Biochemical Characterization of the Human Mediator Complex

by

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A thesis submitted to the

Faculty of the Graduate School of the

University of Colorado in partial fulfillment

of the requirement for the degree of

Master of Science

Department of Chemistry and Biochemistry

2010

This thesis entitled:

Biochemical Characterization of the Human Mediator Complex

written by Yuefeng Gao

has been approved for the Department of Chemistry and Biochemistry

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Date: _____

The final copy of this thesis has been examined by the signatories, and we find that both the content and the form meet acceptable presentation standards of scholarly work in the above mentioned discipline.

Abstract

Gao, Yuefeng (M.S., Department of Chemistry and Biochemistry)

Biochemical Characterization of the Human Mediator Complex

Thesis directed by Dr. Dylan J. Taatjes

Accurate regulation of transcription is essential for normal gene expression and proper organism development. Defects in regulatory components are often related to serious human diseases, and even lethality. The multi-subunit human Mediator complex is required for transcription and regulation of all protein-coding genes. Mediator regulates transcription by mediating signals between activators or repressors and the general transcription machinery. However, most of the molecular mechanisms regarding the Mediator complex remain unclear.

The composition of the Mediator complex can vary and Mediator complexes of different subunit composition can be isolated using different purification procedures. The Mediator complexes usually exist in two forms: larger complexes such as CDK8-Mediator and T/G-Mediator (2.0 MDa) and smaller complexes such as core Mediator (1.2 MDa). These two forms of Mediator complexes regulate transcription positively or negatively. The CDK8-Mediator complex and the T/G-Mediator complex have been shown to have kinase and/or acetyltransferase activities, but whether additional enzyme activities are present is far from clear. During my thesis work I studied assorted enzymatic activities of T/G-Mediator by various approaches. The results indicate that the Mediator complexes may possess additional enzymatic activities, such as methyltransferase and ubiquitin ligase function. Here, we

demonstrate that T/G-Mediator can methylate histone H3 and also that some Mediator subunit(s) are associated with the ubiquitination pathway.

RNA polymerase II coordinates with a wide range of factors and catalyzes DNA transcription to synthesize mRNA. One critical step of transcription initiation is the interaction between the Mediator complex and the carboxy-terminal domain (CTD) of the largest subunit of human RNA polymerase II that consists of 52 repeats of the general consensus sequence YSPTSPS. This Mediator-Pol II CTD interaction is believed to be important in transcriptional regulation, RNA processing and chromatin remodeling. Therefore, elucidating the mechanism of this interaction is crucial for understanding gene expression and developing potential clinical applications. Our lab used a chemical crosslinking method in order to identify which Mediator subunit(s) interact with CTD. The results indicated that there might be two or three Mediator subunits bound to the Pol II CTD. Western blot experiments using antibodies specific for Mediator subunits revealed that Med1 and Med23 likely interact with CTD. In addition, *in vitro* binding assays show that Med1 binds to the first half of CTD (repeats 1-24), but not to the second half (repeats 25-52). The molecular details of the interaction need to be explored using mass spectrometry along with combined approaches of molecular biology and biochemistry.

Acknowledgements

First and foremost I am heartily thankful to my supervisor, Dr. Dylan Taatjes, who has supported me and helped me better understand lots of things about research throughout my thesis with his patience and knowledge. Also, I need to acknowledge all Taatjes Lab members: Jack, Heeyoun, Matt, Chris, Carrie, Hong, Jesse, Carly, Vanessa, Zach, Krista and Autumn for teaching me more or less about experimental details. We are not only labmates, but also friends.

I express my special thanks to the Liu Lab for providing me some chemical reagents and suggestions of experimental procedures. Furthermore, I truly appreciate Dr. Xuedong Liu and Dr. Amy Palmer's advice of my thesis defense. Lastly, I would like to thank my family for its constant support and encouragement to help me go through struggles.

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Chapter 1

Introduction

Proper regulations of transcription and other nuclear events are required for mammalian cell growth, development and survival. Transcription in Eukaryotic cells is a complex biological process that requires numerous factors, including RNA polymerase II, general transcription factors (TFIIA, TFIIB, TFIID, TFIIE, TFIIF, and TFIIH), chromatin remodeling factors, gene-specific transcriptional regulators (activators and repressors), and Mediator (Lewis and Reinberg, 2003; Myers and Kornberg, 2000; Rachez and Freedman, 2001). The Mediator complex is a vital regulatory complex that bridges signals between transcription factors and the general transcription machinery. Detailed study of the Mediator complex will significantly help us understand gene expression and related human diseases.

1.1 Mediator

Mediator is a large multi-subunit complex and was first discovered from yeast (Kim *et al.*, 1994; Thompson *et al.*, 1993). Mediator is required for transcription *in vivo* and regulates activator-dependent transcription *in vitro* (Knuesel *et al.*, 2009; Meyer *et al.*, 2010; Takagi and Kornberg, 2006). Functionally distinct Mediator complexes with different subunit composition can be specifically purified (Bourbon *et al.*, 2004; Conaway *et al.*, 2005; Myers and Kornberg, 2000; Taatjes, 2010). The mammalian Mediator complexes purified in different research groups have diverse yet overlapping subunit compositions (Table 1.1) (Conaway *et al.*, 2005; Jiang *et al.*, 1998, Meyer *et al.*, 2008), and interestingly no known

enzyme activity is associated with Mediator except for acetylation and phosphorylation by T/G-Mediator and CDK8-Mediator (Knuesel *et al.*, 2009; Meyer *et al.*, 2008; Taatjes, 2010).

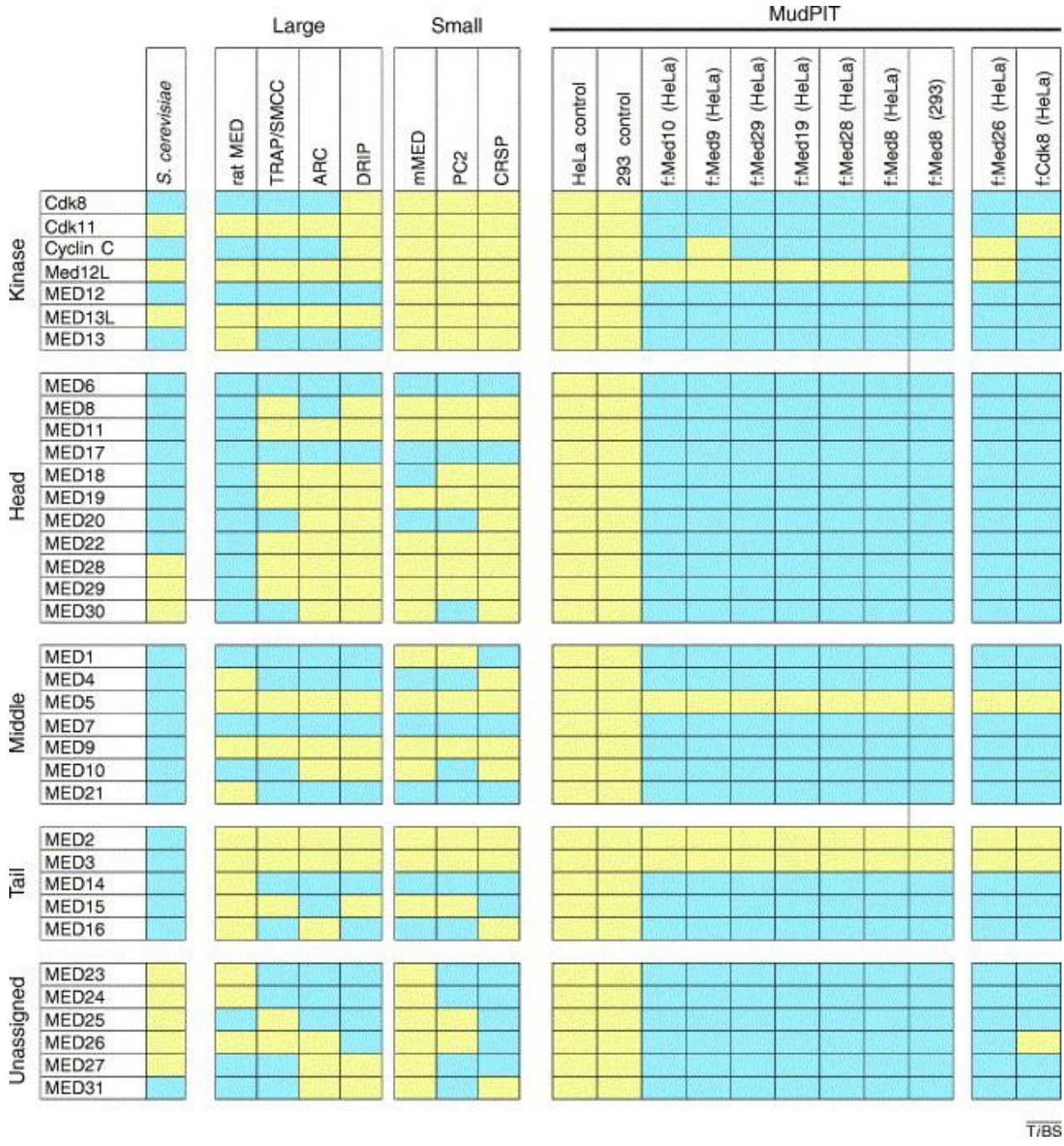

Table 1.1 Comparison of mammalian Mediator-like complexes. Mediator subunits identified in different purifications are indicated with blue; Mediator subunits unidentified are indicated with yellow. Subunits are classified as the head, middle, tail, or unassigned modules (Conaway *et al.*, 2005). Mammalian Mediator complexes and subunit compositions are listed on the left of the table. The subunit compositions of Mediator complexes from HeLa or 293 cells are identified by immunoaffinity chromatography and MudPIT on the right of the table (Boube *et al.*, 2002; Conaway *et al.*, 2005; Sato *et al.*, 2004).

Mediator is essential for expression of protein-coding genes, and plays an important role in activation and repression of eukaryotic transcription. The exact mechanism by which Mediator regulates transcription has not been completely understood. In summary, Mediator serves as a regulatory target to activate transcription by direct interaction with DNA binding transcription factors. Furthermore, substantial evidence reveals that Mediator also can stimulate basal transcription (Baek *et al.*, 2002; Mittler *et al.*, 2001; Taatjes, 2010). Some biochemical studies partially depict many interactions between different activators and Mediator subunits, and also demonstrate how critical some Mediator subunits are in cell development (Table 1.2) (Lewis and Reinberg, 2003).

Human mediator subunit (yeast)	Activator	Mutant subunit defects
MED240 (Srb9)		Eye-antennal disc development and sex-comb identity function (*Drosophila*)
MED230 (Srb8)		See MED240
MED220 (Med1)	TR, VDR, GR, RARα RXRα, PPARγ, ER, AR	Embryonic lethal (mouse); adipocyte-specific differentiation defects (mouse)
MED150 (Rgr1)		
MED130 (Gal11)	E1A	*C. elegans* Sur2: vulval developmental defects via RAS/MAP kinase pathway
MED105 (Med9)		
MED100		Embryonic lethal (mouse)
MED97		
MED95 (Sin4)		
MED78 (Srb4)	p53, VP16	Essential; sex-comb identity defect in combination with dMED240 (*Drosophila*)
MED70 (Med2)		
CDK8 (Srb10)		
MED36 (Med4)		
MED34 (Med7)		Necessary for expression of developmentally regulated genes (*C. elegans*)
MED33 (Med6)		Embryonic lethal (*C. elegans*); necessary for expression of developmentally regulated genes (*C. elegans*)
Cyclin C (Srb11)		
MED28b (Srb5)		
MED17 (Srb7)		Embryonic lethal (mouse)
MED10 (Med10)		Necessary for expression of developmentally regulated genes (*C. elegans*)
(Surf5) (Srb6)		

Table 1.2 Human Mediator complex. Listed in the columns are subunits of human Mediator complex (left), activators interacting with diverse subunits (middle), and defects of the subunit mutations (right) (Lewis and Reinberg, 2003).

