction as

indicated in the figures. PICs were formed by incubation for 45 min at 30ºC in the shaking block (1400 rpm). After PIC formation, the paramagnetic beads were concentrated with a magnet and washed three times with TXN wash buffer. Depending on the experiment, the magnetic beads were then eluted with 2 x SDS-PAGE loading dye in order to analyze the PIC by immunoblot or the template bound PICs were forwarded to a second incubation step under *in vitro* transcription conditions (see section B.1.6.). The buffer for the second incubation step was composed as described in table 2 and the reaction volume was reduced to 50% of that during the PIC formation. At this stage either an *in vitro* transcription experiment was performed (to test the *in vitro* transcription potential of a particular PIC population) or it was analyzed how the PIC protein composition changed if incubated with NTPs as indicated in the respective figures by immunoblot. In order to monitor proteins that remained associated with the DNA template and those that dissociated from it both fractions were analyzed separately by immunoblot. The DNA-bound proteins were eluted with 2 x SDS-PAGE loading dye, the dissociated proteins were analyzed by collecting the supernatant of the second incubation step and adding ¼ volume 5 x SDS-PAGE loading dye to it.

B.1.8. *In vitro* kinase assay

All *in vitro* kinase assays in this study were performed under the same buffer conditions as an *in vitro* transcription reaction (see section B.1.6.). Kinases were immunoprecipitated with appropriate antibodies, washed in BC150 buffer containing 0.1% IGEPAL-CA630 (NP-40) and then applied to the reaction mixture. Up to 10 µl kinase loaded IP-beads were added to a 50 µl kinase assay reaction. In terms of reaction composition the bead volume was calculated as if it would be the same volume BC150/ 0.1% NP-40 buffer. *In vitro* kinase assays were carried out in a first stage that resembled a PIC formation reaction, and a second stage that resembled the conditions of an *in vitro* transcription reaction. During the first stage ATP at a final concentration of 1 µM was included into the reaction (30 min incubation at 30ºC), then NTPs were added to a final concentration of 100 µM for each NTP, and the reaction was incubated for another 30 min at 30ºC. The reaction was stopped by addition of 5 x Laemli buffer and boiled immediately afterwards at 95ºC.

B.1.9. SDS-PAGE, Coomassie staining and Westernblotting

B.1.9.1. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Prior to analysis by Coomassie staining or immunoblot proteins were separated according to their molecular weight by SDS-PAGE. In most cases 12/18% step gels were used, in rare cases gels with an acrylamide content ranging from 8 to 18%. In order to obtain better results when immunoblotting big proteins, an acrylamide mix containing acrylamide and bisacrylamide in a ratio of 170:1 was used. The stacking gel was prepared as a 5% gel. Before loading to the gel, protein samples were supplemented with 5 x SDS-PAGE loading dye such that the loading dye was diluted 5-fold by adding the sample. The samples were boiled at 95ºC, centrifuged briefly, and loaded onto the gel which was submerged in 1 x SDS-PAGE running buffer. Proteins were separated at a current of 25 mA per minigel, or 50 mA for big gels, with the voltage restricted to a maximum of 170V. As a molecular weight standard either Biorad high- or lowrange maker was used.

Gel-mixtures	12%	18%	stacking gel
Gel A (Roth, cat. no.: 3037.1):	4 ml	6 ml	0.85 ml
Gel B (Roth, cat. no.: 3039.1):	0.35 ml	0.52 ml	0.35 ml
1.5 M Tris HCl, pH 8.8	2.5 ml	2.5 ml	-
1 M Tris-Cl, pH 6.8	-	-	0.63 ml
water	2.95 ml	0.78 ml	3.1 ml
10% SDS	0.1 ml	0.1 ml	50 µl
10% APS	0.1 ml	0.1 ml	50 µl
TEMED	4 µl	4 µl	5 µl

5 x SDS-PAGE loading dye:	250 mM Tris-HCl pH 6.8
	10% (v/v) SDS
	50% (v/v) glycerol
	500 mM DTT
	0.2% Bromphenolblue

SDS-PAGE running buffer: 25mM Tris-HCl
190 mM Glycine
1% (w/v) SDS

B.1.9.2. Coomassie staining

To visualize proteins directly in a SDS-PAGE gel the gel was incubated with Coomassie staining solution for at least 1 hr on a rocking platform. After that gels were incubated with destaining solution for at least 1 hr. For documentation gels were scanned and then dried between cellophane films at RT.

Coomassie staining solution: 40% (v/v) Methanol
10% (v/v) Acetic acid
0.025% (w/v) Coomassie R-250

B.1.9.3. Immunoblot

Together with a piece of nitrocellulose membrane (Biorad, cat. no.: 162-0115) that matched the size of the gel, SDS-PAGE gels were equilibrated for 15 min in semi-dry blotting buffer. For the semi-dry protein transfer, the gel and the membrane were sandwiched between 4 pieces of semi-dry buffer soaked thick Whatman papers (2 pieces below, 2 above gel and membrane). The protein transfer was carried out in a Hoeffer TE77 blotting apparatus at a constant voltage of 18 V for 45 min.

After the transfer proteins were visualized by PonceauS staining on the membrane, and the position of the molecular weight standards were indicated with a pencil. Before antibody incubation, the membrane was blocked by incubation in a solution containing 1% dry milk powder in TBS-T for at least 1 hr. Then the membrane was incubated with a specific antibody dissolved in 1% milk TBS-T solution and incubated overnight at 4ºC. Following that incubation the membrane was washed 4 times within 1 hr with plain TBS-T and then incubated with appropriate horse reddish peroxidase (HRP) conjugated secondary antibodies dissolved 1 to 5000 (v/v) in 1 % mild TBS-T solution. After 45 min incubation at room temperature the membrane was washed again 4 times within 1 hr with plain TBS-T. After a brief final wash with water the enzymatic activity associated with

the HRP-conjugated secondary was detected with an enhanced chemiluminescence detection system (Perkin Elmer, cat. no.: NEL105) and the processed membranes were exposed to BioMax MR film (Kodak, cat. no.: 8736936).

Semi-dry blotting buffer: 190 mM glycine
50 mM Tris-Cl
0.25 % (w/v) SDS
20 % methanol

Ponceau S solution: 0.1 % (w/v) PonceauS
5 % (v/v) acetic acid

TBS-T: 150 mM NaCl
100 mM Tris-HCl, pH 7.6
0.1 % (v/v)Tween-20

Secondary Antibodies	Cat. no.	Provider	Conjugate
anti-rat	112-035-044	Jackson Immunoresearch	HRP
anti-goat	Sc-2020	Santa Cruz	HRP
anti-mouse	W402B	Promega	HRP
anti-rabbit	W401B	Promega	HRP

Table 3: Secondary antibodies

B.1.10. DE52 column chromatography

B.1.10.1. Preparation of the column material

DEAE-cellulose DE52 (Whatman, cat. no.: 4057050) was washed three times in water and then equilibrated over night at 4ºC in DE52-buffer. The next day, the column material was washed twice in BC100 containing 1 mM DTT, 0.5 mM PMSF and poured into Biorad Econo column.

DE52 buffer: 0.5 M Tris-HCl pH 7.3 at RT
0.5 M ammonium sulfate, pH 7.9

B.1.10.2. Protein separation by DE52 chromatography

The freshly prepared DE52 column was washed extensively with BC100 / 1 mM DTT, 0.5 mM PMSF and then loaded with 2 mg protein per ml column volume at a flow rate of 1 column volume (CV) per hour. After that the column was washed with 3 CV BC100 / 1 mM DTT / 0.5 mM PMSF and then the KCl concentration was raised in linear gradient over 5 CV to 300 mM KCl. Finally the column was washed with BC300 / 1 mM DTT / 0.5 mM PMSF until no more protein could be detected in the column fractions.

B.1.11. Crosslink-restriction digest immunoprecipitation (CRIP) assay

The CRIP assay, which represents an adaptation of the *in vivo* chromatin immunoprecipitation technique allows to monitor the binding site of a particular epitope on a DNA template in an *in vitro* assay. In brief, a *in vitro* assay is performed on a radiolabelled DNA template, e.g. the formation of a PIC or an *in vitro* transcription reaction, and the reaction is stopped by an formaldehyde crosslink. Then the DNA template is digested with appropriate restriction enzymes, followed by an immunoprecipitation of the epitope of interest. If that epitope is crosslinked to an radiolabelled DNA fragment, it is coprecipitated and can therefore be detected in an autoradiography, without further amplification. Since the restriction pattern of the applied enzyme is known, it can be determined to which part of the DNA template the epitope was crosslinked.

B.1.11.1. Preparation of the radiolabelled DNA-template

Radiolabelled MRG5 promoter templates were generated by PCR and were amplified from the vector pGL2MRG5, that in turn stems from pMRG5 plasmid, which contains a Luciferase expression cassette in place of the G-free cassette of pMRG5. The promoter comprises 5 GAL4 bindings sites immediately upstream of a synthetic HIV/ML core promoter as described previously (Xie et al., 2000). The primers were: 5'- GCA TTC TAG TTG TGG TTT GTC CAA (biotinylated) and 5'- GCC GGG CCT TTC TTT ATG TT.

The PCR reaction was carried out with the following reaction mix:

10 X PCR buffer	5 µl
$MgCl_2$ (25 mM)	3 µl
Template (10 ng/µl)	2 µl
Primer A (10 µM)	2 µl
Primer B (10 µM)	2 µl
Taq (LC)	2 µl
dNTP Mix (cold)	1 µl
γ-^{32}P-dCTP (3000 Ci/mmol)	1 µl
Water	32 µl

dNTP Mix (cold):	25 mM	dATP
	25 mM	dGTP
	25 mM	dTTP
	6.25 mM	dCTP

After the PCR reaction, the templates were purified with a PCR purification kit (Qiagen, cat. no.: 28104) according to the manufacturer's instructions.

B.1.11.2. Preparation of the CRIP IP beads

10 µl antibody loaded sepharose beads were prepared for one immunoprecipitation reaction. Any centrifugation step involving sepharose beads was carried out for 3 min at 700 x g and 4ºC, if not stated otherwise. Protein A (for rabbit and goat antibodies) or protein G sepharose beads (for rat antibodies) were equilibrated by washing the beads three times in 20 column volumes (CV) 1 x TBS, pH 8.0. Unspecific binding sites on the beads were blocked by a 1 hr incubation with 10 CV blocking buffer at 4ºC. Concentrated antisera were added directly to the blocking buffer (2 µg antibody per 10 µl sepharose beads), hybridoma supernatants were added to the beads after removal of the blocking buffer (100 µl supernatant per 10 µl sepharose beads). The antibody binding

reaction was carried out for at least 2 hrs at 4ºC. Finally the beads were washed once in 10 CV 1 x TBS-T, pH 8.0.

B.1.11.3. CRIP assay on PICs

Conditions of PIC formation were chosen similar to the ones used in a typical *in vitro* transcription experiment. For one CRIP assay immunoprecipitation a 25 µl reaction was prepared according to the following table:

Basic components	150 ng (0.6 pmol) radiolabelled promoter DNA template 40-50% (v/v) total BC-buffer content 60 mM total chloride ion concentration 20 mM Hepes-KOH, pH 8.2 5 mM $MgCl_2$ 0.5 mg/ml BSA (Roche, ca. no.: 711454) Maximal 0.025% (v/v) IGEPAL-CA-630 (NP-40) 0.1 mg/ml PEG-8000 40 units RNase inhibitor (Promega, cat. no.: N211A) 25 ng/µl poly(dG:dC) (Amersham, cat. no.: 27-7890-02) 10 mM DTT 0.2 mM PMSF
Optional components	1 µM ATP or 1 µM ATPγS or 500 µM H8 50 ng GAL4-VP16

Table 4: CRIP Conditions

For PIC formation, the reaction mixture was incubated for 45 min at 30ºC. Prior to the formaldehyde crosslink, a TATA-box containing double stranded DNA oligo nucleotide (5'- CCT GAA GGG GGG TAT AAA AGG GGG TGG GGG CGC G) was added to a final concentration of 0.6 mM. Exact 1 min after addition of the competition TATA-oligonucleotide, the reaction was crosslinked by addition of 5% (v/v) formaldehyde solution (diluted in PBS) to a final concentration of 0.25% (v/v) in the reaction. Crosslinking was allowed for 10 min at RT and stopped by addition of one reaction volume 300 mM glycine, pH 7.5. For the restriction digest, the samples were diluted with 3 reaction volumes of water. Then the restriction digest buffer as well as the restriction enzyme indicated in the figures were added to the reaction and incubated for 2 hrs at 37ºC. Finally the samples were placed on ice.

B.1.11.4. CRIP immunoprecipitation

For the immunoprecipitation the samples were diluted 1:5 in 5 x CRIP IP buffer and protease inhibitors (Roche, cat. no.: 11697498001) were added. To reduce unspecific binding of proteins and DNA to the IP beads, each sample was pre-cleared with 10 µl plain protein A or G sepharose beads, which were equilibrated in TBS, pH 8.0. Pre-clearing was carried out for 2 hrs in the cold room on a rotating wheel. For the immunoprecipitation, the pre-cleared samples were transferred to the antibody-coated IP beads and incubated for 16 hrs in the cold room on the rotating wheel. The next day, the IP-beads were washed 3 times with 500 µl ice-cold 1 x TBS-T (CRIP), then once with TE containing 50 mM NaCl. For elution of immunoprecipitated complexes the IP-beads were incubated with 30 µl ChIP-EB buffer containing 0.1 µg/ml proteinase K (Roche, cat. no.: 03115887001) for 15 min at 65ºC in a shaking block. Then the IP-beads were peletted by centrifugation (1 min, 13200 x g, RT) and the supernatant was transferred to a fresh Eppendorf tube. For reversal of the formaldehyde crosslink the samples were incubated overnight at 65ºC. Remaining proteins were then digested by adding 15 µg proteinase K (Roche, cat. no.: 11697498001) to each sample followed by an incubation for 2 hrs at 55ºC. The immuoprecipitated radiolabelled DNA fragments were then separated by native polyacrylamide gel electrophoresis on a 6% gel, the gel was dried and exposed to BioMax MR film (Kodak, cat. no.: 8736936).

TBS, pH 8.0:	150 mM NaCl 100 mM Tris-HCl pH to 8.0
1 X TBS-T (CRIP):	1 X TBS 0.05% Tween-20
Blocking Buffer:	1 X TBS pH 8.0 0.5% BSA

300 mM Glycine pH 7.6:	300 mM Glycine pH adjusted to 7.6
5 x CRIP IP Buffer:	100 mM Hepes-KOH pH 7.5 0.5% NP-40 0.5% Trition X-100 1 M NaCl 0.1% Polyvinylpyrolidone-40,000 0.1% FICOLL400 0.5% BSA 0.1 mg/ml tRNA 300 µg/ml sonicated salmon sperm DNA 2% PEG-8000 55 mM EDTA 200 µg/ml sonicated salmon sperm DNA
ChIP-EB:	50 mM Tris pH 7.5 10 mM EDTA 1% SDS

B.1.12. Protein concentration determination

To determine the total protein concentration of a sample the Bradford assay was used. A serial dilution of a BSA-solution with known concentrations (2 to 0.125 mg/ml) served as a standard in addition to the sample. The assay was carried out in 96 well plates. 5 µl of a sample or standard dilution were supplemented with 195 µl 1 x Bradford reagent solution (Bio-Rad, cat.no.: 500-0006). After 10 min incubation the absorbance at 595 nm was determined on a plate reader (BIO-TEK INSTRUMENTS INC., EL800 universal microplate reader).

B.2. Cell Biology

B.2.1. Cell culture

B.2.1.1. Adherent cells

HeLa adherent cell lines were grown in Dulbecco's modified Eagle medium (DMEM, Invitrogen/Gibco, cat.no.: 15140-122) supplemented with 10% fetal bovine serum (FCS; Invitrogen/Gibco, cat. no.: 15140-122) and Penicillin (1000 u/l)-Streptomycin (100 mg/l) (Invitrogen/Gibco, cat. no.: 15140-122). Cells were cultured in plastic dishes (10 or 15 cm in diameter) or in 6 or 12 well plates (Nunc, cat. no.: 157150, 150350, 140675 and 150628, respectively). Cells were grown in tissue culture incubators in the presence of 5% CO_2 at 37ºC. In order to detach cells from cell culture dishes, the medium was removed, the cells were rinsed with 1 x PBS and then incubated with 0.25% trypsin / 0.2% EDTA (Invitrogen/Gibco, cat. no.: 25050-014) for 3 – 5 minutes. Cells were splitted at a ratio of 1:4.

B.2.1.2. Suspension cells

Jurkat and HeLaS3 cells were grown in RPMI 1640 containing L-glutamine (Invitrogen/Gibco, cat. no.: 21875-034), supplemented with 10% fetal bovine serum (FCS; Invitrogen/Gibco, cat. no.: 15140-122) and Penicillin (1000 u/l)-Streptomycin (100mg/l) (Invitrogen/Gibco, cat. no.: 15140-122). Suspension cells were grown in 25 to 175 cm^2 cell culture flasks and maintained at a density between 3 to 9 x 10^6 cells per milliliter medium.

B.2.1.3. Spinner cultures

HeLaS3 cells were grown in 3 liter glass spinner flasks (Wheaton, cat. no.: 356886) and stirred at a constant rate of 42 rpm. Cells were cultured similar to suspension cells as described in the previous chapter, except that the medium was supplemented with only 5% FCS. Spinner cultures were grown in a humidified incubator in the presence of 5% CO_2.

B.2.1.4. Freezing of eukaryotic cells

0.5 to 1 x 10^7 cells were collected and pelleted by centrifugation (500 x g, room temperature). Cells were resuspended in freezing medium. Cells were frozen in 0.5 to 1 ml aliquots at -80ºC in a freezing box and subsequently transferred to a liquid nitrogen tank for storage.

Freezing medium: 90% (v/v) FCS
10% (v/v) DMSO

B.2.1.5. Thawing of eukaryotic cells

In order to take frozen cells into culture, the vial containing the frozen cells was thawed in a water bath at 37ºC. After completion of the thawing process cells were resuspended immediately in 10 ml of prewarmed cell culture medium.

B.2.2. Luciferase assay

CMV-HeLa cells were tested for reporter gene induction 16 hours after addition of doxycycline to the cell culture medium. For each luciferase assay reading one well of a 12 well plate was used. Immediately prior to cell lysis, the cell culture medium was removed, the cells rinsed once with 1 x PBS and then lysed by addition of 100 µl 1 x passive lysis buffer (Promega, cat. no.: E194A). After 10 min incubation on ice the lysate was collected in an Eppendorf tube and centrifuged for 10 min (16200 x g, RT). 20 µl of this supernatant was used in the luciferase assay according to the manufacturer's instructions (Promega; cat. no.: E1501). Luciferase readings were normalized to the protein content of each sample. The protein concentration of each luciferase sample was measured with a Bradford assay (see section B.1.12.).

B.2.4. Chromatin immunoprecipitation

This ChIP protocol is based on a protocol published by the Young lab (Odom et al., 2004).

B.2.4.1. Crosslinking of adherent cells

CMV HeLa cells were crosslinked on 15 cm diameter cell culture dishes by adding 37% formaldehyde solution (Roth, cat. no.: 4979.1) to a final concentration of 1% (v/v) directly to the medium. After exact 10 min incubation on a rotating platform at RT the crosslink was stopped by addition of 2 M glycine solution to a final concentration of 125 mM in the cell culture dish. All subsequent steps were carried out in the cold room at 4ºC. Crosslinked cells were rinsed 3 times with ice-cold PBS containing 0.2 mM PMSF and then harvested from the cell culture dishes with a rubber scrapper. Cells were collected in polypropylene tubes, peletted by centrifugation (5 min, 500 x g, 4ºC) and then either processed directly or snap-frozen in liquid nitrogen and stored at -80ºC.

B.2.4.2. Cell lysis and sonication

For the lysis, a crosslinked cell pellet comprising about 10^8 cells was resuspended in 5 ml lysis buffer LB1 supplemented with protease inhibitors (Roche, cat. no.: 11697498001). The suspension was incubated on a roller for 10 min at 4ºC. After centrifugation (10 min, 4000 rpm, 4ºC) the pellet was resuspended in LB2 and incubated on a roller for 10 min at 4ºC. Then the cells were pelleted by centrifugation (10 min, 4000 rpm, 4ºC) and resuspended in LB3 supplemented with protease inhibitors (Roche, cat. no.: 11697498001). In addition a spatula tip of acid-washed glass beads (Sigma, cat. no.: G1277) was added to each sample prior to sonication. Sonication of the crosslinked extracts was carried out on a Branson 250 sonifier with the tubes being cooled in a ice/ethanol bath during sonication. The sonication settings were: 40% output amplitude, total sonication time: 6 min, sonication cycles: pulse on: 30 sec, pulse off: 10 sec. After sonication the average DNA fragment size was checked by agarose gel electrophoresis (Before loading the samples onto the agarose gel proteins were digested and the crosslink reverted). Typically, the fragment size was between 300 and 600 bp. The sheared chromatin was then centrifuged (5 min, 5500 rpm, 4ºC) and the supernatants were transferred into fresh Eppendorf tubes. Debris was removed by another centrifugation

step (15 min, 13200 x g, 4ºC), then the double strand (ds) DNA concentration was determined in a spectral photometer and the samples were diluted with LB3 to a ds DNA concentration of 1 mg/ml. Before aliquoting and freezing of the ChIP samples in liquid nitrogen, 80% glycerol was added to each sample such that the final glycerol concentration was 5 % (v/v). ChIP samples were stored at -80ºC.

B.2.4.3. Immunoprecipitation

25 µl antibody-coupled protein A or G sepharose beads were prepared as described in section B.1.3.1. for each chromatin immunoprecipitation sample and equilibrated in LB3 buffer. Crosslinked chromatin extract was allotted such that each chromatin immunoprecipitation contained the equivalent of 200 µg double-strand DNA. Typically, an immunoprecipitation was carried out in a total volume of 400 µl LB3 containing a final concentration of 0.5% (v/v) Triton X-100, 0.25% (v/v) N-lauryl sarkosyl and 0.1% (v/v) deoxycholate. Each chromatin immunoprecipitation mix was supplemented with protease inhibitors (Roche, cat. no.: 11697498001). Before the immunoprecipitation with the specific antibody, each sample was precleared by incubation with 25 µl plain protein A or G sepharose beads equilibrated in LB3 containing 0.5% Triton X-100, 0.25% N-lauryl sarkosyl and 0.1% deoxycholate for 2 hrs at 4ºC on the rotating wheel. After that the supernatant was transferred to the antibody coupled IP beads and incubated overnight at 4ºC on the rotating wheel. The next day the IP beads in each sample were washed 6 times with 1 ml ice-cold ChIP wash buffer supplemented with protease inhibitors. After a final wash with 1 ml TE containing 50 mM NaCl the IP-beads of each sample were eluted by addition of 100 µl ChIP-EB buffer. For the elution, the samples were incubated for 15 min at 65ºC in a thermo mixer. Finally the beads were pelleted by centrifugation (1 min, 13200 x g, RT) and the supernatant was transferred to a fresh Eppendorf tube. For reversal of the crosslink samples were incubated overnight at 65ºC. The next day 1 volume TE was added to each sample. Then RNase A (Ambion, cat. no.: 2272) was added to a final concentration of 10 µg/ml and the samples were incubated at 37ºC for 2 hrs. After that proteinase K (Roche, cat. no.: 03115887001) was added to each sample to a final concentration of 150 µg/ml and the samples were incubated at 55ºC for 2 hrs. Then the immunoprecipitated DNA fragments were prepared by phenol-chloroform

extraction and ethanol precipitation with glycogen as a coprecipitant. Dried DNA pellets were resuspended in 50 µl 10 mM Tris-HCl, pH 8.0 at room temperature.

