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The Role of Histone H2B Ubiquitylation and its Related Factors in Transcriptional Regulation in Mammalian Cells

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THE ROLE OF HISTONE H2B UBIQUITYLATION
AND ITS RELATED FACTORS
IN TRANSCRIPTIONAL REGULATION
IN MAMMALIAN CELLS

A Thesis Presented to the Faculty of
The Rockefeller University
in Partial Fulfillment of the Requirements for
the degree of Doctor of Philosophy

by
Jaehoon Kim
June 2007

The Role of Histone H2B Ubiquitylation and Its Related Factors in Transcriptional Regulation in Mammalian Cells

Jaehoon Kim
The Rockefeller University 2007

Diverse histone modifications such as acetylation, methylation, and phosphorylation play important roles in transcriptional regulation throughout eukaryotes, and recent studies in yeast also have implicated H2B ubiquitylation in the transcription of specific genes. However, a systematic study of H2B ubiquitylation in mammalian cells has been hindered by the lack of information about mammalian homologues of the yeast enzymes responsible for H2B ubiquitylation. I report identification of a functional human homologue, the hBRE1A/B complex, of the yeast BRE1 E3 ubiquitin ligase. hBRE1A, which forms a complex with hBRE1B, specifically increases the global level of H2B ubiquitylation at lysine 120 and enhances activator-dependent transcription *in vivo*. An extensive screening of cognate E2 ubiquitin conjugating enzyme for the hBRE1A/B complex revealed that hRAD6A and hRAD6B specifically interact with the N-terminal region of hBRE1A, and ubiquitylate H2B at lysine 120 in the presence of hBRE1A/B *in vitro*. Moreover, reduction of hBRE1A, hBRE1B and hRAD6 proteins by RNAi decreases endogenous H2B ubiquitylation, activator-dependent transcription, and both H3-K4 and H3-K79 methylation. Of special significance, I show that hBRE1A/B directly interacts with p53 and that it is

recruited to the mdm2 promoter in a p53-dependent manner. These studies suggest that hBRE1A/B is an H2B-specific E3 ubiquitin ligase and that it functions, at least in part, through direct activator interactions, as a transcriptional coactivator. In addition, hBRE1A/B directly interacts with the hPAF complex to bring hRAD6 to the transcription machinery. I also found that a direct interaction between the hPAF complex and the previously characterized transcription elongation factor SII enhances their mutual association with RNA polymerase II. In an *in vitro* transcription assay with highly purified transcription factors, the hPAF complex and SII showed significant synergistic effects on activator- and histone acetyltransferase-dependent transcription from a chromatin template. However, addition of the H2B ubiquitylation factors to the *in vitro* transcription reaction led to an unexpected reduction in transcription. These results suggest that the reconstituted system lacks additional histone modifying and/or chromatin remodeling activities that link H2B ubiquitylation and gene activation. Taken together, my new findings set a stage for studying the molecular mechanisms of H2B ubiquitylation in transcriptional control in mammalian cells.

To My Parents

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gratitude to my parents and sister. I really regret that I cannot show PhD diploma to my father who passed away two years ago. However, I believe that my father, next to God, enjoys watching his son's scientific achievements in peace.

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LIST OF ABBREVIATIONS

Δ	Deletion	Ig	Immunoglobulin
AD	Activation domain	Kb	Kilobases
Anti-	Antibody	kDa	Kilodalton
bp	Basepair	NE	Nuclear extracts
DTT	Dithiothreitol	NP-40	Nonidet P-40
E1	E1 Ubiquitin activating enzyme	PAGE	Poly-acrylamide gel electrophoresis
E2	E2 Ubiquitin conjugating enzyme	PCR	Polymerase chain reaction
E3	E3 Ubiquitin ligase	PMSF	Phenylmethylsulfonyl fluoride
GST	Glutathione-S-transferase	RT	Reverse transcriptase
H2A	Histone H2A	SDS	Sodium dodecyl sulfate
H2B	Histone H2B	TR	Thyroid hormone receptor
H3	Histone H3	WCE	Whole cell extracts
H4	Histone H4	WT	Wildtype
HEPES	N-2-hydroxyethylpiperazine-N-(2-ethanesulfonic acid)		

CHAPTER 1

Introduction

Nuclear DNA in eukaryotic cells is organized within a hierarchical chromatin structure that restricts access to regulatory proteins that activate gene expression or other processes such as DNA replication, recombination and repair (Kornberg and Lorch, 1999). Studies over the past decade have revealed elegant mechanisms that alter nucleosomal structure and position and thereby increase the accessibility of DNA to various transcription factors. These mechanisms involve both nucleosome disruption and repositioning by ATP-dependent chromatin remodeling complexes (Vignali et al., 2000) and chemical modifications of nucleosomes by histone modifying enzymes (Strahl and Allis, 2000). The protruding histone tails are susceptible to a high density of post-translational modifications that include acetylation, methylation, phosphorylation, and ubiquitylation (Figure 1.1). Recent years have seen an explosion of information concerning effects of covalent modifications on transcription (Jenuwein and Allis, 2001). Despite the discovery of histone ubiquitylation over 30 years ago (Ballal et al., 1975), and expectations of prominent effects on chromatin structure and transcription because of the large size (8.6 kDa) of the adducts, mechanistic insights into the role of histone ubiquitylation in transcription are rather recent (Jason et al., 2002).

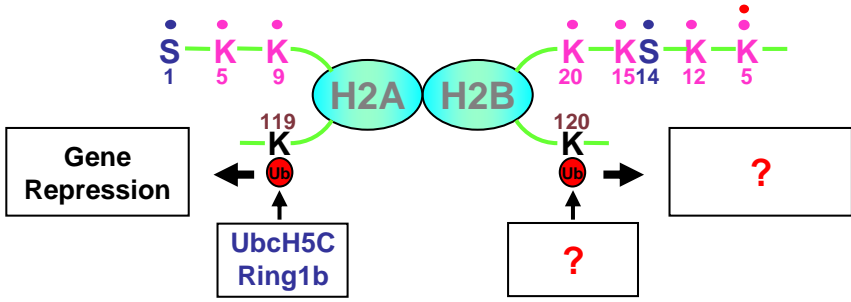
Ubiquitylated Histone Species and Sites of Ubiquitylation

The carboxyl end of ubiquitin can be attached to ϵ -amino groups of H2B lysine 123 (H2B K123) in yeast by mono-ubiquitylation (Robzyk et al., 2000). In mammalian cells, H2A and H2B have been shown to be ubiquitylated at lysine 119 (Nickel and Davie 1989) and lysine 120 (Thorne et al., 1987), respectively (Figure 1.1); and poly-ubiquitylated H3 has been found in elongating spermatids in rat testis (Chen et al., 1998). In relation to the levels of ubiquitylated histones, 5-15% of the total H2A and about 1.5% of total H2B are post-translationally linked to ubiquitin, and these levels are quite variable within different species and during the cell cycle (West and Bonner 1980).

Enzymes Involved in Histone H2B Ubiquitylation

Three classes of enzymes are involved in the conjugation of ubiquitin to proteins (Figure 1.2A). The first of these is the E1 ubiquitin activating enzyme. E1 transfers ubiquitin to the second enzyme in the pathway, the E2 ubiquitin conjugating enzyme. The conjugation of ubiquitin to target proteins requires the additional action of an isopeptide ligase, E3 (Hershko and Ciechanover 1998). Substrate specificity is governed by specific E3s through direct interaction between the substrate and the E3. A given E3 acts with its cognate E2, resulting in both E3 auto-ubiquitylation and substrate ubiquitylation (Figure 1.2B). In the case of protein ubiquitylation systems

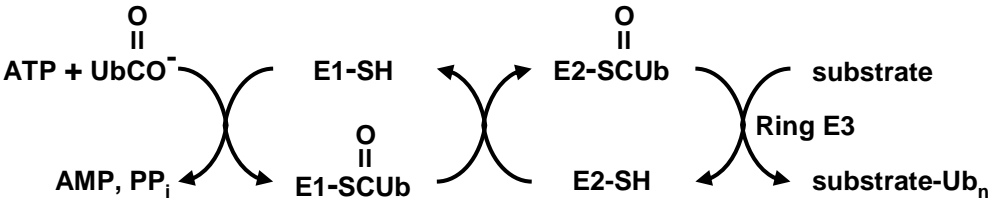
Figure 1.1 Post-Translational Modifications on Histone Tails



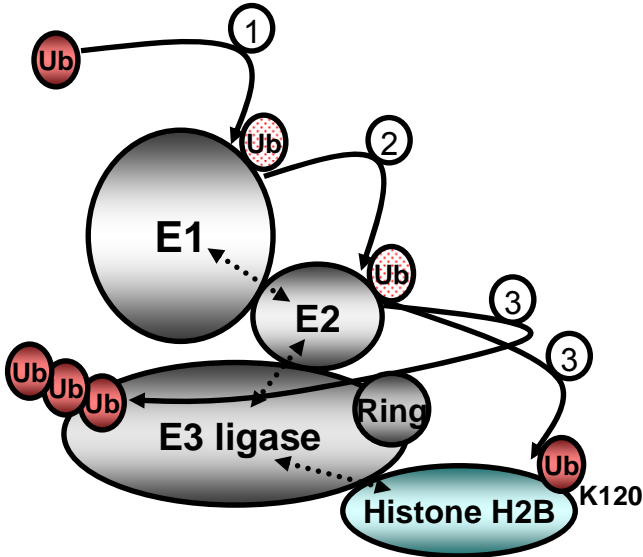
Post-translational histone modifications are indicated at their corresponding sites. The E2 ubiquitin conjugating enzyme and E3 ubiquitin ligase responsible for histone H2A ubiquitylation are shown.

Figure 1.2 Histone H2B Ubiquitylation System

A



B



(A) Enzymatic pathway of ubiquitin conjugation. (B) Histone H2B ubiquitylation by cognate E2 ubiquitin conjugating enzyme and ring-finger E3 ubiquitin ligase. Direct protein interactions are indicated by dashed arrows.

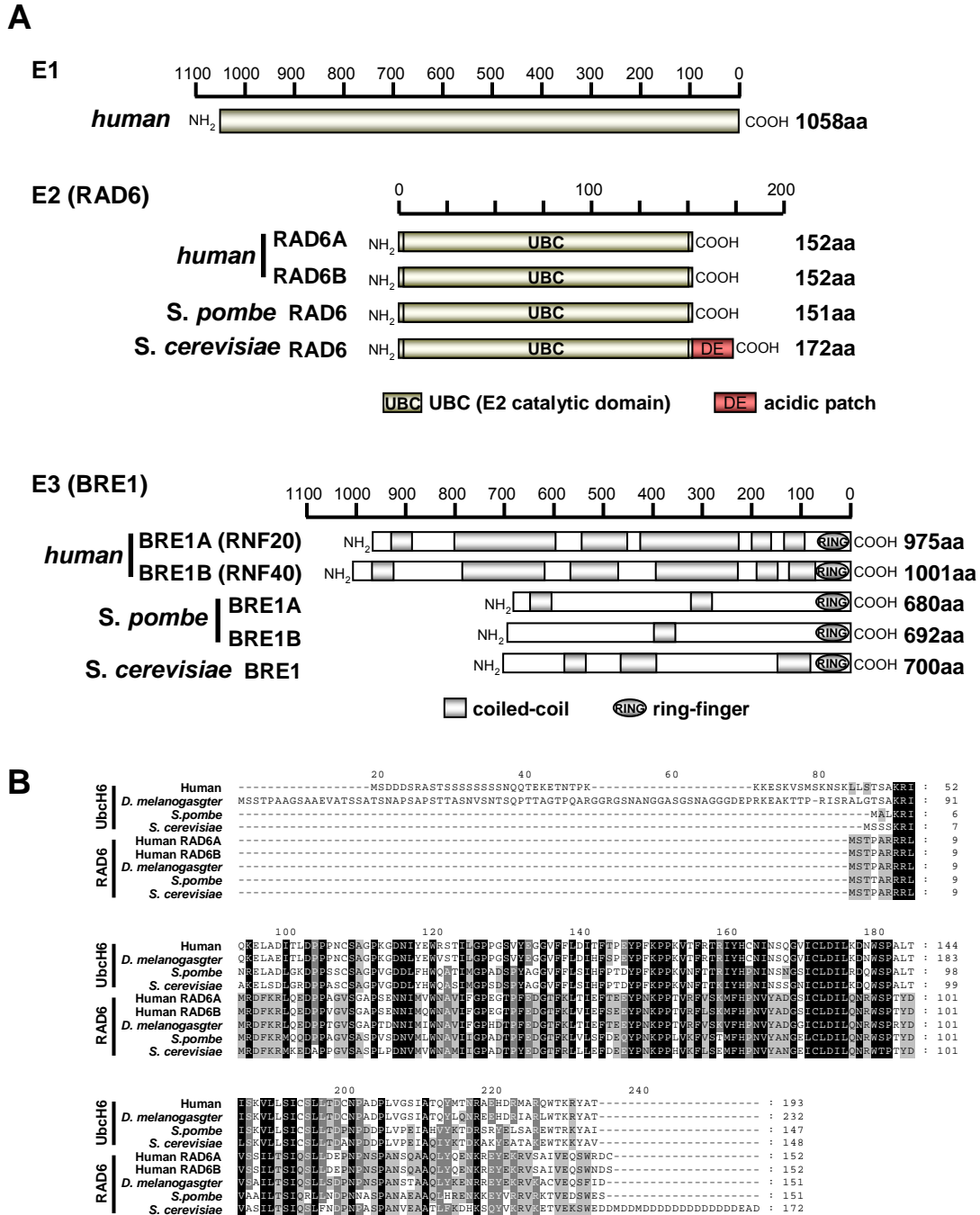
that employ RING finger proteins as E3 ligases, ubiquitin is transferred directly from the E2 to the substrate (Jackson et al., 2000). It is thought that the ring type E3 ubiquitin ligase acts as a bridge molecule between E2 and substrate.

In most organisms there is a single E1, a significant but limited number of E2s, and a much larger number of E3s (Pickart, 2001). Among the several E2s in yeast, RAD6 is responsible for H2B ubiquitylation *in vivo* (Robzyk et al., 2000). Interestingly, it has been shown that RAD6 can ubiquitylate free histones *in vitro* without any need for an E3 (Jentsch et al., 1987). Recently, however, it was shown that BRE1, a ring-finger containing E3 ligase, is required for H2B mono-ubiquitylation *in vivo* (Wood et al., 2003a; Hwang et al., 2003). In humans, two highly conserved homologues (hRAD6A and hRAD6B) of yRAD6 (Koken et al., 1991) and two homologues (hBRE1A and hBRE1B) of yBRE1 (Hwang et al., 2003) were predicted from protein similarity search programs (Figure 1.3A), although there have been no reports of functions for these proteins.

Histone Ubiquitylation and Transcription

Usually, ubiquitylation of proteins marks them for protein degradation (Hershko and Ciechanover 1998; Jennissen 1995). But several studies have shown that the ubiquitylation of histones does not mark them for degradation

Figure 1.3 Homologues of Putative E2 and E3 Enzymes Responsible for H2B Ubiquitylation in Different Species



(A) Domain structures of RAD6 and BRE1 homologues in different species.
 (B) Homology comparison between RAD6 and UbcH6 from different species.

by the 26S proteasome *in vivo* (Seale 1981; Wu et al., 1981), indicating that histone ubiquitylation must serve another purpose. Because of its nature, ubiquitin adduct represents the most bulky histone modification and, as such, would be expected to exert an important role on chromatin structure and transcriptional regulation. However, the role of histone ubiquitylation in transcription has been quite controversial.