1.2 RNA polymerase II Rpb1 CTD structure and function

RNA polymerase II (Pol II) is an essential enzyme for transcription of protein-coding genes in eukaryotic cells. The carboxy-terminal domain (CTD) of the largest subunit Rpb1 of RNA Polymerase II consists of repeating seven amino acid motif $Y_1S_2P_3T_4S_5P_6S_7$. The number of heptapeptide repeats varies depending on the genome complexity. *Drosophila*

CTD has 43 repeats, yeast CTD has 26 repeats, and human CTD has 52 repeats (Figure 1.1) (Palancade and Bensaude, 2003).

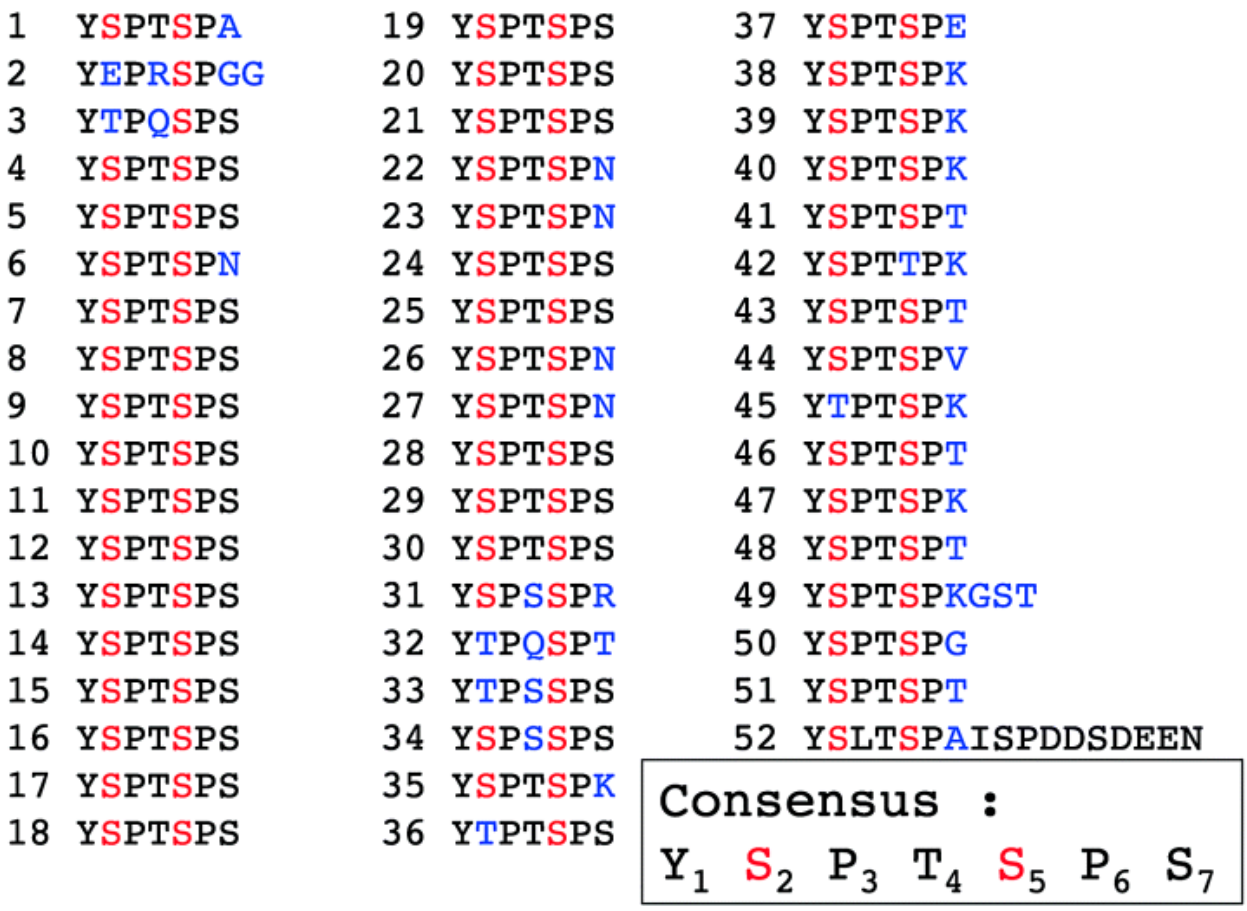

Figure 1.1 The human RNA Pol II CTD sequence (Palancade and Bensaude, 2003)**.**

The Pol II CTD is an inherently unstructured tail-like extension but is highly conserved from yeast to mammals during evolution (Phatnani and Greenleaf, 2006), suggesting its fundamental importance in transcription and regulation. The Pol II CTD is essential for organization of transcription, pre-mRNA processing and chromatin remodeling (Gerber *et al.*, 1995; Hirose and Manley, 2000; Hirose and Ohkuma, 2007; Howe, 2002; Misteli, 2000;

Proudfoot *et al.*, 2002). The partial loss of the CTD repeats causes cell damage, even lethality (Meinhart *et al.*, 2005; Phatnani and Greenleaf, 2006).

The free Pol II CTD structure is highly flexible. NMR studies proposed that the CTD has a population of β-turns at SPXX motifs in the CTD repeats (Meinhart *et al.*, 2005). The CTD serves as a flexible platform to coordinate various nuclear factors for proper regulation of gene expression (Meinhart *et al.*, 2005; Phatnani and Greenleaf, 2006). Indeed, a Pol II CTD repeat has five potential phosphorylation sites (Y1, S2, T4, S5, S7); however, CTD phosphorylation occurs mainly at S2 and S5, which is specifically recognized by different associated factors (Meinhart *et al.*, 2005). The distribution of Pol II CTD phosphorylation plays a major role in transcription and regulation *in vivo*. The dynamic changes in serine phosphorylation of the CTD, referred to as the "CTD code" in regulation of gene expression, are implicated in integrating multiple nuclear events (Figure 1.2) (Phatnani and Greenleaf, 2006).

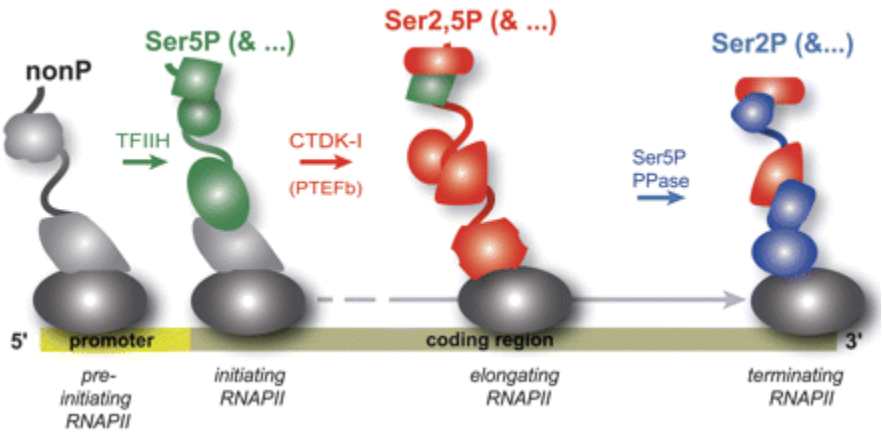

Figure 1.2 RNA Pol II CTD phosphorylation patterns with associated factors (Phatnani and Greenleaf, 2006).

Various kinases have been reported to phosphorylate the CTD (Brès *et al.*, 2008; Govind *et al.*, 2010; Knuesel, *et al.*, 2009; Rother and Strasser, 2007), such as CDK7, CDK8 and CDK9. Under the control of site-specific CTD kinases and phosphatases, the phosphorylation pattern changes during the transcription cycle, which leads to recruitment of specific transcription factors (Phatnani and Greenleaf, 2006). Biochemical studies revealed that CTD phosphorylation at S2 and S5 is functionally different; however, the specificity of CTD phosphorylation remains elusive. Mass spectrometry might be the best tool for the determination of the exact CTD phosphorylation pattern (Geromanos *et al.*, 2000; Han *et al.*, 2006; Hansen and Pergantic, 2006; Haydon *et al.*, 2003).

1.3 RNA Pol II CTD-Mediator interaction

The human Mediator complex is a large multi-protein coactivator that supports transcriptional activation. Mediator serves as a regulatory target of a number of transcriptional factors and interacts with RNA polymerase II, forming a tight and functional complex with distinct conformational states (Lewis and Reinberg, 2003; Myers and Kornberg, 2000; Näär *et al.*, 2002; Taatjes *et al.*, 2004). Mediator usually regulates transcription by bridging signals between transcriptional factors and basal transcriptional machinery. Reversible associations of different nuclear factors with Mediator strongly affect how Mediator regulates transcription (Figure 1.3) (Taatjes, 2010). Significantly, Mediator undergoes a distinct conformational shift when bound to specific nuclear factors, which plays a key role in recruitment mechanisms (Meyer *et al.*, 2010; Taatjes *et al.*, 2002).

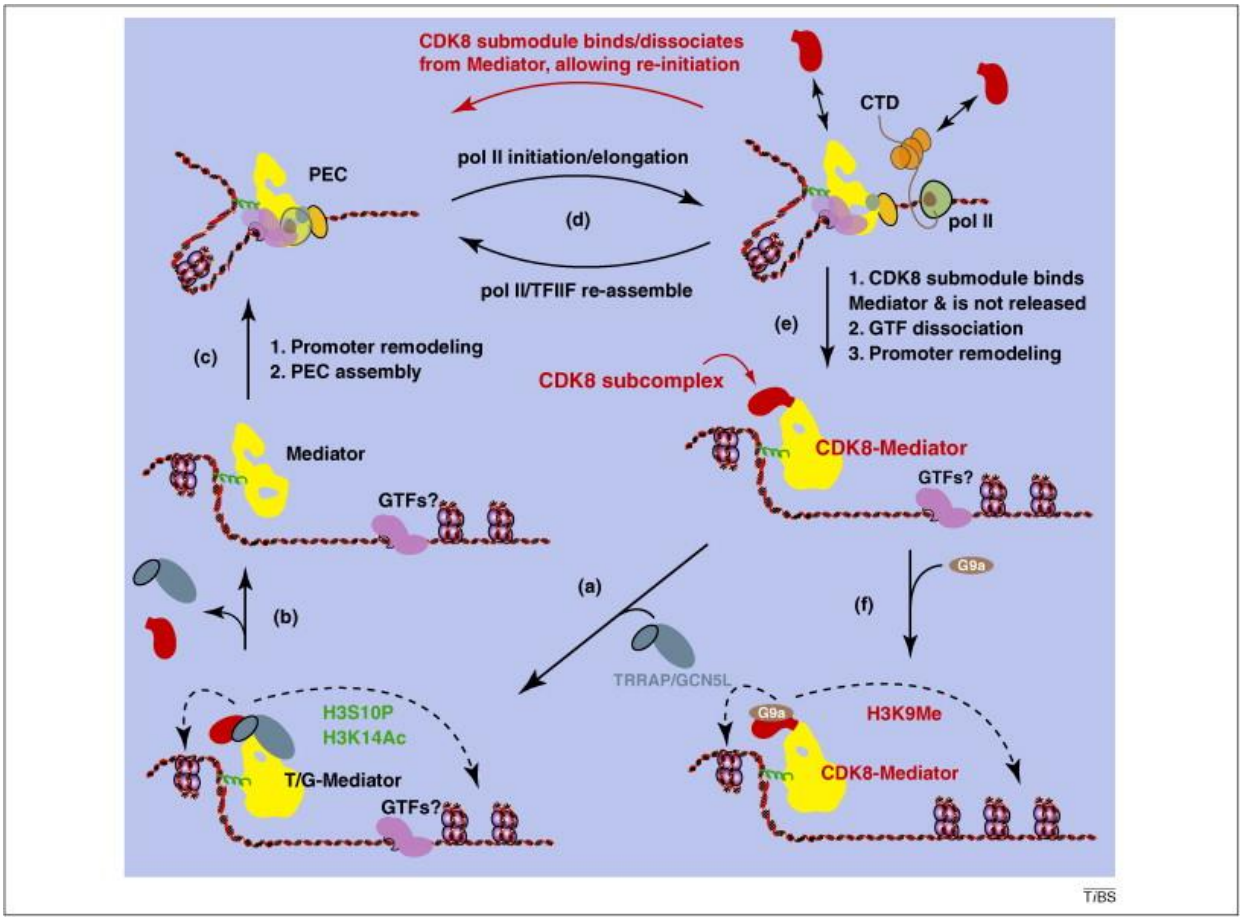

Figure 1.3 Mediator regulates transcription (Taatjes, 2010).