Lysis buffer 1 (LB1):	50 mM Hepes-KOH, pH 7.5 140 mM NaCl 1 mM EDTA 10% (v/v) Glycerol 0.5% (v/v) IGEPAL CA630 (NP-40) 0.25% (v/v) Triton X-100
Lysis buffer 2 (LB2):	200 mM NaCl 1 mM EDTA 0.5 mM EGTA 10 mM Tris-Cl, pH 8.0 at RT
Lysis buffer 3 (LB3):	140 mM NaCl 1 mM EDTA 0.5 mM EGTA 20 mM Tris-Cl, pH 8.0 at RT 0.5% N-lauryl sarkosyl 0.1 % Sodium deoxycholate
ChIP-Wash buffer:	25 mM HEPES-KOH, pH 7.6 0.5 mM EDTA 0.5% (v/v) IGEPAL CA630 (NP-40) 0.25 M LiCl 0.7% (v/v) sodium deoxycholate
ChIP-EB:	50 mM Tris pH 7.5 10 mM EDTA 1% SDS

B.2.5. Quantitative real-time PCR

Quantitative real-time PCR were carried out on a Gene Amp 5700 (Applied Biosystems) machine. The subsequent data analysis was performed with the Microsoft Excel software. A typical 25 µl PCR reaction was composed as follows

4 µl	sample in 10 mM Tris pH 8.0 or TE
12.5 µl	2X SYBR Green PCR Mastermix
	(Applied Biosystems, Cat. no.: 4367659)
1 µl	10 mM Primer Mix
7.5 µl	water

B.3. Basic buffers and solutions

BC-buffers	20 mM Tris-HCl pH 7.3 (RT)
	0.2 mM EDTA, pH 8.0
	0 – 2000 mM KCl
	20% (v/v) glycerol
HEGN	25 mM Hepes-KOH, pH 7.6
	0.2 mM EDTA
	0 – 2000 mM KCl
	10% (v/v) glycerol
	0.1% (v/v) IGEPAL CA630 (NP-40)
10 x TBE	1 M Tris-HCl
	1 M Boric acid
	20 mM EDTA, pH 8.0

10 x PBS	1.37 M NaCl
	78 mM Na_2HPO_4
	15 mM KH_2PO_4
	27 mM KCl
	pH 7.4
1 x TE	10 mM Tris HCl, pH 7.5
	1 mM EDTA, pH 8.0

B.4. Material

B.4.1. Antibodies

Primary antibody	Number	Provider	Species
AF-10	sc-27083	Santa Cruz	goat
CPSF1	sc-17289	Santa Cruz	goat
Brd4	sc-27975	Santa Cruz	goat
CstF-64	sc-16473	Santa Cruz	goat
PARP-1	sc-1561	Santa Cruz	goat
CDK7	sc-529	Santa Cruz	rabbit
CDK8	sc-1521	Santa Cruz	goat
CDK9	sc-8338	Santa Cruz	rabbit
CBP/HAT	6D6	E.Kremmer	rat
cyclin T1	sc-10750	Santa Cruz	rabbit
GAL4-DBD	sc-577	Santa Cruz	rabbit
H3-K79me2	05-835	Upstate	rabbit
H3	ab1791	Abcam	rabbit
H3-K9-me3	07-523	Upstate	rabbit
Ku	sc-466	Santa Cruz	mouse
TRAP220	sc-8998	Santa Cruz	rabbit
TRAP150	sc-5378	Santa Cruz	goat
MED6	sc-9433	Santa Cruz	goat
MED7	3E12	E.Kremmer	rat
MED15	6C9	E.Kremmer	rat
MED15	1H7	E.Kremmer	rat
MED25	VC1	E.Kremmer	rat
NC2-alpah	4G7	E.Kremmer	rat
TRAP95	sc-5366	Santa Cruz	goat
TRAP95	sc-5363	Santa Cruz	goat

TRAP240	sc-5369	Santa Cruz	goat
PolII (N20)	sc-899	Santa Cruz	rabbit
Leo1		T. Albert	rabbit
Sur2	550429	BD	mouse
TFIIB	sc-225	Santa Cruz	rabbit
TFIIB	sc-23875	Santa Cruz	mouse
TFIIH p62	sc-292	Santa Cruz	rabbit
TAFII100	1C2	L.Tora	mouse
TAFII80		L.Tora	mouse
TAFII250	sc-735	Santa Cruz	mouse
TBP SI-1	sc-273	Santa Cruz	rabbit
Cdc2 p34	sc-54	Santa Cruz	mouse
Cdc2 p34	sc-954	Santa Cruz	rabbit
hSpt5	sc-28678	Santa Cruz	rabbit
CTD Ser-7 P	4E12	E.Kremmer/D.Eick	rat
CTD Ser-5 P	3E8	E.Kremmer/D.Eick	rat
CTD Ser-2 P	3E10	E.Kremmer/D.Eick	rat
Paf1		T. Albert	rabbit
PC4		M.Meisterernst	rabbit

Table 5: Primary antibodies

B.4.2. Plasmids

Plasmid	Insert	Purpose	Provider	Reference
pSB1	GST-VP16	Protein expression	T. Stuehler	(Ikeda et al., 2002)
pSB2	GST-H1	Protein expression	T. Stuehler	(Ikeda et al., 2002)
pSB3	GST-H1mt	Protein expression	T. Stuehler	(Ikeda et al., 2002)
pSB4	GST-H2	Protein expression	T. Stuehler	(Ikeda et al., 2002)
pSB5	GST-H2mt	Protein expression	T. Stuehler	(Ikeda et al., 2002)
pSB6	GST-CTD	Protein expression	Dynan lab	(Peterson et al., 1992)

Table 6: Plasmids

B.4.3. Primers

Oligo ID	Name	Sequence	Modi-fication	Use	Provider/ Reference
oSB1	bio-pGL2-MRG5	GCA TTC TAG TTG TGG TTT GTC CAA	5'-Biotin	IMTA	T.Uhlmann
oSB2	Luc-bio-dw-MRG5	GCC GGG CCT TTC TTT ATG TT		IMTA	T.Uhlmann
oSB3	oriP_FW	TGA TAC CCA GTA GTA GAG TGG		ChIP	T.Uhlmann
oSB4	oriP_BW	CAG CAG GAA AAG GAC AAG CAG		ChIP	T.Uhlmann
oSB5	TRE1	AGG CG GTA CGG TGG GAG GCC		ChIP	T.Uhlmann
oSB6	TRE2	AGG CTG GAT CGG TCC CGG TGT		ChIP	T.Uhlmann
oSB7	Bio-H2B-FW	GAC GTG TTG TTG GTT AGG GCT	5'-Biotin	IMTA	T.Albert
oSB8	H2B-SP-Bac	CAGAGG AGG AAT ACA AGC ACC		IMTA	T.Albert
oSB9	MRG5-IMTA_long_3'	AT ACG ACG ATT CTG TGA TTT G		IMTA	T.Uhlmann
oSB10	bio-G5ML-IMTA	CGA TTC ATT AAT GCA GCT GG	5' Biotin	IMTA	Wu et al., 2003
oSB11	G5ML-Reverse-IMTA	AAC TCG ACT GCA GCA TAT GTA TCA TAC ACA TAC G		IMTA	Wu et al., 2003

Table 7: Primers

B.4.4. Cell lines

HeLa S3: HeLa derived cell line that has been adapted for growth in suspension culture.

HeLa: Human epithelial cell line originating from a cervical carcinoma. Adherent cells.

CMV-HeLa: HeLa cell line carrying the episomal plasmid pEBNA-SVP-GL-EμCAG-rtTA

Jurkat J6: Human T lymphoblastoid cell line

B.4.5. Standards

B.4.5.1. DNA ladders

1 kB DNA-ladder (Gene ruler), Fermentas. Fragment lengths in bp: 10 000, 8000, 6000, 5000, 4000, 3500, 3000, 2500, 2000, 1500, 1000, 750, 500, 250.

100 bp DNA-ladder (Gene ruler), Fermentas. Fragment lengths in bp: 1031, 900, 800, 700, 600, 500, 400, 300, 200, 100, 80.

B.4.5.2. Protein standards

SDS-PAGE protein standard high and low range (Biorad):

Molecular weight	Protein	High range (Biorad, cat. no.: 161-0303)	Low range (Biorad, cat. no.: 161-0304)
200	Myosin	x	
116	β-Galactosidase	x	
97	Phosphorylase B	x	x
66	Serum albumin	x	x
45	Ovalbumine	x	x
31	Carboanhydrase		x
21	Trypsininhibitor		x
14	Lysozyme		x

C. Results

C.1. Mediator modules and Mediator subpopulations

The Mediator complex in a nuclear extract does not present itself as a homogeneous entity. Based on its composition several distinct Mediator forms can be distinguished. In the first chapter I describe my efforts to further characterize the Mediator populations defined by the presence of the CDK8 module and the MED25 subunit respectively. In terms of function I focused on the role of Mediator as a basal transcription factor.

C.1.1. Characterization of Mediator subpopulations in nuclear extracts

Chromatin immunoprecipitation (ChIP) experiments revealed that CDK8 is lost at the promoter region upon transcription induction in a doxycycline inducible tet-VP16 model system *in vivo* (Uhlmann et al., 2007). Previous biochemical studies of VP16-Mediator interactions had not revealed a selectivity of VP16 for a CDK8-less Mediator population that seems to mediate transcription activation *in vivo*. (Ito et al., 1999; Mittler et al., 2003). Solutions to this apparent contradiction could be either the substochiometric distribution of CDK8 in Mediator or a mechanism that leads to its removal i.e. during transcription initiation. Monoclonal antibodies directed against MED15 and MED25 and polyclonal antibodies against CDK8 and MED1 were used to deplete standard Dignam HeLa nuclear extracts for the complexes associated with them. TBP served as a loading control (Fig. 3A). MED15 depletion reproducibly removed all MED25 as well as other Mediator core subunits but retained a minor fraction of CDK8. This initially implies that most of the CDK8 exists in a Mediator-bound form. CDK8 depletion, while being highly efficient for the antigen, removed a smaller fraction of MED25 and of other Mediator subunits. MED25 depletion retains a larger fraction of CDK8. MED1 depletion removed all MED7 but retained a fraction of MED15, MED25 and CDK8. These findings indicate that the extracts contain minimally a MED15-MED25 containing subcomplex that lacks MED7. This could be a free head module or another complex. The data further qualitatively suggest that CDK8 is underrepresented in MED25 and MED1 associated Mediators.

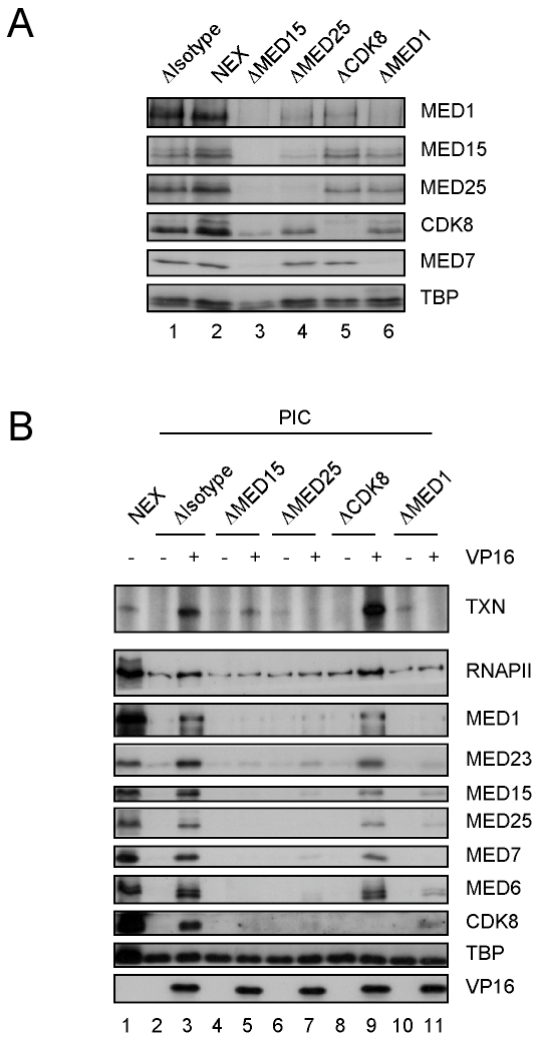

Figure 3: Biochemical and functional analyses of extracts depleted for variant Mediator complexes. *A*, Western blot analysis of immunodepleted HeLa extracts using antibodies against the indicated Mediator subunits. Jurkat nuclear extracts led to an essentially identical picture (data not shown). *B*, Westernblot analysis of factors bound to immobilized templates upon immunodepletion with antibodies against the indicated Mediator subunits in the absence and presence of the activator GAL4-VP16 using Jurkat extracts. Lane 1 (NEX) shows a nuclear extract control. PIC (preinitiation complex) indicates analysis of complexes bound to and washed on the immobilized templates (lane 2 to lane 11). *In vitro* transcription analyses (TXN) of isotype and the indicated Mediator-subunit depleted Jurkat nuclear extracts on a adenovirus major-late promoter carrying five upstream GAL4 binding sites in the presence and absence of Gal4-VP16 as indicated.

C.1.2. CDK8 is dispensable for basal and VP16-dependent *in vitro* transcription

To address the question whether CDK8 is necessary or inhibitory to Mediator recruitment by VP16 I employed the immobilized promoter template technology. Jurkat nuclear extracts were incubated with an immobilized promoter template comprising five consecutive GAL4 binding sites upstream of a synthetic core promoter and part of the

luciferase reporter gene. Transcription complexes were formed in the absence and presence of GAL4-VP16 using extracts (NEX) and extracts depleted for Mediator variants (Δ). Bound complexes were washed, proteins eluted, separated on SDS-PAGE gels and analyzed by immunoblot (Fig. 3B). The data argue for VP16-dependent binding of Mediator in mock-depleted extracts (ΔIsotype) and loss of Mediator binding if the extracts which were depleted with antibodies directed against MED15 or MED25. Conversely, depletion of CDK8 does not prevent recruitment of Mediator. Hence, CDK8 is not necessary but - in the light of the reduced amount of Mediator in the ΔCDK8 extract - the kinase is also not inhibitory to the interaction with VP16. Of note, TBP binding is independent of the activator and the presence of Mediators, and, thus, does serve here as a loading control. Next the analysis of transcription complex formation at immobilized promoter templates was extended to PICs which were exposed to a second incubation step under *in vitro* transcription conditions (Fig. 4B). Like TBP, TFIIH binds promoters independent of the presence of VP16 (a decrease of the TFIIH signal upon addition of VP16 was not generally seen). In agreement with previous studies (Johnson et al., 2004; Malik et al., 2005 and references therein) Mediator binding was strongly induced by VP16. Among the different Mediator subunits, the enrichment of MED25 by VP16 is most pronounced. This may be in part attributed to the fact that this factor is present in free and bound form both of which directly interact with VP16 (Mittler et al., 2003). CDK8, although absent *in vivo*, was co-recruited with Mediator to the template as is MED1.

To investigate the changes occurring in the template bound Mediator population upon transcription initiation washed PICs were incubated in a second step in transcription buffer supplemented with 500 μM NTPs (Fig. 4B). Afterwards the proteins bound to the immobilized templates were washed, eluted with Laemmli buffer and analyzed by immunoblot. The PIC is stable if incubated without any NTPs (Fig. 4B, lane 4). Addition of 500 μM ATP alone is sufficient to dissociate the PIC (lane 5) and leads to a similar result as ATP and CTP (formation of the first phosphodiester bond is permitted; Fig. 4B, lane 6), or ATP, CTP and UTP were added (transcription proceeds till position 29 of the promoter template, Fig. 4B, lane 7) or all four NTPs were added (run-off transcription takes place, Fig. 4B, lane 8). The process of PIC dissociation appears to be independent

of productive transcription. In yeast a re-initiation Mediator has been described, which remains bound to the core promoter after transcription initiation (Yudkovsky et al., 2000). I find that Mediator subunits dissociate to varying degrees. The MED7 component, which is part of the middle module, remains attached to the promoter template. A slight loss is seen for the MED25 subunit. CDK8, a constituent of the

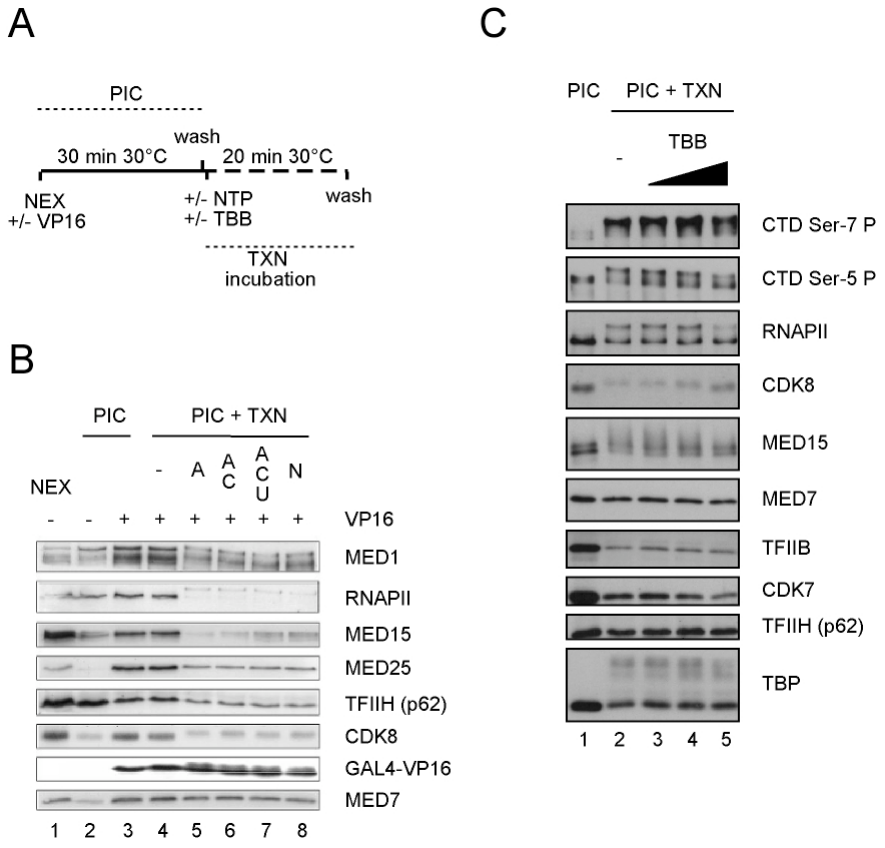

Figure 4: Characterization of transcription complexes formed under the control of GAL4-VP16 on immobilized templates and influence of nucleotides on their composition. *A*, Schematic protocol of the immobilized template assay. PIC, preinitiation complex formation; NEX, nuclear extract. *B*, Western blot analysis of Jurkat extract (NEX) (25% of material relative to the bound fraction, lane 1) and factors bound to immobilized templates (PIC formation) in the absence and presence of saturating amounts of GAL4-VP16 using the indicated antibodies. Several reactions (lanes 4–8) were subjected to a second incubation period with buffer only (lane 4), 500 μM ATP (A, open complex formation, lane 5), 500 μM ATP and 500 μM CTP (AC, allowing transcription to position 2), 500 μM ATP, 500 μM CTP, and 500 μM UTP (ACU, allowing transcription to position 29) and all four NTPs at a final concentration of 500 μM (*N*, 91-bp run-off transcript conditions). *C*, PICs were formed in the presence of 1 μM ATP, washed and incubated in a second step in the presence of 1, 10 and 100 μM of the casein kinase 2 (CKII) inhibitor TBB (lanes 3, 4 and 5 respectively). In this case the second incubation step contained NTPs to a final concentration of 100 μM.

CDK8 module is lost to a larger degree, as is MED15, which is part of the tail module. The amount of promoter bound TBP is modestly affected by NTP addition (Fig. 4C, compare lane 1 and 2). A modification of TBP is observed, which increases the apparent molecular weight of TBP in an SDS-PAGE gel by approximately 10 kDa (Fig. 4C, lanes 2-5). As in the yeast study, a loss of CDK7 and TFIIB is seen, the TFIIH core subunit p62 is lost to a lesser degree (Fig. 4B, C) upon transcription initiation. This leads to the conclusion that in higher eukaryotes the reinitiation Mediator complex consists of the head and middle modules, but is devoid of the CDK8- and tail module components.

C.1.3. The MED25-Mediator is sufficient for *in vitro* transcription

MED25 lacks an apparent homologue in yeast (Blazek et al., 2005; Bourbon, 2008). To the present day the physiological role of this particular Mediator subunit is not understood. Earlier work by a former Ph.D. student, Gerhard Mittler, hinted that there might be two functionally distinct Mediator populations. One dubbed basal Mediator or B-MED, which acts similar to a general transcription factor and a second that is required for activator-dependent transcription (A-MED). It was reasoned, that the MED25 Mediator might represent the A-MED variety (Mittler et al., 2001). I now asked whether the MED25 Mediator by itself is sufficient to support *in vitro* transcription or whether other Mediator complexes are required. A Jurkat nuclear extract was immunodepleted for all Mediator populations. Subsequently immunopurified MED25-Mediator was added back to the extract and tested for its ability to form a PIC and to support *in vitro* transcription (Fig. 5). The presence of the MED25 Mediator in a nuclear extract is sufficient to form a functional PIC (as measured by TFIIB and TFIIH recruitment) and to support *in vitro* transcription at levels comparable to an extract which had been mock-depleted (ΔIso). This suggests that the MED25-associated complexes contain all functional elements required for VP16-dependent transcription initiation, even if washed under stringent (800 mM KCl) conditions.

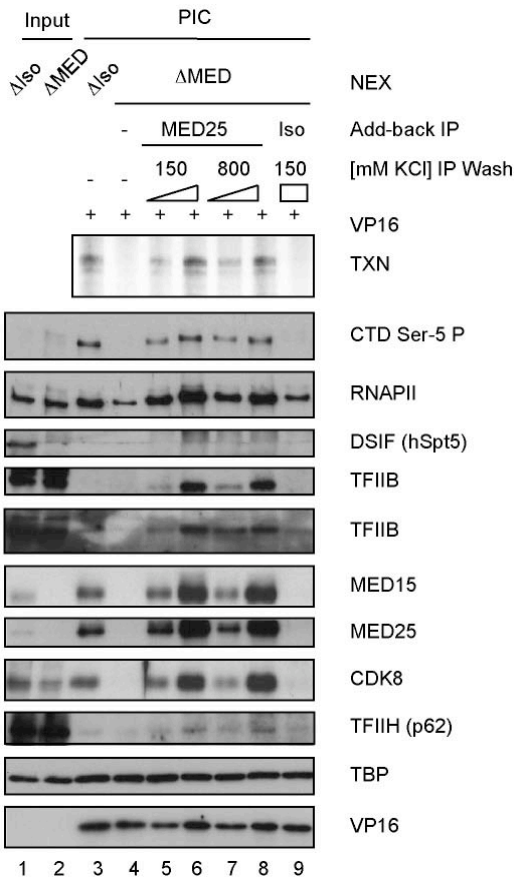

Figure 5: The MED25-Mediator is sufficient for transcription *in vitro*. *A*, Immunoblot and *in vitro* transcription analysis (TXN) of immobilized promoter templates (lanes 3-9). Nuclear extracts were either mock-treated (ΔIso) or Mediator-depleted (ΔMED) and supplemented with immunoprecipitated MED25-Mediator (MED25) or isotype immunoprecipitation (IP-) beads (Iso). IP-beads were washed either with low (150 mM KCl) or high stringency (800 mM KCl).