Several lines of evidence suggest that H2B ubiquitylation is associated with actively transcribed genes. For instance, ubiquitylated H2B is preferentially localized in transcriptionally active chromatin in bovine thymus and chicken erythrocytes and is enriched in transcriptionally active *Tetrahymena* macronucleus (Davie and Murphy 1990; Nacheva et al., 1989; Nickel et al., 1989). Recent functional studies have shown that the transcription of several inducible genes (i.e. *GAL1*, *SUC2*, and *PHO5*) is impaired in the absence of ubiquitylated H2B (Kao et al., 2004). Moreover, the level of ubiquitylated H2B is transiently increased around the *GAL1* core promoter region upon galactose activation (Henry et al., 2003), implying that histone ubiquitylation is linked to activator-dependent transcription.

In contrast to the correlation between H2B ubiquitylation and transcription, it has been reported that H2A ubiquitylation has different relationships. For example, early studies in rat liver nuclei correlated the disappearance of

ubiquitylated H2A with increased transcription (Ballal et al., 1975) and the most recent reports have shown that hUbcH5C/Ring1b-mediated H2A ubiquitylation (Figure 1.1) plays a role in Polycomb silencing (Wang et al., 2004) and X chromosome inactivation in female cells (Fang et al., 2004).

Cross Talk between Histone H2B Ubiquitylation and H3-K4/K79 Methylation

Histone ubiquitylation in yeast also provides the first example of a trans-tail modification (Fischle et al., 2003). Methylation of H3-K4 by SET1 and H3-K79 by DOT1 requires ubiquitylation of H2B by RAD6-BRE1 (Sun and Allis 2002; Ng et al., 2002). A more recent study has revealed that histone ubiquitylation controls processive methylation (di-, and tri-) of H3-K4 and by SET1 (Shahbazian et al., 2005). Since H3-K4 tri-methylation marks actively transcribed genes (Santos-Rosa et al., 2002), it has been proposed that H2B ubiquitylation acts as a master switch for gene regulation through a trans-histone pathway that leads to the appropriate patterns of histone methylation. Although methylation of H3-K36 by SET2 is also known to be linked to active transcription, H2B ubiquitylation is not prerequisite for this modification (Ng et al., 2003a), indicating that H2B ubiquitylation functions selectively in H3 methylation pathways. However, the mechanisms of how H2B ubiquitylation directs H3 methylation and how ubiquitylation mark activates histone methylases have been unknown.

Although SET1 serves as the sole H3-K4 methyltransferase in yeast, there are several enzymes that have H3-K4 methyltransferase activity in human cells (Sims et al., 2003). In case of H3-K79 methylation, only one methyltransferase (DOT1) has been reported in mammalian cells (Feng et al., 2002). At the start of this study, it remained to be determined whether H2B ubiquitylation controls H3-K4 and H3-K79 methylation and which methyltransferase(s) is responsible for H2B ubiquitylation-directed H3-K4 methylation in mammalian cells.

Transcription Elongation Factors

Eukaryotic RNA polymerase II-mediated transcription is a highly coordinated process with three major steps: initiation, elongation and termination. The nucleosome is the primary structural unit of chromatin in eukaryotic cells and comprises two turns of DNA wrapped with a histone octamer that contains an H3-H4 heterotetramer and two H2A-H2B dimers. The nucleosome forms a strong barrier for RNA polymerase II to transcribe *in vitro* (Knezetic and Luse 1986; Lorch et al., 1987; Workman and Roeder 1987). During the transcription elongation process in living cells, specific factors associate with RNA polymerase II and help to overcome restriction resulting from transient pausing or nucleosome structures (Li et al., 2007; Shilatifard et al., 2003). Eukaryotic factors with functions in transcription

elongation include SII (TFIIS), TFIIF, DSIF, Elongin, ELL, NELF, the PAF complex and the histone chaperone FACT (Shilatifard et al., 2003). These factors have been shown to be associated with RNA polymerase II *in vivo*, and different *in vitro* studies have indicated direct functions in transcription (Uptain et al., 1997). However, there has been little direct proof of function in transcription from nucleosomal template *in vitro*.

Very recently, the Roeder lab has identified SII, through biochemical fractionation, as a functional transcription elongation factor that enhances transcription from reconstituted chromatin templates. After activator-dependent pre-initiation complex assembly, SII strongly stimulates transcription elongation through nucleosomes in a p300- and acetyl-CoA-dependent manner (Guermah et al., 2006). A single polypeptide SII was initially identified by its ability to stimulate transcription by a purified RNA polymerase II (Sekimizu et al., 1976) and then shown to stimulate transcription elongation (Reinberg and Roeder 1987). Later studies showed, first, that prolonged pauses (due to abnormal DNA sequences, intercalated drugs and bound proteins) can result in arrested RNA polymerase II due to backtracking and displacement of the growing RNA chain from the active site and, second, that SII could relieve the arrest by stimulating digestion of 3' RNA sequences by an exonuclease activity in RNA polymerase II (Reines et al., 1993). Similarly, when RNA polymerase transcribes into nucleosomal

templates, it also arrests at certain sites to constraints created by DNA-histone contacts (Bondarenko et al., 2006; Kireeva et al., 2005), but can be reactivated by SII for transcription through the nucleosome (Kireeva et al., 2005).

A role for the histone chaperone complex FACT, which is composed of subunits p140 and SSRP1, in transcription elongation is supported by the finding that FACT associates with known elongation factors (Krogan et al., 2002) and elongating RNA polymerase II (Mason and Struhl 2003; Saunders et al., 2003). In addition, biochemical and genetic experiments suggest that FACT can also facilitate RNA polymerase II transcription through nucleosomes (Reinberg and Sims, 2006). However, its mechanism is different from that of SII. The requirement for stoichiometric amounts of FACT equivalent to nucleosomal templates initially suggested that FACT might act as a histone chaperone (Orphanides et al., 1999) and function in both disassembly and reassembly of H2A/H2B dimers during transcription (Belotserkovskaya et al., 2003). However, other studies favor a mechanism involving FACT function, predominantly in nucleosomal reassembly, rather than disassembly during transcription (Schwabish and Struhl 2003). Besides the roles in elongation process, several groups suggested the alternative roles of FACT, which include not only transcription initiation but also DNA repair

and DNA replication (Biswas et al., 2005; Li et al., 2005; VanDemark et al., 2006).

The PAF complex was originally identified as an RNA polymerase II-associated factor (Wade et al., 1996) in yeast and minimally contains CTR9, LEO1, RTF1, PAF1 and CDC73 (Mueller et al., 2002). It is of special interest because it has been implicated in transcription elongation and plays a role in specific histone modifications. It has been reported that the RTF1 component of the PAF complex is required for H2B ubiquitylation (Ng et al. 2003a; Wood et al. 2003b) and for downstream H3-K4 methylation by SET1 (Ng et al. 2003b) and H3-K79 methylation by DOT1 (Wood et al., 2003b) in yeast. These studies further showed that the RAD6-BRE1 complex travels with RNA polymerase II via RTF1 (Xiao et al., 2005). The SET1 complex is also recruited to the transcription machinery through the PAF complex and the resulting H3-K4 methylation by SET1 provides memory of recent transcription (Ng et al., 2003b). These results suggest that the histone modification machinery participates in transcriptional regulation by forming a complex with the transcription elongation factor. Beyond the roles for the PAF complex in histone modifications, genetic and biochemical evidence in yeast suggest key roles at various stages of the gene expression pathway, including transcript site selection (Stolinski et al., 1997), transcriptional elongation (Pokholok et al., 2002; Rondon et al., 2004; Squazzo et al., 2002)

and, more recently, poly-A length control and the coupling of transcriptional and posttranscriptional events (Mueller et al., 2004).

CHAPTER 2

*Identification of hBRE1A as an E3
Ubiquitin Ligase Responsible for Histone
H2B Ubiquitylation and its Roles in
Transcription in Mammalian Cells*

2.1 PREFACE: ENZYMES INVOLVED IN HISTONE H2B UBIQUITYLATION AND THEIR EFFECT ON TRANSCRIPTION IN MAMMALIAN CELLS

Most of the recent progress regarding histone ubiquitylation has been achieved by yeast genetics. However, progress has been impeded due to the lack of appropriate assay systems to study histone ubiquitylation. Therefore, an ability to establish joint *in vitro* and *in vivo* histone ubiquitylation assays on chromatin templates would provide a powerful approach to understand the minimal requirements for chromatin ubiquitylation, its effect on transcriptional regulation, and whether there is a broader histone code mechanism involving histone ubiquitylation.

2.2 RESULTS

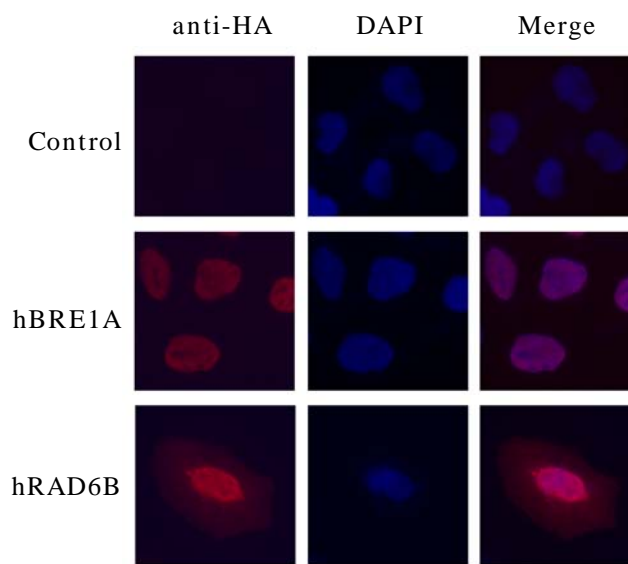
Human BRE1A Is a Nuclear Protein and Binds to Histones *in Vitro* and *in Vivo*

To investigate the role of histone ubiquitylation in transcriptional regulation, I first subcloned human homologues of yBRE1, an H2B specific E3 ubiquitin ligase. Although there are two reported human homologues of yBRE1, I first focused on hBRE1A (RNF20) since it shows a higher sequence homology to yBRE1 than does RNF40. To gain insight into the function of hBRE1A, I first examined its subcellular localization. Because available anti-hBRE1A antibodies failed to show significant signals in immunofluorescence studies, presumably because the antibody does not recognize native hBRE1A, I

generated a HeLa cell line that stably expresses HA-tagged hBRE1A and performed immunofluorescence staining using an anti-HA antibody. As shown in Figure 2.2.1, HA-hBRE1A was mainly localized in the nucleus. I also tested the subcellular localization of HA-tagged hRAD6B, the putative human homologue of the yeast E2 ubiquitin conjugating enzyme yRAD6. HA-hRAD6B was shown to be located in both the cytoplasm and nucleus.

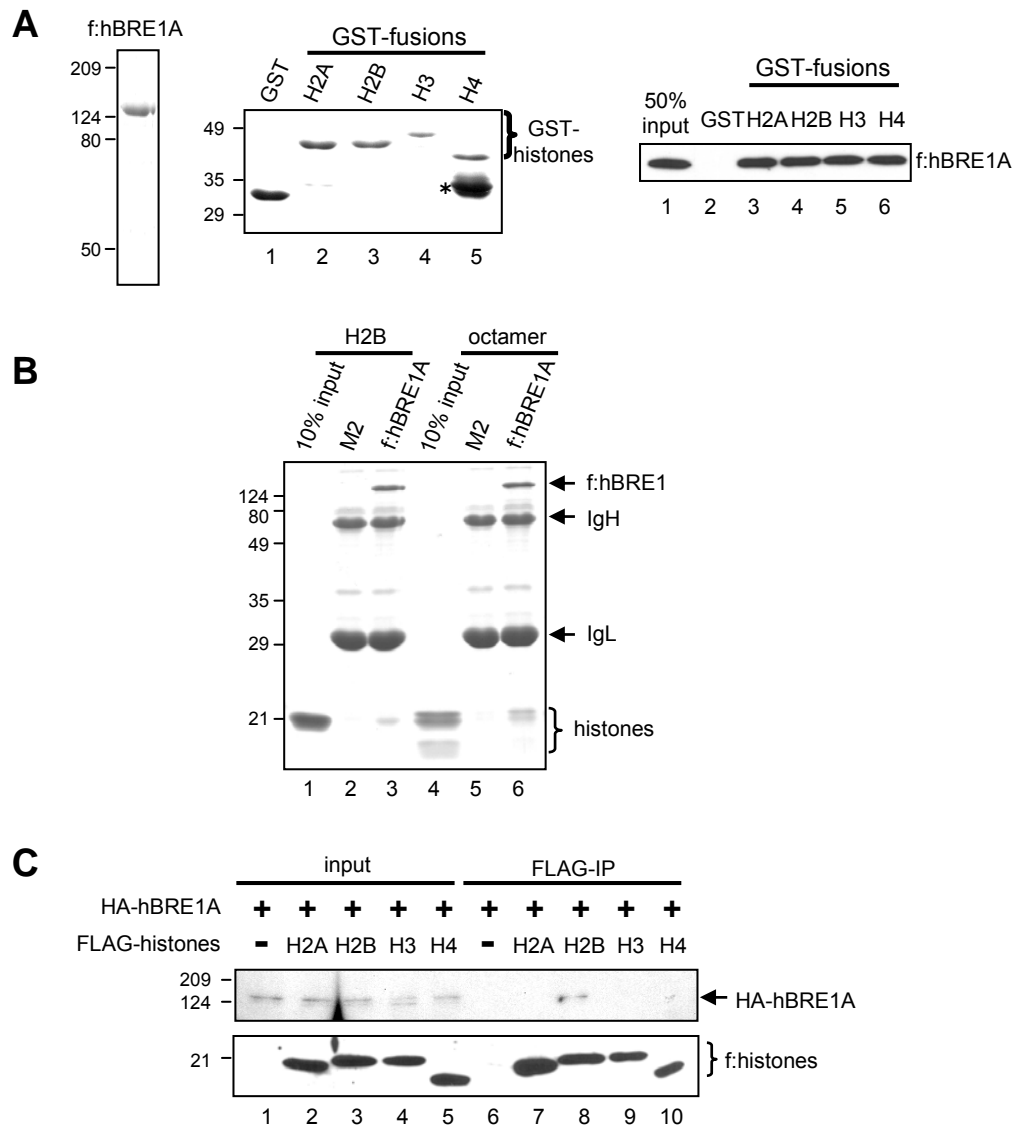
In a protein ubiquitylation system, substrate recognition is governed by direct interaction of the E3 ubiquitin ligase (Pickart, 2001). Given that hBRE1A is localized in the nucleus and related in sequence to histone ubiquitin ligase yBRE1, I first examined interactions of purified FLAG-hBRE1A with GST-histone fusion proteins (Figure 2.2.2A). hBRE1A showed a direct interaction with all four core histones. In a reciprocal approach, FLAG-hBRE1A protein was immobilized on M2-agarose beads and incubated with purified histones (Figure 2.2.2B). Both H2B alone (lane 3) and core histones within an octamer (lane 6) bound selectively to M2 agarose-immobilized FLAG-hBRE1A, relative to M2 alone (lanes 2 and 5), confirming a direct interaction between purified hBRE1A and core histone proteins *in vitro*. Next, I examined intracellular interactions between hBRE1A and core histones following ectopic expression of HA-hBRE1A and FLAG-tagged

Figure 2.2.1 Human BRE1A Is a Nuclear Protein



HeLa-derived HA-hBRE1A and HA-hRAD6B cell lines were stained with antibodies against HA-antibody. DNA was visualized with DAPI.