A critical step of transcription initiation is the interaction between Mediator and the unphosphorylated Pol II CTD. The CTD-Mediator interaction is essential for Mediator functions in transcriptional regulation (Meinhart *et al*., 2005). In the absence of the CTD repeats, Mediator cannot stimulate transcription. In fact, transcription will not occur without the Pol II CTD. Studies from electron microscopy along with 3D reconstruction revealed that Mediator usually consists of three distinct domains, termed head, middle and tail (Dotson *et al.*, 2000). The Pol II CTD may bind between the head and middle domains (Kang *et al.*, 2001; Meinhart *et al*., 2005). To date, it is believed that Mediator

strongly binds the unphosphorylated CTD, and CTD phosphorylation dissociates this interaction (Max *et al.*, 2007). However, the Mediator subunit(s) that interacts with the Pol II CTD remains unknown. Identification of CTD-interacting subunit(s) will greatly help elucidate relevant mechanisms of the CTD-Mediator interaction, and furthermore better understand how the CTD functions in transcription.

1.4 Enzymatic activities of Mediator

A protein is often chemically modified by various enzymes after translation, called post-translational modification, which is an important step in biological regulation. The posttranslational modifications of amino acids greatly extend the diversity of protein functions by covalently attaching chemical functional groups, such as phosphate, acetate, or a methyl group. The posttranslational modifications can change the properties of the protein, and often correlate with structural changes. Enzymes attach these covalent modifications, which can be classified into different types, such as phosphorylation, methylation, ubiquitination and acetylation. These chemical modifications are reversible by the action of a modifying enzyme that possesses the opposite role.

Subunits of the core Mediator complex possess no identifiable functional motifs for enzymatic activities according to bioinformatics studies (Taatjes, 2010). However, it was surprising that there are little known enzymatic activities in Mediator subunits, because Mediator has important functions in cells, such as serving as a target site recognized by nuclear factors and facilitating assembly and disassembly of the cellular complexes (Ebmeier and Taatjes, 2010; Meyer, *et al.*, 2010; Myers, *et al.*, 1999; Tóth-Petróczy, *et al.*, 2008). Recently, substantial evidence has revealed that large Mediator complexes function as

enzymes, and some Mediator subunits are involved in protein modifications in biological pathways (Akoulitchev *et al.*, 2000; Brower, *et al.*, 2002; Knuesel, *et al.*, 2009; Meyer, *et al.*, 2008). It is possible that some subunits of this large and highly conserved protein complex Mediator may possess enzymatic activities.

1.5 Histone modification

Histone proteins are highly basic and were discovered in eukaryotic cell nuclei, packaging the DNA into chromatin with around 10,000 fold DNA compacting. The core histones (H2A, H2B, H3 and H4) are structurally similar and highly conserved in evolution. Histones are essential in gene regulation (Grant, 2001; Luger *et al.*, 1997; Wu and Grunstein, 2000).

It is well known that post-transcriptional modifications of histones play a significant role in nuclear events, which facilitates biological process such as DNA repair, replication and transcription (Brian *et al.*, 2000; Grant, 2001; Singh *et al.*, 2010). An unstructured amino-terminal tail of the core histone provides many sites for a variety of posttranslational modifications, including acetylation, phosphorylation, methylation and ubiquitination (Figure 1.4) (Higashi *et al.*, 2010; Kouzarides, 2007; Meyer *et al.*, 2008).

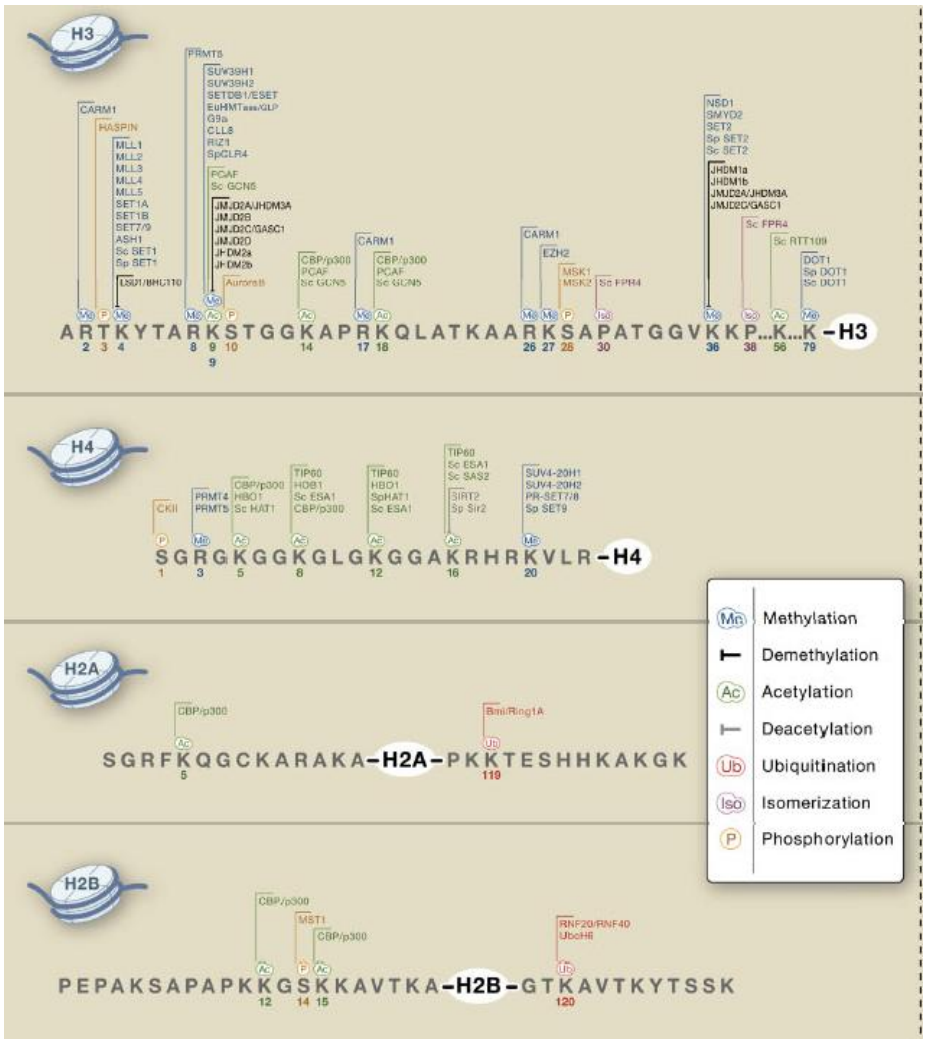

Figure1.4 Histone-modifying enzymes (Kouzarides, 2007).

Clearly the histone modifications demonstrate the interactions between histones and DNA or other nuclear factors, which regulates chromatin structure. Identification of the multitude of histone modifications and enzymes generating them fundamentally help us to understand replication and transcription.

1.6 Statement of Purpose

The first purpose of this thesis is to uncover RNA Pol II CTD-bound Mediator subunits, and further determine the interaction mechanism. Apparently Pol II CTD-Mediator plays a key role in transcription regulation, however, little is known about the interaction details. Here, a chemical crosslinking method is employed to determine which Mediator subunit can bind the Pol II CTD. The bait protein Pol II CTD must have a free sulfhydryl group and react with the crosslinking reagent, therefore, several cysteine mutants of Pol II CTD were made in different positions of the repeats. After crosslinking, Pol II CTD-interacting Mediator subunits were disassociated by DTT and urea treatments, and identified by Western blot and mass spectrometry. Elucidating the mechanism of the Pol II CTD-Mediator interaction will provide important insight into how transcription is specifically regulated.

The second purpose of this thesis was to study potential new enzymatic activities of the Mediator complexes. Protein sequence analysis of the core Mediator subunits indicates that there are no known enzymatic domains. But it is possible that the Mediator complexes harbor enzymatic activities that cannot be predicted based upon polypeptide sequence alone. T/G-Mediator purified from HeLa nuclear exacts is utilized to characterize potential Mediator enzymatic activities, such as phosphorylation, methylation and ubiquitination. Pol II CTD and histone proteins are essential in transcription, and these proteins are employed as the substrates modified by the Mediator complex. The major goal of the second project is to identify whether the purified Mediator complex possesses enzymatic activities, and which subunit catalyzes the modification, and what's the modification profile. Discovering potential

enzymatic activities of the Mediator complexes will significantly help us to better understand the scope of Mediator's functions in transcription and regulation.

Chapter 2

Materials and Methods

2.1 Expression and purification of various recombinant proteins from bacteria

Human RNA Pol II CTD (repeats 1-52) and its deletion and cysteine mutants were subcloned into the Glutathione S-transferase gene fusion vector pGEX-4T-1 (Figure 2.1) (GE healthcare). We got all CTD constructs from the Tjian lab at the University of California at Berkeley. All CTD plasmids were transformed into BL21 cells. Individual fresh colonies were picked from LB agar plates containing antibiotics, and grown overnight for around 16 hours in the 37 ℃ shaker. Each 5ml overnight culture was added to one liter of fresh LB media contain 100 mg/ml ampicillin. Flasks were shaken at 37 ℃ until the $(OD)_{600}$ reached 0.6 to 0.8. Then 0.5 mM isopropyl- β -D-thiogalactopyranoside (IPTG) was added into the flask, and induced gene expression for 6 to 8 hours at 30 ℃. For other proteins such as GST-SREBP, IPTG-induction time was 3 to 4 hours. Cells were harvested at 5,000 RPM for 15 minutes at 4 ℃. The supernatants were removed and the cell pellets were placed at -80 ℃ freezer after washing using 1x PBS with protease inhibitors.

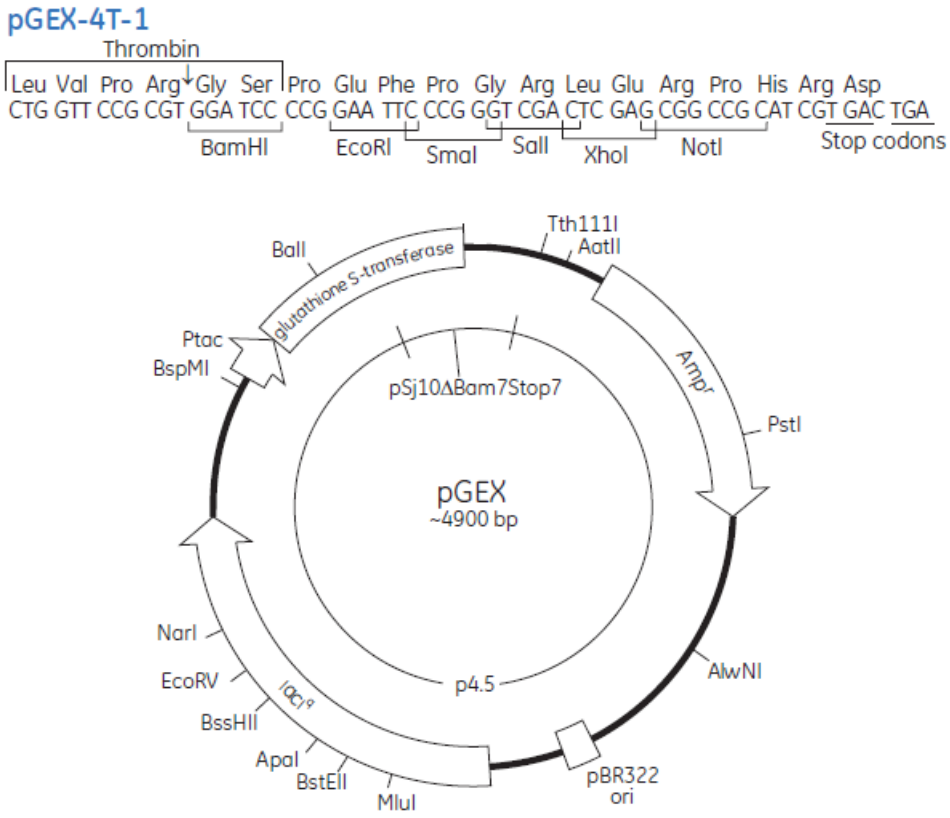

Figure 2.1 Map of the Glutathione S-transferase gene fusion vector pGEX-4T-1 (GE healthcare).