C.1.4. Basal Mediator

In previous studies our laboratory discovered that Mediator is absolutely required for activator-independent basal transcription in the nuclear extract *in vitro* transcription system. This requirement is not observed in a minimal *in vitro* transcription system composed of highly purified and recombinant components (Mittler et al., 2001). I set out to analyze the molecular mechanism behind this requirement and found that the basal Mediator acts at two distinct steps: in the nuclear extract transcription system Mediator acts on TFIIB, TFIIH, RNAPII recruitment and also is required to establish a ser-5 phosphorylation of the IIa form of the CTD of RNAPII before complex opening. Notably, the nuclear extract transcription system is CTD-phosphorylation dependent, whereas the recombinant/purified transcription system is not (Serizawa et al., 1993).

C.1.4.1. Mediator promotes GTF recruitment to a TATA-TBP complex

In the nuclear extract system the presence of Mediator is required for TFIIB and TFIIH recruitment to the promoter even though TFIIB is present in the nuclear extract in large excess compared with the amount finally recruited to the template ((Baek et al., 2006) and Fig. 5, compare lanes 3 and 4; Fig. 6A, compare lanes 4 and 5). First I compared the recruitment efficiency of TFIIB to a TATA-TBP complex using either a nuclear extract or recombinant TBP (rTBP) and recombinant TFIIB (rTFIIB) in similar concentrations as they are present in a nuclear extract (Fig. 6C, lanes 1 and 2). An immobilized template assay revealed that TFIIB is recruited in much larger amounts in case only rTFIIB/rTBP is used in the assay. Although the nuclear extract contains similar amounts of TBP/TFIIB, a significant lower amount of TFIIB is recruited to the immobilized promoter template (Fig. 6C, lanes 3 and 4). Furthermore I found that components of the 0.1 M P11 phosphocellulose column fraction were able to interfere with recruitment of rTFIIB to an rTBP-TATA complex (Fig. 6C, lanes 5-8). However this effect could be due to unspecific DNA binding proteins present in the 0.1 M P11 fraction. In transcription system composed of nothing but recombinant and highly purified factors GTFs are recruited sequentially. To test whether this mechanism applies in this case I incubated the promoter DNA template with recombinant TBP and recombinant TFIIB prior to addition of mock-treated (ΔIso) or Mediator-depleted (ΔMED) nuclear extract (Fig. 6B). Even if massive amounts of TFIIB were bound to the promoter DNA template recruitment of TFIIH remained Mediator-dependent. This indicates that Mediator not only acts on TFIIB recruitment, but rather facilitates recruitment of GTFs such as TFIIB and TFIIH. This would be in line with the predictions of the holoenzyme model which states that Mediator, RNAPII and the GTFs TFIIB, TFIIE, TFIIF and TFIIH are recruited to an promoter in form of a holoenzyme complex. One can envision two modes for this GTF-recruitment limiting mechanism that imposes the Mediator requirement on basal transcription: (i) Mediator is required so that TBP can adopt a conformation on the promoter that allows recruitment of TFIIB and TFIIH or (ii) Mediator counteracts the effect of a factor that blocks access of GTFs to their respective binding sites on the TBP-DNA complex. To distinguish between the two possibilities I performed an immobilized template assay with the major-late promoter template in which I incubated the template

with either recombinant TBP (rTBP) alone (Fig. 6B *lanes* 12 and 13), or with TBP plus varying amounts of recombinant TFIIB (rTFIIB, Fig. 6B lanes 6-11). The amount of rTBP offered to the promoter template resembled that offered by a nuclear extract (see input, compare Fig. 6B *lanes* 1 and 2 to 3). The rationale behind this was that in the purified system rTBP binds without difficulties to the TATA box. If this binding is established prior to the presence of a nuclear extract factor that represents an obstacle to proper TBP positioning, TFIIB/TFIIH recruitment and *in vitro* transcription should become independent of a basal Mediator activity. After the pre-incubation step the templates were washed and incubated in a PIC formation reaction either with mock- (ΔIso) or Mediator-depleted (ΔMED) Jurkat nuclear extract in the presence of 1 μM ATP. After the PIC formation reaction, immobilized templates were washed and eluted in part with Laemmli buffer for immunoblot analysis of the PIC or were subjected to an *in vitro* transcription reaction (TXN). Binding of rTBP to the promoter template in a pre-incubation step did not alleviate the Mediator requirement for TFIIB and TFIIH recruitment as well as the Mediator-requirement for basal transcription (compare Fig. 6B lanes 4 and 5 to l2 and 13). This led to the conclusion that TBP positioning is unlikely to be affected by a negative acting nuclear extract factor. Next it was tested whether TFIIB is actively recruited by basal Mediator or whether basal Mediator helps to overcome a factor that blocks TFIIB recruitment. In another PIC formation assay it was tested whether TFIIB recruitment might be dependent on a kinase or helicase activity. PICs were formed on an immobilized major-late promoter template in the presence of 500 μM of the broad spectrum kinase inhibitor H8, 1 μM of the helicase inhibitor ATPγS or 1 μM of ATP. TFIIB and CDK7 of TFIIH were recruited in similar quantities in each situation (Fig. 10A). Notably endogenous TFIIB was recruited by Mediator even if similar amounts of rTFIIB were recruited to the template bound rTBP during the pre-incubation (Fig. 6B, lanes 8 and 9). rTFIIB ran on a SDS page gel at a higher molecular weight due to its His-tag. Thus even massive recruitment of TFIIB to the promoter template in a TBP-TFIIB complex could not substitute for the Mediator-requirement for recruitment of TFIIH and basal transcription, as it would be predicted by the sequential GTF assembly model. This particular results rather argue in favor of the holoenzyme model.

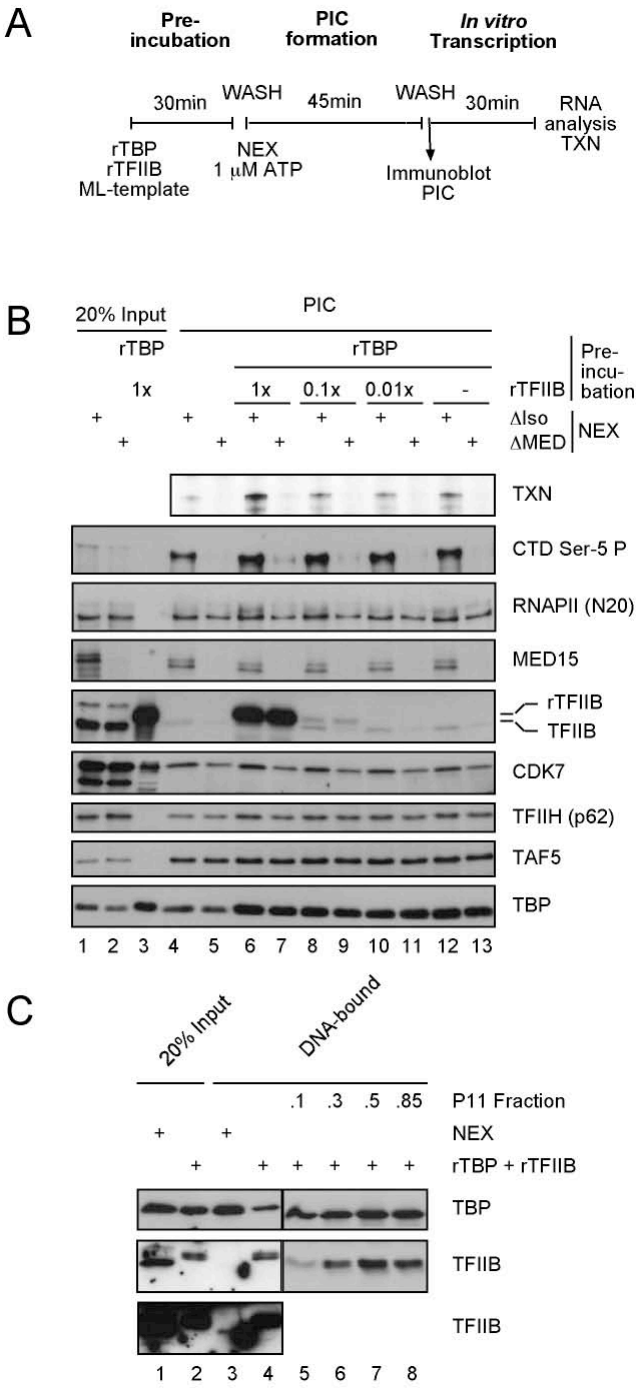

Figure 6: Basal Mediator is required for TFIIB/TFIIH recruitment, establishment of CTD ser-5 hypophosphorylation during PIC formation and transcription. *A*, Schematic protocol of the immobilized template assay. *PIC*, preinitiation complex formation. *B*, Immunoblot analysis and *in vitro* transcription (TXN) analysis of PICs formed on major-late promoter templates with mock depleted (ΔIso) or Mediator-depleted (ΔMED) Jurkat nuclear extracts in the presence of 1 μM ATP. Preincubations of the template with rTBP (TBP concentration similar to that found in a nuclear extract) and rTFIIB was carried out as indicated. Input lanes 1-3 display 20% of the protein offered to the immobilized templates analyzed in lanes 4-13. *C*, Immobilized template assay. PICs were formed with rTBP/rTFIIB, Jurkat nuclear extract (NEX) or the indicated P11 fractions supplemented with rTBP/rTFIIB.

C.1.4.2. Establishment of CTD ser-5 phosphorylation on the low-mobility form of RNAPII Rpb1 during PIC formation is a distinct basal Mediator function

As will be discussed in detail in sections C.2 I found that phosphorylation of the high mobility form of RNAPII (CTD hypophosphorylation) was established prior to complex opening by TFIIH and did correlate with the transcription potential of a PIC. In the above mentioned experiment it became evident, that CTD ser-5 hypophosphorylation was established in a strictly Mediator-dependent manner. Even recruitment of large amounts of TFIIB to the template did not bypass this dependency arguing that CTD ser-5 hypophosphorylation did not occur by default after TFIIB recruitment. This provides evidence for the notion that CTD ser-5 hypophosphorylation is established only in the context of a transcription competent PIC. To further investigate the properties of Mediator as a general transcription factor I asked whether the transcription activation domains of VP16 and Sp1 are dependent on a basal Mediator function. I formed PICs on a major-late promoter template comprising five GAL4 binding sites with either mock- (ΔIso) or Mediator-depleted (ΔMED) nuclear extracts in the absence of activator (basal; Fig. 7, lanes 1 and 2) or in the presence of GAL4-VP16 (Fig. 7, lanes 3 and 4) or GAL4-Sp1 (Fig. 7 lanes 5 and 6). Template bound VP16 led to an increase in template recruited Mediator and in turn to an increase in TFIIB and TFIIH recruitment. The increase of Mediator at the promoter template exceeded the one seen for TFIIB and TFIIH (Fig. 7, lanes 3 and 4). Sp1 did not lead to an increased Mediator recruitment, but efficient PIC formation remained Mediator-dependent (Fig. 7, lanes 5 and 6). CTD ser-5 hypophosphorylation correlated with the amount of TFIIB and TFIIH recruited to the promoter template. TAF4 has been reported to be an essential cofactor of Sp1 transcription activation (Hoey et al., 1993). A modest, Mediator-independent increase of TAF4 recruitment was seen in the presence of both activators. In summary, transcription activation by Sp1 did not lead to an increased Mediator recruitment, however, Sp1-activated transcription proved to be dependent on a basal Mediator function that Sp1-recruited factors could not bypass.

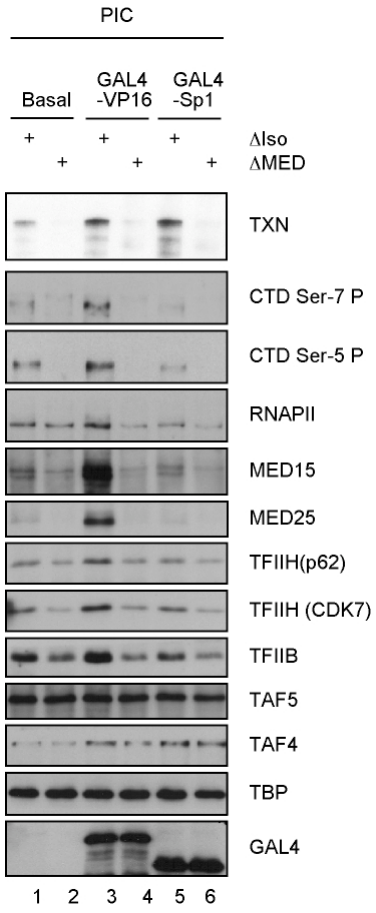

Figure 7: Recruitment of TFIIB and TFIIH to a PIC as well as establishment of CTD ser-5 hypophosphorylation is dependent on Mediator in the presence of GAL4-VP16 and GAL4-Sp1. PICs were formed in immobilized template assays on the major-late promoter (ML) with mock- (ΔIso) or Mediator-depleted (ΔMED) nuclear Jurkat extracts. PICs were washed and either analyzed for their *in vitro* transcription potential (TXN) or by immunoblot. In lanes 3 and 4 the activator GAL4-VP16, in lanes 5 and 6 the activator GAL4-Sp1 was included. The amount of both activators recruited to the five GAL4 binding sites on the ML-promoter template were comparable as shown by the GAL4 immunoblot.

C.1.4.3. Negative cofactor 2 (NC2) is not responsible for the Mediator requirement in basal transcription

A yeast genetic study found NC2 as well as the CCR4-NOT complex act as antagonists of the positive Mediator function (Lee et al., 1998). For NC2 it has been demonstrated with recombinant proteins that it has the ability to compete with TFIIA and TFIIB recruitment to a TATA-TBP complex *in vitro* (Goppelt et al., 1996). On this basis I tested the hypothesis that NC2 does impose the Mediator-requirement on basal transcription in

the nuclear extract system. Nuclear extracts were immunodepleted for Mediator (ΔMED), NC2 (ΔNC2), and the combination of both (ΔIsoΔMED). With these extracts PICs were formed in the presence of 1 μM ATP and the extent of TFIIB and TFIIH (CDK7) recruitment as well as the appearance of CTD ser-5 hypophosphorylation was scored by immunoblot (Fig. 8A). In parallel *in vitro* transcription reactions were carried out following the same scheme (Fig. 8, TXN). If NC2 would be the GTF-recruitment limiting factor, one would expect that taking out NC2 leads to increased TFIIB and TFIIH recruitment, and that in the absence of both, Mediator and NC2, the positive as well as the negative transcription cofactor, basal *in vitro* transcription should be Mediator-independent. Immunodepletion of NC2 indeed led to an increase of TFIIB and TFIIH recruitment to the promoter template in the basal as well as VP16-activated situation (Fig. 8A, compare lanes 5 and 7 as well as 9 and 11). Correlated with NC2 immunodepletion an increase in basal and VP16-activated transcription was observed. However, immunodepletion of NC2 together with Mediator did not led to levels of functional PIC formation as were seen in the presence of both factors (Fig. 8A, compare lanes 5 to 8 and 9 to 12). Thus transcription was even in the absence of NC2 Mediator-dependent. To further investigate how NC2 modulates TFIIB and TFIIH recruitment during PIC formation I performed an additional PIC formation assay and in parallel an *in vitro* transcription experiment in which I doubled the amount of NC2 in the nuclear extract by adding recombinant NC2 (rNC2) complexes. Addition of this extra amount of NC2 led to a reduction of TFIIB and TFIIH (CDK7) recruitment during PIC formation under basal and VP16-activated conditions. (Fig. 8B, compare lanes 3 and 7 and lanes 4 and 8 respectively). In summary, NC2 appeared to fine tune GTF recruitment to the PIC. The fact that adding extra NC2 could suppress TFIIB and TFIIH recruitment might be explained by competition of TFIIB and NC2 for the binding to TATA-TBP complexes. This fact is supported by the notion that the recruitment kinetics of TFIIB as well as of NC2 were both much slower than that of Mediator or TFIID (Fig. 9). Notably, the amount of the CTD ser-5 hypophosphorylation correlated with the amount of transcripts produced by a given PIC (Fig 8A/B).

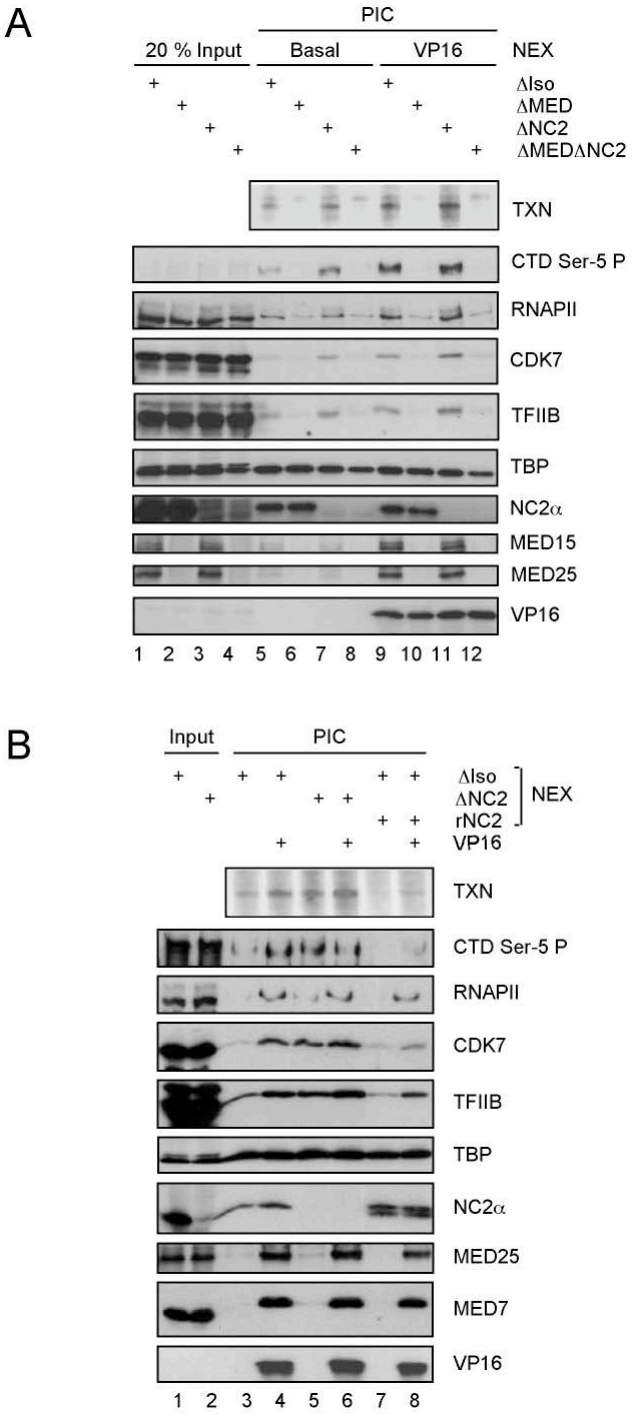

Figure 8: TFIIB and TFIIH (CDK7) recruitment during PIC formation is influenced by NC2, but NC2 does not impose the Mediator requirement on TFIIB/TFIIH recruitment. Immobilized template assays on MRG5 promoter templates. *A,* PICs were formed under basal conditions or in the presence of GAL4-VP16 using mock-treated (ΔIso), NC2-depleted (ΔNC2), Mediator depleted (ΔMED) or Mediator and NC2 depleted (ΔMEDΔNC2) nuclear extracts. In parallel an *in vitro* transcription experiment was carried out (TXN). After NC2 removal, TFIIB and CDK7 recruitment is still Mediator dependent in the basal (compare lanes 5 to 8) as well as the VP16-activated situation (compare lanes 9 to 12). *B,* PICs were formed in the absence of NC2 (lane*s* 5 and 6) or after doubling the amount of NC2 in the nuclear extract by adding recombinantly expressed NC2 (rNC2) complexes (lanes 7 and 8).

C.1.4.4. Basal Mediator activity is physically associated with MED25

Next I wanted to address whether basal activity is physically associated with MED25 or whether an additional Mediator population is required for transcription. The experiment I performed was to immunodeplete all Mediator forms from a nuclear extract and to add-back immunoprecipitated MED25 Mediator, the mediator population that binds to the transcription activator VP16. If there would be a requirement for another Mediator population *in vitro* transcription should be abolished in such a setting. In order to minimize the effect unspecifically MED25-associated proteins I included a stringent wash at 800 mM KCl before adding back the MED25-immunoprecipitation to the Mediator-depleted nuclear extract (Fig. 5). Proteins associated with a MED25 immunoprecipitation were sufficient to restore TFIIB and TFIIH recruitment to a PIC, establishment of CTD ser-5 hypophosphorylation and transcription in a Mediator-depleted nuclear extract (Fig. 5). Thus the basal Mediator function was physically associated with MED25 and no additional Mediator population was required for transcription initiation.

C.2. Function of CTD ser-5 phosphorylation in transcription initiation

CTD ser-5 phosphorylation is known to correlate with transcription initiation. However the exact timepoint at which this mark is established during PIC formation or transcription initiation is unknown. My data suggest that phosphorylation of the high-mobility form of RNAPII at ser-5 does correlate with active PIC formation in various experimental settings. To characterize the underlying process further, I analyzed the conditions under which the mark is established. The kinase that mediates CTD phosphorylation at ser-5 of the high- but notably not of the low-mobility form of RNAPII *in vitro* is CDK7 (Fig. 15B). In the following chapters I provide evidence that *in vitro* at least two distinct forms of CTD ser-5 phosphorylation can be distinguished. In addition I provide evidence, that CDK7-dependent phosphorylation of the high-mobility form of RNAPII at ser-5 (CTD ser-5 hypophosphorylation) was established already before complex opening by TFIIH (Fig. 9B and 10A) as it is insensitive to the helicase inhibitor ATPγS. I found that CTD ser-5 hypophosphorylation correlates with the transcription potential of an *in vitro* formed PIC (Figures 5, 6B, 8, 11B, 12B, 13, 17, and 20).