Figure 2.2.2 Human BRE1A Directly Binds to Histones



(A) Interaction of hBRE1A with core histones. Purified FLAG-hBRE1A (left panel), GST and GST-tagged *Xenopus* histones (middle panel) were analyzed by SDS/PAGE and Coomassie blue staining. FLAG-hBRE1A (right panel) was tested for binding to GST-histones, and bound proteins were scored by anti-FLAG immunoblot. (B) Recombinant H2B and histone octamers were tested for binding to mock or FLAG-hBRE1A proteins. Bound proteins were analyzed by SDS/PAGE with Coomassie blue staining. (C) HA-hBRE1A was co-expressed with the indicated FLAG-histones in 293T cells. Cell extracts were incubated with M2-agarose and immunoprecipitated proteins were analyzed by anti-HA (top panel) and anti-FLAG (bottom panel) antibodies, respectively.

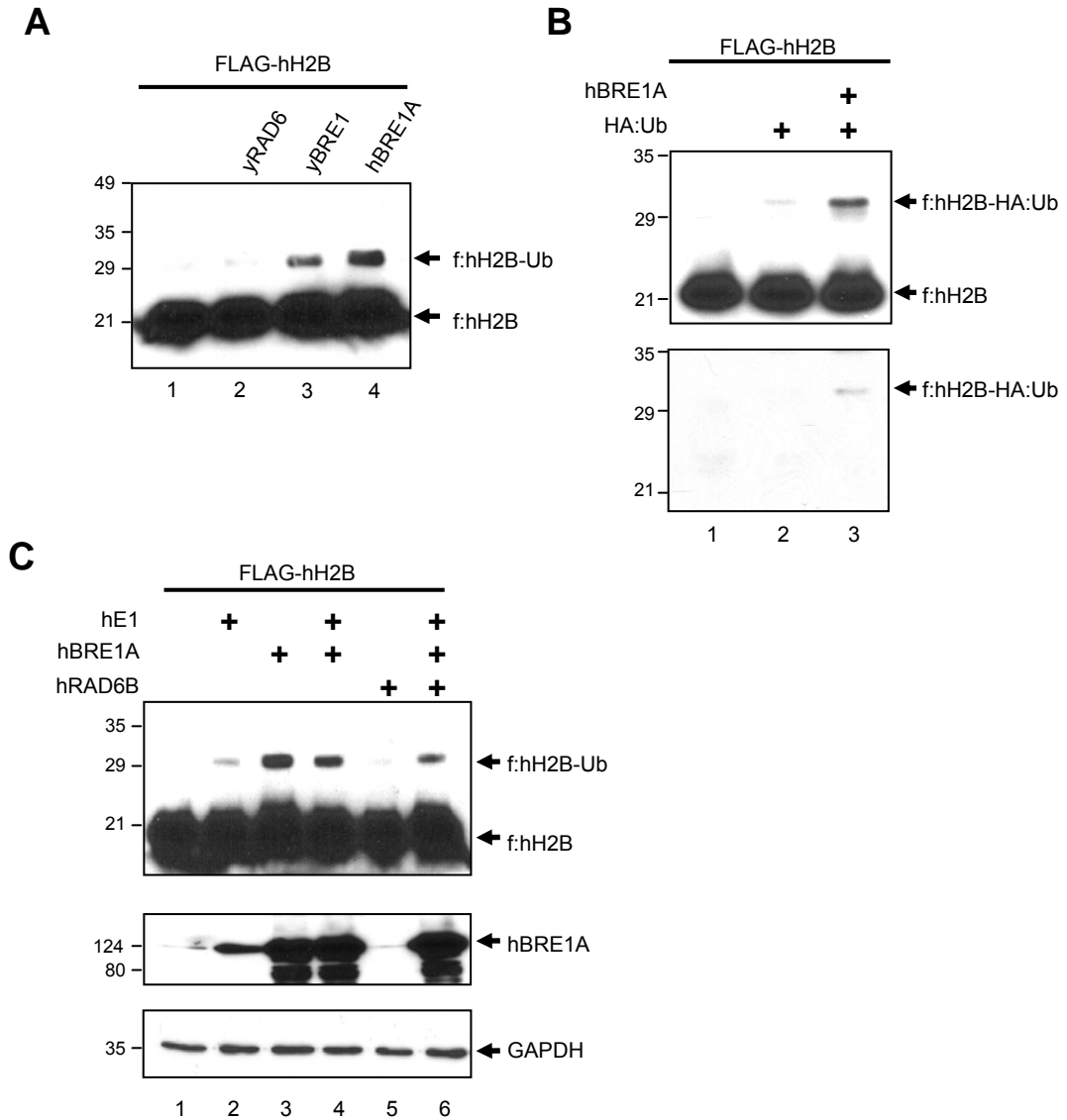
histones (Figure 2.2.2C). In contrast to what was observed with the purified histones, and consistent with its clear *in vivo* role in H2B ubiquitylation (below), HA-hBRE1A protein was co-immunoprecipitated specifically with the FLAG-H2B (lane 8). The observed specificity for H2B in this assay contrasts with the lack of specificity for purified core histones in Figure 2.2.2A, but may reflect related (partially conserved) interaction surfaces between H2B and other core histones and the absence in the *in vitro* assay of natural constraints that give selectivity *in vivo*. Taken together, these observations imply that hBRE1A is localized mainly in the nucleus, and can directly bind to its natural H2B substrate.

Human BRE1A Elevates Global Histone H2B Mono-Ubiquitylation at Lysine 120 *in Vivo*

Since endogenous histones are tightly bound to DNA, I first asked whether ectopically expressed FLAG-tagged histones are incorporated into chromatin. After transfecting cells with histone expression vectors, small-scale biochemical fractionations were performed. All FLAG-tagged core histones (H2A, H2B, H3 and H4) were found exclusively in the chromatin fraction (data not shown). Consistent with these results, other groups also reported that epitope-tagged histones are localized exclusively within chromatin (Tagami et al., 2004). Therefore, it is expected that FLAG-histones will behave like endogenous histones.

To test whether hBRE1A can increase H2B ubiquitylation *in vivo*, 293T cells were transfected with a vector expressing FLAG-H2B alone or in combination with vectors for yRAD6, yBRE1 and hBRE1A. Cell lysates were immunoprecipitated with M2 agarose under denaturing conditions followed by anti-FLAG immunoblotting (Figure 2.2.3A). Modification of FLAG-H2B with ubiquitin was readily detectable because of the slow migration of the ubiquitylated histone species. Both yBRE1 and hBRE1A overexpression resulted in a discrete increase in the ubiquitylated H2B level (lanes 3 and 4), implying that hBRE1A has the same intrinsic enzyme activity as yBRE1. However, yRAD6 overexpression did not increase the H2B ubiquitylation level (lane 2). Immunoblot assays with several anti-ubiquitin antibodies failed to confirm that the slow migrating band is indeed ubiquitylated H2B, presumably due to the restricted sensitivity of anti-ubiquitin antibodies (data not shown). As an alternative approach, cells were transfected with a vector encoding FLAG-H2B in combination with vectors for HA-ubiquitin and hBRE1A (Figure 2.2.3B). The shifted band was recognized by both anti-FLAG (lane 3, upper panel) and anti-HA (lane 3, bottom panel) antibodies, thus confirming that hBRE1A does enhance H2B ubiquitylation. We then tested the effect of other putative human histone ubiquitylation-related proteins for H2B ubiquitylation (Figure 2.2.3C). The hE1 ubiquitin-activating enzyme resulted in a moderate increase of ubiquitylated H2B (lane 2), whereas the

Figure 2.2.3 Human BRE1A Elevates Global H2B Mono-Ubiquitylation

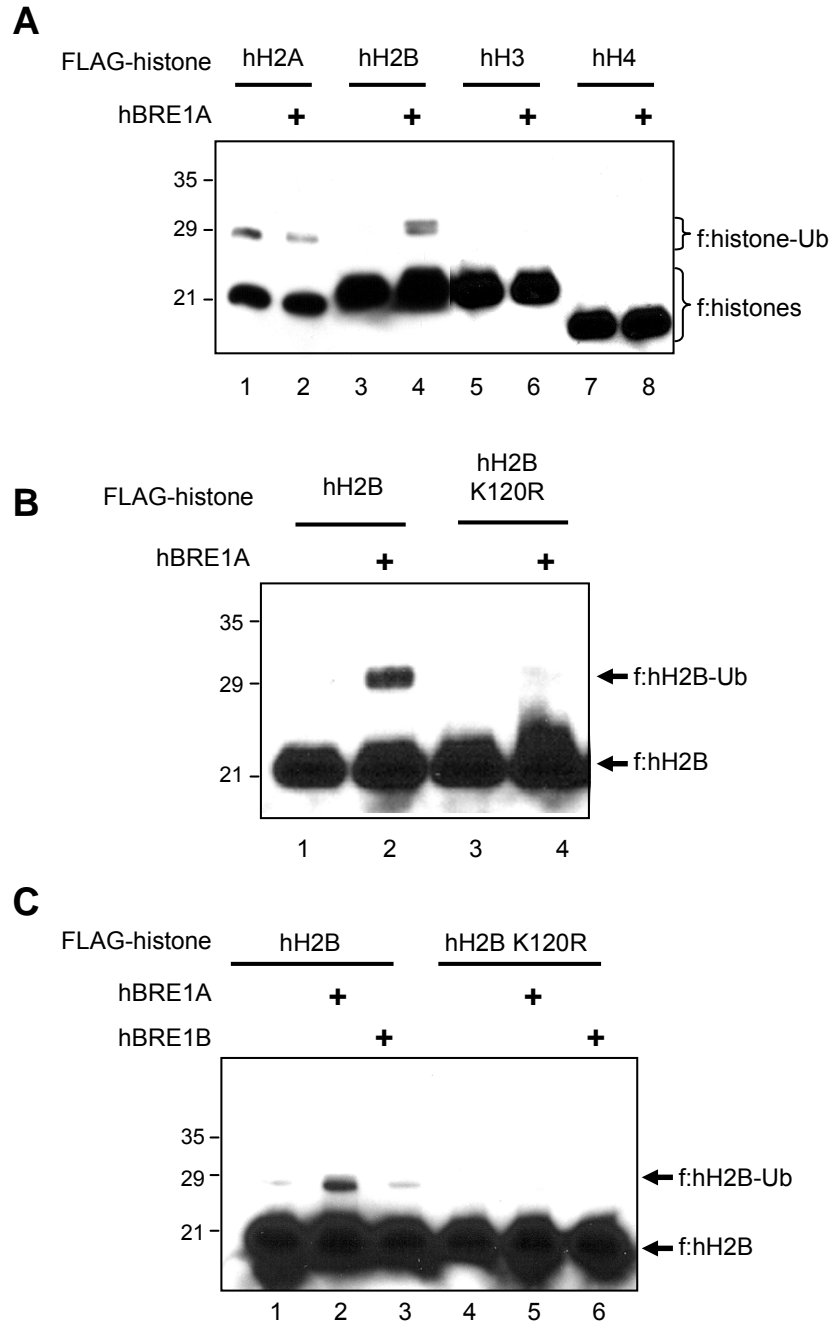


(A-C) FLAG-H2B was co-expressed with yRAD6, yBRE1, and hBRE1A (A), indicated combinations of hBRE1A and HA-ubiquitin (B), and indicated combinations of hE1, hBRE1A and hRAD6B (C) in 293T cells. Cell extracts were immunoprecipitated with M2 agarose and subjected to immunoblot analysis with anti-FLAG and anti-HA (B, bottom panel) antibodies. Immunoblot of whole-cell extracts for hBRE1A and GAPDH (C, middle and bottom panels, respectively).

robust increase of H2B ubiquitylation in response to hBRE1A (Figure 2.2.3A) was reproduced (lane 3). However, the combination of hE1 and hBRE1A did not result in a synergistic or additive effect on H2B ubiquitylation (lane 4). Like yRAD6, neither hRAD6B (lane 5) nor hRAD6A (data not shown) affected the level of H2B ubiquitylation. Interestingly, overexpression of hE1 caused an enhancement of the endogenous hBRE1A level (lane 2, middle panel), indicating that hE1 might be linked somehow to hBRE1A.

Since the substrate specificity is determined by the E3 ubiquitin ligase, I tested the substrate specificity of hBRE1A for each of the 4 core histones. To this end, 293T cells were transfected with vectors expressing FLAG-tagged histones alone or in combination with hBRE1A expression vectors (Figure 2.2.4A). Consistent with previous reports showing that 5~15% of histone H2A is ubiquitylated (West and Bonner, 1980), we found that a significant amount of FLAG-H2A is ubiquitylated without hBRE1A overexpression (lane 1). Additionally, hBRE1A did not result in a further increase in the level of H2A ubiquitylation (lane 2). I did not observe ubiquitylation of histones H3 and H4 in untransfected or hBRE1A-overexpressing cells, suggesting that hBRE1A does not play a role in the ubiquitylation of these particular histones (lanes 5-8). On the other hand, hBRE1A overexpression resulted in a large increase in H2B ubiquitylation (lanes 3-4), implying that hBRE1A affects the ubiquitylation level of H2B. The appearance of ubiquitylated

Figure 2.2.4 Human BRE1A Is an H2B K120 Specific E3 Ubiquitin Ligase



(A-C) FLAG-core histones (A) and FLAG-H2B and the H2BK120R mutant (B and C) were transiently expressed in the presence or absence of hBRE1A and hBRE1B in 293T cells, as indicated. Cell extracts were immunoprecipitated with M2 agarose and subjected to immunoblot analysis with anti-FLAG.

histones (lane 4) as a doublet has also been described (Davie and Murphy, 1990). Next, I tested the site specificity of hBRE1A on H2B. In yeast, K123 in H2B is the sole ubiquitylation site and corresponds to K120 in human H2B. In order to test whether K120 is the only ubiquitylation site on H2B, I generated a FLAG-H2B mutant (H2BK120R) with K120 changed to arginine (R). The robust increase of H2B ubiquitylation by hBRE1A was not observed in H2BK120R mutant cells (Figure 2.2.4B, lane 4). Next, I also tested H2B ubiquitylation activity of hBRE1B. Compared to significant effect by hBRE1A, hBRE1B did not result in increase of ubiquitylated H2B (Figure 2.2.4C). Taken together, these data indicate that hBRE1A promotes the specific ubiquitylation of K120 in H2B.