The frozen cell pellets were thawed and resuspended in 0.15M HEGN buffer (50mM HEPES pH7.6, 0.15M KCl, 0.1mM EDTA, 10% glycerol, 0.02% NP-40) with protease inhibitors (1mM DTT, 1 mM Benzamidine, 0.25 mM PMSF, 1 mM Sodium Metabisultate, 1x Aprotinin) on ice. The cells were lysed by sonication. Lysates were cleared through centrifugation at 40,000 RPM for 30 minutes at 4 ℃. The supernatants were incubated with pre-equilibrated glutathione sepharose 4B beads (GE healthcare) for an hour at 4 ℃. The beads were washed five times with high salt buffer (50mM Tris-HCl pH7.9, 1M NaCl, 8mM CHAPS, 0.5mM EDTA, 10% glycerol, 0.5% NP-40), and then washed twice with 0.15M

HEGN. Finally, GST tagged CTD proteins were eluted using elution buffer (20 mM Tris, 0.1 mM EDTA, 10% glycerol, 0.15M KCl) containing fresh 30mM GSH. The purified CTD proteins were dialyzed in 0.15M HEGN buffer at 4 ℃, and snap frozen and stored in small aliquots at -80 ℃ freezer.

2.2 Purification of Mediator from HeLa cells

Different Mediator complexes with distinct subunit composition are purified and characterized according to various preparation procedures. Both the core and T/G-mediator complexes were purified from HeLa cells (Figure 2.2). HeLa nuclear exacts were loaded into a pre-equilibrated P11 phosphocellulose column (Whatman). 0.5 M HEGN (20 mM HEPES pH 7.9, 0.1 mM EDTA, 10% glycerol, 0.1% NP-40, 0.5 M KCl) and 1 M HEGN (20 mM HEPES pH 7.9, 0.1 mM EDTA, 10% glycerol, 0.1% NP-40, 1 M KCl) elutes (P0.5M and P1M) were dialyzed against 0.15 M HEGN to decrease the salt content. P0.5M flow-through fractions through HiTrap Q column were incubated with GST-SREBP bound GSH resins after ammonium sulfate precipitation, and finally the T/G-Mediator complexes were obtained after the GSH elutes were run on a linear 15%-40% glycerol gradient at 55,000K RPM for 6h at 4 ℃. P1M were loading into Poros Q column, and 1M HEMG (20 mM HEPES pH 7.9, 0.1 mM EDTA, 10% glycerol, 2mM $MgCl_2$, 1 M KCl) elutes (Q1M) were incubated with GST-SREBP bound GSH resins. After pull-down assays, the core Mediator complexes were obtained from 15%-40% glycerol gradient fractions of the GSH elutes after 55,000 RPM spinning for six hour at 4 ℃. Both gradient fractions were collected from the top of ultracentrifuge tubes in 100 μl aliquots. Both Mediator complexes' profiles were examined by silver stained gel.

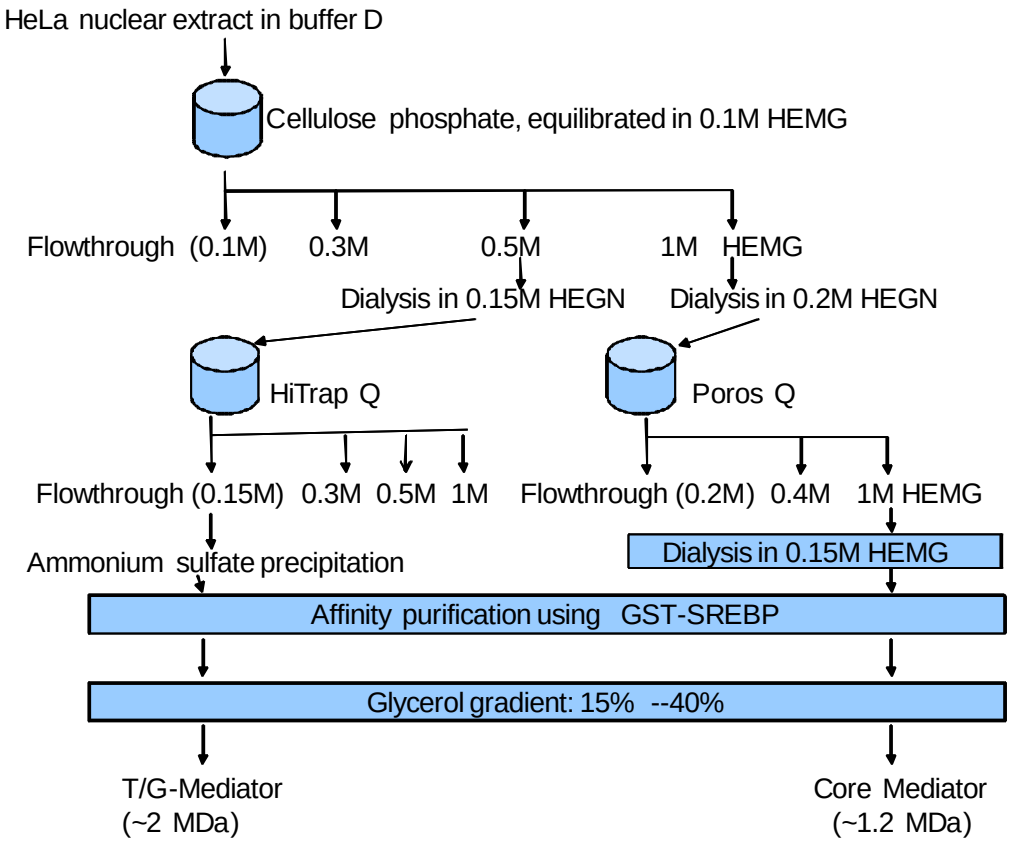

Figure 2.2 Purification schemes of the endogenous core and T/G-Mediator

2.3 Identification of Pol II CTD-interacting Mediator subunits using chemical crosslinking

Identification of protein-protein interactions is critical for understanding the dynamics of cellular signaling. Chemical crosslinking is a powerful and effective method to study the interactions. Generally, chemical crosslinkers can work for both transient and stable protein-protein interactions. There are many chemical crosslinking reagents commercially available.

The Mts-Atf-Biotin label transfer reagent from Pierce was utilized to study RNA Pol II CTD-Mediator interaction. This reagent has three functional groups: biotin group that can transfer to the unknown interacting protein for affinity detection and purification, methanethiosulfonate (Mts) group that is sulfhydryl-reactive and can label the bait protein in the reduced cysteine residue, and tetrafluorophenyl azide reactive group that is activated by UV light and can nonspecifically attach to the side chain and backbone of the interacting protein (Figure 2.3).

Molecular Weight: 839.95
Biotin-Mts Spacer Arm: 29.3 Å
Biotin-Atf Spacer Arm: 30.7 Å
Mts-Atf Spacer Arm: 11.1 Å

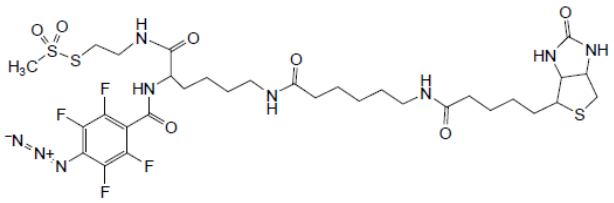

Figure 2.3 Mts-Atf-Biotin (2-[*N2*-(4-azido-2,3,5,6-tetrafluorobenzoyl)-*N6*-(6-biotinamidocaproyl)-L-lysinyl] ethyl methanethiosulfonate) (Pierce).

Here, for crosslinking experiments, the bait protein is the CTD cysteine mutants, and the prey protein is the unknown subunits of the Mediator complex. The crosslinking experiments were carried out according to the following procedure modified from the manufacturer's instructions (Figure 2.4): prepared the GSH resins containing distinct CTD cysteine mutants according to the procedure 2.1, and incubated the GST-CTD cysteine mutant bound GSH resins in 1x PBS (0.1 M phosphate, 0.15 M NaCl; pH 7.2); dissolved 1-2 mg Mts-Atf-Biotin reagent in 25-50 µl of dimethyl sulfoxide (DMSO), and added the reagent into the tube to achieve around five fold molar excess over the CTD cysteine mutants, and gently mixed and incubated for 4 hours at 4 ℃; removed free crosslinking reagent by washing with

1x PBS; thrombin digested to release labeled CTD cysteine mutants from the GST tag, and used NeutrAvidin beads to capture the biotin-labeled bait protein, and washed the beads with 1x PBS; added the Mediator complexes (Dialyzed P1M elutes) into the biotin-labeled bait protein tube, and rocked the mixture for 4 hours in cold room, and washed with 0.5M PBS (0.1 M phosphate, 0.5 M NaCl; pH 7.2); photoactivated the Atf moiety with a UV light at 300-370 nm; reduced disulfide bond using 50 mM DTT and dissociated the Mediator complex using 3 M Urea; finally, loaded the biotin-modified interacting Mediator subunits on SDS-PAGE gels, and detected by silver stain and Western blot. Most of the above steps were conducted in subdued light or in the dark at 4 ℃.

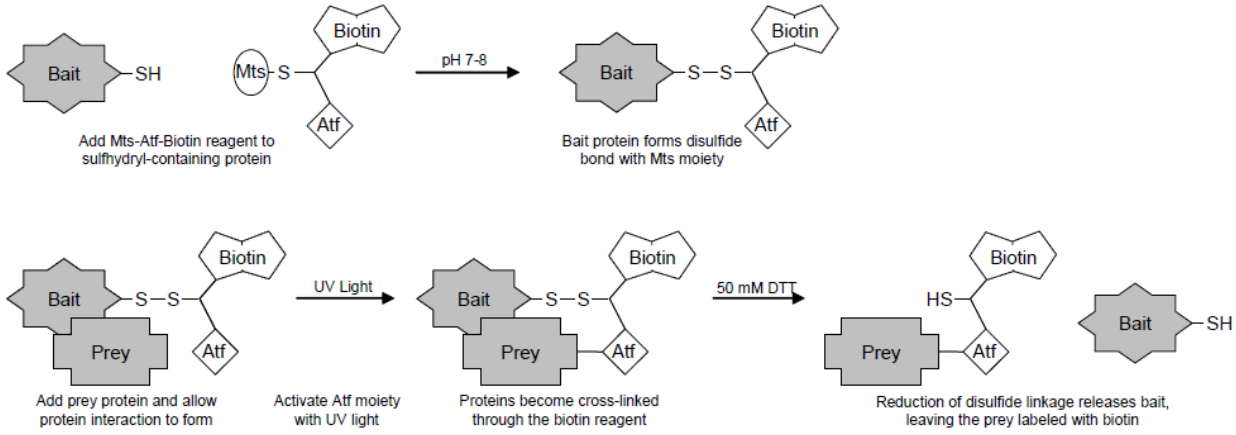

Figure 2.4 Chemical crosslinking and label-transfer scheme (Pierce).

2.4 *In vitro* enzymatic activity assays (Kinase, methyltransferase, and ubiquitin ligase) by Mediator

In vitro modification assays are powerful tools to test protein's enzymatic activities. These assays use highly purified components for distinct tests, which is easier to handle. In

this thesis, I tested three Mediator complexes' enzymatic activities: phosphorylation on RNA Pol II CTD, methylation and ubiquitination on histone proteins. Detailed procedures are summarized in the following sections.

2.4.1 *In vitro* kinase assay by the core and T/G-Mediator

Kinase assay used 2.5 μCi of γ-^{32}P ATP for 10 μl reaction, together with the Mediator complex and substrate in kinase buffer (25mM Tris-HCl pH 8.0, 0.1mM ATP, 0.1M KCl, 10mM MgCl2, 2mM DTT). Reaction usually incubated at 30 ℃ for 30 minutes, and then quenched with SDS loading buffer and separated on SDS-PAGE gel. The dried gel was exposed on the storage phosphor screen (GE healthcare) and scanned with the Typhoon 9400 (GE healthcare).