C.2.1. RNAPII CTD ser-5 hypophosphorylation is established before complex opening by the TFIIH associated helicase

The Eick laboratory has recently generated a set of new monoclonal antibodies against several phosphorylated RNAPII CTD residues (Chapman et al., 2007). In this work I used a monoclonal antibody against phosphorylated CTD ser-5 to determine the timepoint at which CTD ser-5 phosphorylation is established. Given that it has been reported that small amounts of ATP or ATPγS present during PIC formation do facilitate the subsequent *in vitro* transcription reaction (Safer et al., 1985; Serizawa et al., 1997) I made use of this reagents to determine the exact timepoint of CTD ser-5 phosphorylation. To address this question I made use of the immobilized template assay using major-late promoter templates and Jurkat nuclear extracts. PICs were prepared in presence of 500 μM of the kinase inhibitor H8, 1 μM of the helicase inhibitor ATPγS or 1 μM of ATP. PICs were washed and either analyzed directly by immunoblot (Fig. 9B lanes 2 to 4) or subjected to a second incubation step in transcription buffer supplemented with 100 μM NTPs (Fig. 9B lanes 5 to 7); 100 μM ATPγS (Fig. 9B lanes 8 to 10); or 100 μM ATP (Fig. 9A lanes 11 to 13). Afterwards the proteins that had dissociated during the incubation were collected and analyzed separately by immunoblot (Fig. 9C). The promoter templates were washed and also analyzed by immunoblot (Fig. 9B). Most notably CTD ser-5 phosphorylation appeared to be established on the RNAPII high-mobility form (CTD ser-5 hypophosphorylation) in the presence of 1 μM ATPγS although somewhat less efficient than in the presence of 1 μM ATP (Fig. 9B). ATPγS can not be hydrolyzed by helicases, but kinases can use it as a substrate and form a thiophosphorylated product on the target protein (Stelzer et al., 1994; Tazi et al., 1993). Since ATPγS efficiently inhibits the complex opening by TFIIH (Holstege et al., 1997; Stelzer et al., 1994) this is an indication that CTD ser-5 hypophosphorylation occurs before complex opening. Upon incubation of PICs with 100 μM NTPs most CTD ser-5 and ser-7 phosphorylation is seen on IIo-like low-mobility forms of RNAPII (CTD ser-5 hyperphosphorylation). CTD ser-7 phosphorylation was also seen on the high-mobility form of RNAPII but appeared to be established predominantly after complex opening,

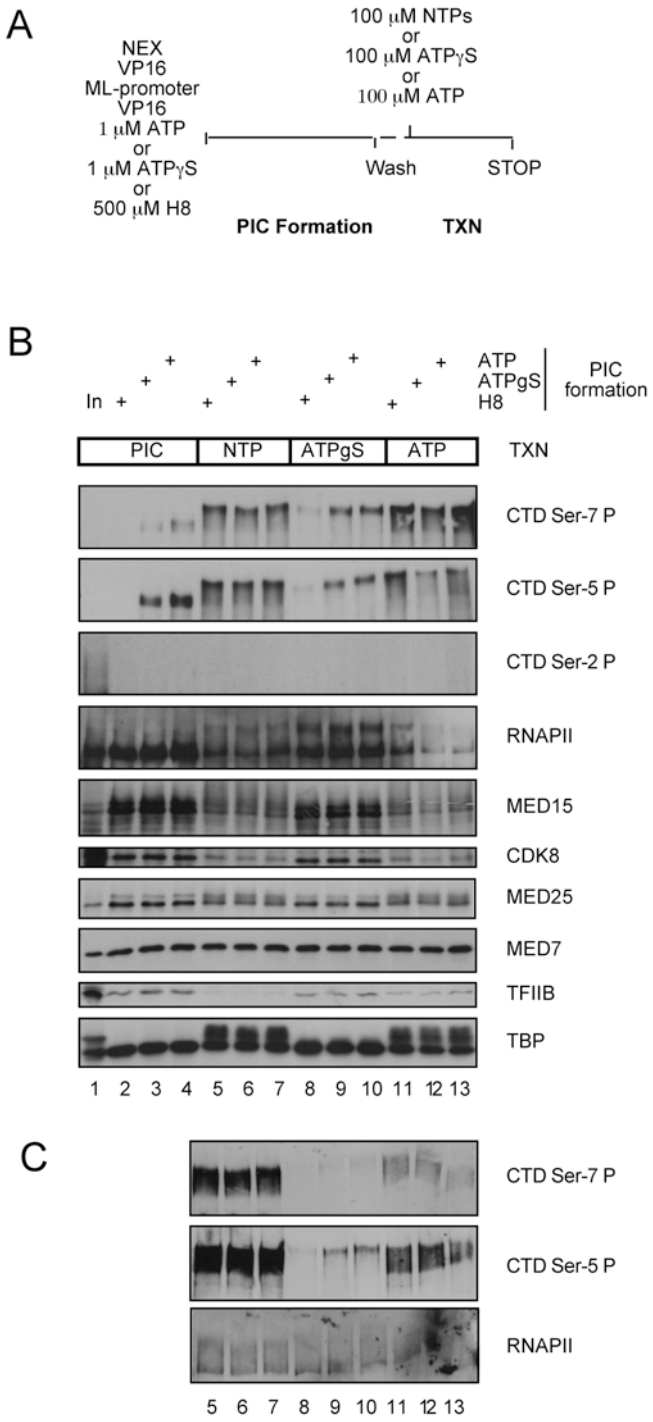

Figure 9: PIC dissociation and CTD ser-5/7 hyperphosphorylation were dependent on an ATPase and occur before formation of the first phosphodiester bond. *A,* Reaction scheme. *B,* PICs were formed in the presence of 500 μM H8 (lanes 2, 5, 8 and 11), 1 μM ATPγS (lanes 3, 6, 9, 12) or 1 μM ATP (lanes 4, 7, 10, and 13) on a major-late promoter template. PICs were washed and either analyzed directly by immunoblot (lanes 2-5) or incubated in transcription buffer together with 100 μM NTPs (lanes 5-7), 100 μM ATPγS (lanes 8-10) or 100 μM ATP (lanes 11-13). The DNA-templates were washed and analyzed by immunoblot. *C,* The supernatant of the second incubation step (TXN) was also analyzed by immunoblot to monitor the RNAPII fraction that dissociated from the template during the TXN reaction. In: 20% input.

as it can be inhibited efficiently by the helicase inhibitor ATPγS (Fig. 9B/C and 10A). CTD ser-5 and -7 hyperphosphorylation was significantly decreased if PICs were incubated in the presence 100 μM ATPγS, in particular if during PIC formation establishment of CTD ser-5/7 hypophosphorylation was suppressed by the kinase inhibitor H8. Incubation of PICs in the presence of 100 μM ATP during the second incubation step gave rise to more CTD ser-5/7 hyperphosphorylation, but the overall amount remained below that seen when incubated in the presence of 100 μM NTPs. Nuclear extracts contain a small amount of NTPs. Therefore the observed CTD phosphorylation during PIC formation could be established by transcription engaged polymerases during the PIC formation reaction. To rule out this possibility I prepared PICs in the presence of the transcription inhibitor α-amanitin. Again PICs were formed without any supplement or in the presence of 500 μM H8, 1 μM ATPγS or 1 μM ATP during PIC formation (Fig. 10A, lanes 1-4). The incubations with 1 μM ATP and 1 μM ATPγS were also supplemented with 2 μg/ml α-amanitin (Fig. 10A, lanes 5 and 6). The presence of α-amanitin did not alter the initial results, leading to the conclusion that hypophosphorylation of the RNAPII Rpb1 high-mobility form at ser-5 and -7 did indeed occur independent of processive transcription. Next I determined whether CTD ser-5/7 hyperphosphorylation is established in a transcription-dependent fashion. *In vitro* transcription reactions were carried out without and with 2 μg/ml α-amanitin in the reaction. After the reaction was completed, the whole reaction was loaded onto an SDS-PAGE gel and analyzed by immunoblot. Treatment with α-amanitin, which was tested in parallel for its ability to inhibit *in vitro* transcription, did not alter the pattern of generated CTD ser-5/7 phosphorylation in the reaction (Fig. 10B, compare lanes 3 and 4), indicating that these marks were established independent of processive *in vitro* transcription. Under the given experimental conditions I could not detect a significant increase in CTD ser-2 phosphorylation upon transcription initiation (Fig. 10B). In summary this experiments extends the timepoint window during which CTD ser-5 phosphorylation is established. Phosphorylation of the high- and low mobility-form of RNAPII at ser-5 and -7 was established in this *in vitro* assay independent of processive transcription before transcription initiation.

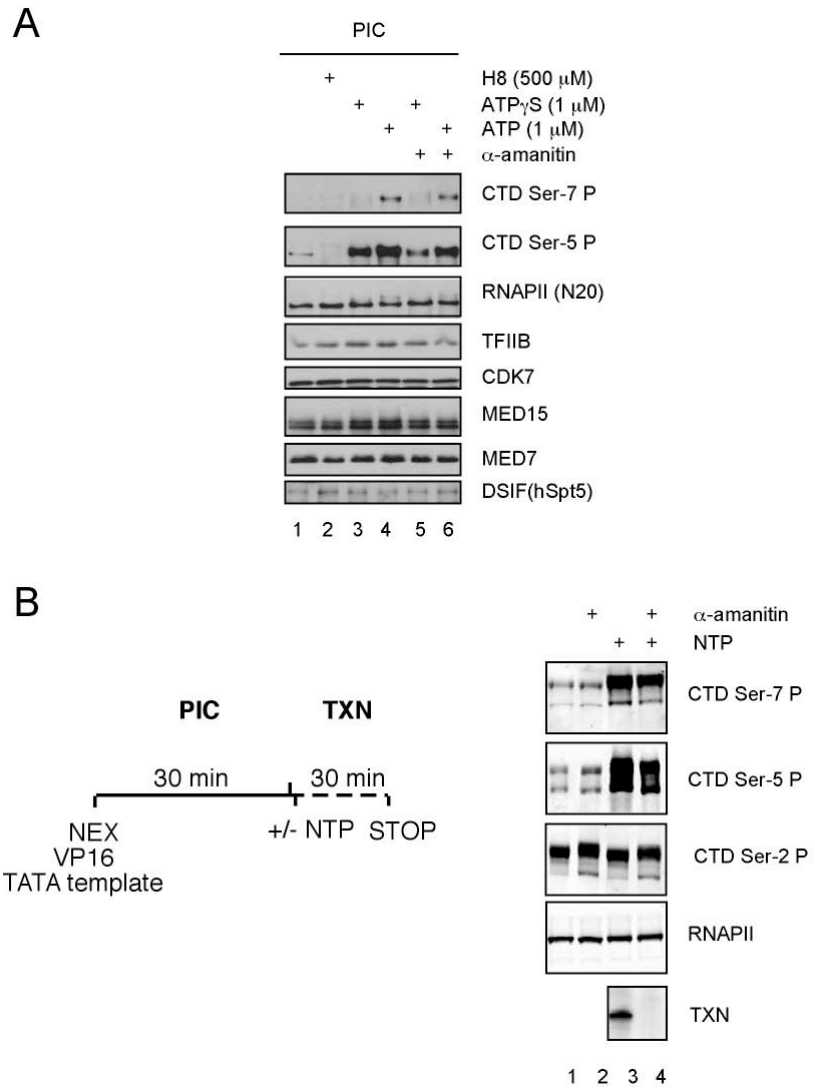

Figure 10: CTD ser-5 and -7 hypo and hyperphosphorylation are established before RNAPII commits to processive transcription. *A,* PICs were formed for 45 min in the presence of the indicated supplements and the activator GAL4-VP16 (lanes 1-6), washed extensively and analyzed by immunoblot. α-amanitin was used at a concentration of 2 μg/ml. *B,* Standard *in vitro* transcription reactions were carried out on major-late promoter templates as indicated in the presence of 1 μM ATP. At the end the reactions were stopped by addition of Laemmli buffer and loaded onto a SDS-PAGE gel for protein analysis. The inhibitory effect of *a*-amanitin under these experimental conditions was verified by a RNA analysis of the resulting transcripts (TXN). Reactions in lane 1 and 2 were loaded on the gel without NTP incubation, reactions in lanes 3 and 4 were incubated with NTPs as indicated in the panel on the left.

C.2.2. Establishment of CTD ser-5 hypophosphorylation follows a slow kinetic and correlates with the transcription potential of a PIC

In figures 6B and 7 it is shown that CTD ser-5 hypophosphorylation was established in a Mediator-dependent fashion *in vitro*. Next I examined the kinetics with which this modification is established during PIC formation. I carried out an immobilized template assay on major-late promoter templates and allowed PIC formation in the presence of 1 μM ATP for 5 to 45 minutes. After PIC formation PICs were washed and either analyzed by immunoblot or forwarded to an *in vitro* transcription reaction. Recruitment of TFIIB and NC2 and establishment of CTD ser-5 hypophosphorylation followed a slow kinetic as compared to that of recruitment of TFIID (TBP, TAF5) and Mediator (Fig. 11B). Furthermore the amount of CTD ser-5 hypophosphorylation correlated to the amount of transcript produced by the respective PIC (Fig. 11). Thus CTD ser-5 hypophosphorylation may serve as a mark for an active, transcription competent PIC.

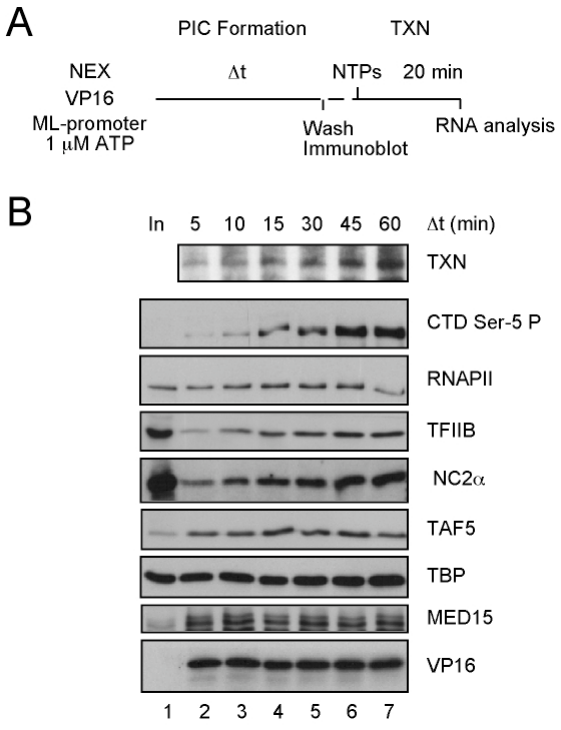

Figure 11: TFIIB recruitment and formation of an active PIC is slow as compared to the recruitment of activator, TFIID and Mediator. CTD ser-5 hypophosphorylation correlates to transcription potential of the respective PIC. *A,* Schematic representation of the reaction. *B*, PICs were formed for the indicated periods of time on a major-late promoter templates in the presence of 1 μM ATP, washed and analyzed in part by immunoblot and in part probed in an *in vitro* transcription reaction. In: 20% input.

C.2.2.1. CTD ser-5 hypo phosphorylation is dependent on TFIIB

In figure 6 I had shown that CTD ser-5 hypophosphorylation was dependent on Mediator even if TFIIB was recruited to the promoter template. Next I wanted to determine whether CTD ser-5 hypophosphorylation is dependent on TFIIB. I immunodepleted a Jurkat nuclear extract for TFIIB and tested in an immobilized template assay its capacity to establish CTD ser-5 hypophosphorylation. After TFIIB depletion a significant reduction in CTD ser-5 hypophosphorylation was observed during PIC formation (Fig 12A, compare lanes 3 and 4 to 5 and 6). The absence of TFIIB during PIC formation led to decreased TFIIH recruitment (CDK7 and p62) in the VP16-activated situation. Overall NC2 recruitment was not affected by TFIIB depletion. Mediator was not co-depleted with TFIIB (Fig. 12A, compare lanes 1 and 2) and Mediator recruitment to the promoter template was not affected in the absence of TFIIB (Fig. 12A, compare lanes 3 and 4 to 5 and 6).

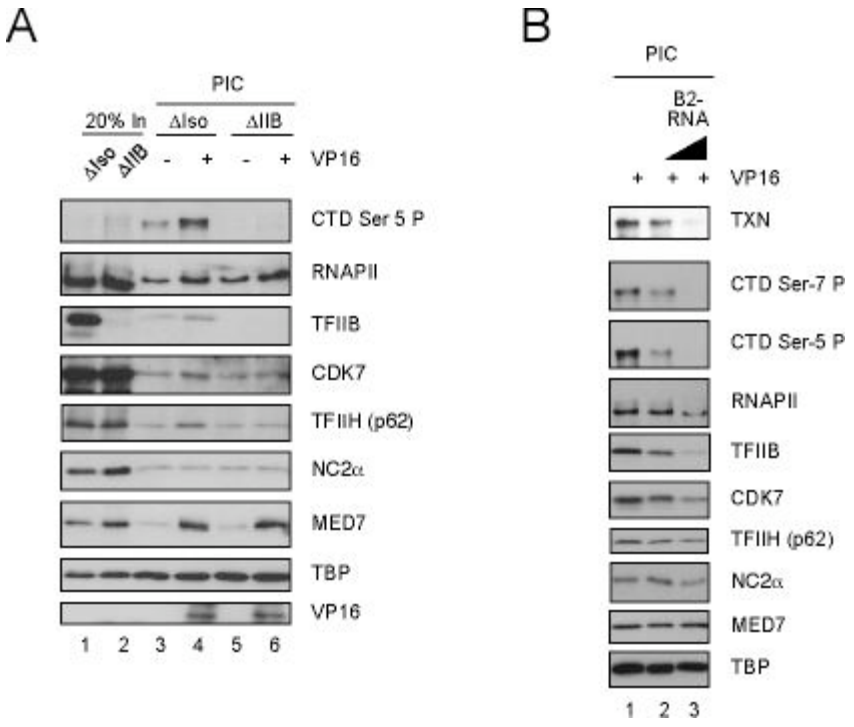

Figure 12: CTD ser-5 hypophosphorylation is dependent on TFIIB. *A,* Immobilized template assay on MRG5 promoter templates were carried out under basal conditions or in the presence of GAL4-VP16 with either mock-treated or TFIIB-depleted nuclear extracts (lanes 3-6). TFIIB-depletion did not co-deplete Mediator (compare lanes 1 and 2). In: 20% input. *B,* PICs were prepared on major-late promoter templates in the presence of 0.1 and 1 μM B2-RNA (lanes 2 and 3). PICs were washed and analyzed either by immunoblot or probed for their transcription potential (TXN).

Of note RNAPII associated mostly independent of TFIIB with the promoter template, both in the absence and presence of VP16. In addition I used the small non-coding B2-RNA, which inhibits *in vitro* transcription in a transcription system composed of recombinantly expressed and purified factors prior to complex opening in the context of a PIC (Espinoza et al., 2004). In the nuclear extract *in vitro* transcription system the B2-RNA present in a PIC formation reaction supplemented with 1 μM ATP interfered with TFIIB recruitment, CDK7 recruitment and establishment of the CTD ser-5 and -7 hypophosphorylation states (Fig. 12B, compare lanes 1 to 2 and 3). Presence of the B2-RNA during PIC formation also diminished the *in vitro* transcription potential of the formed PIC. Apparently, the B2-RNA limits TFIIB and TFIIH recruitment to the PIC but has no influence on TBP or Mediator recruitment. The B2-RNA used in this experiments was a kind gift of Florian Brueckner of the Cramer laboratory. These experiments provide further evidence that the CTD ser-5 hypophosphorylation state correlates to an active, transcription competent state of the PIC.

C.2.2.2. CTD ser-5 hypophosphorylation is dependent on TBP and a TATA box

Next I asked whether establishment of CTD ser-5 hypophosphorylation is dependent on a TATA box in an immobilized promoter template assay. PICs were formed on MRG5 promoter templates comprising five upstream GAL4 binding sites and a portion of the luciferase gene downstream of the core promoter. PICs were formed either on templates containing a wild type TATA box or on templates containing a mutated TATA box, which no longer supports basal as well as activated *in vitro* transcription (Fig. 13). PICs were formed under basal conditions or in the presence of the activator GAL4-VP16. CTD ser-5 hypophosphorylation was significantly reduced if a mutated TATA box was present in the core promoter. Again CTD ser-5 hypophosphorylation was seen to correlate with the *in vitro* transcription potential of the respective PICs. Also recruitment of RNAPII, TFIIB and TFIIH (p62, CDK7) correlated to the amount of transcript produced by the respective PIC. Although the functional relevance of the TATA-box mutation became evident in terms of *in vitro* transcription no influence on TBP recruitment was observed. However, the promoter template contains several DNA sequence elements to which TBP can bind unspecifically. Immunodepletion of TFIID (TBP together with TBP-associated

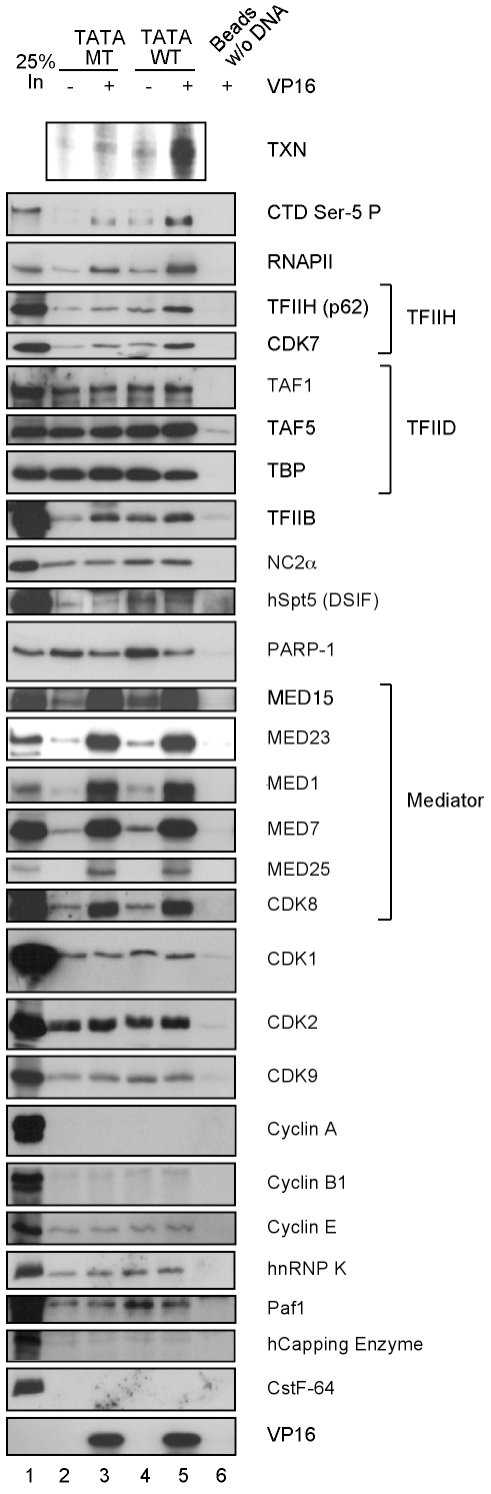

Figure 13: CTD ser-5 hypophosphorylation is dependent on a TATA box. PICs were formed on immobilized MRG5-promoter templates with a wildtype (TATA WT, lanes 4 and 5) or mutated TATA box (TATA MT, lanes 2 and 3) and after washing analyzed by immunoblot or for their transcription potential (TXN). Reactions were formed under basal conditions (lanes 2 and 4) or in the presence of the activator GAL4-VP16 (lanes 3 and 5). A reaction with column material without an attached promoter template served as specificity control for the used antibodies (beads w/o DNA, lane 6).

factors (TAFs)) did not lead to an establishment of CTD ser-5 hypophosphorylation in PICs formed under basal conditions (Fig. 20, compare lanes 3 and 4). A PIC formation assay performed with a TFIID-depleted nuclear extract into which recombinantly expressed TBP was added back restored establishment of CTD ser-5 hypophosphorylation during PIC formation (Fig. 20, compare lanes 1 and 3), indicating that TBP but not TAFs are essential for establishment of CTD ser-5 hypophosphorylation.

C.2.2.3. CTD ser-5 hypophosphorylation is established close to the transcription start site

In order to figure out in which region of the promoter DNA template CTD ser-5 hypophosphorylation was established I performed a chromatin-immunoprecipitation restriction digest immunoprecipitation (CRIP) assay (Schluesche et al., 2007), which was designed as depicted in Fig. 14A: A PIC was formed on radiolabelled MRG5-promoter DNA templates containing five activator binding sites (GAL sites) upstream of a modified ML core promoter. PICs were formed with nuclear extracts treated with an isotype antibody or immunodepleted for MED15 (removes all Mediator populations), MED25 (removes the VP16 interacting Mediator) or for NC2. Formed PICs were crosslinked with formaldehyde and the templates were then subjected to a restriction digest with restriction enzymes as indicated in figure 14B. Afterwards an immunoprecipitation was carried out with a CTD ser-5 phosphate specific monoclonal antibody. After protein removal with proteinase K DNA fragments that co-immunoprecipitated with the crosslinked proteins were analyzed on a native PAGE gel by autoradiography. As shown in figure 14C the CTD ser-5 phosphorylation is found primarily close to the transcription start site whereas flanking DNA fragments have a lower ratio. Restriction digests with different enzymes confirmed this observation (Fig. 14D). Of note removal of NC2 increased the amount of CTD ser-5 phosphorylation. This confirmed the result of a similar immobilized template assay experiment (Fig. 8). This finding further supports the notion that CTD ser-5 hypophosphorylation is established only on RNAPII that is associated with an transcription competent PIC.