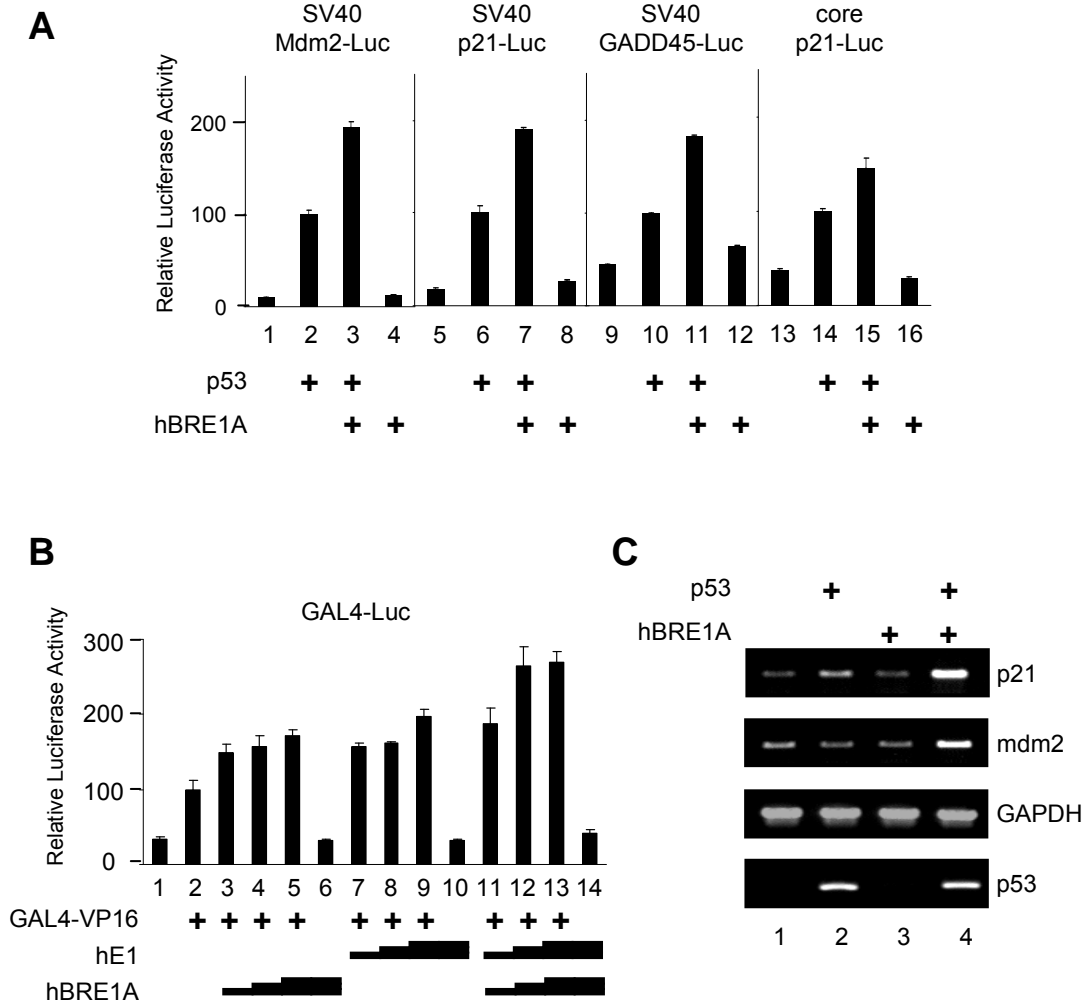
Human BRE1A Functions as a Versatile Coactivator in Activator-Dependent Transcription

Several lines of evidence indicate that histone ubiquitylation is linked to transcriptional activation (Osley, 2004) and further dependent (by an undetermined mechanism) on a DNA-binding activator, as has been shown in *Drosophila* (Bray et al., 2005) and yeast (Kao et al., 2004). To test whether hBRE1A influences transcription in mammalian cells, p53-deficient H1299 cells were jointly transfected with luciferase reporter genes bearing responsive elements from several p53 target genes (mdm2, p21, and GADD45), in addition to SV40 promoter elements, and vectors expressing

p53 and hBRE1A. As shown in Figure 2.2.5A, at a limiting concentration of p53, ectopic expression of hBRE1A produced 1.9-fold (mdm2), 1.9-fold (p21) and 1.8-fold (GADD45) increases in the levels of luciferase expression in the presence of p53. Although hBRE1A moderately enhanced transcription in the absence of p53 in some cases (compare lanes 9 and 12), similar results were observed for other p53 coactivators such as p300, PRMT1 and CARM1 (data not shown) and most likely reflects the action of hBRE1A with other transcription factors that operate on the strong SV40 promoter. In a further analysis with a pWWP-luciferase reporter bearing only the natural p21-core promoter (Nakano et al., 1997), ectopic expression of hBRE1A resulted in a smaller, but significant, effect on transcription in the presence of p53 (lane 15), but showed no effect on transcription in the absence of p53 (compare lanes 13 and 16).

To investigate the effect of histone ubiquitylation on transcription from a chromosomal (rather than episomal) gene, I employed a cell line (GAL4-293T) that contains an integrated luciferase reporter bearing five copies of a GAL4-responsive element. Ectopic expression of hE1 (Figure 2.2.5B, lanes 7-9) and hBRE1A (lanes 3-5) resulted in modest dose-dependent increases of luciferase activity, whereas co-expression of both proteins showed a near additive effect (lanes 11-13). The limited (approximately 2-fold), but nonetheless significant, stimulatory effects of ectopic hE1 and hBRE1A in

Figure 2.2.5 Human BRE1A Functions as a Versatile Coactivator in Activator-Dependent Transcription



(A and B) H1299 cells were transfected with luciferase reporters containing p53-responsive elements from different p53 target genes in combination with vectors expressing p53 and/or hBRE1A, as indicated (A). The GAL4-293T cell line was transfected, as indicated, with expression plasmids for GAL4-VP16, hE1 and hBRE1A (B). Cell extracts were subjected to luciferase analyses. The levels of activation observed in cells transfected with activator alone were arbitrarily set at 100%. (C) H1299 cells were transiently transfected with p53 and hBRE1A expression plasmids as indicated. Total RNAs were subjected to RT-PCR analyses for p21, mdm2, GAPDH, and p53 mRNA levels, as indicated.

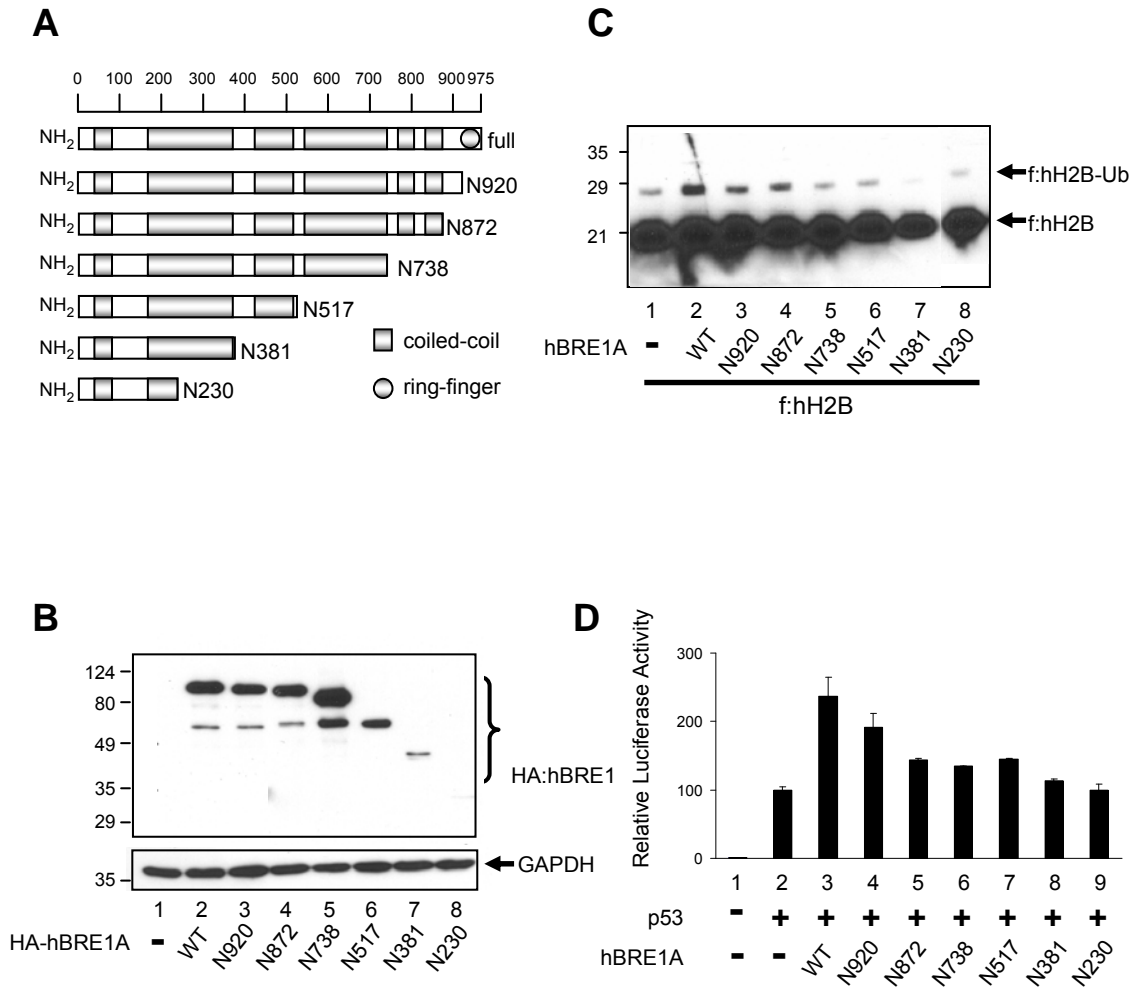
these experiments, and in those of Figure 2.2.5A, likely reflect the presence of significant levels of endogenous hE1 and hBRE1A. The coactivator function of hBRE1A in these assays is dependent on DNA-binding activators, because hBRE1A alone did not enhance transcription significantly (Figure 2.2.5B, lanes 10 and 14). I also tested the effects of hRAD6A and hRAD6B on p53- and GAL4-VP16-dependent transcription, but these enzymes either had no effect or moderately decreased transcription (data not shown).

To investigate whether hBRE1A affects transcription of an endogenous p53-responsive gene, H1299 cells were transfected with an hBRE1A expression vector in the presence or absence of a p53 expression vector, and transcripts were measured by RT-PCR. At a limiting concentration of the p53 vector, p53 expression alone resulted in a moderate increase of p21 mRNA (Figure 2.2.5C, top panel, lane 2) and no detectable change of mdm2 mRNA (second panel, lane 2). However, whereas expression of hBRE1A in the absence of p53 did not change the p21 and mdm2 mRNA levels (lane 3), co-expression of p53 and hBRE1A resulted in large increases in p21 and mdm2 mRNA levels (lane 4). Taken together, these results indicate that hBRE1A functions as a coactivator for at least certain DNA-binding activators tested in this assay.

The Human BRE1A C-Terminus, Including Its Ring-Finger Domain, Is Required for Optimal Coactivator Function and Histone H2B Ubiquitylation

To determine the region(s) in hBRE1A that is required for H2B ubiquitylation and coactivator function, I generated serial deletion mutants of hBRE1A (Figure 2.2.6A). 293T cells were transfected with HA-hBRE1A expression vectors and ectopic expression of each protein was confirmed by anti-HA immunoblot (Figure 2.2.6B). The protein expression levels for all deletion mutants other than N381 and N230 were comparable to that of wild type (wt). Small-scale cell fractionation experiments revealed that N381 and N230 mutants are mainly localized in the cytoplasm, whereas WT and all other mutant proteins are exclusively localized in the nucleus (data not shown). This indicates that a nuclear localization motif resides between 381 and 517 amino acids in hBRE1A and that nuclear localization is important for the stability and/or efficient expression of hBRE1A protein. The C-terminal deletion mutants N920 and N872 showed only moderate increases in H2B ubiquitylation relative to the large increase effected by wt hBRE1A (Figure 2.2.6C, lanes 1-4), whereas the other nuclear-localized deletion mutants (N738 and N517) showed no increases when compared to the level in mock-transfected cells (compare lane 1 vs. lanes 5-6). In functional assays, H1299 cells were transfected with an mdm2 luciferase reporter and vectors expressing hBRE1A and corresponding deletion mutants (Figure 2.2.6D).

Figure 2.2.6 The Human BRE1A C-terminus, Including Its Ring-Finger Domain, Is Required for Optimal Coactivator Function and H2B Ubiquitylation



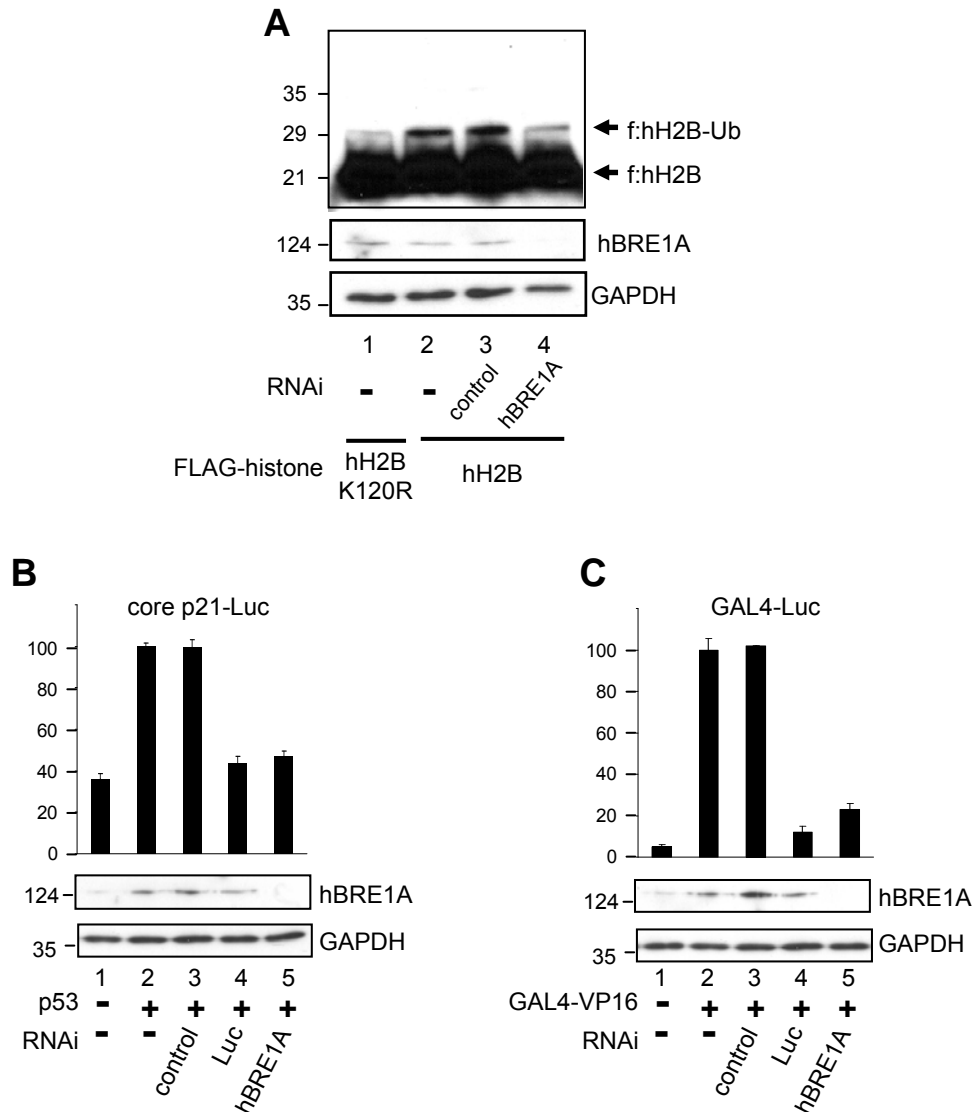
(A) Schematic diagram of wild type and mutant hBRE1A. (B) Expression levels of ectopic wild type and mutant hBRE1A proteins following transfection of 293T cells with corresponding vectors. Expressed proteins were probed by immunoblot using anti-HA antibody. (C and D) Wild type and mutant hBRE1A were tested for H2B ubiquitylation activities following transfection of 293T cells with corresponding vectors (C) and for coactivator functions following transfection of H1299 cells with an mdm2 reporter and vectors expressing p53 and hBRE1A as indicated. (D)

Deletion of the ring-finger domain in hBRE1A (N920) resulted in lower transcription enhancement relative to that observed with wt hBRE1A (Figure 4D, lanes 2-4). The other nuclear-localized mutants showed even less stimulatory activity (lanes 5-7), whereas the cytoplasmic-localized N381 and N230 mutants showed none (lanes 8 and 9). These data indicate that the ring finger-containing C-terminus is required for optimal stimulation by ectopic hBRE1A of both H2B ubiquitylation and coactivator functions, and also indicate a correlation between these activities.