2.4.2 *In vitro* methylation assay by the T/G-Mediator complex

The methylation assay used 0.12-0.16 μCi of ^{14}C-SAM (Adenosyl-L-methionine, S-[methyl-^{14}C]) as the methyl donor in 25 μl of methyltransferase buffer (50mM Tris-HCl pH 9.0, 5mM MgCl2, 4mM DTT), together with the Mediator complex and substrate histone proteins made by Dr. Meyer in the Taatjes Lab. Reaction was carried out for an hour at room temperature. Protein samples were separated by SDS-PAGE. Coomassie blue-stained gel was use to check the equal loading of the substrate. Then the dried gel was exposed on the storage phosphor screen (GE healthcare) and scanned with the Typhoon 9400 (GE healthcare).

2.4.3 *In vitro* ubiquitination assay by the T/G-Mediator complex

The ubiquitination assays were performed by incubating the Mediator complex with 0.5 μM E1, 5 μM UbcH5a (E2), 5 μM ubiquitin (BostonBiochem), 100 μM methylated ubiquitin (BostonBiochem), 1 μM ubiquitin aldehyde (BostonBiochem) in reaction buffer (10 mM ATP, 20 mM HEPES pH 7.4, 10 mM MgOAc, 300 mM creatine phosphate, and 0.5 mg/ml creatine phosphokinase). The reactions were incubated for 2 hr at 30 ℃, and quenched by SDS loading buffer, and analyzed by SDS-PAGE. The samples were detected by Western blot using the antibody against ubiquitin. If Biotin-labeled ubiquitin was used, Streptavidin-HRP was employed to check the assay products.

Chapter 3

Results and Discussion

3.1 RNA Pol II CTD-Mediator interaction

Generally, the Pol II CTD is functionally conserved through evolution in eukaryotes, but the number of CTD repeats and the length of the CTD varies markedly across species. So far, the functional importance of the Pol II CTD remains poorly understood. There is an accumulation of evidence that the CTD-Mediator interaction is very dynamic and plays important roles in nuclear events. The Mediator complexes are strongly bound to the unphosphorylated CTD, and dissociate upon CTD phosphorylation (Max *et al.*, 2007; Meinhart *et al*., 2005; Phatnani and Greenleaf, 2006). The CTD-Mediator interaction facilitates transcription initiation and also mediates signals between activators or repressors and the general transcriptional machinery.

3.1.1 Preparation of GST tagged Pol II CTD first half, second half and full length

GST fused Pol II CTD first half (repeats 1-24), the second half of the CTD (repeats 25-52) and full length Pol II CTD (repeats 1-52) were successfully expressed and purified from *E. coli* BL21 cells for the CTD-Mediator interaction assay.

Mammalian RNA Pol II CTD consists of 52 repeats of the consensus sequence YSPTSPS, which is difficult for subcloning and purification. Interestingly, repeats 25-52 are more diverse than repeats 1-24 (Figure 1.1).

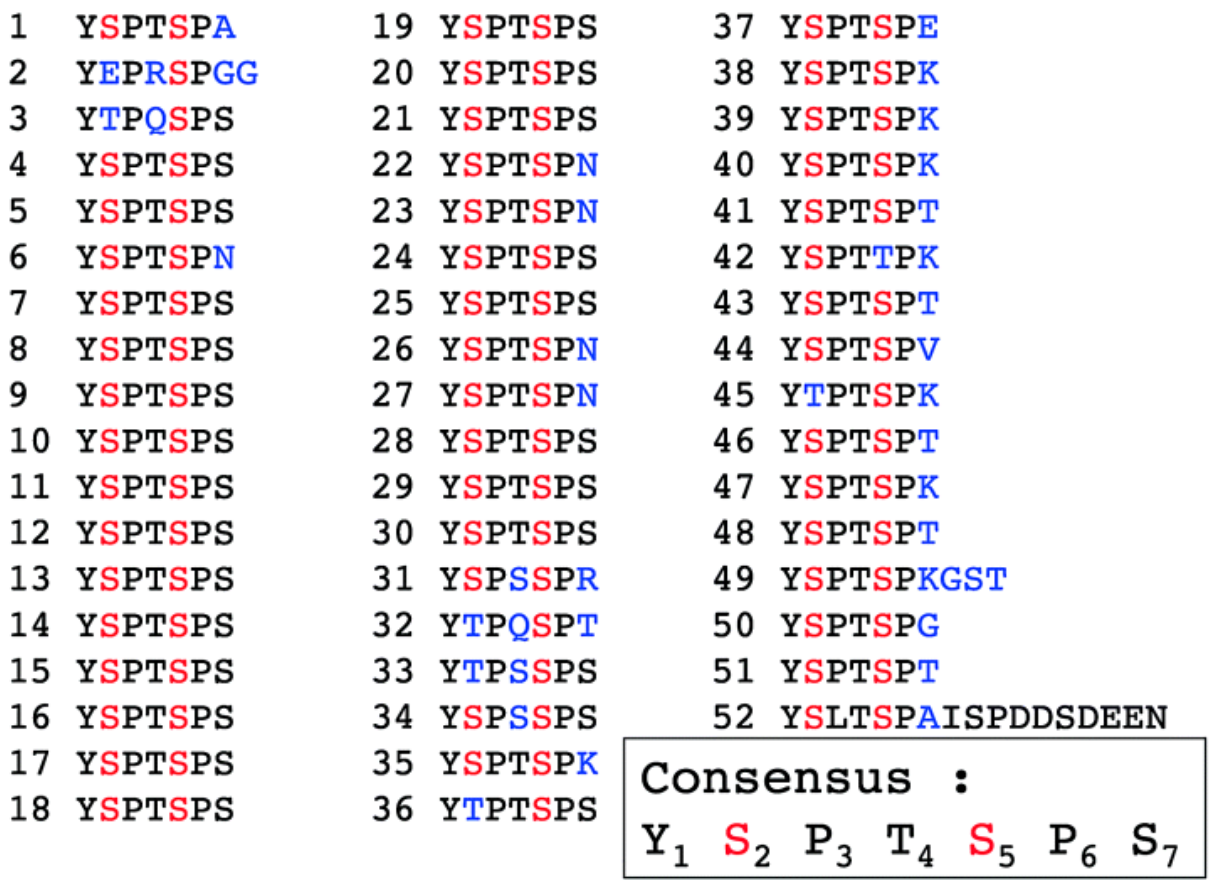

Figure 1.1 The human RNA Pol II CTD sequence (Palancade and Bensaude, 2003). Serine at position 7 is not conserved in the second half of Pol II CTD. Serines at position 2and 5are major phosphorylation sites indicated in red, and unconserved amino acids are indicated in blue.

What does this imply? Perhaps the first half is functionally different from the second half. To test the interaction with Mediator complexes, I prepared three proteins: CTD full length (repeats 1-52), the first half (repeats 1-24) and the second half of the CTD (repeats 25-52) (Figure 3.1).

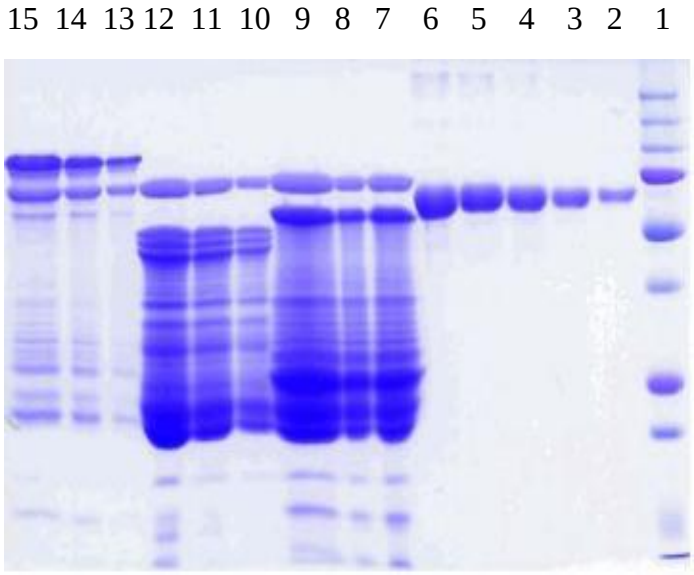

Figure 3.1 Purified Pol II CTD first half, second half, and full length by GST affinity chromatography. Lane 1:Precision plus protein dual color standards (Bio-Rad); lanes 2,3,4,5,6: BSA; lanes 7,8,9: GST-CTD (1-24); lanes 10,11,12: GST-CTD (25-52); lanes 13,14,15: GST-CTD full length.

3.1.2 Preparation of GST tagged Pol II CTD cysteine mutants

GST fused Pol II CTD cysteine mutants (CTDr24C, r31C, r38C, r52C) were successfully expressed and purified from BL21 cells for chemical crosslinking experiments.

The chemical crosslinking Mts-Atf-Biotin label transfer reagent (Pierce) was used to capture Pol II CTD-interacting Mediator subunits. This reagent requires the bait protein with a free sulfhydryl group reacted with the Mts moiety. The wild type CTD repeats don't have any free sulfhydryl group, therefore, four cysteine mutants (CTDr24C, r31C, r38C, r52C) of the Pol II CTD were made in the seventh amino acid residue of the repeat 24, 31, 38, 52, respectively. These Pol II CTD cysteine mutants were provided by the Tjian Lab at the

University of California at Berkeley. The CTD cysteine mutants were expressed and purified for the bait protein of chemical crosslinking experiments (Figure 3.2).

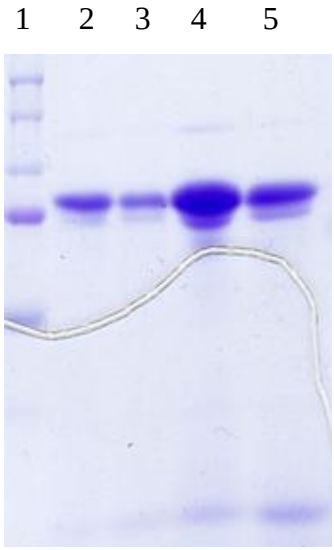

Figure 3.2 Purified Pol II CTD cysteine mutants by GST affinity chromatography. Lane 1: Precision plus protein dual color standards (Bio-Rad); lanes 2 and 4: GST-CTDr24C; lanes 3 and 5: GST-CTDr52C.

3.1.3 Preparation of untagged Pol II CTD cysteine mutants by Thrombin digestion

Untagged Pol II CTD cysteine mutants were obtained directly by Thrombin digestion on protein bound GSH resins. To enhance efficiency of chemical crosslinking and reduce interference of the GST tag, the endoprotease Thrombin (Sigma-Aldrich) was utilized to remove the tag directly from GST-CTD cysteine mutants bound GSH resins (Figure 3.3). The digestion was performed in PBS buffer for more than four hours in the cold room. Untagged Pol II CTD cysteine mutants interact with the Mediator complex for high quality crosslinking.

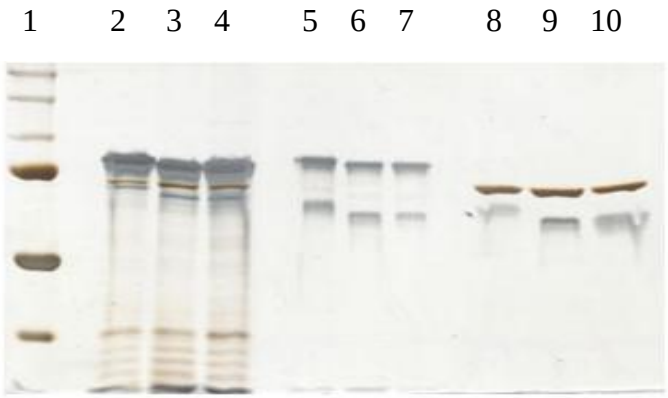

Figure 3.3 Thrombin digestion of GST-CTD cysteine mutants. Lane 1: Precision plus protein dual color standards (Bio-Rad); lanes 2: GST-CTDr31C; lanes 3: GST-CTDr38C; lanes 4: GST-CTDr52C; lanes 5: Thrombin digestion of GST-CTDr31C; lanes 6: Thrombin digestion of GST-CTDr38C; lanes 7: Thrombin digestion of GST-CTDr52C; lanes 8: CTDr31C; lanes 9: CTDr38C; lanes 10: CTDr52C.