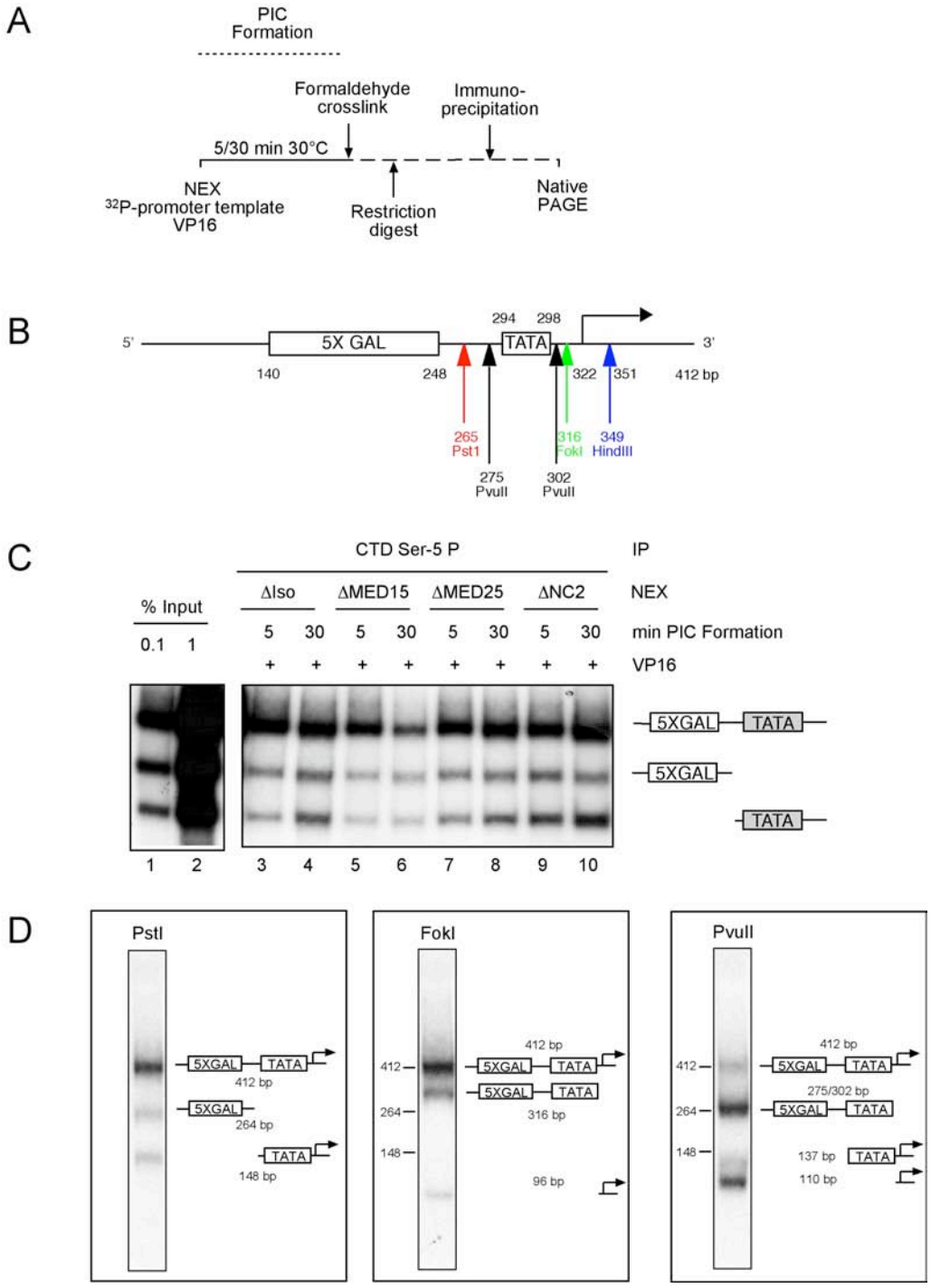

Figure 14: CTD ser-5 phosphorylation is established close to the transcription start site during PIC formation. *A,* Scheme of a CRIP (crosslink-restriction-immunoprecipitation) assay. *B*, Diagram indicating the position of the restriction sites on a MRG5-promoter template. *C*, PIC formation reactions were carried out as depicted in A in the presence of 1 µM ATP. Jurkat nuclear extracts were either mock-treated (ΔIso) or immunodepleted for all Mediator populations (ΔMED15), for the MED25 Mediator (ΔMED25) or for NC2α (ΔNC2). *D,* PICs were formed with Jurkat nuclear extract in the presence of GAL4-VP16 and 1 µM ATP and analyzed in CRIP assays with the indicated restriction enzymes. A Mediator-dependent CTD-Ser5 phosphorylation was established during PIC formation predominantly on the DNA fragments containing the transcription start site.

C.2.2.4. CDK7 established CTD ser-5 hypophosphorylation but did not influence establishment of CTD ser-5 hyperphosphorylation *in vitro*

CTD ser-5 phosphorylation has been attributed to the TFIIH associated kinase CDK7. Here I wished to analyze the contribution of TFIIH/CDK7 to CTD ser-5 hypo and hyperphosphorylation respectively. I prepared PICs with Jurkat nuclear extracts immunodepleted for CDK7, CDK8 or CDK9 in the presence of 1 μM ATP and found that depletion of CDK7, but not depletion of CDK8 or CDK9, resulted in a complete loss of CTD ser-5 hypophosphorylation (Fig. 15B, compare lane 6 to lanes 5, 7 and 8). The reduction in transcription observed could relate to either diminished CTD ser-5 hypophosphorylation levels or to a co-depletion of TFIIH with CDK7. Remarkably, CTD ser-5 hyperphosphorylation was, if at all, marginally affected by the absence of CDK7 (Figure 15B, compare lanes 9 and 10). This holds true for RNAPII bound to the template, as well as for the population that dissociated from the promoter during the 20 minutes of transcription incubation (Fig. 15C, compare lanes 2 and 3). Note that levels of CTD ser-2 phosphorylation were below detection level. CTD ser-2 phosphorylation could be detected in the RNAPII population dissociating from the promoter template during transcription incubation. However, this might represents a background, as the input sample gives rise to an equal amount of CTD ser-2 phosphorylation signal (Figure 15C, compare Input/Experiment ratio for CTD ser-2, 5 and 7P). If CDK8 was immunodepleted a reduction in CTD ser-5 hyperphosphorylation was observed (Fig. 15B, compare lanes 9 and 11). As the CDK8 immunodepletion had removed most CDK8 from the nuclear extract this may mean that another kinase than CDK7 and CDK8 is involved in establishment of CTD ser-5 hyperphosphorylation. In summary CTD ser-5 hypophosphorylation was established dependent on the TFIIH-associated kinase CDK7. CTD ser-5 hyperphosphorylation was established at least in part dependent on CDK8.

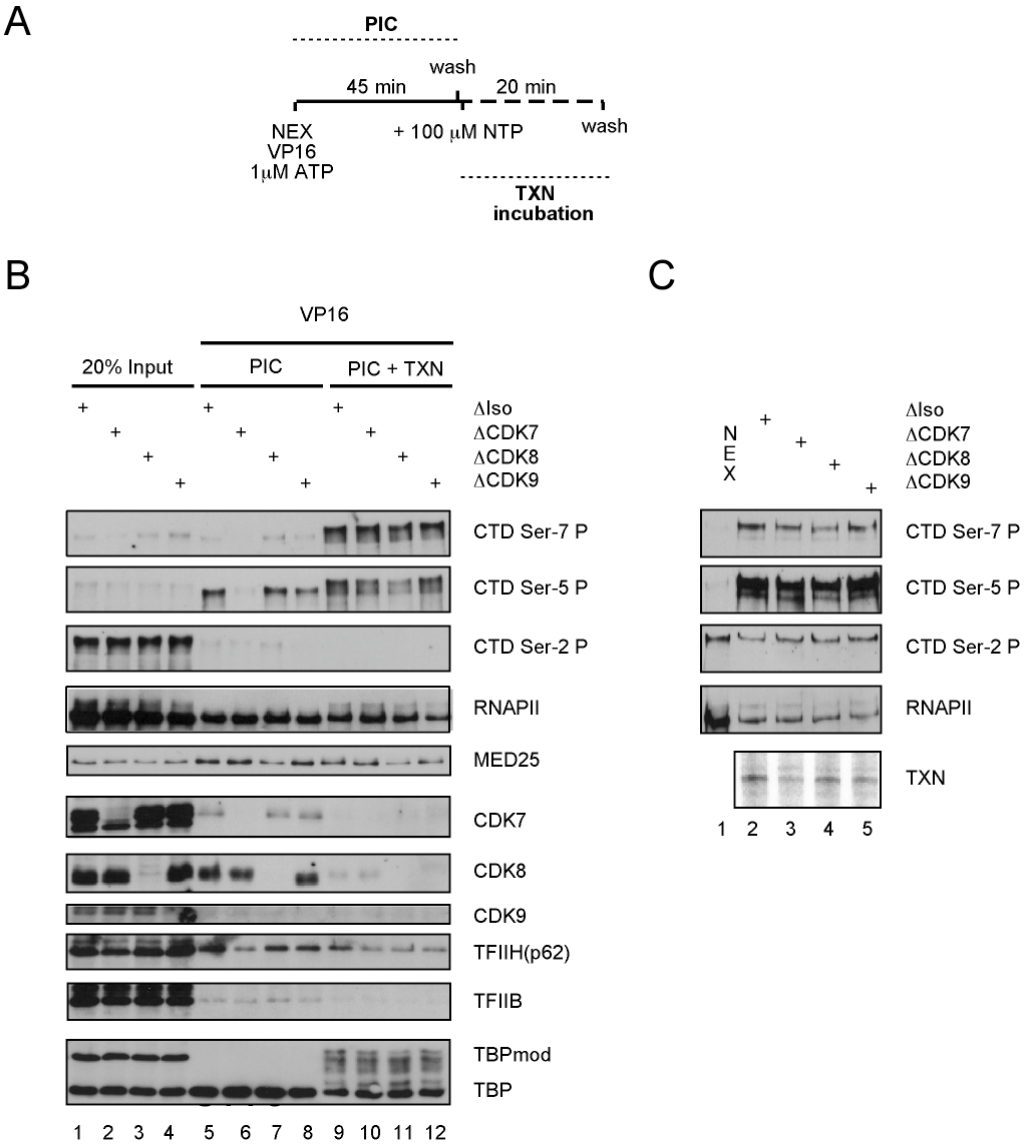

Figure 15: CTD ser-5 hypophosphorylation, but not CTD ser-5 and -7 hyperphosphorylation, is dependent on CDK7. *A,* Reaction scheme. *B,* Immobilized template assays were carried out on MRG5-promoter templates with nuclear extracts that were mock-treated (ΔIso) or immunodepleted for CDK7 (ΔCDK7), CDK8 (ΔCDK8) or CDK9 (ΔCDK9) respectively. PICs were after extensive washing either analyzed directly by immunoblot (PIC) or were subjected to a second incubation under *in vitro* transcription conditions and then analyzed (PIC+TXN). *In vitro* transcription reactions (TXN) were carried out in parallel under the same conditions as in the immobilized template assays. *C,* RNAPII that either ran off or dissociated from the promoter template during transcription was analyzed by immunoblot.

C.3. Biochemical characterization of RNAP II CTD ser-7 phosphorylation.

The Eick laboratory discovered that ser-7 of a consensus repeat of the CTD of RNAPII is a phosphorylation site *in vivo* (Chapman et al., 2007). Furthermore it could be established that CTD ser-7 phosphorylation is observed at messenger RNA (mRNA) coding genes and required for the recruitment of the integrator complex to genes coding for the U2 snRNA (Chapman et al., 2007; Egloff et al., 2007). Starting from there I asked whether CTD ser-7 phosphorylation is generated in an inducible model gene system by the tet-VP16 activator *in vivo* and whether CTD ser-7 phosphorylation can be recapitulated in an immobilized promoter template assay *in vitro*.

C.3.1. CTD ser-7 phosphorylation is detected *in vivo* and *in vitro* upon transcription initiation

To characterize CTD ser-7 phosphorylation *in vivo*, I used the previously described EBV-based CMV reporter gene system (Uhlmann et al., 2007). I monitored CTD ser-5 and -7 phosphorylation before (transcription off) and after induction of the reportergene with doxycycline by chromatin immunoprecipitation (ChIP). In order to be able to normalize the amount of CTD modification to the amount of RNAPII, I also monitored the amount of RNAPII associated with the promoter, the start of the coding region and the control region oriP. The epitope recognized by the RNAPII N20 antibody is located outside the CTD of RNAPII. Induction of transcription in the reporter gene system correlated with increased recruitment of RNAPII and an increase in CTD ser-5 and 7 phosphorylation of the RNAPII CTD. Note that RNAPII was detected in the non-transcribed oriP region above background level (Fig 16, isotype IP) but this RNAPII population was not phosphorylated at CTD ser-5 or -7 upon addition of doxycycline. This *in vivo* experiment confirmed the results of the *in vitro* experiments presented in the previous chapter. Establishment of CTD ser-7 phosphorylation correlates to transcription initiation *in vitro* and *in vivo*.

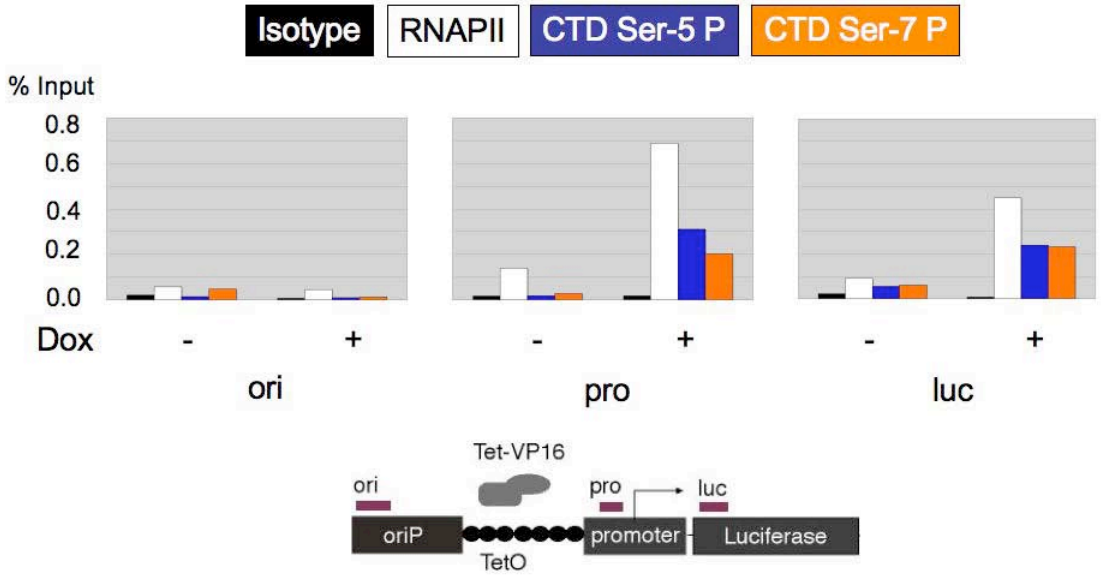

Figure 16: CTD ser-5 and -7 phosphorylation does occur upon transcription initiation *in vivo*. Chromatin immunoprecipitation experiments were carried out with an isotype antibody (black bars) or antibodies specific for RNAPII (white bars), phosphorylated CTD ser-5 (blue bars) or CTD ser-7 (orange bars). Transcription was induced in the tet-VP16 system in CMV-vector transfected HeLa cells by addition of doxycycline to the cells as indicated. The oriP region served as a negative control for the ChIP experiment. The promoter (pro)-PCR amplicon included the TATA box, the luc amplicon was located immediately downstream of the transcription start site. The distance between TATA box and transcription start site in this system is 166 nucleotides.

C.3.2. Establishment of CTD ser-7 phosphorylation can be recapitulated *in vitro* and the CTD ser-7 kinase is part of an isolated PIC

As already discussed in context of CTD ser-5 phosphorylation in the previous section, CTD ser-7 phosphorylation was observed during PIC formation and transcription initiation. I observed CTD ser-7 hypophosphorylation on the high-mobility form of RNAPII Rpb1, which was established during PIC formation (Fig. 9B and 10A). The mechanisms responsible for establishment of CTD ser-5 and ser-7 hypophosphorylation appear to be distinct, since ser-5 hypophosphorylation was observed already before complex opening, whereas ser-7 hypophosphorylation appeared to be established after complex opening (Fig. 10A). Establishment of CTD ser-7 hypophosphorylation could be suppressed by immunodepletion of CDK7 (Fig. 15 and data not shown). CTD ser-5 and ser-7 hyperphosphorylation were seen at the same stage of transcription initiation after incubation of a preformed PIC in the presence of 100 μM ATP or 100 μM NTP. Generation of CTD ser-5 and ser-7 hyperphosphorylation was insensitive to the

transcription inhibitor α-amanitin, indicating that this modification occurred before formation of the first phosphodiester bond (Fig 10B). Furthermore CTD ser-5/7 hyperphosphorylation could be initiated in the presence of 100 μM ATP but not in the presence of 100 μM ATPγS, indicating that in addition to a kinase a ATPγS sensitive enzyme such as a helicase might be involved in this process (Eckstein, 1985).

C.3.3. CTD ser-7 phosphorylation is dependent on Mediator

In section C.2. I showed that CTD ser-5 hypophosphorylation is dependent on Mediator and CDK7. Here I asked whether Mediator is required for the establishment of CTD ser-5 and -7 hyperphosphorylation. Given the above results that CTD ser-5 and -7 hyperphosphorylation did not correlate to processive transcription (Fig. 9B and 10B) I also included the negative cofactor 2 (NC2) into the analysis. I made use of the immobilized template assays in combination with Jurkat nuclear extracts that were either mock-treated with an isotype antibody (ΔIso) or immunodepleted for Mediator (ΔMed), NC2 (ΔNC2) or the combination of both factors (ΔMED/ΔNC2). PICs were formed with the depleted extracts, washed extensively and then either analyzed directly by immunoblot (Fig. 17B, lanes 5 to 8) or subjected to a second incubation step under *in vitro* transcription conditions. CTD modifications established during the transcription incubation step were analyzed by immunoblot in two fractions: The first contained RNAPII that had not dissociated from the promoter template during the transcription incubation (Fig. 17B, lanes 9-12), the second contained RNAPII that had dissociated from the promoter template during the transcription reaction (Fig. 17C, lanes 2-5). Importantly this type of analysis can not discriminate between RNAPII that was engaged in productive transcription and RNAPII that dissociated in a non-productive fashion from the promoter template. Under the used assay conditions RNAPII was associated with the promoter template even in the absence of Mediator. However the amount of RNAPII recruited in the absence of Mediator was lower than in the presence of Mediator. In the course of the *in vitro* transcription reaction (second incubation step) PIC associated RNAPII was partially hyperphosphorylated at CTD ser-5 and -7. RNAPII did partially dissociate from the promoter template during the transcription reaction and also the dissociated RNAPII population was found to be hyperphosphorylated at

CTD ser-5 and -7 (Fig. 17B/C). This experiment further demonstrated that NC2 had no influence on establishment of CTD ser-5/7 hyperphosphorylation. It indicates however that Mediator might be required for CTD ser-5/7 hyperphosphorylation.

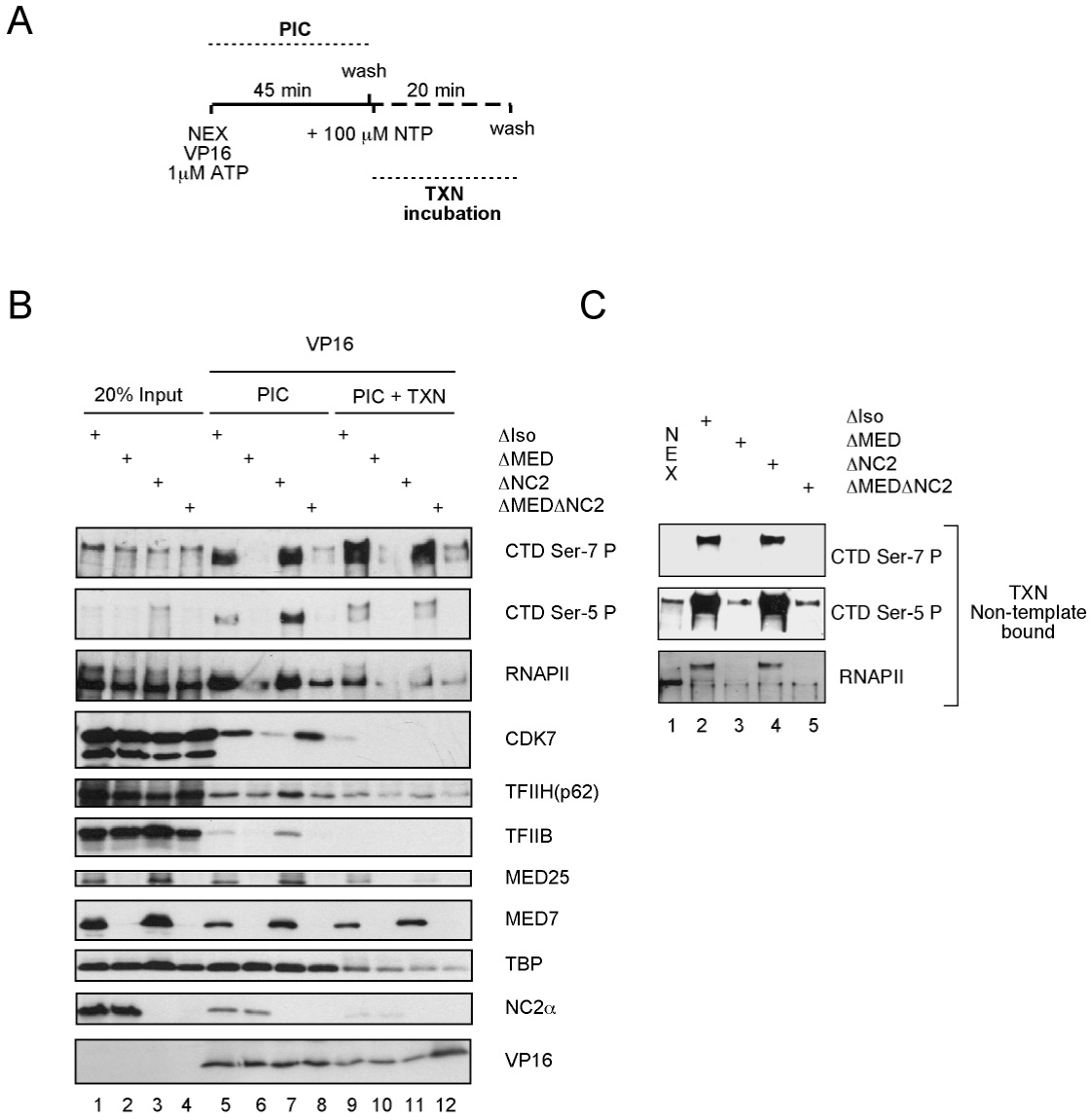

Figure 17: CTD ser-5 and -7 hyperphosphorylation is Mediator dependent. *A,* Reaction scheme *B,* Immobilized template assays were carried out on MRG5 promoter templates with mock-treated (ΔIso), Mediator depleted (ΔMED), NC2 depleted (ΔNC2) or Mediator/NC2 depleted (ΔMEDΔNC2) Jurkat nuclear extract. PICs were extensively washed and either analyzed directly or subjected to a second incubation under *in vitro* transcription conditions in the presence of 100 μM NTPs. CTD phosphorylation was analyzed on the template bound RNAPII population *C,* as well as on the RNAPII population that dissociated from the template during the second incubation step.