Human BRE1A Is Required for Histone H2B Ubiquitylation and Efficient Activator-Dependent Transcription

To determine whether endogenous hBRE1A is essential for H2B ubiquitylation, I performed RNAi experiments in 293T cells. Immunoblotting confirmed the specificity and efficacy of the siRNA-mediated hBRE1A reduction, with the hBRE1A level being effectively lowered and GAPDH level remaining unaltered (Figure 2.2.7A, middle panels). In control cells, ectopic FLAG-H2B showed a high level of ubiquitylation while a FLAG-H2BK120R mutant showed a dramatically reduced level of ubiquitylation (top panel, compare lanes 1 and 2). Importantly, hBRE1A siRNA lowered H2B ubiquitylation to a level comparable to that of the H2BK120R mutant (lane 4 versus lane 1). This inhibition was specific, because the unrelated control siRNA had no effect (lane 3).

Figure 2.2.7 Human BRE1A Is Required for H2B Ubiquitylation and Efficient Activator-Dependent Transcription



(A) 293T cells treated with hBRE1A siRNA and nonspecific (control) siRNA were transfected with FLAG-H2B and FLAG-H2BK120R expression vectors. Cell extracts were immunoprecipitated with M2 agarose and subjected to immunoblot analysis with anti-FLAG (B and C) H1299 cells (B) and GAL4-293T cells (C), previously treated with siRNA targeting nonspecific (control), firefly luciferase (Luc) and hBRE1A as indicated, were transfected with core p21-luciferase reporter and p53 (B) and with GAL4-VP16 (C), respectively. Cell extracts were subjected to luciferase analysis (top panels). The activation observed from non-siRNA treated cells was arbitrarily set as 100%.

To test the role of endogenous hBRE1A in activator-dependent transcription *in vivo*, RNAi treated H1299 cells were transfected with a core p21-luciferase reporter and p53 expression vector (Figure 2.2.7B). hBRE1A siRNA reduced the level of p53-dependent transcriptional activation observed in the mock-transfection (no siRNA) control by 85% (lane 5 versus lanes 2 and 1). A requirement for endogenous hBRE1A in GAL4-VP16-mediated transcription was also tested in GAL4-293T cells using hBRE1A siRNA. In this case, knockdown of hBRE1A resulted in an 80% inhibition of GAL4-VP16-dependent transcription (Figure 2.2.7C, compare lane 5 with lanes 2 and 1). An unrelated control siRNA had no effect (lanes 3 in Figure 2.2.7B and 2.2.7C) and an siRNA specific for firefly luciferase resulted in more than 70% inhibition of activation (lanes 4 in Figure 2.2.7B and 2.2.7C), thus confirming the efficacy of siRNA-mediated inhibition. These data indicate and confirm that hBRE1A is required both for H2B ubiquitylation and for optimal activator-dependent transcription.

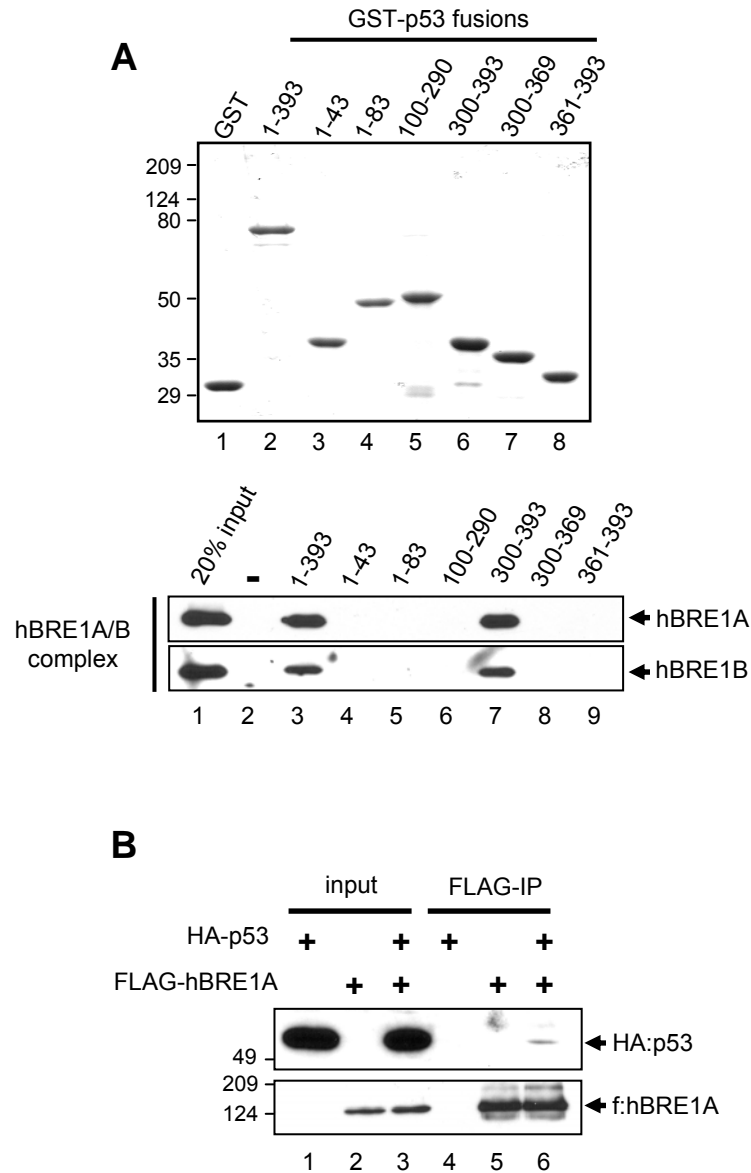
Human BRE1A Physically Binds to p53 and Is Recruited to the Promoter in a p53-Dependent Manner

To test the possibility that the coactivator function of hBRE1A might reflect a direct binding to DNA-binding activators, I examined the interactions of purified FLAG-hBRE1A/B complex (See Chapter 3 for the details about

hBRE1A/B complex) and GST-p53 fusion proteins. To determine the region(s) in p53 that is required for interaction to hBRE1A/B, I also generated serial deletion mutants of p53 in GST-fusion form (Figure 2.2.8A, top panel). hBRE1A/B showed a direct interaction with full-length and C-terminal 300-393 of p53 (bottom panel, lanes 3 and 7). In a test for intracellular interactions, ectopically expressed HA-p53 protein was co-immunoprecipitated by anti-FLAG antibody (M2 agarose) only in the presence of FLAG-hBRE1A (Figure 2.2.8B). These observations imply that hBRE1A directly binds to p53 *in vitro* and *in vivo*.

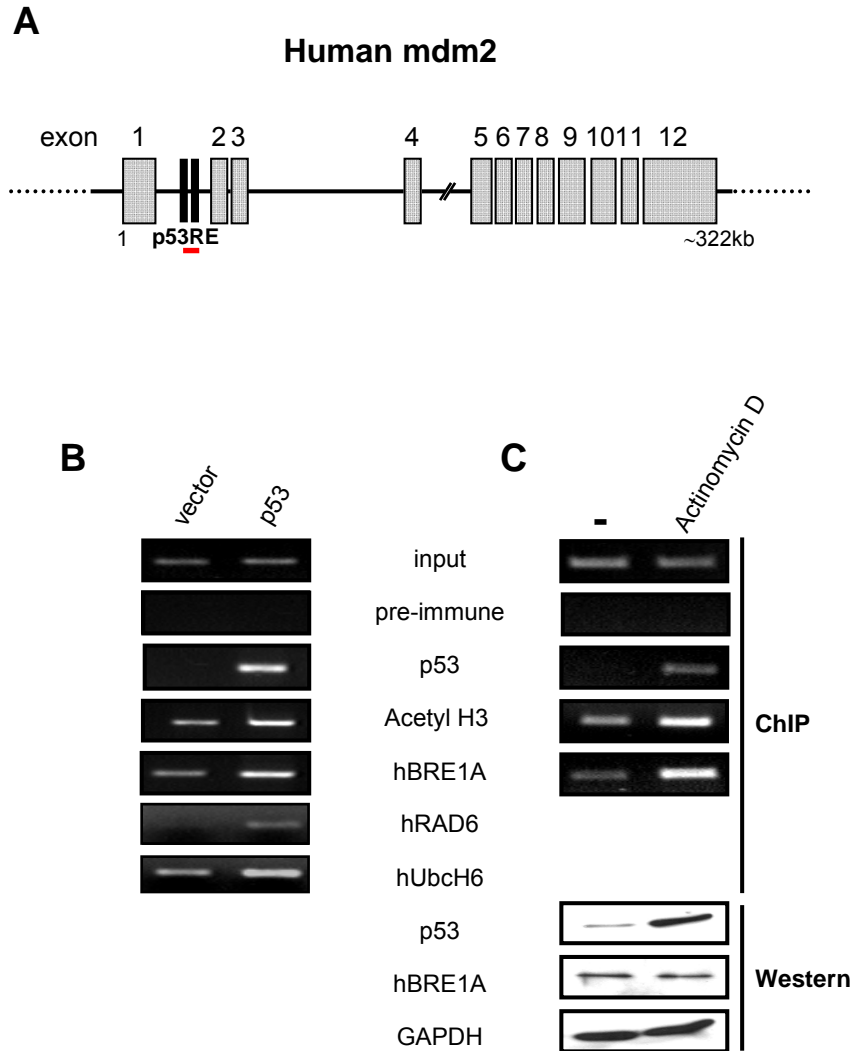
My observation that hBRE1A and p53 interact directly raised the question of whether hBRE1A is recruited to the promoter of p53-responsive genes in a p53-dependent manner. To test this possibility, I transiently transfected H1299 cells with control and p53 expression vectors and performed a chromatin immunoprecipitation (ChIP) analysis with primers for the mdm2 promoter (Figure 2.2.9A). This analysis showed that the hBRE1A level on the mdm2 promoter is significantly increased following ectopic expression of p53 (Figure 2.2.9B). The ChIP analysis also showed a p53-dependent accumulation of H3 acetylation in agreement with previous results (Kaesler and Iggo, 2004). Although I failed to see any function of hRAD6B with hBRE1A *in vivo*, a ChIP analysis also showed accumulation of hRAD6 on the mdm2 promoter in response to p53. Furthermore, to test whether activation

Figure 2.2.8 Human BRE1A Physically Binds to p53 *in Vitro* and *in Vivo*



(A) Analysis of purified GST and GST-p53 serial-deletion proteins by SDS/PAGE and Coomassie blue staining (top panel). Recombinant FLAG-hBRE1A/B was tested for binding to GST or GST-p53 proteins, and bound proteins were scored by anti-FLAG immunoblot (bottom panel). (B) 293T cells were transfected with vectors expressing HA-p53 and/or FLAG-hBRE1A proteins, as indicated. Cell extracts were incubated with M2-agarose, and bound proteins were visualized by immunoblot with anti-HA (top panel) and anti-FLAG (bottom panel) antibodies.

Figure 2.2.9 Human BRE1A and RAD6 Are Recruited to the Human Mdm2 Promoter in a p53-Dependent Manner



(A) Schematic diagram of human mdm2 gene. The region containing internal p53 binding sites and probed by ChIP analysis is indicated by red bar. (B and C) H1299 cells were transiently transfected with p53 expression vector (B) and U2OS cells were treated with actinomycin D (C). Chromatin immunoprecipitation and immunoblot analyses were performed with indicated antibodies.

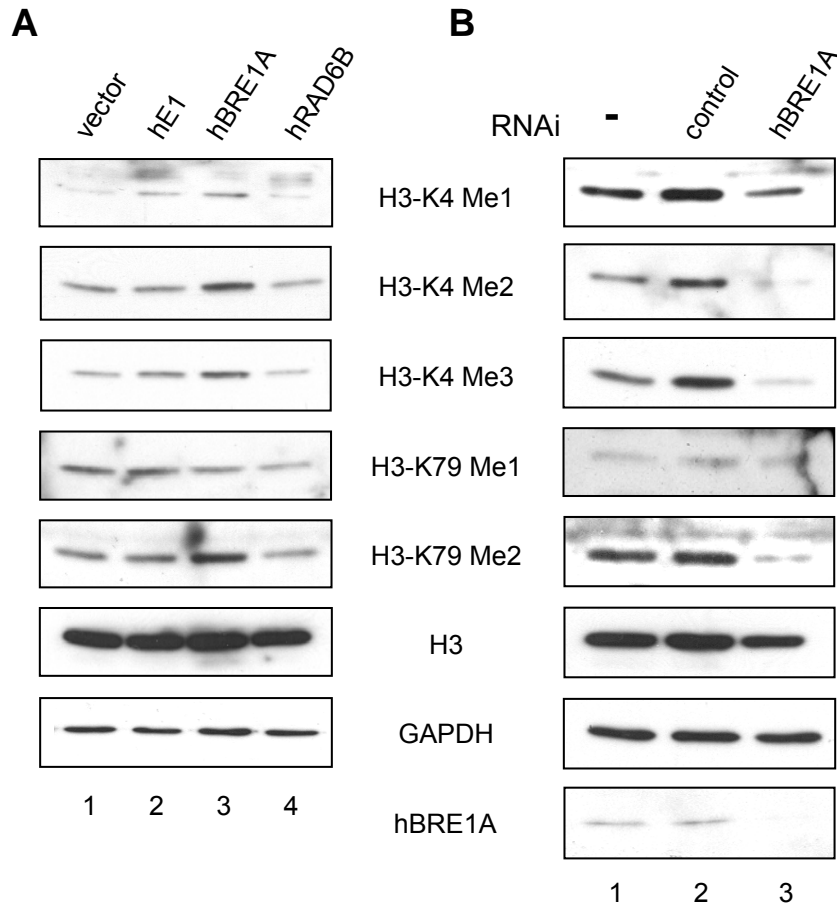
of endogenous p53 enhances accumulation of hBRE1A on the mdm2 promoter, U2OS cells were treated with actinomycin D and then subjected to a ChIP analysis (Figure 2.2.9C). A 5 hour actinomycin D treatment resulted in elevation of endogenous p53, with no effect on hBRE1A level, and a ChIP analysis showed that hBRE1A occupancy on the mdm2 promoter was dramatically increased concomitant with activation of p53.

Human BRE1A Is Linked to Histone H3-K4 and K79 Methylation

H2B ubiquitylation is the first known histone modification indicative of a cross-talk between histone modifications and, in yeast, is a prerequisite for H3-K4 and K79 methylation, but not for K36 methylation (Sun and Allis, 2002; Ng et al., 2002). Based on my observation that hBRE1A affects the global H2B ubiquitylation level, I tested whether hBRE1A also influences global H3-K4 and K79 methylation in human cells. To this end, 293T cells were transfected with hE1, hBRE1A, and hRAD6B expression vectors. Total cell lysates then were probed with anti-H3-methyl K4 and anti-H3-methyl K79 antibodies. As shown in Figure 2.2.10A, ectopic hBRE1A expression resulted in moderate increases in di-, and tri-methylated H3-K4 and di-methylated H3-K79 whereas ectopic hRAD6B had no effect.

To investigate the effect of endogenous hBRE1A on H3-K4 and K79 methylation, I performed RNAi experiments. A clear reduction of

Figure 2.2.10 Human BRE1A Is Linked to H3-K4 and K79 Methylation



(A and B) 293T cells were transiently transfected with control, hE1, hBRE1, or hRAD6B expression plasmids (A), and 293T cells were treated with siRNA targeting nonspecific (control) and hBRE1A as indicated (B) Total cell lysates were subjected to immunoblot analysis with antibodies as indicated.

endogenous hBRE1A by hBRE1A siRNA, but not by a control siRNA, resulted in significant concomitant decreases of di-, and tri-methylated H3-K4 and di-methylated H3-K79 levels with little or no changes in mono-methylated H3-K4 and H3-K79 levels (Figure 2.2.10B). These data indicate that the reduction of hBRE1A protein level down-regulates H3-K4 di-, and tri-methylation and H3-K79 di-methylation, and thus imply that hBRE1A-mediated H2B ubiquitylation by hBRE1A also plays a role in subsequent H3-K4 and K79 methylation in mammalian cells.