3.1.4 Determination of the Mediator complex interacting with the first half or the second half of the Pol II CTD

Both the core and T/G-Mediator was confirmed to bind the first half and the second half of the Pol II CTD by GST pull-down assay.

Numerous data have shown that Pol II CTD full length strongly binds the Mediator complex. To investigate the Pol II CTD-Mediator interaction mechanism, it is good to know whether there is any difference between them because the second half of the Pol II CTD is more divergent than the first half. I did the *in vitro* binding assays between the Mediator complex and the first half or the second half of the Pol II CTD. Preliminary results showed that both the first half (repeats 1-24) and the second half (repeats 25-52) of the Pol II CTD can bind P0.5M containing larger Mediator complexes (Figure 3.4), and also to Q1M containing

the core Mediator complexes (Figure 3.5), like Pol II CTD full length. This result implies that there are multiple Pol II CTD-Mediator interaction sites or this interaction may be quite flexible.

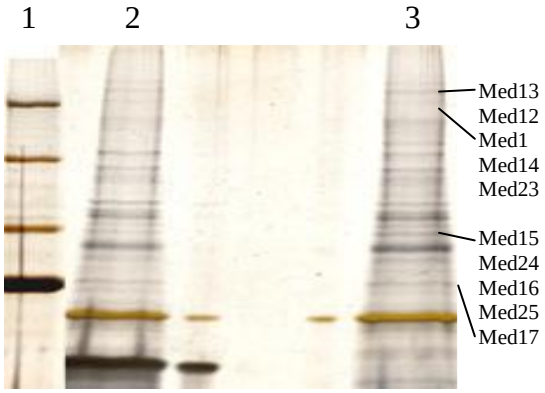

Figure 3.4 P0.5M interacting with the first half or the second half of the Pol II CTD.

Lane1: Precision plus protein dual color standards (Bio-Rad); lanes 2: P0.5M interacting with the first half of the Pol II CTD; lane 3: P0.5M interacting with the second half of the Pol II CTD.

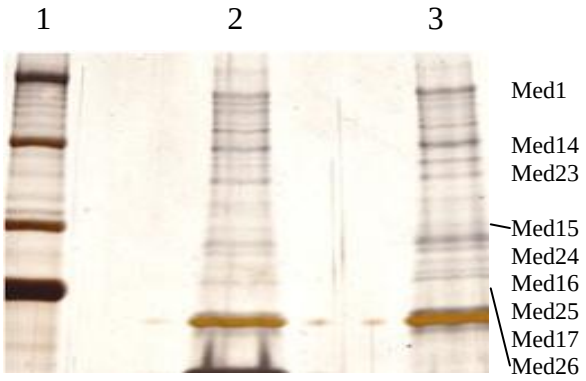

Figure 3.5 Q1M interacting with the first half or the second half of the Pol II CTD.

Lane1: Precision plus protein dual color standards (Bio-Rad); lanes 2: Q1M interacting with the first half of the Pol II CTD; lane 3: Q1M interacting with the second half of the Pol II CTD.

3.1.5 Identification of Pol II CTD-interacting Mediator subunits by chemical crosslinking

Med1 and Med23 were uncovered to be the Pol II CTD-interacting Mediator subunits using four different Pol II CTD cysteine mutants by chemical crosslinking and western blot .

One of critical steps in transcription is the RNA Pol II CTD-Mediator interaction. Here, the Mts-Atf-Biotin label transfer reagent was employed to capture RNA Pol II CTD-interacting Mediator subunits. First, two RNA Pol II CTD cysteine mutants GST-CTDr24C and GST-CTDr52C were labeled by the crosslinking reagent. After Thrombin digestion, NeutrAvidin beads were used to bind untagged Biotin-labeled Pol II CTD cysteine mutants, and then interacted with P1M containing the core Mediator complex. Following photo-activation, the Pol II CTD-interacting subunits were released from the bait protein and the Mediator complex by DTT and urea treatments. The crosslinking product was examined by silver stain (Figure 3.6) and Western blots.

1 2 3 4 5 6 7 8 9

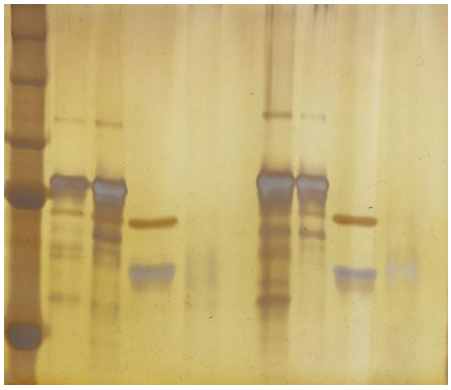

Figure 3.6 Silver-stained gel for the chemical crosslinking using CTDr24C and CTDr52C. Lane1: Precision plus protein dual color standards (Bio-Rad); lane 2: GST-CTDr24C; lane 3: labeled GST-CTDr24C; lane 4: labeled CTDr24C; lane 5: final crosslinking product after the CTDr24C-P1M interaction; lane 6: GST-CTDr52C; lane 7: labeled GST-CTDr52C; lane 8: labeled CTDr52C; lane 9: final crosslinking product after the CTDr52C-P1M interaction.

In the chemical crosslinking, the Biotin label can transfer to the interacting Mediator subunit. Therefore, Streptavidin-HRP was used to detect Biotin-labeled products with high affinity and sensitivity (Figure 3.7). Based on Streptavidin-HRP detection, the result was further examined by western blots using the antibody against the Mediator subunit Med9 or Med23 (Figure 3.8).

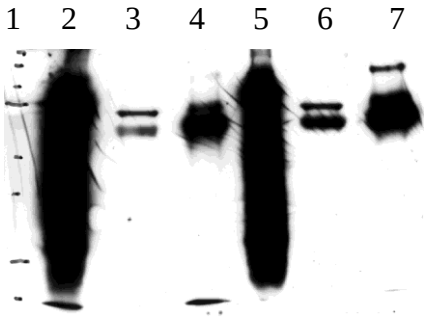

Figure 3.7 Streptavidin-HRP detection for the chemical crosslinking using CTDr24C and CTDr52C. Lane1: Precision plus protein dual color standards (Bio-Rad); lane 2: labeled GST-CTDr24C; lane 3: labeled CTDr24C; lane 4: final crosslinking product after the CTDr24C-P1M interaction; lane 5: labeled GST-CTDr52C; lane 6: labeled CTDr52C; lane 7: final crosslinking product after the CTDr52C-P1M interaction.

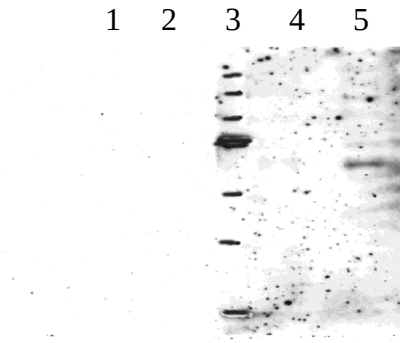

Figure 3.8 Immunoblots with the antibody against Med9 or Med23 for the chemical crosslinking using CTDr24C and CTDr52C. Lane 1: final crosslinking product after the CTDr24C-P1M interaction (Anti-Med9); lane 2: final crosslinking product after the CTDr52C-P1M interaction (Anti-Med9); lane3: Precision plus protein dual color standards (Bio-Rad); lane 4: final crosslinking product after the CTDr24C-P1M interaction (Anti-Med23); lane 5: final crosslinking product after the CTDr52C-P1M interaction (Anti-Med23).

The resulting amount of the crosslinking product is around silver staining detection limit and is hard to visualize. However, Streptavidin-HRP detection showed there are several specific bands ranging from 60 kD to 150 kD. Combined with the profile of the Mediator complex, two antibodies against Med9 and Med23 were used to examine these specific bands. The immunoblot results showed there's nothing for Med9 and there might have the ambiguous signal for Med23. Based on western blots, the crosslinking result using CTDr52C was more specific and better than the result using CTDr24C. Very likely the cysteine mutant position in the repeats affects the chemical crosslinking. I did the crosslinking experiment again using the other two cysteine mutants CTDr31C and CTDr38C as well as CTDr52C. I did the same crosslinking procedure as indicated above for CTDr31C, CTDr38C, and CTDr52C. Then the results were first detected by Streptavidin-HRP (Figure 3.9).

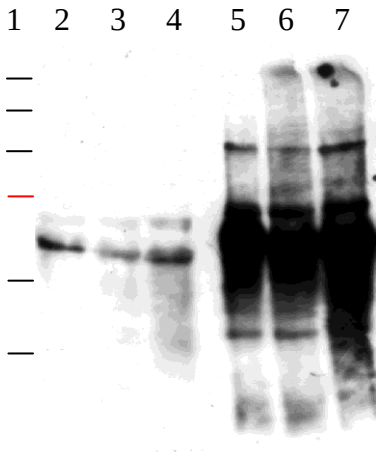

Figure 3.9 Streptavidin-HRP detection for the chemical crosslinking using CTDr31C, CTDr38C and CTDr52C. Lane1: Precision plus protein dual color standards (Bio-Rad); lane 2: labeled CTDr31C; lane 3: labeled CTDr38C; lane 4: labeled CTDr52C; lane 5: final crosslinking product after the CTDr31C-P1M interaction; lane 6: final crosslinking product after the CTDr38C-P1M interaction; lane 7: final crosslinking product after the CTDr52C-P1M interaction.

The data shown in figure 3.9 indicated there were clearly several particular bands. Next, according to the Mediator subunit composition, three antibodies against Med1, Med23, and Med26 were specifically selected to identify these potential interacting subunits (Figures 3.10, 3.11, and 3.12).

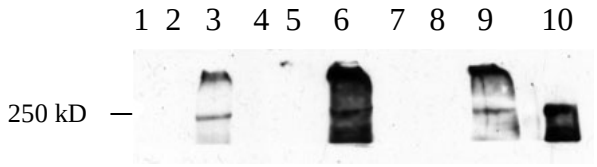

Figure 3.10 Immunoblots with the antibody against Med1 for the chemical crosslinking using CTDr31C, CTDr38C and CTDr52C. Lane 1: labeled GST-CTDr31C; lane 2: labeled CTDr31C; lane 3: final crosslinking product after the CTDr31C-P1M interaction; lane 4: labeled GST-CTDr38C; lane 5: labeled CTDr38C; lane 6: Final crosslinking product after the CTDr38C-P1M interaction; lane 7: labeled GST-CTDr52C; lane 8: labeled CTDr52C; lane 9: final crosslinking product after the CTDr52C-P1M interaction; lane 10: P1M.

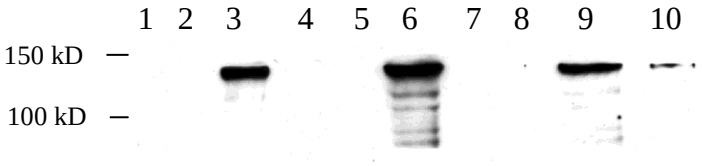

Figure 3.11 Immunoblots with the antibody against Med23 for the chemical crosslinking using CTDr31C, CTDr38C and CTDr52C. Lane 1: labeled GST-CTDr31C; lane 2: labeled CTDr31C; lane 3: final crosslinking product after the CTDr31C-P1M interaction; lane 4: labeled GST-CTDr38C; lane 5: labeled CTDr38C; lane 6: final crosslinking product after the CTDr38C-P1M interaction; lane 7: labeled GST-CTDr52C; lane 8: labeled CTDr52C; lane 9: final crosslinking product after the CTDr52C-P1M interaction; lane 10: P1M.