C.3.4. Mediator is required for CTD ser-5 and -7 hyperphosphorylation

In order to further investigate whether Mediator is required for CTD ser-5/7 hyperphosphorylation I performed in addition to the assay presented in the previous chapter an alternative assay. I assembled standard *in vitro* transcription reactions using either isotype-depleted (ΔIso) or Mediator-depleted Jurkat nuclear extract (ΔMED) and performed standard *in vitro* transcription reactions. But instead of analyzing the RNA at the end of the reaction I loaded the whole reaction on a SDS-PAGE and analyzed it by immunoblot. As a reference I also loaded transcription reactions on the SDS-PAGE that had not been incubated with NTPs (To situation). Thus I was able to determine the amount of CTD phosphorylation that was established during the transcription reaction by comparing the two situations (To vs. NTP incubated). This allowed me to detect the differences in the CTD phosphorylation status between a mock-depleted nuclear extract (ΔIso) and an Mediator-depleted (ΔMED) nuclear extract. In lanes 1 and 2 of figure 18B reaction mixtures prepared with mock- or Mediator-depleted nuclear extract were loaded onto the SDS-PAGE gel without any incubation, in order to monitor the amount of CTD ser-5/7 phosphorylation present at the beginning of the reaction (To). In the presence of a promoter template and incubation with 100 μM NTPs establishment of CTD ser-5/7 hyperphosphorylation was diminished in the absence of Mediator (Fig. 18B, lanes 5 and 6). Although a 10 fold excess (w/w) of poly(dG:dC) competitor DNA over promoter template DNA was present in the reaction CTD ser-5/7 hyperphosphorylation was maximal in the presence of a promoter DNA-template. Incubation with 100 μM ATP alone allowed for CTD ser-5/7 hyperphosphorylation (Fig. 18B, lanes 9 and 10) whereas incubation with 100 μM ATPγS did not led to CTD ser-5/7 hyperphosphorylation (Fig. 18B, lanes 11 and 12). ATPγS is a nucleotide analogue that can be used by kinases, but not ATPases such as helicases (Stelzer et al., 1994; Tazi et al., 1993). Therefore a helicase might be involved in the establishment of CTD ser-5/7 hyperphosphorylation. The experiment depicted in figure 9 points in the same direction. In summary establishment of CTD ser-5 and -7 hyperphosphorylation was dependent on Mediator, DNA and an ATPase in this assay. Next I wanted to investigate whether a particular Mediator species is required for establishment of CTD ser-5 and -7 hyperphosphorylation. To do so, I used mock-treated and Mediator depleted nuclear

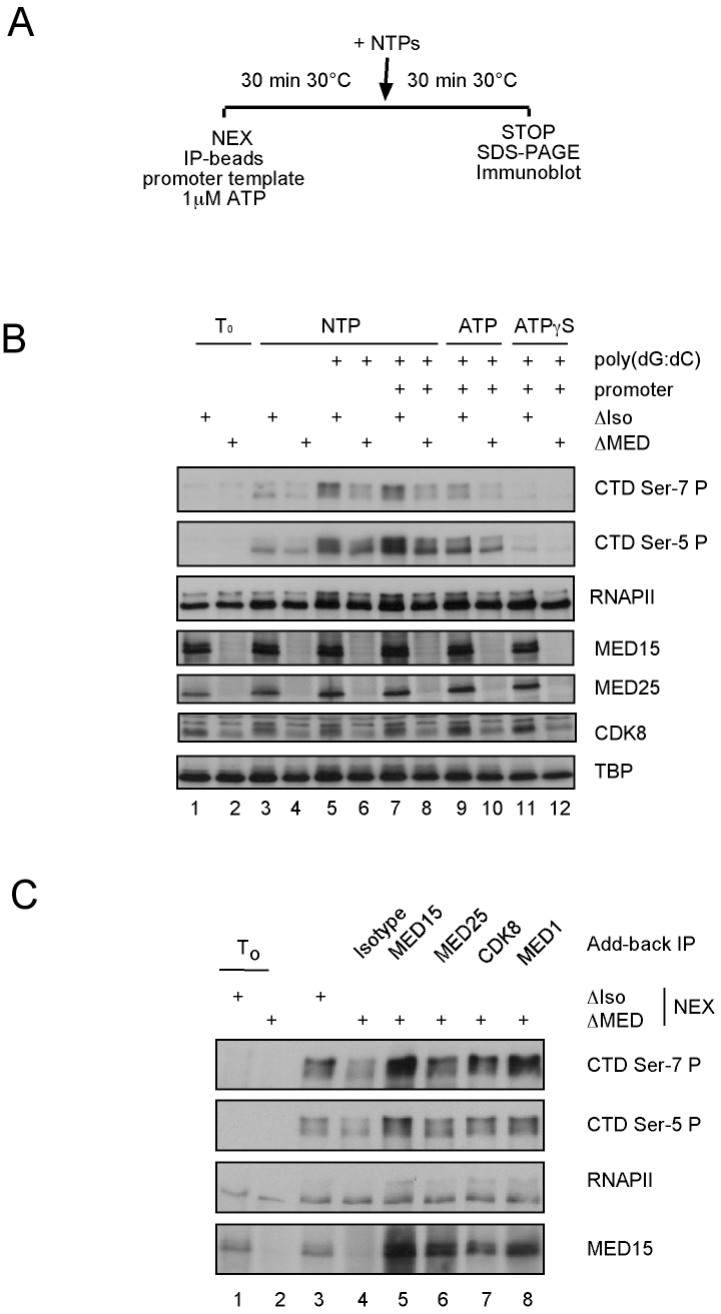

Figure 18: CTD ser-5 and -7 hyperphosphorylation are Mediator and DNA dependent. *A,* Standard *in vitro* transcription reactions were carried out as depicted in the scheme, but instead of analyzing the RNA, the complete reactions were analyzed by SDS-PAGE and immunoblot. *B,* Reactions were carried out either with mock-treated (ΔIso) or Mediator depleted (ΔMED) nuclear extract. Some reactions were carried out in the absence of promoter-DNA, in some reactions poly(dG:dC) DNA was omitted, which was added in a 10 fold excess over the promoter DNA fragment. The second transcription incubations were carried out in the presence of 100 μM NTPs, 100 μM ATP or 100 μM ATPγS as indicated. In lanes 1 and 2 reactions were loaded without incubation at 30°C and NTP addition (*To*). *B,* SDS-PAGE *in vitro* transcription assays were carried out as depicted in A. Mediator-depleted (ΔMED) Jurkat nuclear extract was supplemented with immunoprecipitations carried out with isotype-, MED15-, MED25-, CDK8- and MED1-specific antibodies (lanes 4-8 respectively). The MED15 lanes serves as an indicator for the amount of Mediator complex added back to the respective reactions.

extract in a SDS-PAGE *in vitro* transcription assay. Reactions containing Mediator-depleted nuclear extracts were supplemented with various Mediator species which were immunoprecipitated from nuclear extracts with the respective antibodies (Fig. 18C, lanes 4 to 8). Complementation of the reaction with isotype IP beads (Fig. 18C, lane 4) led to establishment of significant less CTD ser-7 phosphorylation than in the reference reaction (Fig. 18C, lane 3). Complementation of the Mediator-depleted nuclear extract with immunoprecipitated MED15-, MED25-, CDK8 or MED1- Mediator complexes restored the amount of CTD ser-5 and -7 hyperphosphorylation established during the *in vitro* transcription reaction. Thus all tested Mediator subpopulations had the potential to facilitate CTD ser-5 and -7 hyperphosphorylation *in vitro*.

C.3.5. Mediator preparations are associated with a kinase activity towards RNAPII CTD ser-5 but not with a kinase activity towards CTD ser-7

Since I found CTD ser-7 phosphorylation to be dependent on Mediator I tested whether the CTD ser-7 kinase is associated with Mediator. I performed *in vitro* kinase assays using GST-CTD as a substrate. For the kinase assay I used either immunoprecipitated Mediator (immunoprecipitated with the monoclonal MED15 antibody 6C9) or Mediator that associated with GST-VP16 or GST-H2 columns in pulldown experiments (Fig. 19B and 19C respectively). *In vitro* kinase assay reactions with immunoprecipitated Mediator were assembled and either analyzed without any incubation (Fig. 19B, lane 1, To) or incubated according to the scheme indicated in Fig. 19A. Presence of immunoprecipitated Mediator in the reaction led to an increase in CTD ser-5 phosphorylation on RNAPII (which was co-precipitated with the Mediator complex) as well as on the GST-CTD substrate (Fig 19B, compare lanes 1 and 2). No increase in the amount of CTD ser-7 phosphorylation was detected. In a second experiment Mediator was enriched by GST-VP16 or GST-H2 pulldown experiments from Jurkat nuclear extracts. As control served a pulldown with a GST-H2 mutant (GST-H2mt) column (Ikeda et al., 2002). *In vitro* kinase assay reactions were assembled and either analyzed without any incubation (Fig. 19C, lanes 1 to 3, To) or incubated with 100 μM NTPs under *in vitro* transcription conditions as indicated in the scheme in Fig. 19A. The GST-VP16 pulldown contains kinase activities that led to establishment of CTD ser-7

phosphorylation on the co-precipitated RNAPII as well as to slight ser-7 phosphorylation of GST-CTD. CTD ser-5 phosphorylation was observed on RNAPII as well as on GST-CTD for both the GST-VP16 and the GST-H2 pulldown, but importantly, not with the control GST-H2mt pulldown. In summary, a CTD ser-7 kinase activity was precipitated with a GST-VP16 pulldown, but no detectable CTD ser-7 kinase activity was co-precipitated with MED15 or GST-H2. Consistent with this finding is that immunodepletion of the Mediator-associated kinase CDK8 abolished CTD ser-5 hyperphosphorylation in part (Fig. 15B).

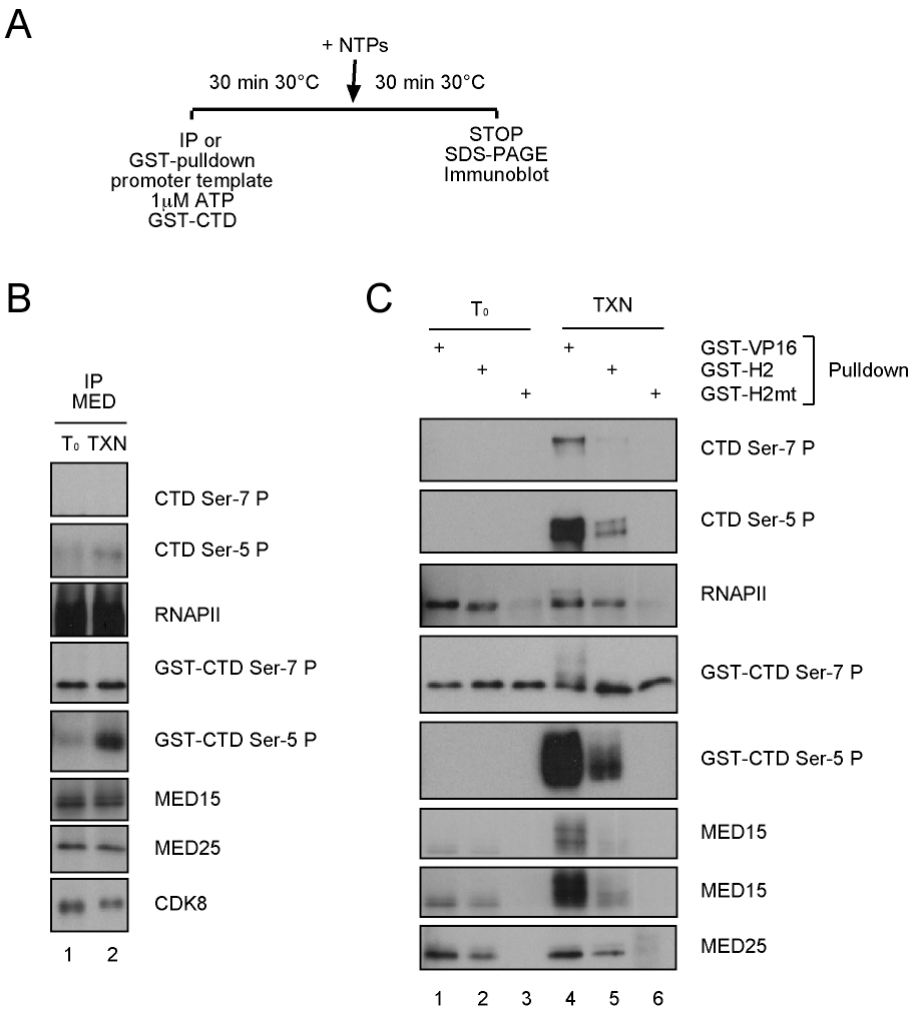

Figure 19: Mediator is associated with CTD ser-5 but not ser-7 kinase activity. *A,* Schematic representation of this *in vitro* kinase assay carried out under *in vitro* transcription conditions. *B*, *In vitro* kinase assay reaction with GST-CTD as a substrate supplemented with immunoprecipitated Mediator. *C, In vitro* kinase assay with GST-CTD as substrate supplemented with a GST-VP16, GST-H2 or a GST-H2mt pulldown. To: Immunoblot of the reaction without incubation, TXN: Immunoblot of the reaction after incubation as indicated in A.

C.3.6. CTD ser-7 kinase activity is independent on TBP and a TATA box

Next I determined whether the general transcription factor TFIID has any influence on the CTD ser-5/7 hyperphosphorylation activity. To do so I used immobilized template assays on major-late promoter templates in combination with either mock-depleted (ΔIso) or TBP-depleted (ΔTBP) Jurkat nuclear extracts. In addition to TBP the TBP-associated factors (TAFs) were removed from TBP-depleted nuclear extracts (Fig 20B, compare lanes 1 and 2). In order to analyze whether CTD ser-7 hyperphosphorylation is dependent

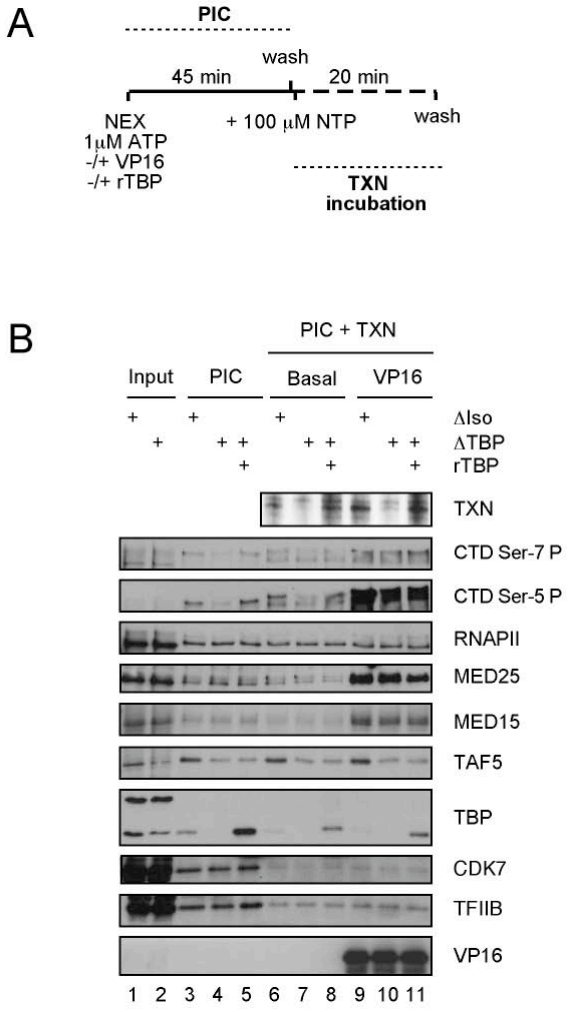

Figure 20: CTD ser-5 hypophosphorylation was dependent on TBP but not on TAFs; CTD ser-5 and -7 hyperphosphorylation was independent of TBP and TAFs. *A*, Reaction scheme. *B,* Immobilized template assays were carried out on major-late promoter templates in combination with mock-treated (ΔIso) or TBP-depleted (ΔTBP) nuclear extract. Recombinant human TBP (rTBP) was added to TBP-depleted nuclear extract as indicated. The amount of added rTBP was chosen such that the amount of endogenous TBP present in the initial extract was resembled. The activator GAL4-VP16 was present in the indicated reactions all other reactions were performed under basal conditions. Input: 20% input was loaded

on TBP or TFIID (TBP plus TAFs) I reconstituted some of the TBP/TAF depleted nuclear extract with recombinant human TBP (rTBP). The amount of added TBP equaled the amounts of TBP that were present in the nuclear extract before TBP/TAF depletion. Although the TBP/TAF-depletion was not complete few TBP was recruited to PICs formed with TBP/TAF-depleted extract (Fig. 20B, lane 4). *In vitro* transcription experiments (TXN) confirmed that most transcription relevant TBP was removed from the nuclear extract (Fig. 20B, compare transcription (TXN) in lanes 6 and 9 to that observed in lanes 7 and 10). I*n vitro* transcription could be reconstituted by adding recombinant human TBP to TBP/TAF-depleted nuclear extracts (Fig. 20B, lanes 8 and 11). This experiment showed in addition that CTD ser-5 hypophosphorylation was dependent on TBP but not on TAFs (Fig. 20B, lanes 3-5). This result is in agreement with the previous finding that a mutated TATA-box did abolish the appearance of CTD ser-5 hypophosphorylation during PIC formation (Fig. 13). Establishment of CTD ser-5 and -7 hyperphosphorylation on preformed PICs during the transcription incubation with 100 μM NTPs was not affected by the absence of TBP and TAFs (Fig. 20B, lanes 6-11). Again, establishment of CTD ser-5 and -7 hyperphosphorylation occurred independent of productive transcription. Interestingly, Mediator, TFIIB and CDK7 were recruited to the promoter template in the basal situation even in the absence of TBP and TAFs. However this was not sufficient of initiate transcription (Fig. 20B, lanes 3-5). In summary I found establishment of CTD ser-5 hypophosphorylation during basal PIC formation to be critically dependent on TBP but independent of TAF presence. Furthermore PIC associated CTD ser-5 and -7 hypophosphorylation correlated with the transcription potential of the respective PIC. In contrast CTD ser-5 and ser-7 hyperphosphorylation were established independent of TBP/TAFs and productive transcription in this assay.

C.3.7. PIC analysis by mass spectrometry

As described earlier, I could show that the CTD ser-7 kinase activity is part of a purified PIC assembled on an immobilized promoter template (Fig. 9B). Given that the CTD ser-7 kinase activity appeared to be dependent on a ATPase (Fig. 9B) and was not associated in its active form with Mediator complexes (Fig. 19) I reasoned that the ser-7 kinase activity might be active only in the context of a larger protein network, e.g. a PIC. During classical column chromatography the CTD ser-7 kinase complex might disintegrate and therefore loose its activity. The laboratory of Gerhard Mittler at the Max-Planck Institute for Immunobiology in Freiburg I performed for me a mass spectrometric analysis of an *in vitro* formed PIC. I screened the resulting protein list manually for kinases and could identify 13 kinases. These kinases were grouped into three categories: (i) Kinases which had been reported in the literature to posses the ability to phosphorylate RNAPII or GST-CTD as a substrate, (ii) kinases which were functionally linked to the transcription processes and (iii) kinases which did not fit into one of the other two groups. In this thesis the seven PIC associated kinases identified in the first group were tested for their ability to contribute to CTD hyperphosphorylation at ser-5 and -7. The first group comprised the kinases DNA-PK, CDK1, CDK11, CKI, CKII, CDK7 and CDK9. Investigation of the kinases identified in the other two groups takes place in an ongoing project.

C.3.8. CDK7, CDK8 and CDK9 are not CTD Ser-7 kinases

In order to test the transcription related kinases CDK7, 8 and 9 for their contribution to CTD ser-5/7 hyperphosphorylation during an *in vitro* transcription reaction I used the immobilized template assay in combination with immunodepleted nuclear extracts. The immunoblot confirmed that all kinases were depleted to a significant degree (Fig 15B, lanes 1-4). Analysis of the PICs in the presence of 1 μM ATP revealed that CDK7 was responsible for the CTD ser-5 and -7 hypophosphorylation during PIC formation (Fig. 15B, lanes 5 to 8 and data not shown). In a subsequent step washed PICs were incubated for 20 min under *in vitro* transcription conditions in the presence of 100 μM NTPs. At the end of the transcription incubation RNAPII was analyzed in two fractions. First, RNAPII that remained associated to the promoter template was analyzed (Fig. 15B, lanes 9-12) and second, RNAPII that had dissociated during the second incubation was analyzed by

immunoblot (Fig. 15C, lanes 2-5). In the absence of CDK7, I observed a significant reduction in CTD ser-5 hypophosphorylation during PIC formation, but moderate if any reduction in CTD ser-5 and -7 hyperphosphorylation. Absence of CDK8 led to a reproducible decrease in CTD ser-5, but not ser-7 hyperphosphorylation established during transcription initiation. The data do not provide evidence for an involvement of CDK7, CDK8 or CDK9 in CTD ser-7 hyperphosphorylation.

C.3.9. Evidence that DNA-PK, CKI, and CKII are not CTD ser-7 kinases in a PIC

To determine the contribution of DNA-protein kinase (DNA-PK), casein kinase (CKI) and casein kinase 2 (CKII) to CTD ser-5/7 hyperphosphorylation I made use of the DNA-PK specific inhibitor wortmannin, the CKI specific inhibitor D4476 and the CKII specific inhibitor TBB (4,5,6,7-tetrabromobenzotriazole). I prepared PICs with Jurkat nuclear extracts in the presence of GAL4-VP16 and 1 μM ATP on immobilized major-late promoter templates, washed the PICs and either analyzed them directly by immunoblot (Fig. 21 lane 1) or subjected them to a second incubation in *in vitro* transcription buffer supplemented with 100 μM NTPs and the indicated inhibitors (Fig. 21, lanes 2-5 and 6-16). Roscovitine, an inhibitor of CDK1, CDK2 and CDK5 led to a slight reduction of CTD ser-7 hyperphosphorylation upon transcription initiation (Fig. 21, compare lanes 6 to 15 and 16). The DNA-PK inhibitor wortmannin was able to suppress the appearance of post-translationally modified TBP species that migrated with an up to 10 kDa higher molecular weight than unmodified TBP (Fig. 21, compare lanes 6 to 11 and 12). This experiment suggests that CTD ser-5 and -7 hyperphosphorylation is not significantly mediated by CKI, CKII, DNA-PK and CDK9 (CDK9 is inhibited by DRB; lanes 13 and 14). However the predominantly CDK1, CDK2 and CDK5-specific inhibitor roscovitine led to a decrease in CTD ser-7 hyperphosphorylation.