2.3 DISCUSSION

A role for yBRE1 in H2B ubiquitylation, and subsequent H3-K4 and K79 methylation and selective gene activation, has been well established in yeast. In an extension of these studies to mammalian cells, the present study reports the identification of hBRE1A as an H2B-specific E3 ubiquitin ligase from human cells. Studies in which hBRE1A was either overexpressed or depleted (by RNAi) have documented a role for hBRE1A in H2B ubiquitylation and a transcriptional coactivator function that appears to involve enhanced promoter recruitment of hBRE1A through direct activator (p53 and GAL4-VP16) interactions. A role for hBRE1A in global H3-K4 and K79 methylation, as observed in yeast, is also documented. These studies indicate that histone ubiquitylation, like histone acetylation and methylation, actively participates in transcriptional regulation in higher eukaryotes.

Enzymes Involved in Histone Ubiquitylation in Mammalian Cells

The conjugation of ubiquitin to proteins involves the ubiquitin activating enzyme E1, an E2 ubiquitin conjugating enzyme, and an E3 isopeptide ligase that binds both to its specific substrate and to its cognate E2 enzyme and thus determines substrate specificity (Pickart 2001). Hwang et al. (2003) reported that yBRE1 functions as an H2B-specific E3 ubiquitin ligase and identified two candidate human homologues, RNF20 (designated hBRE1A in this study) and RNF40, that show 47% and 43% sequence similarity, respectively, to yBRE1. My current analysis shows that hBRE1A is localized to the nucleus and interacts with H2B both *in vivo* and *in vitro*. Although the lack of a corresponding functional E2 enzyme (below) has precluded analysis of hBRE1A-dependent H2B ubiquitylation *in vitro*, hBRE1A overexpression and depletion (by RNAi) have been found, respectively, to increase and decrease global H2B ubiquitylation. In addition, an H2B mutational analysis has shown that K120 is the dominant (potentially exclusive) hBRE1A-dependent ubiquitylation site. These results strongly imply that hBRE1A is a functional homologue of yBRE1. In contrast, a similar test of RNF40 revealed no apparent effects on H2B ubiquitylation.

Relevant to the roles of hBRE1A in H2B ubiquitylation, overexpression of hE1 was found to enhance the endogenous hBRE1A level, implying that they

are somehow linked and act physiologically in same pathway. Thus, the observed enhancement of H2B ubiquitylation, as well as GAL4-VP16-dependent transcription and H3-K4 methylation (below), by hE1 may be exerted by up-regulation of hBRE1A by a yet unknown mechanism.

Among the several E2 enzymes in yeast, yRAD6 is required for H2B ubiquitylation *in vivo* (Robzyk et al., 2000). In human, two candidate homologues (hRAD6A and hRAD6B) of yRAD6 were reported (Koken et al., 1991). Although they show very strong sequence similarity (~70% sequence identity) to yRAD6, it is not clear whether either is involved in histone ubiquitylation in mammalian cells (Baarends et al., 1999). The studies presented here suggest that, unlike hBRE1A, ectopic hRAD6A and hRAD6B do not affect H2B ubiquitylation, activator-dependent transcription, or histone H3 methylation. A further analysis confirmed that yBRE1 interacts directly with yRAD6, but, under the same binding conditions, hBRE1A failed to show any interaction with either hRAD6A or hRAD6B--thus implying that hRAD6 proteins might not be cognate E2 enzymes for hBRE1A (data not shown) or that my assay might not be relevant to screen E2 enzyme for hBRE1A. The problem is compounded by the presence of multiple E2 enzymes--11 in yeast and even more in higher organisms. Interestingly, it recently has been reported that the E3 Ring1b utilizes Ubc5C, but not hRAD6, as an E2 enzyme for H2A ubiquitylation *in vitro* (Wang et al., 2004),

further implying that hRAD6 is not involved in all histone ubiquitylation pathways in higher organisms. Thus, identification of the cognate E2 enzyme(s) for hBRE1A remained an important objective of further studies of hBRE1A-dependent H2B ubiquitylation mechanisms and role(s) in transcriptional regulation.

Role of hBRE1A as a Versatile Transcriptional Coactivator in Mammalian Cells

Beyond several studies implicating yBRE1 in transcriptional activation of specific genes (Osley 2004), there is a single recent report indicating a role for *Drosophila* BRE1 in a Notch-dependent signaling pathway (Bray et al., 2005). Here, I have documented an intracellular coactivator function for hBRE1A through analyses of effects of hBRE1A overexpression or depletion (RNAi) on activator-dependent transcription of both endogenous genes and ectopic (transfected) reporters. A mutational analysis of hBRE1A has shown a correlation between H2B ubiquitylation and transcription, and further indicated that the ring finger domain is required for optimal function of hBRE1A. Apart from the coactivator functions for p53 and GAL4-VP16 that are demonstrated here, a coactivator function for thyroid hormone receptors also has been demonstrated (data not shown). Hence, hBRE1A has the potential for broad coactivator functions.

In relation to mechanisms for hBRE1A recruitment and function, data presented here show both *in vivo* and direct *in vitro* interactions of hBRE1A with p53. hBRE1A has also been found to interact directly with GAL4-VP16 and thyroid hormone receptor (data not shown). Hence, it is likely that DNA-bound activators are involved directly in hBRE1A recruitment. Consistent with this possibility, p53-enhanced accumulation of hBRE1A on p53 target gene has been demonstrated in this report.

Relevant to my observations, several reports in yeast have shown that yBRE1-dependent H2B ubiquitylation within chromatin is dependent on an activator and is required for optimal gene transcription. For example, both yBRE1/yRAD6 recruitment and H2B ubiquitylation around the *GAL1* promoter are dependent on galactose induction and precede recruitment of the histone acetyltransferase-containing SAGA complex (Kao et al., 2004). The fact that SAGA is one of earliest factors recruited to the *GAL1* promoter during galactose induction (Bhaumik and Green, 2001) raises the possibility of a direct interaction between activator (yGAL4p) and the histone ubiquitylation machinery. This would also be consistent with the observation that yBRE1 is indispensable for yRAD6 recruitment to the promoter (Wood et al., 2003a). Nonetheless, despite the detailed information on yBRE1 function in yeast, and in contrast to my demonstration of activator interactions with

hBRE1A, there are as yet no reports of activator interactions with yBRE1 that are involved in yBRE1/yRAD6 recruitment.

Other Aspects of hBRE1A Function in Transcription in Mammalian Cells

My analyses have shown stronger effects on transcription by RNAi-induced knockdown of hBRE1A than by hBRE1A overexpression, presumably due to the presence of significant level of endogenous hBRE1A. In addition, and possibly related, both the H2B ubiquitylation activity of yBRE1/yRAD6 and the H2B deubiquitylation activity of the Ubp8 component of SAGA are required for efficient transcription in yeast, implying a dynamic histone ubiquitylation state during transcription (Zhang, 2003). If this were also the case in mammalian cells, persistent histone ubiquitylation around promoter regions as a result of hBRE1A overexpression might restrict the overall level of transcriptional enhancement. Thus, identification of a potential ubiquitin hydrolysase for ubiquitylated H2B and its role in transcriptional activation remain an important problem in higher organisms.

My analyses show that hBRE1A depletion results in large decreases in global H3-K4 di- and tri-methylation and H3-K79 di-methylation. Relevant to my findings, and in extension of earlier studies showing that H3-K4 methylation is dependent upon yBRE1 (Wood et al., 2003a), a recent study in yeast has

revealed that histone ubiquitylation controls processive methylation (di-, and tri-) of H3-K4 and by SET1 and H3-K79 by DOT1 (Shahbazian et al., 2005). These observations imply that the role of histone ubiquitylation in mediating trans-tail histone modifications is conserved from yeast to humans. In yeast, SET1 is recruited to the transcription machinery through the PAF complex (below) and the resulting H3-K4 methylation provides memory of recent transcription (Ng et al., 2003). Although SET1 serves as sole H3-K4 methyltransferase in yeast, there are several enzymes (SET1, SET7/9, MLL-1, MLL-2, MLL-3, ALR) that have H3-K4 methyltransferase activity in human cells (Sims et al., 2003). Hence, although an MLL-1 complex has been shown to effect activator-dependent H3-K4 methylation of a nucleosomal template in the absence of H2B ubiquitylation (Dou et al., 2005), it remains to be determined whether H2B ubiquitylation by hBRE1A is important for efficient H3-K4 methylation by the other H3-K4 methyltransferases or whether it may enhance H3-K4 methylation by the MLL-1 complex.

Several findings have demonstrated that the yeast PAF complex is required for H2B ubiquitylation (Sims et al., 2004; Xiao et al., 2005). Thus, deletion of either the RTF1 subunit or the PAF1 subunit results in drastic loss of H2B ubiquitylation. Furthermore, RTF1 enables the yBRE1/yRAD6 complex to associate, and travel, with RNA polymerase II during transcription elongation. However, unlike yBRE1, neither RTF1 nor PAF1 are required for

the recruitment of RAD6 to the promoter of active genes (Wood et al., 2003b). Hence, it is thought that the histone ubiquitylation machinery associates with the promoter and with RNA polymerase II through separate processes-- i.e. that yBRE1/yRAD6 is recruited to the promoter via an activator (as suggested by the present data) and then transferred to the transcription machinery via the PAF complex (Hampsey and Reinberg, 2003). These studies raise interesting questions regarding the mechanism by which the histone ubiquitylation machinery communicates with the transcription machinery for efficient transcription in mammalian cells. The present report of a key component (hBRE1A) of this machinery sets the stage for analyses of this question.

CHAPTER 3

*Identification of Human RAD6 as E2
Ubiquitin Conjugating Enzyme
Responsible for Histone H2B
Ubiquitylation in Mammalian Cells*

3.1 PREFACE: E2 ENZYME FOR HISTONE H2B UBIQUITYLATION IN MAMMALIAN CELLS

Among the several E2 enzymes in yeast, yRAD6 is responsible for H2B ubiquitylation *in vivo* (Robzyk et al., 2000). In human, two candidate homologues (hRAD6A and hRAD6B) of yRAD6 were reported (Koken et al., 1991). Although they show very strong sequence similarity (~70% sequence identity) to yRAD6, the published studies discussed in Chapter 2 showed that, unlike hBRE1A, ectopic hRAD6A and hRAD6B do not affect H2B ubiquitylation or activator-dependent transcription. These observations suggested that hRAD6 and hBRE1A might not be in same physiological pathway or that ectopic expression of hRAD6 proteins might not be a relevant approach because these enzymes are not limiting in the protein ubiquitylation process *in vivo*.

The ring-finger E3s bridge E2s and substrates through direct protein interactions. These relationships result in not only substrate ubiquitylation but also E3 auto-ubiquitylation (Joazeiro and Weissman 2000; Lorick et al., 1999). This self-ubiquitylation activity is thought to act as a regulatory mechanism that controls the abundance of E3s by marking them for degradation (Brown et al., 2002; Fang et al., 2000). Therefore, these two criteria, i.e. direct E2-E3 interaction and auto-ubiquitylation of E3 by specific E2, are widely used in screening methods to elucidate functional cognate E2/E3 pairs.

Based on the above notion, a further analysis in Chapter 2 confirmed that yBRE1 interacts directly with yRAD6, but, under the same binding conditions, hBRE1A failed to show any interaction with several tested E2s-- thus precluding the systematic *in vitro* approach for the role of H2B ubiquitylation in transcription (data not shown).

At the almost same time that the studies in Chapter 2 were completed, the Reinberg lab reported an interesting finding. Through the affinity purification of FLAG-RNF20 (hBRE1A) from cell line, they showed that hBRE1A and hBRE1B form a heterodimeric complex and that the resulting purified complex has *in vitro* H2B ubiquitylation activity in conjunction with E2 hUbcH6 (Zhu et al., 2005b).

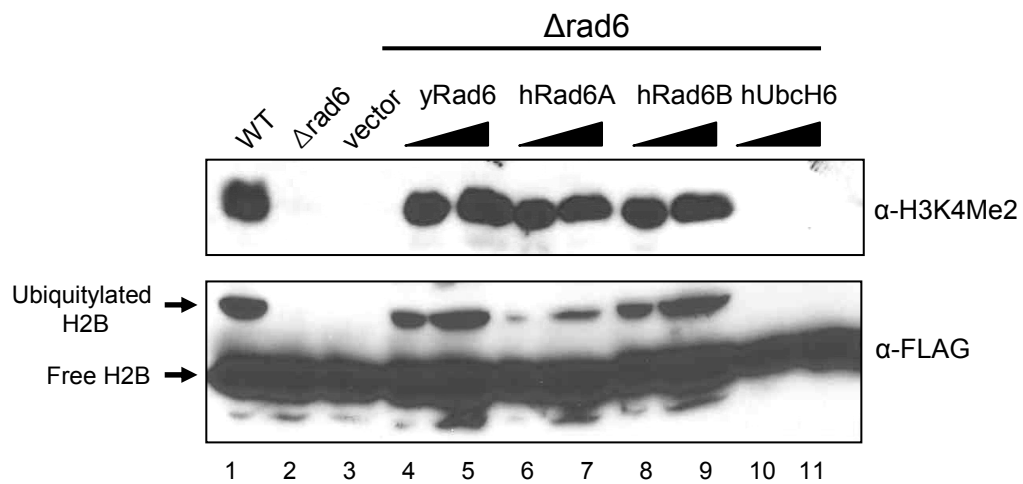
However, in an extension of my H2B *in vitro* ubiquitylation study, I have found a large discrepancy between my results and those in the Reinberg report. This chapter describes the identification of hRAD6 proteins as cognate E2s for hBRE1A/B complex, the mechanism by which the H2B ubiquitylation factors communicate with each other and the establishment of an *in vitro* H2B ubiquitylation assay.