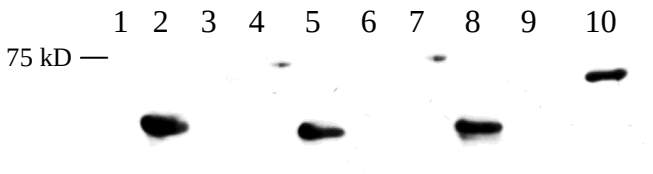

Figure 3.12 Immunoblots with the antibody against Med26 for the chemical crosslinking using CTDr31C, CTDr38C and CTDr52C. Lane 1: labeled GST-CTDr31C; lane 2: labeled CTDr31C; lane 3: final crosslinking product after the CTDr31C-P1M interaction; lane 4: labeled GST-CTDr38C; lane 5: labeled CTDr38C; lane 6: final crosslinking product after the CTDr38C-P1M interaction; lane 7: labeled GST-CTDr52C; lane 8: labeled CTDr52C; lane 9: final crosslinking product after the CTDr52C-P1M interaction; lane 10: P1M.

Western blot results revealed that both Med1 and Med23 might be RNA Pol II CTD-interacting Mediator subunits, but Med26 was not. Three different Pol II CTD cysteine mutants had the same results, which further proved that this interaction may have multiple binding sites. Absolutely, more experiments are needed for verifying the interacting subunits, such as using mass spectrometry to identify the crosslinking products, generating recombinant proteins for *in vitro* binding assays, and using different chemical crosslinking reagents.

3.2 Examining enzymatic activities of Mediator

Based on protein sequence analysis, subunits of the core Mediator possess no predicted functional motifs. However, it is hard to believe it because Mediator is a large 1.2 MDa multi-protein complex and different Mediator complexes play distinct roles *in vivo* and *in vitro*. Another reason is that the assembly of the large Mediator complex is dynamic in cells. The Mediator complex may catalyze enzymatic activities for specific regulation in the biological

pathway. Recently, some interesting findings have indicated that the Mediator complex has enzymatic activities, such as kinase and acetyltransferase activities, and some Mediator subunits are assembled in the enzyme complex, such as Med8 involved in the ubiquitin ligase (Brower, *et al.*, 2002; Knuesel, *et al.*, 2009; Meyer, *et al.*, 2008). Exploring enzymatic activities of Mediator will provide new insight into how Mediator regulates transcription in cells.

3.2.1 Isolation of different Mediator complexes

Both core Mediator and T/G-Mediator were successfully isolated from HeLa cells, and then confirmed by silver stain and Western blot.

A good purification for endogenous Mediator is necessary for studying the structure and function of the Mediator complex, because Mediator is a large multi-protein complex and it's almost impossible to assemble *in vitro*. The Taatjes lab has well-established purification procedures for different Mediator complexes, such as CDK8-Mediator, the core Mediator and T/G-Mediator. A detailed purification procedure was outlined in the figure 2.2. A typical preparation begins with nuclei isolated from 100 liters of HeLa cells. For the affinity purification step, the activator SREBP was used to pull-down the Mediator complex (Figure 3.13). Then GSH elutes were run on a linear 15%-40% glycerol gradient. Glycerol gradient fractions containing Mediator were aliquoted and stored at -80 ℃. The T/G-Mediator fractions were purified from P0.5M fraction (Figure 3.14), and the core Mediator fractions were from the P1M fraction (Figure 3.15).

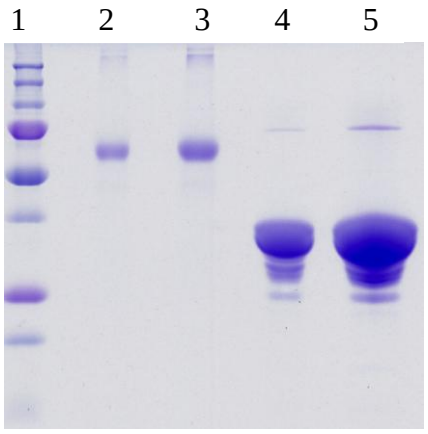

Figure 3.13 Purified GST-SREBP by GST affinity chromatography . Lane 1: Precision plus protein dual color standards (Bio-Rad); lanes 2 and 3: BSA; lanes 4 and 5: GST-SREBP.

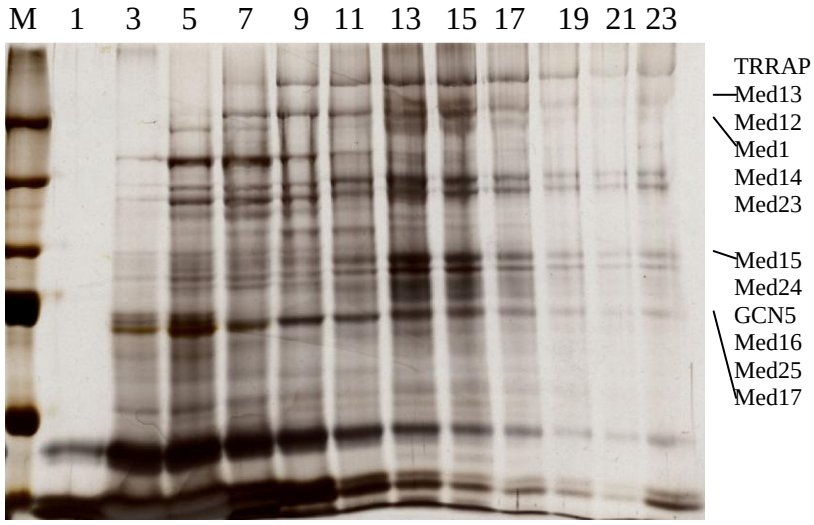

Figure 3.14 Glycerol gradient fractions for T/G-Mediator. Lane M: Precision plus protein dual color standards (Bio-Rad); other lanes show glycerol gradient fractions (Fraction number is indicated at the top of each gel lane).

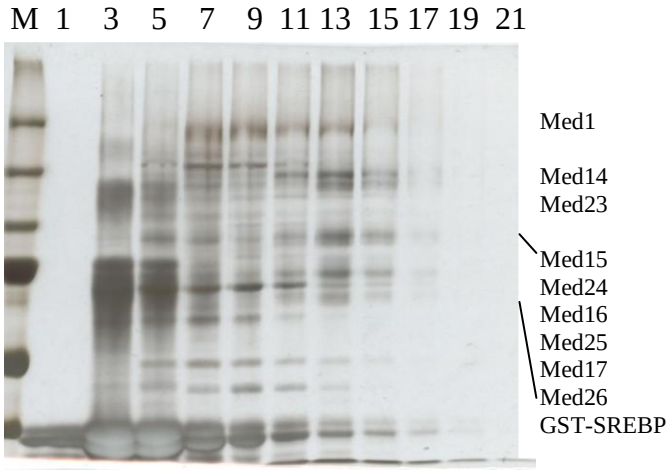

Figure 3.15 Glycerol gradient fractions for the core Mediator. Lane M: Precision plus protein dual color standards (Bio-Rad); other lanes show glycerol gradient fractions (Fraction number is indicated at the top of each gel lane).

According to the silver stained gels together with the Mediator subunit composition, the best glycerol gradient parts for both the core and T/G-Mediator complexes are fractions 13, 14 and 15. I used these fractions to test enzymatic activities of Mediator.

3.2.2 RNA Pol II CTD phosphorylation by Mediator

In vitro kinase assays revealed that both the core and T/G-Mediator can phosphorylate the first half, the second half and full length of the RNA Pol II CTD. Moreover, there was no significant difference between the first half and the second half.

It is known that RNA Pol II CTD phosphorylation plays central roles in transcription and other nuclear events, and different sets of factors recognize specific phosphorylation patterns for distinct biological functions (Hirose and Ohkuma, 2007; Meinhart *et al*., 2005; Phatnani and Greenleaf, 2006) such as transcription initiation, splicing, mRNA cleavage

and RNA degradation. To date, several cyclin-dependent kinases have been discovered that phosphorylate the Pol II CTD, such as CDK7, CDK8 and CDK9. Surprisingly, in mammalian cells, the CDK/cyclin pairs alone cannot phosphorylate the targets, which suggests that other subunit(s) associated with the CDK/cyclin pair regulate activities of cyclin dependent kinases. I tested the core and T/G-Mediator's kinase activity on different CTD templates. We want to know whether there are any phosphorylation difference between the CTD full length and the CTD deletion mutants by the core and T/G-Mediator complexes. TFIIH contains the CDK7/cyclin H pair and can phosphorylate the Pol II CTD. Here, I examined the core Mediator's kinase activity together with TFIIH on different CTD templates, the full length, the first half and second half of the Pol II CTD (Figure 3.16). Then T/G-Mediator was tested using the same procedure and same templates indicated above (Figure 3.17).

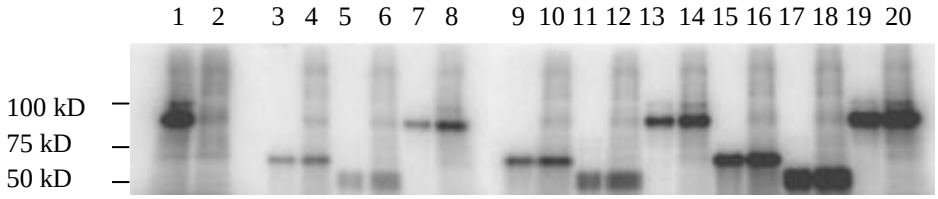

Figure 3.16 Pol II CTD phosphorylation by the core Mediator and TFIIH. Lane 1: the CTD full length together with the core Mediator; lane 2: TFIIH together with the core Mediator; lanes 3, 9 and 15: the CTD(1-24) together with TFIIH for 0.5, 2 and 8 minutes reaction time, respectively; lanes 4, 10 and 16: the CTD(1-24) together with TFIIH and the core Mediator for 0.5, 2 and 8 minutes reaction time, respectively; lanes 5, 11 and 17: the CTD(25-52) together with TFIIH for 0.5, 2 and 8 minutes reaction time, respectively; lanes 6, 12 and 18: the CTD(25-52) together with TFIIH and the core Mediator for 0.5, 2 and 8 minutes reaction time, respectively; lanes 7, 13 and 19: the CTD full length together with TFIIH for 0.5, 2 and 8 minutes reaction time, respectively; lanes 8, 14 and 20: the CTD full length together with TFIIH and the core Mediator for 0.5, 2 and 8 minutes reaction time, respectively.

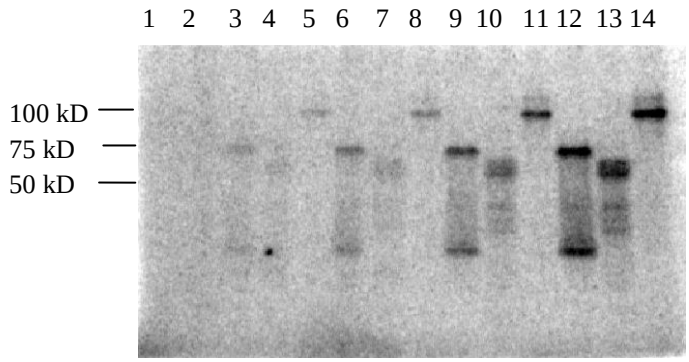

Figure 3.17 Pol II CTD phosphorylation by T/G-Mediator. Lane 1: the CTD full length; lane 2: T/G-Mediator; lanes 3, 6, 9 and 12: the CTD(1-24) together with T/G-Mediator for 5, 10, 30 and 60 minutes reaction time, respectively; lanes 4, 7, 10 and 13: the CTD(25-52) together with T/G-Mediator for 5, 10, 30 and 60 minutes reaction time, respectively; lanes 5, 8, 11 and 14: the CTD full length together with T/G-Mediator for 5, 10, 30 and 60 minutes reaction time, respectively.

The results revealed that both the core and T/G-Mediator can phosphorylate the Pol II CTD, and there were no significant difference between the first half and the second half of the CTD repeats. Perhaps the kinases mainly catalyze the position of amino acid residues in the repeat, not in specific repeats. Or there might be the different result in cells because of the complicated environment. Subsequent to these studies it was determined that core Mediator examined in Figure 3.16 contained a small amount of co-purifying TFIIH. This was attributed to the phosphorylation observed for "core Mediator."

3.2.3 Histone H3 methylation by T/G-Mediator

T/G-Mediator can methylate histone H3, but not H2A, H2B and H4. *In vitro* methyltransferase assays also showed that methylation occurred mainly in the amino-terminal tail of histone H3.