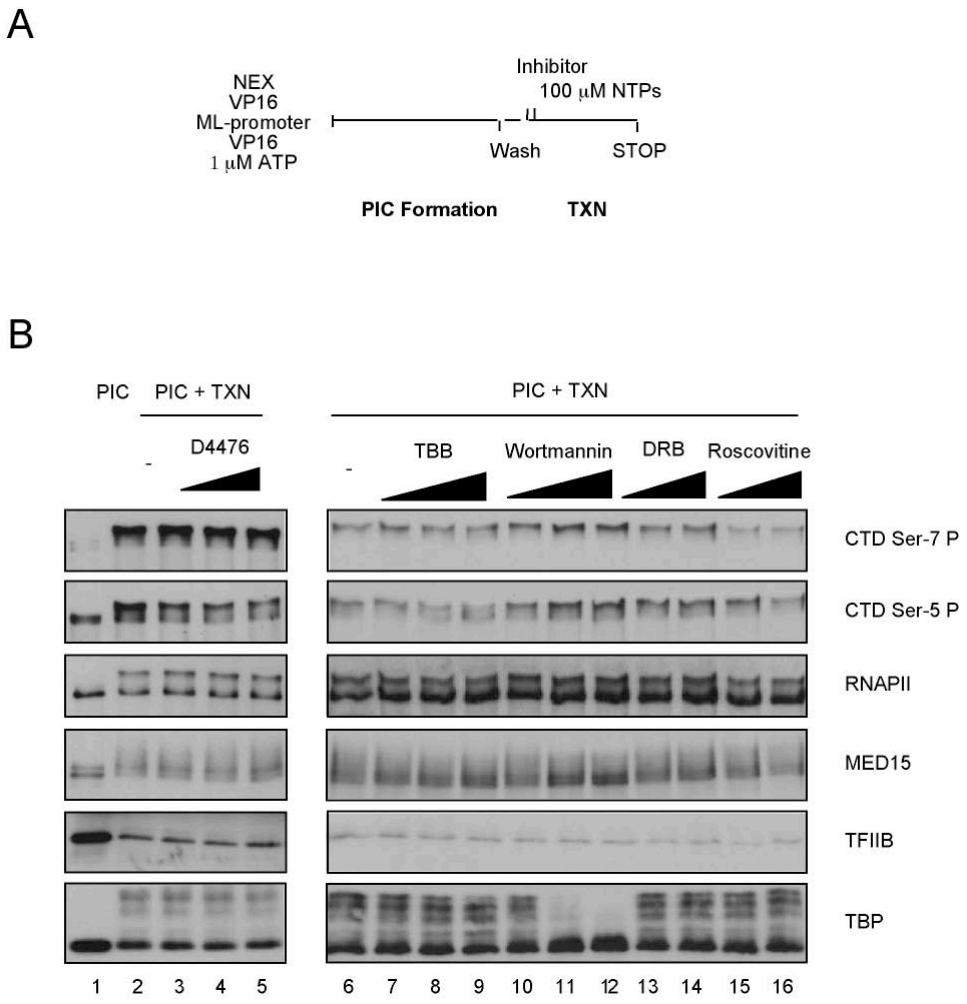

Figure 21: Effects of the kinase inhibitors D4476, TBB, wortmannin, DRB and roscovitine on CTD ser-5 and -7 hyperphosphorylation. *A,* Reaction scheme. *B,* PICs were formed on major-late promoter templates in the presence of 1 μM ATP, washed extensively and then subjected to a second incubation step in transcription buffer containing no inhibitors (lanes 2 and 6) or the kinase inhibitors D4476 (0.5, 5, 50 μM, lanes 3-5); TBB (1, 10 and 100 μM, lanes 7-9); wortmannin (0.1, 1 and 10 μM, lanes 10-12); DRB (50, 100 μM, lanes 13 and 14) or roscovitine (10, 100 μM, lanes 15 and 16). PIC: Immunoblotanalysis of a PIC that had not been subjected to the second incubation step. The TBP post-translational modifications led to an apparent shift of about 10 kDa in molecular weight in SDS-PAGE gels.

C.3.10. CDK1 phosphorylates GST-CTD substrates at ser-5 and -7, but is dispensable for CTD ser-5 and -7 hyperphosphorylation in the PIC context

CDK1 as well as a CDK1-related kinase, CDK11 were identified in the mass spectrometric PIC analysis. In addition roscovitine showed in the above and several additional experiments the propensity to inhibit CTD ser-7 hyperphosphorylation. In order to determine whether CDK1 could be the PIC-associated CTD ser-7 kinase I

performed *in vitro* kinase assays with immunoprecipitated CDK1 and the CDK1-related kinase CDK11. A GST-CTD fusion protein was offered as substrate. CDK1 was able to phosphorylate GST-CTD at ser-5 and -7 whereas CDK11 showed no activity toward GST-CTD ser-5 or -7 (Fig. 22A). Note that the monoclonal CTD ser-7 antibody did recognize plain recombinant GST-CTD. Extensive λ-phosphatase treatment did not abolish this recognition. Jurkat nuclear extract was used as a positive control in this assay (Fig 22A, lane 5). A CDK1 immunoprecipitation led to a modest increase in GST-CTD ser-7 phosphorylation and a robust phosphorylation at GST-CTD ser-5 (Fig. 22A, lane 2). CDK11 did not facilitate GST-CTD ser-5 or -7 phosphorylation (Fig. 22A, lane 3). Also the combination of both kinases in one kinase assay reaction did not boost phosphorylation activity towards CTD ser-5 and -7 (Fig. 22A, lane 4). Thus CDK1, a kinase that is recruited to an *in vitro* formed PIC in a Mediator-independent fashion (Fig. 13) showed the tendency to phosphorylate GST-CTD at ser-5 and -7. In parallel to the PIC mass spectrometric analysis I tried to purify the CTD ser-7 kinase by classical column chromatography. On a DE52 column the CTD ser-7 phosphorylation activity towards GST-CTD could be spitted into two activities as determined by an *in vitro* kinase assay with the respective column fractions on GST-CTD as substrate (Fig. 22B). One fraction contained an activity towards GST-CTD ser-5 and -7 and a second peak contained predominant a kinase activity towards GST-CTD ser-7 (Fig. 22B, fractions A and D/E respectively). While characterizing CTD ser-5/7 hyperphosphorylation I never observed one hyperphosphorylated state in the absence of the other. Therefore I focused on the activity in fraction A. Immunoblot analysis revealed that CDK1 is present in fraction A (Fig. 22B, lane 4). In order to further investigate CDK1 as potential CTD ser-5 and -7 hyperphosphorylation kinase in the PIC context I immunodepleted the kinase from Jurkat nuclear extract and performed immobilized template assays on major-late promoter templates (Fig. 22C). Immunodepletion of CDK1 abolished CTD ser-5 and -7 phosphorylation as well as the *in vitro* transcription potential of the PIC (Fig. 22C, compare lanes 3 and 4). However, in the course of the study it became evident that most of the Mediator complex was co-depleted by the polyclonal CDK1 antibody which was used for the CDK1 depletion (compare MED15 and MED25 recruitment to the PIC in lane 3

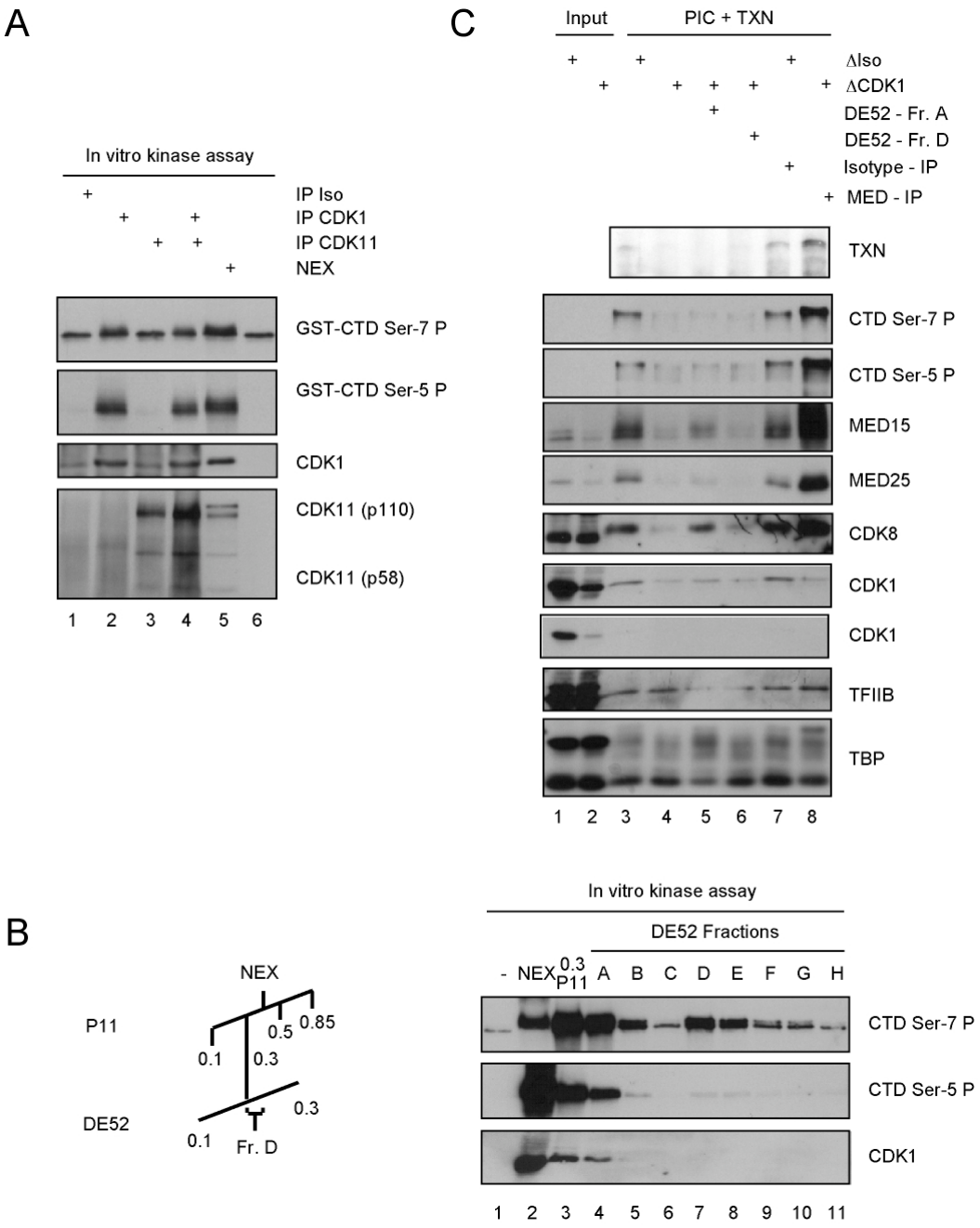

Figure 22: CDK1 phosphorylates GST-CTD at ser-5 and -7 but appears not to be the PIC associated kinase responsible for CTD ser-5 or 7 hyperphosphorylation. *A, In vitro* kinase assay with GST-CTD as substrate. CDK1 or CDK11 immunoprecipitations were probed for their potential to phosphorylate GST-CTD at ser-5 and -7 (lanes 2 and 3 respectively). Jurkat nuclear extract served as a positive control (lane 5). *B, In vitro* kinase assay probing DE52 column fractions for their CTD ser-5 and -7 phosphorylation activity towards GST-CTD. *C,* Immobilized template assay on major-late promoter templates. PICs were formed with mock-treated (ΔIso) or CDK1-depleted (ΔCDK1) Jurkat nuclear extract as indicated. PICs were formed in the presence of the activator GAL4-VP16 and 1 μM ATP, washed afterwards and subjected to a second incubation in *in vitro* transcription buffer containing 100 μM NTPs. The DNA-bound proteins were analyzed by immunoblot, in addition the *in vitro* transcription potential of the respective PICs was probed (TXN). PIC formation reactions were supplemented with DE52 fractions A and D, as well as with an isotype or a Mediator (MED) immunoprecipitation (IP) as indicated.

and 4 as well as the amount of MED15 and MED25 present in the input nuclear extracts, lane 1 and 2). On the other hand, Mediator immunoprecipitations did not co-precipitate CDK1 in detectable quantities. To dissect whether the defect in CTD ser-5 and -7 hyperphosphorylation was due to the absence of Mediator or CDK1, I reconstituted the CDK1 depleted nuclear extract with DE52 fraction A, which contained CDK1 (Fig. 22C, lane 5). Adding back CDK1 from this fraction to the PIC formation reaction did not restore the CTD ser-5/7 hyperphosphorylation activity. Next, immunoprecipitated Mediator, that was not associated with appreciable levels of CDK1, was added-back into a CDK1-depleted nuclear extract and an immobilized template assay was performed on major late promoter templates. Mediator, although added-back in excess to the PIC formation reaction, fully restored the CTD ser-5 and -7 hyperphosphorylation activity and also restored the *in vitro* transcription potential of the nuclear extract (Fig. 22C, lane 8). Thus, although CDK1 is able to phosphorylate GST-CTD at ser-5 and to a lesser extent at ser-7 in an *in vitro* kinase assay (Fig. 22A) I did not find convincing evidence that CDK1 is the major PIC associated kinase responsible for CTD ser-7 hyperphosphorylation upon transcription initiation.

D. Discussion

D.1. Mediator Modules

Several distinct Mediator subpopulations can be isolated from nuclear extracts. This finding could reflect two scenarios: (i) a particular set of target genes is regulated by a particular Mediator subpopulation or (ii) the Mediator subpopulations present in a nuclear extract are generated sequentially at a promoter during transcription initiation. In this scenario one would expect that Mediator acts in a similar fashion for all Mediator-responsive genes.

D.1.1. Characterization of the CDK8 Mediator subpopulation

The Tjian laboratory discovered that two basic classes of Mediator complexes can be distinguished *in vitro*: A small Mediator termed 'cofactor required for Sp1 activation' (CRSP), which stimulates transcription strongly and a large Mediator termed 'activator-recruited cofactor-L' (ARC-L) which is transcriptionally inactive. The main difference between the two complexes is the presence of a CDK8 Mediator module (CDK8, cyclin C, MED12, MED13). It is present in the ARC-L but not in CRSP complex (Taatjes et al., 2002). CDK8 has been reported to phosphorylate and thereby inactivate the cyclin H/CDK7 kinase module of TFIIH (Akoulitchev et al., 2000). A genome wide ChIP-on-chip study in yeast found the CDK8-Mediator and RNAPII to be located at promoter regions of inactive genes *in vivo* (Andrau et al., 2006). In combination with the above molecular mechanism a model results in which the CDK8-module of Mediator keeps a RNAPII holoenzyme complex in a poised state allowing rapid activation of the respective gene upon loss of the inhibitory CDK8-Mediator module. Loss of the CDK8-module or CDK8-Mediator *in vivo* has been shown to dependent on PARP-1 (Pavri et al., 2005). Tom Uhlmann, a former graduate student in the lab, showed that the CDK8-module is lost in a tet-VP16 inducible model gene system upon transcription initiation at the promoter *(Uhlmann et al., 2007)*. Regarding CDK8 function this study was guided by the following questions: Does the CDK8 module dissociate from Mediator upon transcription initiation and can this be recapitulated *in vitro*? Has the CDK8 module any positive function in transcription initiation? I found that the CDK8 module together with the tail subunit MED15 did dissociate from an *in vitro* formed PIC upon incubation with

nucleotides (Fig. 4B and 9B). CKII is known to be associated with transcription complexes *in vitro*, as it is required for *in vitro* transcription from downstream promoter element (DPE) containing promoters (Lewis et al., 2005). The CKII specific kinase inhibitor TBB did prevent partially post-translational modification and/or dissociation of CDK8 upon transcription initiation (Fig 4C). Further investigation will be required to determine whether dissociation of the CDK8 module from a PIC is triggered by CKII. It turned out that only a small fraction of all Mediators in a nuclear extract were associated with CDK8 (Fig. 3A). Immunodepletion of CDK8 from a nuclear extract did not reduce the *in vitro* transcription potential of a nuclear extract. In addition I showed that CDK8 is responsible in part for the establishment of CTD ser-5 hyperphosphorylation upon transcription initiation (Fig. 15B). Both findings provide evidence that neither CDK8 or CDK8-mediated CTD ser-5 hyperphosphorylation have essential positive functions in transcription initiation *in vitro*.

D.1.2. Further characterization of MED25

The MED25 Mediator is essential for VP16-mediated gene activation (Mittler et al., 2003). A basal Mediator has been described which acts similar to a general transcription factor in a nuclear extract *in vitro* transcription system (Mittler et al., 2001). On this background I wished to determine whether a basal Mediatorfunction is associated with MED25 or a MED25 Mediator. I found that immunoprecipitated MED25, even if washed under stringent conditions, is sufficient to support VP16-activated transcription *in vitro*. In addition I find that a MED15 immunodepletion removes all MED25 from a nuclear extract. I conclude that the basal Mediator function is physically associated with a MED25 immunoprecipitation and that all MED25 present in a nuclear extract is physically associated with Mediator.

D.1.3. Characterization of the human equivalent of the yeast reinitiation Mediator

It has been shown that after a first round of *in vitro* transcription a scaffold complex is formed at promoter template in the yeast system. This scaffold or reinitiation complex contains factors like TBP and the Mediator complex. Other factors such as TFIIB and CDK7 were reported to dissociate from the PIC upon addition of ATP or NTPs to a PIC (Yudkovsky et al., 2000). When I incubated preformed and washed PICs with ATP or

NTP I observed a partial dissociation of the PIC in human nuclear extracts. As reported for the yeast system, I observed dissociation of TFIIB and CDK7. Interestingly, TBP appeared to be post-translationally modified during this process (Fig. 4B/C, Fig. 9). Appearance of the post-translational modification can be suppressed by the inhibitor wortmannin (Fig. 21), which is specific for DNA-PK. DNA-PK is a immobilized promoter template associated kinase *in vitro* (Peterson et al., 1992). Furthermore a DNA-PK phosphorylation sites have been discovered in TBP (Chibazakura et al., 1997). The TBP post-translational modification was established in the presence of 100 μM ATP, but not in the presence of 100 μM ATPγS, an ATP analogue that can be used by kinases but not by helicases (Stelzer et al., 1994; Tazi et al., 1993), indicating that also a helicase might be involved in the process leading to post-translational modification of TBP. DNA-PK is a kinase that is not conserved from yeast to man. In the yeast nuclear extract *in vitro* transcription system multiple round transcription is observed, whereas this is not the case in the human nuclear extract *in vitro* transcription system. The post-translational modification of template associated TBP may provide an explanation for this difference. Similar to the TBP modification, the PIC dissociation appears to be dependent on a helicase. Very little TFIIB dissociation was observed if a PIC was incubated with 100 μM ATPγS. Incubation of a PIC with 100 μM ATP or 100 μM NTPs led to TFIIB dissociation Fig 9B. PIC dissociation and TBP post-translational modification also occurred if CDK7, CDK8 or CDK9 were depleted from nuclear extracts (Fig. 15B). In the yeast system it was reported that Mediator remains associated with the DNA promoter template, however the analysis in that study put emphasis on the head subunits MED6, MED17 and MED20 (Yudkovsky et al., 2000). Upon addition of 100 μM ATP or NTP to preformed PICs the tail subunit MED15 as well as the CDK8 module subunit CDK8 did dissociate from immobilized promoter templates, whereas the middle submit MED7 remained associated with the promoter templates (Fig. 4B/C, Fig. 9B). An interesting question for the future will be whether the middle/head scaffold Mediator, that remains associated with an immobilized promoter template after transcription initiation is sufficient to recruit GTFs such as TFIIB and TFIIH for another round of transcription, or whether the tail region is required for that to happen. In summary PIC dissociation upon transcription initiation appeared to be dependent on a yet to be identified ATPase such as

a helicase. Furthermore I found that the head/middle part of Mediator remained associated to the promoter template upon transcription initiation whereas the tail and CDK8 module were dissociating.

D.2. Basal Mediator

Gerhard Mittler, a former graduate student in the laboratory, discovered that Mediator acts like to a basal transcription factor in a nuclear extract *in vitro* transcription system (Mittler et al., 2001). In the minimal *in vitro* transcription system composed of recombinant or highly purified proteins many regulatory mechanisms are neglected due to the simplicity of the system. In comparison in the nuclear extract *in vitro* transcription system several additional transcription regulatory mechanism are in place and therefore it represents the more physiological transcription system. For instance, the minimal transcription system acts independently of RNAPII CTD phosphorylation, whereas in the nuclear extract transcription system this mode of regulation can be recapitulated (Serizawa et al., 1993). In the course of this work the Roeder laboratory published that Mediator is required in the nuclear extract transcription system for efficient recruitment of TFIIB to the PIC. In this article it was demonstrated that the Mediator requirement can be overcome by adding excessive amounts of recombinant TFIIB to a Mediator-deficient nuclear extract (Baek et al., 2006). Starting from these points I investigated how Mediator is involved in GTF recruitment and what influence Mediator has on RNAPII CTD phosphorylation. As discussed in more detail below, I found that Mediator is essential for TFIIB and TFIIH recruitment and that Mediator is also required for the establishment of several CTD ser-5 and -7 phosphorylation states.

D.2.1. Basal Mediator is required for TFIIB and TFIIH recruitment in a nuclear extract *in vitro* transcription system

In the literature two models have been proposed to explain the recruitment of general transcription factors to a promoter to form a functional PIC. The first model represents an extrapolation from the situation found with recombinant and purified transcription factors. In this *in vitro* transcription system there is evidence that the GTFs and RNAPII are recruited sequentially in the following order: TFIID > TFIIA > TFIIB > RNAPII/TFIIF > TFIIE > TFIIH. The second model, the so called holoenzyme pathway,

is based on research carried out with crude nuclear extract transcription systems and states that a PIC is recruited to a promoter in two bulk segments: the TFIID/TFIIA complex and the holoenzyme consisting of RNAPII, Mediator, the SWI/SNF complex, TFIIB, TFIIE, TFIIF and TFIIH (reviewed in (Thomas and Chiang, 2006)). A study by the Roeder lab argued that also in the nuclear extract *in vitro* transcription system the sequential assembly model applies (Baek et al., 2006). Notably, a basal Mediator complex is not required for GTF recruitment in the recombinant/purified *in vitro* transcription system. On this background I investigated the nature of the GTF-recruitment limiting mechanism in the nuclear extract transcription system.

D.2.1.1. What kind of mechanism could limit TFIIB and TFIIH recruitment to a promoter in the nuclear extract transcription system?