3.2 RESULTS

Human RAD6 Proteins Are Functional Homologues of yRAD6

Based on the fact that highly conserved RAD6 proteins are present in almost all organisms and involved in many common cellular processes (Koken et al., 1991; Prakash et al., 1993) and the fact that H2B ubiquitylation is conserved from yeast to human, we (collaboration with Shilatifard Lab in Stowers institute) first tested whether hRAD6 proteins can substitute the yRAD6 E2 activity in yeast. To this end, several E2 proteins including yRAD6, hRAD6A, hRAD6B and hUbc6 were expressed from the yeast *ADH1* promoter in the *rad6Δ* strain YGL058W (Robzyk et al., 2000). Genetic complementation tests revealed that both hRAD6A and hRAD6B can carry out yRAD6-mediated H2B ubiquitylation and subsequent H3-K4 dimethylation (Figure 3.2.1, lanes 4-9), indicating that the function of RAD6 in mediating these histone modifications is highly conserved. The identical observation showing conserved function of mouse RAD6 in H3-K4 dimethylation has also been reported (Sun and Allis 2002). The yRAD6 contains a long-acidic chain at its C-terminus which mediates poly-ubiquitylation of free histones (Sung et al., 1988), but hRAD6 proteins lack this domain. Therefore, our observation also suggests that C-terminal acidic patch in yRAD6 protein is not essential for H2B ubiquitylation *in vivo*. In contrast to the clear activity of hRAD6 proteins, hUbcH6 could not substitute with yRAD6 (lanes 10-11), thus

Figure 3.2.1 Human Rad6 Proteins Complement the Activity of Yeast RAD6 for H2B Ubiquitylation and H3-K4 Methylation



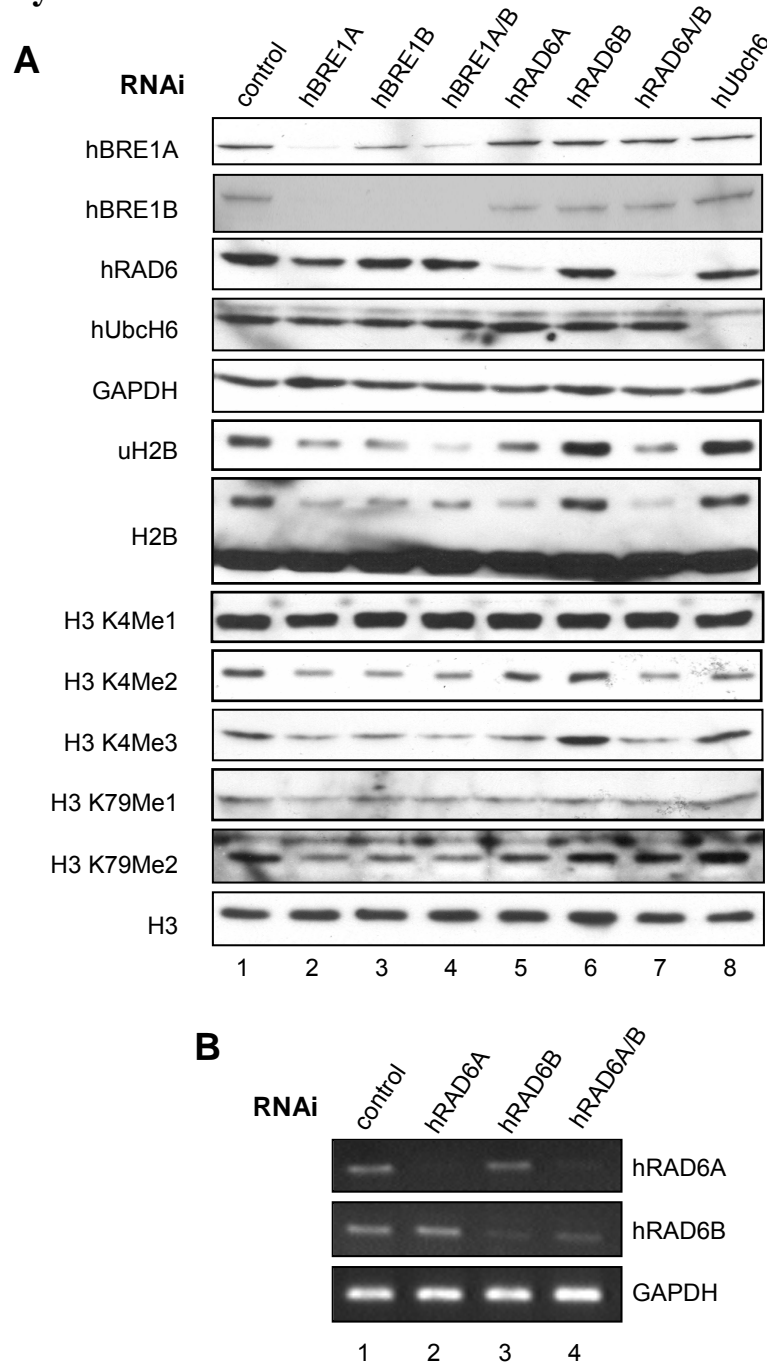
Yeast whole cell extracts from wild type (WT), $\Delta rad6$ and $\Delta rad6$ strains containing plasmids encoding the indicated E2 ubiquitin conjugating enzyme driven by *ADH1* promoter were assessed for H3K4 di-methylation (top panel) and H2B ubiquitylation (bottom panel), respectively.

indicating that hUbcH6 can not substitute for the yRAD6 activity in effecting H2B ubiquitylation and subsequent H3 methylation in yeast.

Human RAD6 Is Linked to Histone H2B Ubiquitylation and H3-K4/K79 Methylation in Mammalian Cells

I previously showed that reduction of endogenous hBRE1A by hBRE1A siRNA resulted in significant decreases of di-, and tri-methylated H3-K4 and di-methylated H3-K79 levels (Kim et al., 2005). A clear complementation activity of hRAD6 proteins for yRAD6 in histone modifications in yeast (Figure 3.2.1) let us to further examine the possible role of hRAD6 proteins in H2B ubiquitylation and subsequent H3 methylation in human cells. To investigate effects of all known H2B ubiquitylation-related factors on histone modifications, I performed RNAi experiments. First, I tested RNAi efficiency by western blot analysis. A clear reduction of endogenous hBRE1A resulted in a concomitant, near complete decrease in hBRE1B (Figure 3.2.2A, second panel, lane2), indicating that hBRE1B is not stable in the absence of hBRE1A and further indicating that hBRE1A and hBRE1B are somehow linked in cells. Interestingly, hBRE1A reduction also resulted in a significant decrease in hRAD6 (third panel, compare lanes 1 and 2), but had no effect on hUbcH6 (fourth panel, compare lanes 1 and 2). hBRE1B RNAi treatment also resulted in large decrease in the hBRE1B level (second panel, lane 3) but, in contrast to effect of hBRE1A on hBRE1B, did not affect the hBRE1A level (first panel,

Figure 3.2.2 Human BRE1A/B and Human RAD6 Are Linked to H2B Ubiquitylation and H3-K4/K79 Methylation



(A and B) 293T cells were treated with combinations of nonspecific (control), hBRE1A, hBRE1B, hRAD6A, hRAD6B and hUbcH6 siRNA as indicated. Total cell extracts were subjected to immunoblot analysis with antibodies as indicated (A). Total RNAs were subjected to RT-PCR analyses for hRAD6A, hRAD6B and GAPDH mRNA levels, as indicated.

lane 3). Thus, the stability of hBRE1B is dependent on the presence of hBRE1A, but not vice versa. Furthermore, hRAD6A RNAi treatment resulted in a clear reduction of the endogenous hRAD6 level, whereas hRAD6B RNAi did not result in any obvious change the RAD6 level (third panel, lanes 5 and 6). To test whether the latter result reflects inefficiency of hRAD6B RNAi treatment, I quantitated hRAD6A and hRAD6B transcript levels by RT-PCR analysis after RNAi treatments. This analysis showed that there is no big difference between the hRAD6A and hRAD6B RNAi efficiencies (Figure 3.2.2B). Next, I learned that the hRAD6 antibody that was developed against full-length hRAD6B by me does not discriminate between recombinant hRAD6A and hRAD6B proteins (data not shown) due to highly conserved sequences between hRAD6A and hRAD6B. Therefore, I concluded that hRAD6A is the dominant population in the total intracellular hRAD6 protein pool and that the band intensities in the anti-hRAD6 western blots represent the overall hRAD6 protein level. My speculation is also supported by a previous report indicating that hRAD6A is more abundant than hRAD6B proteins in human cells (Koken et al., 1996). I also effected an almost complete loss of hUbcH6 protein by hUbcH6 RNAi treatment, but also found that this did not cause any changes in the levels of the other proteins tested (fourth panel, lane 8).

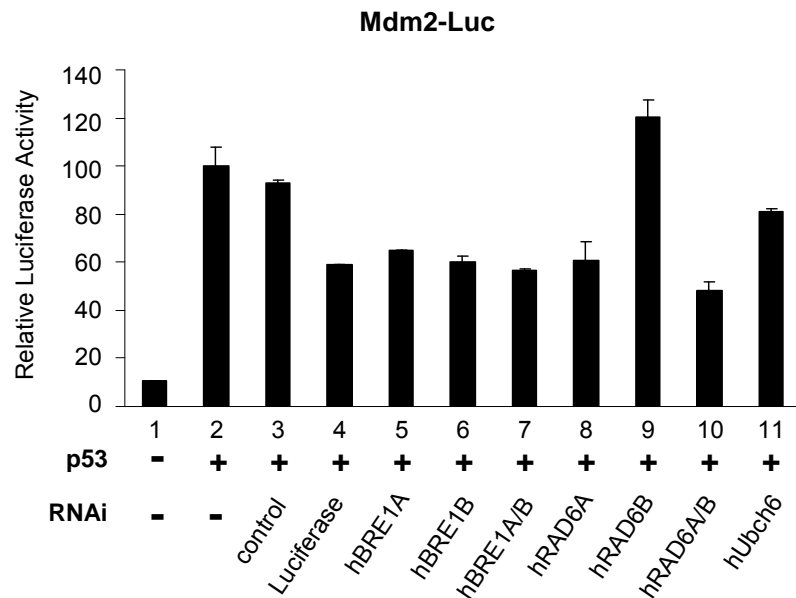
Next, I tested the effects of reductions in each of the H2B ubiquitylation-related factors on histone modifications. Consistent with our previous results (Kim et al., 2005), a clear reduction of endogenous hBRE1A by hBRE1A siRNA resulted in significant concomitant decreases of H2B ubiquitylation, di-, and tri-methylated H3-K4 and di-methylated H3-K79 levels but little or no changes in mono-methylated H3-K4 and H3-K79 levels (Figure 3.2.2A, compare lanes 1 and 2). Interestingly, hBRE1B RNAi and hBRE1A/B dual RNAi treatments also caused almost the same effects with hBRE1A RNAi treatment on examined histone modifications (lanes 3 and 4), thus suggesting that hBRE1A and hBRE1B might both play a role as E3s in the H2B ubiquitylation process. More importantly, hRAD6A and hRAD6A/B dual RNAi treatments resulted in significant decreases in H2B ubiquitylation and H3-K4 tri-methylation and a moderate decrease in H3-K79 di-methylation (lanes 5 and 7). However, hRAD6B RNAi treatment, which did not affect the global hRAD6 level, did not cause any changes in tested histone modifications, strongly indicating a clear correlation between hRAD6 and H2B ubiquitylation and H3-K4/K79 methylation levels. Significantly, in light of the conclusion of a previous study (Zhu et al., 2005b), reduction of endogenous hUbcH6 by hUbcH6 RNAi did not result in any changes in histone modifications tested (lane 8).

Taken together, these data indicate that a reduction of the hRAD6 protein level down-regulates H2B ubiquitylation, H3-K4 tri-methylation and H3-K79 di-methylation. They thus imply strongly that hRAD6 play a role in H2B ubiquitylation in mammalian cells, presumably by acting as an E2 ubiquitin conjugating enzyme in conjunction with hBRE1A and hBRE1B.

Human RAD6 Level Correlates with Efficient p53-Dependent Transcription *in Vivo*

In a previous report, I suggested a role for endogenous hBRE1A in activator-dependent transcription *in vivo* (Kim et al., 2005). To expand this study for E2s, RNAi treated H1299 cells were transfected with an mdm2-luciferase reporter and a p53 expression vector (Figure 3.2.3). hBRE1A, hBRE1B, hBRE1A/B dual, hRAD6A and hRAD6A/B dual siRNAs (lanes 5-8 and 10) reduced the level of p53-dependent transcriptional activation observed in the mock-transfection control (no siRNA, lane 2) to a level comparable level to that of the positive control treated with siRNA specific for firefly luciferase. An unrelated control siRNA had no effect (lane 3). However, hUbcH6 (lane 11) and hRAD6B (lane 9) siRNAs resulted, respectively, in a moderate decrease and a moderate increase in the level of p53-dependent transcriptional activation. These data indicate and confirm a correlation between hRAD6 level and activator-dependent transcription, and thus imply that hRAD6 is required for optimal activator-dependent transcription.

Figure 3.2.3 Human RAD6 Level Correlates with Efficient p53-Dependent Transcription



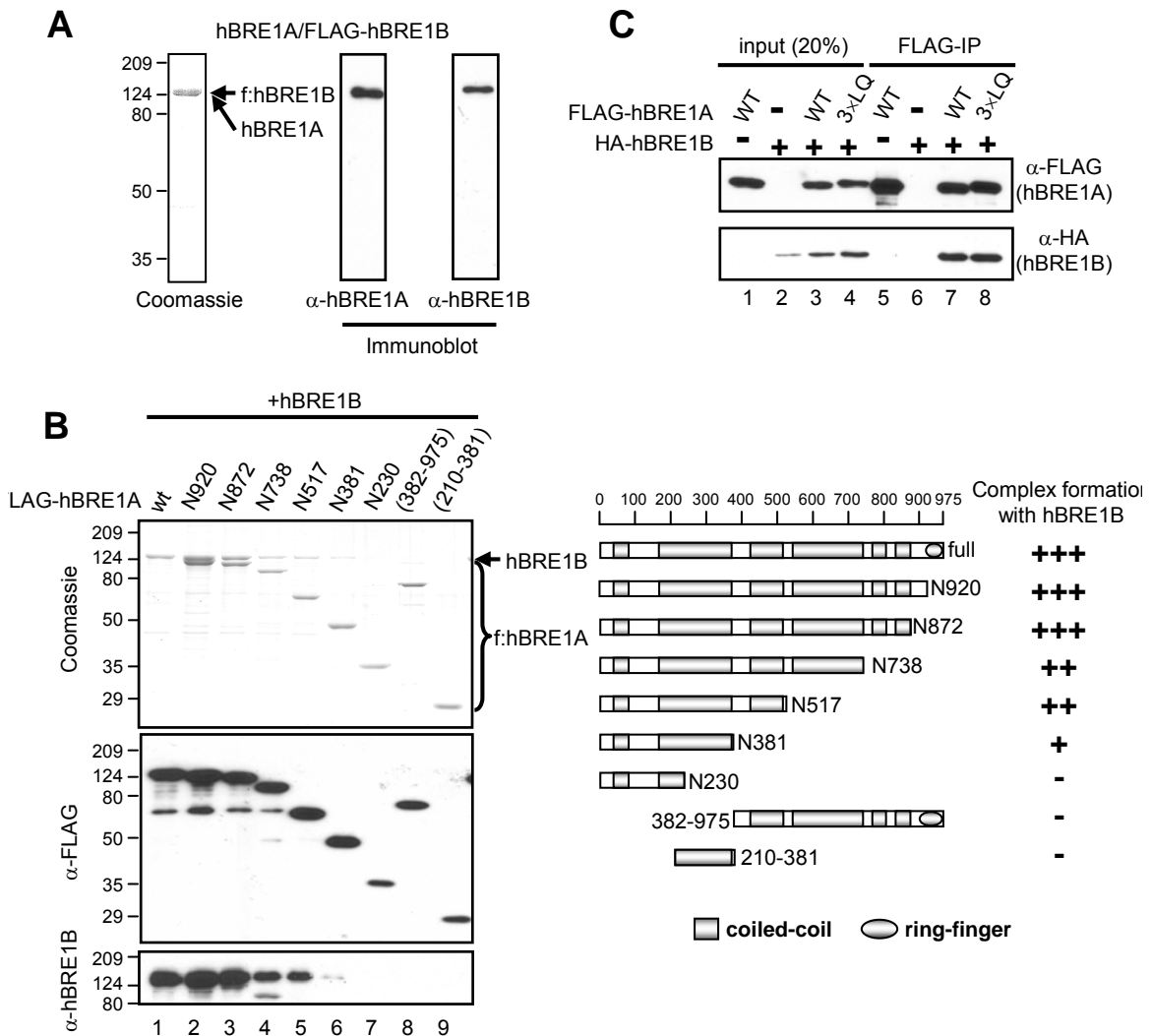
H1299 cells, previously treated with siRNA targeting nonspecific (control), firefly luciferase (Luc) hBRE1A, hBRE1B, hRAD6A, hRAD6B and hUbcH6 as indicated, were transfected with mdm2-luciferase reporter and p53. Cell extracts were subjected to luciferase analysis. The activation observed from non-siRNA treated cells was arbitrarily set as 100%.