Histone proteins have a wide variety of post-translational modifications, such as acetylation, methylation, phosphorylation, and ubiquitination. Histone modifications can alter the electrostatic charge of the protein and result in the structural change, which may affect DNA binding or the protein module recognized by a specific factor. Histone methylation is one of several key modifications implicated in transcriptional regulation. Both Mediator and histone proteins are essential and closely related in transcription.

Some evidence indicated that the Mediator complex can phosphorylate and acetylate Histone proteins. Here, I examined whether the Mediator complex possesses additional enzymatic activities for Histone proteins. First of all, endogenous core histones were used for *in vitro* methyltransferase assays by T/G-Mediator (Figure 3.18). The Coomassie blue-stained gel in the left of the figure was used to check the equal loading of substrate amount.

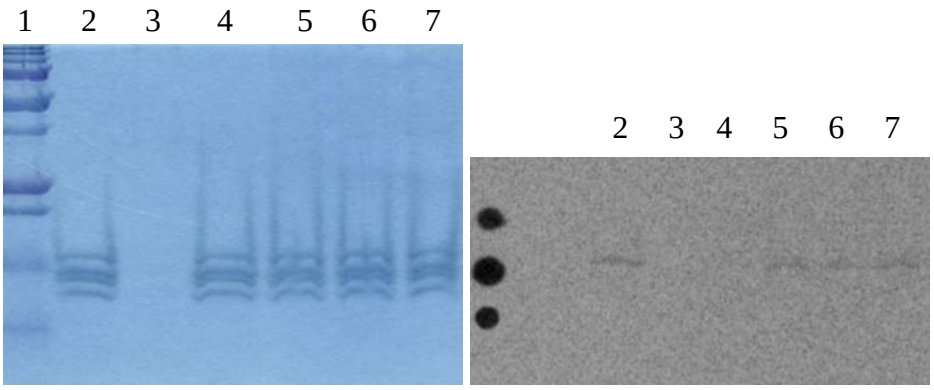

Figure 3.18 Methylation of endogenous core histone by T/G-Mediator. Lane 1: Precision plus protein dual color standards (Bio-Rad); lane2: endogenous core histone together with G9a (positive control); lanes 3: T/G-Mediator; lane 4: endogenous core histone; lanes 5, 6 and 7: endogenous core histone together with 7.5 µl, 5 µl and 11 µl of T/G-Mediator, respectively.

The data in figure 3.18 clearly showed that T/G-Mediator can methylate H3 of the endogenous core histones based upon the fact that there was the band at the same position for the positive control (G9a) and nothing for two negative controls. The histone modifications mainly occur within the amino-terminal tails. Therefore GST tagged H3(1-37) was next tested as the methylation target (Figure 3.19).

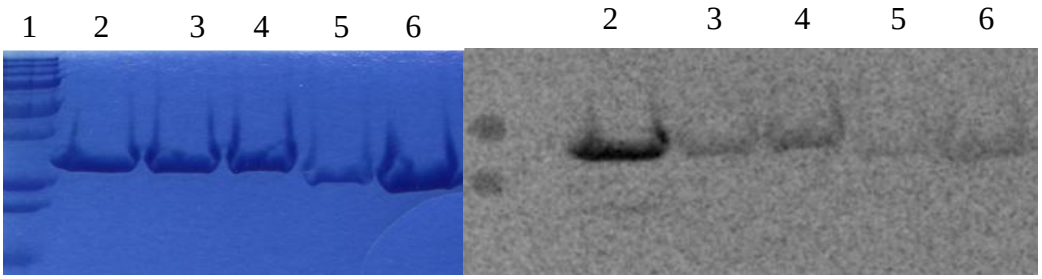

Figure 3.19 Methylation of GST-H3(1-37) by T/G-Mediator. Lane 1: Precision plus protein dual color standards (Bio-Rad); lane2: GST-H3(1-37) together with G9a; lanes 3 and 4: GST-H3(1-37) together with 2 µl and 8 µl T/G-Mediator, respectively; lanes 5 and 6: different amount of GST together with T/G-Mediator.

T/G-Mediator can methylate H3(1-37), although there is some background signal for GST alone. In addition, substantial data have revealed there is cross-talk between histone modifications, and one modification can be induced or strengthened by other modifications. T/G-Mediator can phosphorylate and acetylate histone H3, therefore, I specifically examined GST-H3(1-37)'s methylation with the addition of ATP and Acetyl-CoA in the assay (Figure 3.20). The result showed there was no difference for the addition of ATP and Acetyl-CoA. Perhaps phosphorylation and acetylation didn't occur in the methyltransferase buffer, or those modifications are not functionally related to the H3(1-37) methylation by T/G-Mediator. To obtain the best substrate for mass spectrometry, I compared the methylation between H3(1-37) and H3 full length (Figure 3.21). The H3 full length didn't exhibit stronger signal than H3(1-37). This makes sense, because the histone modification sites are almost exclusively positioned in the amino-terminal tail.

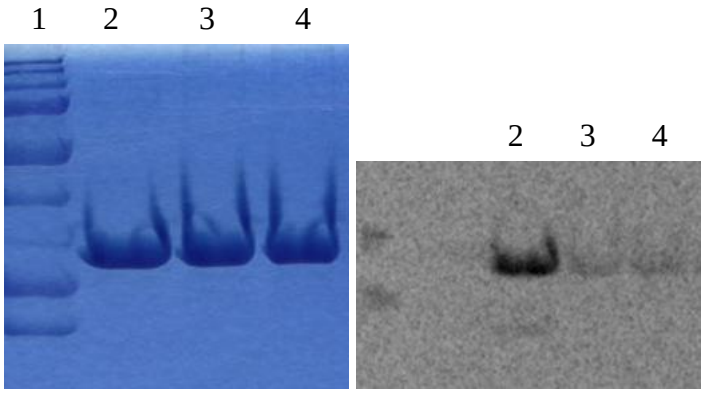

Figure 3.20 Methylation of GST-H3(1-37) by T/G-Mediator with the addition of ATP and Acetyl-CoA. Lane 1: Precision plus protein dual color standards (Bio-Rad); lane2: GST-H3(1-37) together with G9a; lane 3: GST-H3(1-37) together with T/G-Mediator; lane 4: GST-H3(1-37) together with T/G-Mediator and ATP and Acetyl-CoA.

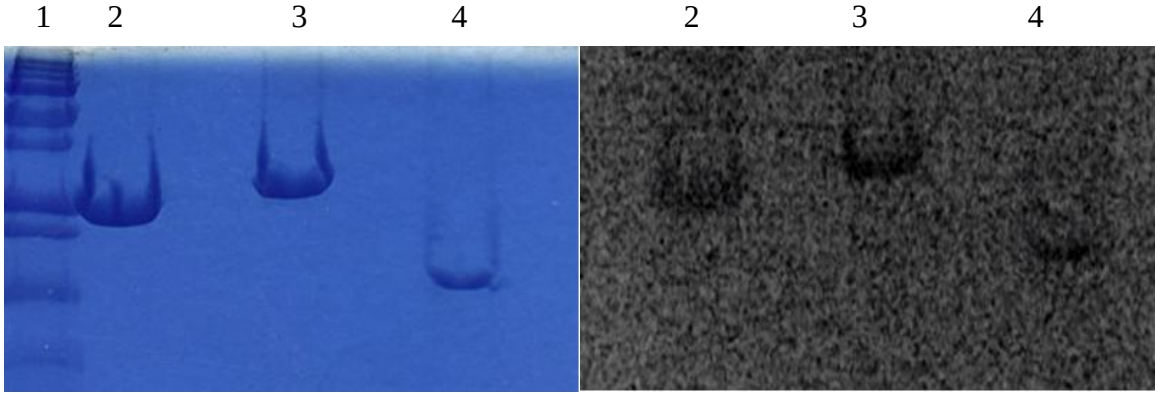

Figure 3.21 Methylation of GST-H3(1-37) and H3 full length by T/G-Mediator. Lane 1: Precision plus protein dual color standards (Bio-Rad); lane2: GST together with T/G-Mediator; lane 3: GST-H3(1-37) together with T/G-Mediator; lane 4: H3 full length together with T/G-Mediator.

3.2.4 Determination of ubiquitin ligase activity of T/G-Mediator

Some interesting bands were obtained from *in vitro* ubiquitination assay using T/G-Mediator when the E1 and E2 existed in the reaction, suggesting that the T/G-Mediator complex may include some subunit with ubiquitin ligase activity.

Recently, H2A and H2B ubiquitination has been proven to play important roles in nuclear events. Some ubiquitin-conjugating enzymes (E2) and ubiquitin ligases (E3) have been discovered for histone ubiquitination. Here, I used ubiquitin-E1 and UbcH5a to identify whether T/G-Mediator contains relevant ubiquitin ligase activity (Figure 3.22). Interestingly, the western blot using the antibody against ubiquitin showed there might be several specific bands for T/G-Mediator, which likely implies that some subunit(s) of the Mediator complex possess ubiquitin ligase function. The western blot signal of the specific band increased when more T/G-Mediator was added in the reaction, suggesting that the band from the ubiquitination assay is tightly related to T/G-Mediator.

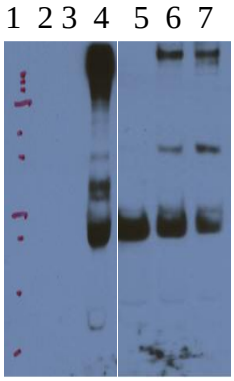

Figure 3.22 Immunoblots of endogenous core histone ubiquitination by T/G-Mediator. Lane 1: Precision plus protein dual color standards (Bio-Rad); lane2: endogenous core histone; lane 3: TRF1; lane 4: TRF1 ubiquitination (positive control); lane 5: endogenous core histone ubiquitination without T/G-Mediator; lanes 6 and 7: endogenous core histone ubiquitination with 4 µl and 8 µl of T/G-Mediator, respectively.

3.3 Discussion and the future work

The first work accomplished in this thesis was to design and carry out the crosslinking experiment for identifying RNA Pol II CTD-interacting Mediator subunits. The final goal of this project will elucidate the molecular mechanism of the Pol II CTD-Mediator interaction, and further investigate how both of them function in transcription. First of all, I compared CTD binding with the Mediator complex between the first half and the second half of the Pol II CTD. There is no significant difference between them, which increases the difficulty of studying the interaction mechanism. The Pol II CTD cysteine mutants were successfully used for the crosslinking experiment. However, different Pol II CTD cysteine mutants affect the experimental result. CTDr31C, CTDr38C and CTDr52C worked well, but CTDr24C did not. The result detected by Streptavidin-HRP indicated there were several specific bands. Together

with the Mediator subunit profile, Med1 and Med23 were identified to be the interacting subunits by western blot with the antibody against specific Mediator subunits. These data suggest that the Pol II CTD-Mediator interaction may be dynamic, and has multiple interaction sites. More work is needed for investigating the interaction mechanism, such as repeating the same procedure using different crosslinking reagents, using mass spectrometry to exactly analyze the crosslinking products, performing *in vitro* binding assays using recombinant deletion mutant proteins, and testing the functional relevance of the interaction.

The second work accomplished in the thesis was to examine Mediator's enzymatic activities. Both T/G-Mediator highly purified from HeLa cells and related substrates were used for testing. Some Mediator complex was shown to have enzymatic activities, but the study about it still remains unclear. Mediator is a large, 26-subunit protein complex that can adopt different compositions in cells. It is possible that Mediator may possess enzymatic activities to accurately implement its functions. Different activity assays indicated that T/G-Mediator can almost equally phosphorylate the first half and the second half of the Pol II CTD, and that T/G-Mediator can methylate histone H3, and that some subunits of T/G-Mediator may possess the ubiquitin ligase activity. The above data suggest that Mediator itself may have enzymatic activities for its master regulation in transcription, or that the Mediator complex with distinct subunit composition plays important role by interacting with other enzyme modules. The next experiment will identify the modification pattern by mass spectrometry, and also uncover which Mediator subunit possesses the enzymatic activity. This work provides new insight into why Mediator is a master regulator in transcription.

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