Recruitment of TFIIB and TFIIH to a PIC appears to be inhibited in a nuclear extract transcription system. Despite a large excess of these GTFs, in particular of TFIIB, present in a nuclear extract, Mediator was absolutely required for their recruitment (Fig. 6B) to an PIC. One possibility is that in a nuclear extract transcription system a negative acting factor competes with TBP for TATA-box binding. Therefore it could be reasoned that Mediator helps TBP to bind properly to the TATA box. Thereby appropriate binding sites for TFIIB and TFIIH recruitment are generated within the PIC. After figuring out that TFIIB recruitment to a PIC is indeed limited – directly or indirectly - by a factor present in the nuclear extract (Fig. 6C) I incubated a promoter DNA-template with recombinant TBP, and subsequently incubated it with either mock-depleted or Mediator deficient nuclear extract. In case TBP-binding would be the GTF-recruitment limiting mechanism one would expect in this case Mediator-independent recruitment of TFIIB to the PIC. However, this is not the case, TFIIB recruitment was still Mediator-dependent (Fig. 6B). Remarkably, TFIID and Mediator recruitment followed a much faster kinetic than the recruitment of TFIIB to a PIC (Fig. 11B). Obviously generating the GTF binding surface within the PIC is a slow process. One could envision that Mediator is required to bring TBP in a position or confirmation that allows TFIIB recruitment to the PIC, e.g. by dissociating TBP from TAFs. However, prebinding of recombinant TBP to the promoter DNA template should alleviate the Mediator-requirement for TFIIB in that case, which

was not the case (Fig. 6B, lanes 12 and 13). For this reason the TFIIB/TFIIH-recruitment limiting mechanism may act downstream of functional TBP recruitment to the TATA-box. Another possibility is that the GTF-binding sites within the PIC are physically blocked by a negative acting protein or complex that has to be displaced by Mediator in order to allow GTF recruitment. In genetic screens two factors turned up as suppressors of MED17 (Srb4) Mediator mutations: mutations in alleles belonging to the NC2 and the CCR4-NOT complexes (Lee et al., 1998). It is well described that NC2 affects and competes with TFIIA and TFIIB for recruitment to a PIC if recombinant proteins are used (Goppelt et al., 1996). Testing whether NC2 imposes the Mediator-requirement on basal transcription revealed that NC2 competes with TFIIB for recruitment to the PIC but also that basal transcription was Mediator-dependent even if a NC2-deficient nuclear extract was used (Fig. 8A/B). Interestingly, if TFIIB recruitment was limited by increased amounts of NC2 also the recruitment of the TFIIH component CDK7 was impaired (Fig. 8B, lanes 7 and 8). NC2 appears to be a fine-tuning mechanism for TFIIB and TFIIH recruitment, but is not the major TFIIB/TFIIH-recruitment limiting factor that is counteracted by Mediator in basal *in vitro* transcription (Fig. 8A/B).
A recent publication claimed that adding the DSIF heterodimer to a *in vitro* transcription system consisting of recombinant and purified transcription factors leads to a Mediator requirement for basal *in vitro* transcription. Importantly the used transcription system did not contain pTEFb or CDK9 which are required to overcome the transcription block imposed by DSIF 20 to 50 nucleotides downstream of the transcription start site (Malik et al., 2007). It will be interesting to address in further investigations whether the CCR4-NOT complex or the DSIF complex are components of the TFIIB-recruitment limiting mechanism. Furthermore it will be interesting to further purify the TFIIB-recruitment limiting factor from the 0.1M P11 column fraction (Fig. 6C).

D.2.1.2. TFIIB recruitment to a PIC is slow

What is the nature of the TFIIB/TFIIH-recruitment limiting mechanism? A remarkable feature of TFIIB recruitment to the PIC was its slow recruitment during PIC formation in comparison to recruitment of TFIID, activator and Mediator (Fig. 11). This may indicate that the TFIIB binding site within the PIC has to be generated by an ATPase such as a

helicase. However PIC formation in the presence of 1 μM of the helicase inhibitor ATPγS does not lead to reduced TFIIB recruitment (Fig. 9 and 10A). The kinase inhibitor H8 was found to inhibit CTD ser-5 and -7 phosphorylation of the RNAPII CTD during PIC formation (Fig. 9 and 10A). In yeast TFIIB recruitment to a *in vitro* formed PIC was found to be dependent on the RNAPII CTD (Ranish et al., 1999). However, TFIIB recruitment was in the assay system used in this study independent of CTD ser-5 and -7 hypophosphorylation or a kinase that is sensitive to H8 (Fig. 9 and 10A).

D.2.1.3. Recruitment of GTFs in the context of the holoenzyme

Mediator was recruited to an *in vitro* formed PIC even in a TBP-depleted nuclear extract. Strikingly TFIIB and TFIIH component CDK7 were found to be associated with the promoter template even in the absence of promoter bound TBP arguing that they were recruited in the context of a holoenzyme complex (Fig. 20, lanes 3-5). Significant amounts of TFIIB were also recruited to a promoter template that contained a mutated TATA-box and therefore did not provide a functional TBP binding site as it is required for functional PIC formation (Fig. 13). In support of this notion it was found in yeast that TFIIB is recruited by GAL4-VP16 to a DNA template that does not contain a promoter (Ranish et al., 1999). If a promoter template was incubated with recombinant TBP/rTFIIB prior to the PIC formation reaction that was carried out with nuclear extract, additional endogenous TFIIB was recruited to the promoter template in a Mediator-dependent fashion (Fig. 6B; rTFIIB contains a His-tag, and therefore has higher molecular weight than the endogenous TFIIB). One possibility is that in a nuclear extract transcription system only the Mediator-recruited TFIIB is transcriptionally active, but not the TFIIB that had been bound to the TATA-TBP complex during the preincubation step. The sequential assembly model would predict that successful recruitment of one GTF is sufficient to recruit the following GTF. However I observed that prebinding of large amounts of rTFIIB to a promoter template – TPB complex did not led to increased TFIIH recruitment (Fig. 6B). On the other hand, if TFIIB recruitment was limited due to an increased amount of NC2 or the B2-RNA during PIC formation, also TFIIH recruitment was diminished (Fig. 8B and 12B respectively). Notably, I did not find any evidence that a holoenzyme complex containing the GTFs TFIIB, TFIIE, TFIIH

and TFIIF in conjunction with Mediator and RNAPII is present as a preformed entity in a nuclear extract or that such a complete holoenzyme is recruited to the PIC in one piece. E.g. TFIIB was not found to be associated with Mediator in measurable quantities in a nuclear extract (Fig. 6B, lanes 1 and 2, Fig. 12A, lanes 1 and 2). Taken together this results indicate that the holoenzyme does not exist as a pre-formed entity, but is rather created in a slow process during PIC formation.

D.2.2. Basal Mediator is required to establish several CTD phosphorylation states

As will be discussed in the next chapter in detail, I found that a particular form of CTD ser-5 phosphorylation correlated with the transcription potential of a PIC. Basal *in vitro* transcription in the nuclear extract system is sensitive to the kinase inhibitor H8, whereas transcription in the system composed of recombinantly expressed and purified transcription factors is not (Serizawa et al., 1993; Stelzer et al., 1994). Furthermore the first description of Mediator provided evidence that Mediator stimulates the kinase activity of the TFIIH associated kinase CDK7 (Kim et al., 1994). I found that CDK7 mediates CTD ser-5 hypo- but not CTD ser-5 hyperphosphorylation (Fig. 15B) and also could show that CTD ser-5 hypophosphorylation is Mediator-dependent (Fig. 8A). In addition to this CDK7-dependent mechanism Mediator is also required for the establishment of CTD ser-5 and -7 hyperphosphorylation, which is generated in a CDK7-independent fashion (Fig. 15B). In summary, establishment of various kinds of CTD phosphorylation is a basal Mediator function.

D.3. Two distinct modes of CTD ser-5 phosphorylation

Two kinases have been identified in conjunction with transcription as RNAPII CTD ser-5 kinases: CDK7 and CDK8. CDK7 is described as a factor that supports transcription whereas CDK8 is described as a factor that inhibits transcription (Hengartner et al., 1998). CTD ser-5 phosphorylation is generally believed to be predominantly associated with the low-mobility IIo form of Rpb1. However a recent study by the Fisher laboratory revealed that highly specific inhibition of CDK7 in a human cell line had no effect on the amount of CTD ser-5 hyperphosphorylation observed in the lysate of inhibitor treated cells (Larochelle et al., 2007). Also upon inactivation of CDK7 in *D. melongaster* only a modest drop in total CTD ser-5 phosphorylation was observed (Schwartz et al., 2003) *in*

vivo. I discovered that CTD ser-5 phosphorylation comes in two distinct classes as CTD ser-5 hypo- and hyperphosphorylation (CTD ser-5 phosphorylation of the IIa and IIo form of RNAPII Rpb1 respectively). Appearance of CTD ser-5 hypophosphorylation was dependent on CDK7, TFIIB, TBP, a TATA box, and Mediator, was established before complex opening by the TFIIH associated helicase and did correlate to the transcription potential of a given PIC (see chapter C.2). However a recent study challenged the view that the CDK7 kinase activity is critically required for transcription to occur in yeast (Kanin et al., 2007). I observed that a fraction of CTD ser-5 hyperphosphorylation, that occurred upon transcription initiation *in vitro*, could be linked to CDK8 (Fig. 15B). CTD ser-5 hypophosphorylation was entirely independent of CDK8. My data provide evidence for the notion that another kinase then CDK7 and CDK8 participates in establishing CTD ser-5 hyperphosphorylation upon transcription initiation. CTD ser-5 and -7 hyperphosphorylation could not be separated in any experiment and might therefore be linked or established by the same mechanism. However, CTD ser-7 hypophosphorylation is preceded by CTD ser-5 hypophosphorylation, arguing that each of these two marks is established by a distinct mechanism (Fig. 10A). In summary I provide evidence that CTD ser-5 phosphorylation occurs in two distinct modes, of which one, CTD ser-5 hypophosphorylation is established in a tightly controlled fashion, whereas the other, CTD ser-5 hyperphosphorylation, appears to be established independent of TBP or the transcription potential of a PIC. Based on this data one can envision the following model: CTD ser-5 hypophosphorylation is established only at *bona fide* transcription start sites if all factors are recruited to the PIC and thus marks an transcription competent PIC. CTD ser-5 hyperphosphorylation is then established to dissociate RNAPII from the PIC in order to start productive transcription. If however an RNAPII holoenzyme is bound to a non-promoter region, e.g. an accessible DNA region in an open reading frame, no functional PIC is formed and thus no CTD ser-5 hypophosphorylation established. If now CTD ser-5 or -7 hyperphosphorylation is established, this RNAPII may be hyperphosphorylated and thereby dissociate in a non-productive fashion from the inappropriate binding site within the genome (Fig. 23). What could be the physiological function of CTD ser-5 hypophosphorylation? A recent study found that specific inhibition of yeast CDK7/Kin28 *in vivo* does not lead to diminished mRNA synthesis but

to decreased mRNA capping (Kanin et al., 2007). Also it was reported that the yeast homologue of the human MLL histone methyltransferase Set1 is recruited to promoters in a CDK7/Kin28-dependent fashion (Ng et al., 2003). In a genome-wide ChIP-on-chip study it was demonstrated that most transcription start sites of active genes are decorated with a highly localized peak of histone H3-lysine-4 trimethylation (Guenther et al., 2007). The mechanism by which this precise localization is achieved is unknown. A possible mechanism would be that the histone methyltransferase is active only in combination with RNAPII that bears the CTD ser-5 hypophosphorylation mark and is devoid of CTD ser-7 phosphorylation. As soon as CTD ser-7 phosphorylation is established after complex opening the histone methyltransferase would be inactivated. The result would be a highly localized histone methylation mark.

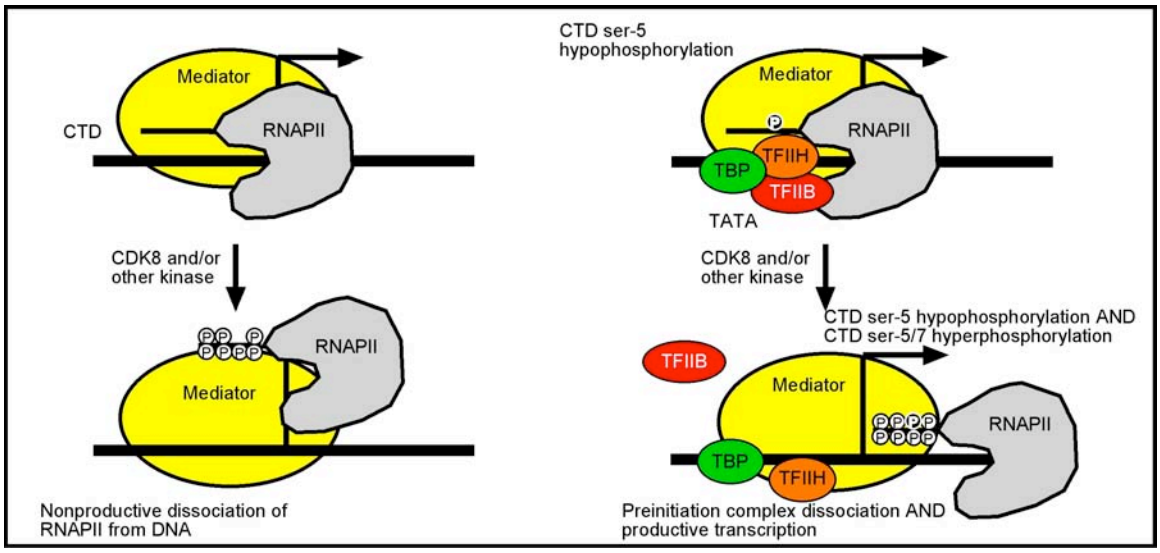

Figure 23: Model CTD phosphorylation (for details see text above)

D.4. Characterization of CTD ser-7 phosphorylation

The Eick laboratory raised monoclonal antibodies specific for the RNAPII CTD phosphorylated at ser-7 and found that this modification is observed *in vivo* (Chapman et al., 2007). After figuring out that CTD ser-7 phosphorylation did appear upon transcription initiation *in vivo* (Fig. 16) and *in vitro* (Fig. 9), I set out to characterize and identify the kinase that mediates this modification.

D.4.1. Establishment of CTD ser-5 and -7 hyperphosphorylation is independent of processive transcription

First I investigated whether CTD ser-7 phosphorylation is established in a transcription-dependent fashion *in vitro* and found that this modification was established before formation of the first phosphodiester bond. Incubation of a PIC with 100 µM ATP under *in vitro* transcription conditions was sufficient to establish similar amounts of CTD ser-7 hyperphosphorylation than were generated by incubation of PICs with 100 µM NTPs. Incubation with NTPs, in contrast to incubation with ATP alone, does allow productive *in vitro* transcription to occur (Fig. 9). Also incubation of an *in vitro* transcription reaction with 100 µM NTPs in the presence of the transcription inhibitor α-amanitin gave rise to the same levels of CTD ser-7 hyperphosphorylation as in the uninhibited situation (Fig. 10B). α-Amanitin is known to inhibit transcription before formation of the second phosphodiester bond (de Mercoyrol et al., 1989). In support of this notion it has been reported previously that CTD hyperphosphorylation occurs before the formation of the first phosphodiester bond (Laybourn and Dahmus, 1990). Immunodepletion of CDK7 or TBP in a nuclear extract did abolish CTD ser-5 hypophosphorylation but left the amount of generated CTD ser-7 hyperphosphorylated RNAPII unaltered as compared to the mock-depleted situation. In both cases the amount of transcript produced by the immunodepleted PICs was reduced (Fig. 15B and 20B respectively). Thus, CTD ser-7 phosphorylation was established at an early stage of transcription initiation and did not show any correlation to the amount of processive transcription from the respective PIC. Appearance of CTD ser-5 hyperphosphorylation generally correlated with the establishment of CTD ser-7 hyperphosphorylation. Immunodepletion of CDK8 led to a reduction in CTD ser-5 but not CTD ser-7 hyperphosphorylation (Fig. 15B). CTD ser-5 and -7 hyperphosphorylation was observed in a double-strand DNA-dependent fashion. Presence of the poly(dG:dC) DNA polymer in an *in vitro* transcription reaction that lacks template DNA still led to the establishment of CTD ser-5 and -7 hyperphosphorylation (Fig. 18B). The amount of established CTD ser-5 and -7 hyperphosphorylation did not increase much if a major-late promoter template was included into the reaction (Fig. 18B). I conclude that the protein kinase that establishes these modifications acts in a DNA but not promoter-dependent fashion.

Establishment of CTD ser-5 and -7 hyperphosphorylation could be triggered by incubating a PIC with 100 μM ATP but not by incubation with 100 μM ATPγS. ATPγS is an ATPase inhibitor but can be used by kinases as a substrate (Eckstein, 1985). Therefore it is likely that the CTD ser-7 hyperphosphorylation kinase is functionally linked to an ATPase such as a helicase (Fig 9B and Fig. 18B). It has been reported that hyperphosphorylation of the CTD of RNAPII leads to dissociation of a RNAPII-Mediator complex (Max et al., 2007; Svejstrup et al., 1997). Taking this into account it is possible that CTD ser-5 and -7 hyperphosphorylation leads to a dissociation of the PIC. Dissociation of a DNA-bound Mediator RNAPII complex could have two consequences: Productive transcription is initiated or RNAPII dissociates from the DNA in a non-productive fashion. In the future it will be interesting to determine whether presence of CTD ser-5 hypophosphorylation in combination with CTD ser-5 and ser-7 hyperphosphorylation leads to productive transcription whereas presence of CTD ser-5 and -7 hyperphosphorylation only leads to non-productive dissociation of RNAPII from DNA as depicted in Fig. 23.

D.4.2. Establishment of CTD ser-5 and -7 hyperphosphorylation is Mediator-dependent

Immunodepletion of Mediator in nuclear extracts led to a loss of CTD ser-5 and -7 hyperphosphorylation in both, the immobilized template assay as well as in an SDS-PAGE *in vitro* transcription assay (Fig. 17 and 18B respectively). CTD ser-5 and -7 hyperphosphorylation could be restored in a Mediator-depleted nuclear extract by adding back Mediator immunoprecipitated with antibodies against the MED15, MED25, CDK8 or MED1 Mediator subunits. Mediator preparations did phosphorylate a GST-CTD substrate at ser-5 but did not increase GST-CTD ser-7 phosphorylation in an *in vitro* kinase assay (Fig. 19). Therefore Mediator is not associated with the active form of the CTD ser-7 hyperphosphorylating kinase. This could be explained by the fact that additional factors are required for the activation of the CTD ser-7 kinase. Alternatively the CTD ser-7 kinase might not be physically associated with Mediator outside the PIC. In the future it will be necessary to determine how Mediator triggers the kinase activity responsible for CTD ser-7 hyperphosphorylation. It will be interesting to determine

whether this kinase also contributes to establishment of CTD ser-5 hyperphosphorylation upon transcription initiation.

D.4.3. Characterization of the kinase responsible for CTD ser-7 phosphorylation

In order to identify the CTD ser-7 kinase I made use of the fact that the functionally active kinase is part of an *in vitro* formed PIC. Mass spectrometric analysis of the isolated PIC identified 13 kinases as constituents of the PIC. In a first step I analyzed those seven kinases in detail which had been reported as CTD kinases in the literature. Given the functional characteristics of the CTD ser-7 kinase a particularly interesting candidate was DNA-protein kinase (DNA-PK). DNA-PK is able to phosphorylate short CTD peptides at ser-7 (Trigon et al., 1998). It forms a complex with the Ku80/Ku86 proteins which are DNA-helicases (Tuteja et al., 1994). I had observed that CTD ser-7 phosphorylation is established in a DNA- and helicase-dependent fashion (Fig. 18B). I probed DNA-PK directly in an immobilized promoter template assay with the inhibitor wortmannin and did not find a reduction in CTD ser-7 hyperphosphorylation (Fig. 21). Importantly, I observed that the DNA-PK inhibitor suppressed TBP post-translational modifications, that occurred in that type of assay (Fig. 21), indicating that the inhibitor was working properly. In parallel I started a biochemical purification of the CTD ser-7 kinase and found that CDK1 copurified with a kinase activity that was able to phosphorylate GST-CTD at ser-5 and -7 (Fig. 22A). Also CDK1 was identified by mass spectrometry as a PIC associated kinase. This result could be confirmed by an immunoblot analysis of a PIC (Fig. 13). In addition the inhibitor roscovitine was able to suppress CTD ser-7 hyperphosphorylation in an immobilized template assay (Fig. 21). A further investigation by immunodepletion of CDK1 was carried out. At first glance immunodepletion of CDK1 led to a loss of CTD ser-5 and -7 hyperphosphorylation in immobilized template assays and also led to a reduction in the transcription potential of the respective PICs (Fig. 22B). However it turned out that during the CDK1 immunodepletion with a polyclonal antiserum Mediator was co-depleted together with CDK1 (Fig 22C, compare lanes 1 and 2). Since immunoprecipitated Mediator was not associated with detectable amounts of CDK1 I conclude that the co-depletion of Mediator with CDK1 occurred due to an unspecific antibody cross-reaction and not due to a specific interaction between the

two components. To compensate for the loss of Mediator, I added back immunoprecipitated Mediator to a CDK1-depleted nuclear extract. This treatment fully restored the ability of the PIC to hyperphosphorylate CTD ser-5 and -7. Also it did restore the *in vitro* transcription potential of the PIC. In contrast, add-back of the CDK1 containing column chromatography fraction to a CDK1-depleted nuclear extract did not restore CTD ser-5 and -7 hyperphosphorylation (Fig. 22C). In the light of these results I conclude that CDK1 is unlikely to be the PIC associated kinase responsible for CTD ser-7 hyperphosphorylation. I tested all other CTD-kinases that were detected in the PIC either by immunodepletion (CDK7, CDK8, CDK9; Fig. 15), by probing the immunoprecipitated kinase in an *in vitro* kinase assay on GST-CTD (CDK11; Fig. 22A) or by using chemical inhibitors (CKI, CKII; Fig. 21). Furthermore I found that roscovitine is able to suppress the activity of the CTD ser-7 kinase in an immobilized template assay, but I could not gather convincing evidence that CDK1, a roscovitine sensitive kinase, is the PIC associated CTD ser-7 kinase. Other kinases that are sensitive to roscovitine are CDK2 and CDK5 (Filgueira de Azevedo et al., 2002). CDK2 could be detected as specific constituent of an *in vitro* formed PIC (Fig. 13). Further investigation will be required in this direction. In summary, the CTD ser-7 kinase activity appears to be connected to an ATPase activity and therefore it is possible that the PIC associated kinase responsible for CTD ser-7 hyperphosphorylation is active only in a complex with a helicase and DNA.

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Abbreviations

ATP	adenosine 5'-triphosphate
ATPγS	adenosine 5'-O-(3-thio)triphosphate
BRE	TFIIB recognition element
BSA	bovine serum albumin
CAK	cyclin-dependent kinase activating kinase
Cat. no.	catalogue number
ChIP	chromatin immunoprecipitation
CKI	casein kinase I
CKII	casein kinase II
CRIP	crossling-restriction digest immunoprecipitation assay
CTD	C-terminal domain of the largest subunit of RNAPII Rpb1
D4476	4-(4-(2,3-Dihydrobenzo[1,4]dioxin-6-yl)-5-pyridin-2-yl-1H-imidazol-2-yl)benzamide
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNA-PK	DNA protein kinase
dNTP	deoxynucleotide triphosphate
DPE	downstream promoter element
DTT	dithiothreitol
EDTA	ethylenediamine tetraacetic acid
EGTA	ethylenebis(oxyethylenenitrilo)tetraacetic acid
Fig.	figure
GTF	general transcription factor
GST	gluthathione S-transferase
H8	N-[2-(Methylamino)ethyl]-5-isoquinolinesulfonamide hydrochloride
Hepes	N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)
His	hexahistidine tag
INR	initiator element
IP	immunoprecipitation

ML	major-late
NC2	negative cofactor 2
NEX	nuclear extract
PC	positive cofactor
PCR	polymerase chain reaction
PIC	preinitiationcomplex
RNA	ribonucleic acid
RNAPII	RNA polymerase II
SDS	sodium dodecysulphate
SDS-PAGE	sodium dodecysulphate polyacrylamide gel electrophoresis
ser	serine
TAF	TBP-associated factor
TBB	4,5,6,7-tetrabromobenzotriazole
TBP	TATA binding protein
Tris	Tris(hydroxymethyl)aminomethane
TXN	transcription
VP16	virion protein 16