Human BRE1A and BRE1B Form a Heterodimeric Complex

Recently, the Reinberg lab reported that, based on affinity purification of FLAG-RNF20 (hBRE1A) from a cell line, hBRE1A and hBRE1B form a heterodimeric complex (Zhu et al., 2005b). My results showing that both hBRE1A and hBRE1B affect the H2B ubiquitylation, H3 methylation and activator-dependent transcription (Figure 3.2.2 and Figure 3.2.3) also imply that these two proteins work together as E3s in H2B ubiquitylation related pathways. For a systemic study of the function of each component in H2B ubiquitylation machinery, the hBRE1A/B complex was reconstituted by coinfection of insect cells with recombinant baculoviruses that individually express hBRE1A and hBRE1B. A stable complex was then purified via the N-terminal Flag-epitope on hBRE1B. Analysis of the purified complex by SDS-PAGE and Coomassie blue staining revealed two polypeptide bands (doublet) of equal intensity, thus implying stoichiometric hBRE1A and hBRE1B levels in the complex. The presence of each protein was also confirmed by anti-hBRE1A and anti-hBRE1B antibodies, respectively (Figure 3.2.4A).

To determine the region(s) in hBRE1A that is required for complex formation with hBRE1B, I generated baculovirus vectors for serial deletion mutants of hBRE1A (Figure 3.2.4B, right panel). Insect cells were coinfecting with baculoviruses expressing each FLAG-hBRE1A and untagged hBRE1B and the resulting cell extracts were subjected to M2-agarose affinity purification.

Figure 3.2.4 Human BRE1A and BRE1B Form a Hetrodimer Complex



(A) Purified hBRE1A/FLAG-hBRE1B complex (left panel) was analyzed by SDS/PAGE and Coomassie blue staining and probed with anti-hBRE1A (middle panel) and anti-hBRE1B (right panel), respectively. (B) Baculoviruses expressing wild type and serial deletion mutant hBRE1A proteins were co-infected with hBRE1B expressing baculovirus in insect cells. Purified FLAG-hBRE1A/B complexes were analyzed by SDS/PAGE and Coomassie blue staining (top panel) and probed with anti-hBRE1A (middle panel) and anti-hBRE1B (bottom panel) antibodies, respectively. The efficiency of complex formation was summarized on right panel. (C) FLAG-hBRE1A was co-expressed with HA-hBRE1B, as indicated, in 293T cells. Cell extracts were incubated with M2-agarose and immunoprecipitated proteins were analyzed by anti-FLAG (top panel) and anti-HA (bottom panel) antibodies, respectively.

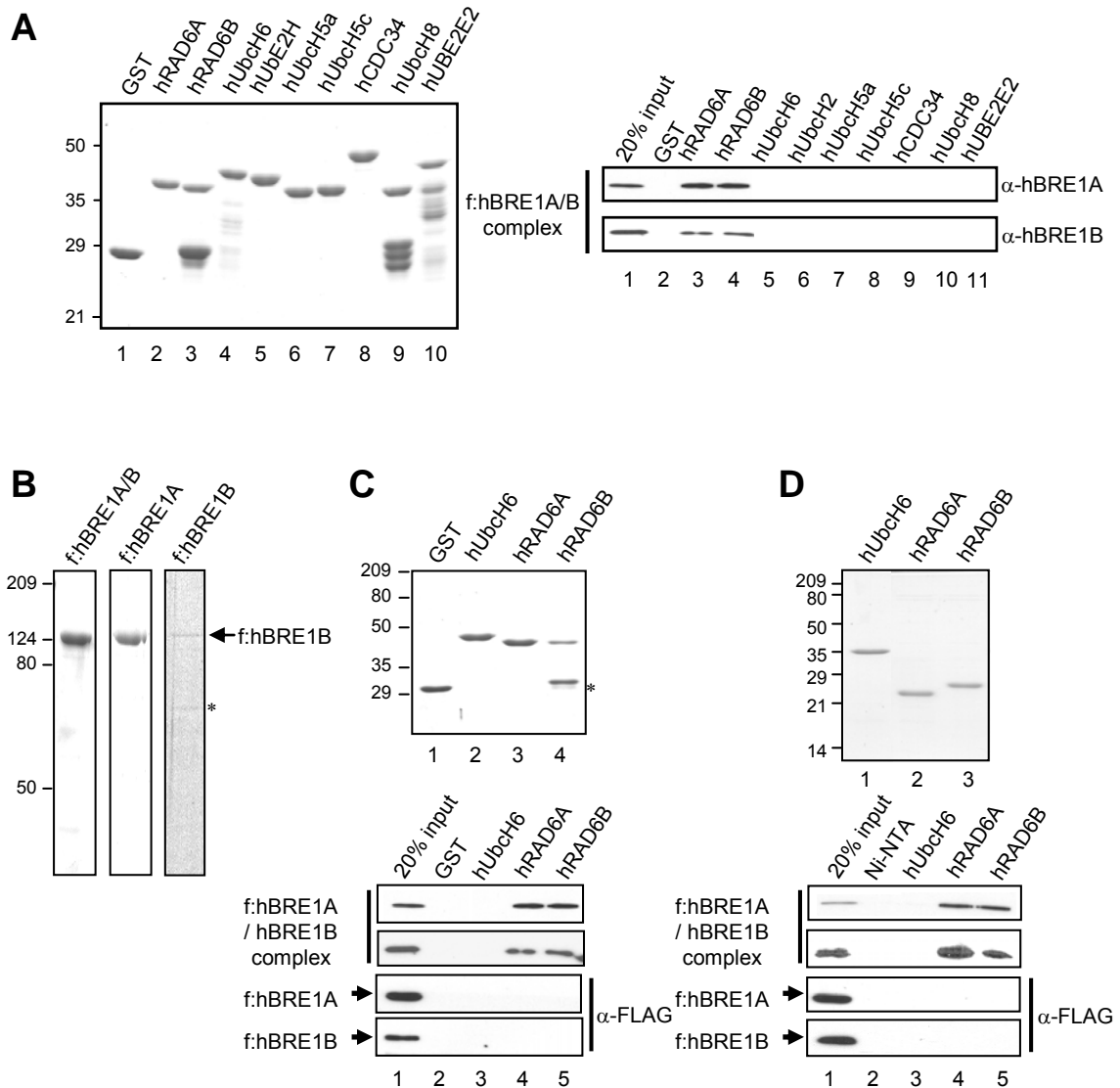
The purified complexes were analyzed by Coomassie blue staining and by anti-FLAG and anti-hBRE1B immunoblots (Figure 3.2.4B, left panels). Since the protein expression and purification yields differed for each deletion FLAG-hBRE1A mutant, I normalized protein loading to equal amounts of FLAG-hBRE1A after complex purification (top and middle panels). The C-terminal deletion mutants FLAG-hBRE1A N920 and N872 co-immunoprecipitated amounts of untagged hBRE1B equivalent to that of the wild type (wt) (bottom panel, lanes 1 to 3). The hBRE1B amounts were decreased in the complex with hBRE1A N738 and even lower in the complex with hBRE1A N381 (bottom panel, lanes 1 to 6), indicating that the regions between 872 and 738 and between 381 and 230 control the efficacy of hBRE1A/B complex formation. A further deletion mutant hBRE1A N230 completely lost the association with hBRE1B (lane 7). These results indicate that a motif critical for complex formation with hBRE1B resides between amino acids 230-381 in hBRE1A. However, this region alone seems to be insufficient for complex formation since the fragment encoding amino acids 210-381 of hBRE1A failed to co-immunoprecipitate hBRE1B (lane 9). In a test for intracellular interactions, ectopically expressed HA-hBRE1B protein was co-immunoprecipitated by anti-FLAG antibody (M2 agarose) only in the presence of FLAG-hBRE1A (Figure 3.2.4C). These observations imply that hBRE1A, through its N-terminal region, forms a stable stoichiometric complex with hBRE1B *in vivo*.

Human BRE1A/B Complex Specifically Interacts with hRAD6A and hRAD6B

Although my previous study had identified hBRE1A as an E3 responsible for H2B ubiquitylation *in vivo*, I could not pursue further systematic *in vitro* studies since I failed to identify cognate E2 enzyme for hBRE1A (Kim et al., 2005). However, the finding that hBRE1A forms a complex with hBRE1B in the cells enabled us to resume the screening of cognate E2 for hBRE1A/B complex.

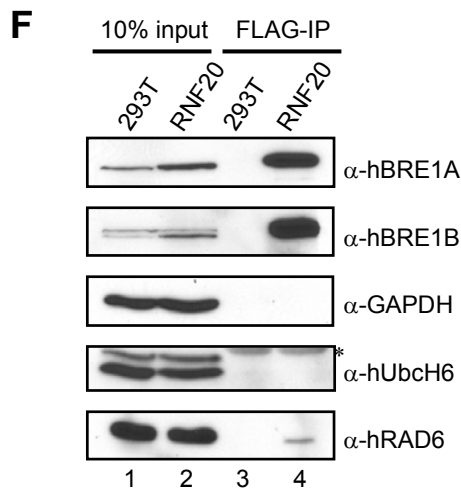
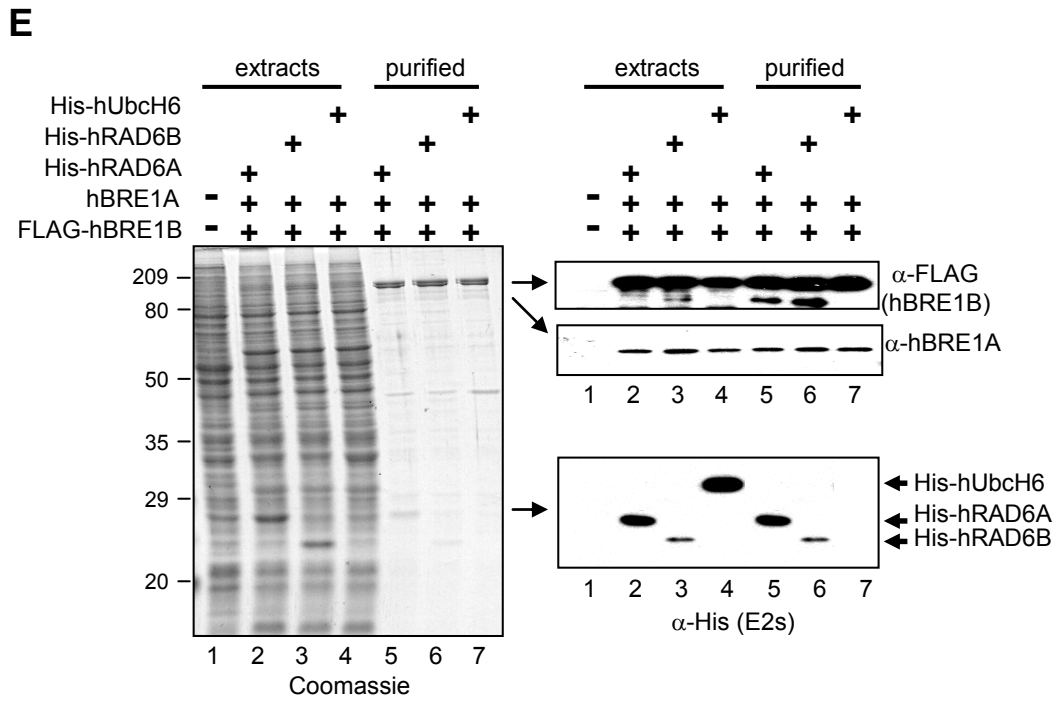
It is generally known that E3 specifically and directly binds to its cognate E2 to carry out substrate ubiquitylation (Pickart, 2001). To identify an E2 that directly interacts with hBRE1A/B, I first purified several recombinant GST-tagged human E2 enzymes (Figure 3.2.5A left panel) and examined their interactions with the purified FLAG-hBRE1A/B complex. Among 9 different E2 enzymes tested, only hRAD6A and hRAD6B showed direct interactions with hBRE1A/B complex (Figure 3.2.5A right panel). Surprisingly, I detected no interaction of hBRE1A/B with hUbcH6, which was claimed to be an E2 for hBRE1A/B by the Reinberg group (Zhu et al., 2005b). Repeated protein interaction studies with different binding conditions (varying salt and detergent concentrations) consistently revealed hRAD6A and hRAD6B as the only E2 proteins binding to hBRE1A/B (data not shown). To test whether

Figure 3.2.5 Human BRE1A/B Complex Specifically Interacts with hRAD6A and hRAD6B



(A) Purified GST and GST- tagged E2 enzymes were analyzed by SDS/PAGE and Coomassie blue staining (left panel). FLAG-hBRE1A/B complex was tested for binding to GST-E2 proteins, and bound proteins were scored by anti-hBRE1A and anti-hBRE1B immunoblots (right panel). (B-D) Purified FLAG-hBRE1A/B complex, FLAG-hBRE1A and FLAG-hBRE1B were analyzed by Coomassie blue staining (B) and were tested for binding to GST-E2 proteins (C) and to His-E2 proteins (D). Bound proteins were scored by anti-hBRE1A, anti-hBRE1B and anti-FLAG antibodies as indicated.

Figure 3.2.5 (Continued) Human BRE1A/B Complex Specifically Interacts with hRAD6A and hRAD6B



(E) Combinations of baculoviruses expressing FLAG-hBRE1B, hBRE1A, His-hRAD6A, His-hRAD6B and His-hUbcH6 were infected in insect cells as indicated. Total cell extracts and purified complexes using M2-agarose were analyzed by SDS/PAGE and Coomassie blue staining (left panel), and probed with anti-FLAG, anti-hBRE1A and anti-His antibodies (right panel), as indicated. (F) Cell extracts from wild-type (293T) and FLAG-tagged hBRE1A (RNF20) 293T cell line were incubated with M2-agarose and immunoprecipitated proteins were analyzed by indicated antibodies.

hBRE1A/B complex formation is required for interaction with hRAD6, individual FLAG-tagged hBRE1A and hBRE1B were purified (Figure 3.2.5B). As shown in Figure 3.2.5C, compared to the strong interaction of the hBRE1A/B complex with hRAD6 proteins, neither FLAG-hBRE1A nor FLAG-hBRE1B alone bound to hRAD6 proteins. Use of the nickel agarose pull-down assay that was employed by the Reinberg lab also showed selective binding of hRAD6 proteins, and not hUbc6, to the hBRE1A/B complex (Figure 3.2.5D).

Based on the strong selective interaction of the hBRE1A/B complex with hRAD6 proteins, I co-infected insect cells with baculoviruses expressing combinations of hBRE1A, FLAG-hBRE1B and different E2s and tested for co-purification (Figure 3.2.5E). Whereas both hRAD6A and hRAD6B efficiently co-purified with hBRE1A/FLAG-hBRE1B (right panel, lanes 5 and 6), no detectable level of hUbcH6 was seen in the same experiment (lane 7). Finally, in a test for intracellular interactions, endogenous hRAD6, but not endogenous hUbcH6, was co-immunoprecipitated by anti-FLAG antibody (M2 agarose) from cell extracts prepared from a FLAG-RNF20 (hBRE1A) cell line (gift from the Reinberg lab) (Figure 3.2.5F). These results are consistent with the observations described above. The less efficient interaction between hBRE1A/B and hRAD6 *in vivo* compared to *in vitro* studies might be due to more stringent condition used for cell extract preparation and binding/washing procedures.

Human RAD6A and RAD6B Specifically Ubiquitylate hBRE1A/B Complex

Direct physical interactions between ring-finger E3 and E2 proteins result in substrate ubiquitylation as well as E3 auto-ubiquitylation (Joazeiro and Weissman 2000; Lorick et al., 1999). More related to histone ubiquitylation, the auto-ubiquitylation of Ring1B, an E3 responsible for mammalian H2A ubiquitylation by its cognate E2 UbcH5C, has been recently reported (Ben-Saadon et al., 2006). Therefore, to test whether hBRE1A/B-hRAD6 can function as an active enzyme, it is important to examine whether hBRE1A/B can be ubiquitylated specifically by hRAD6.

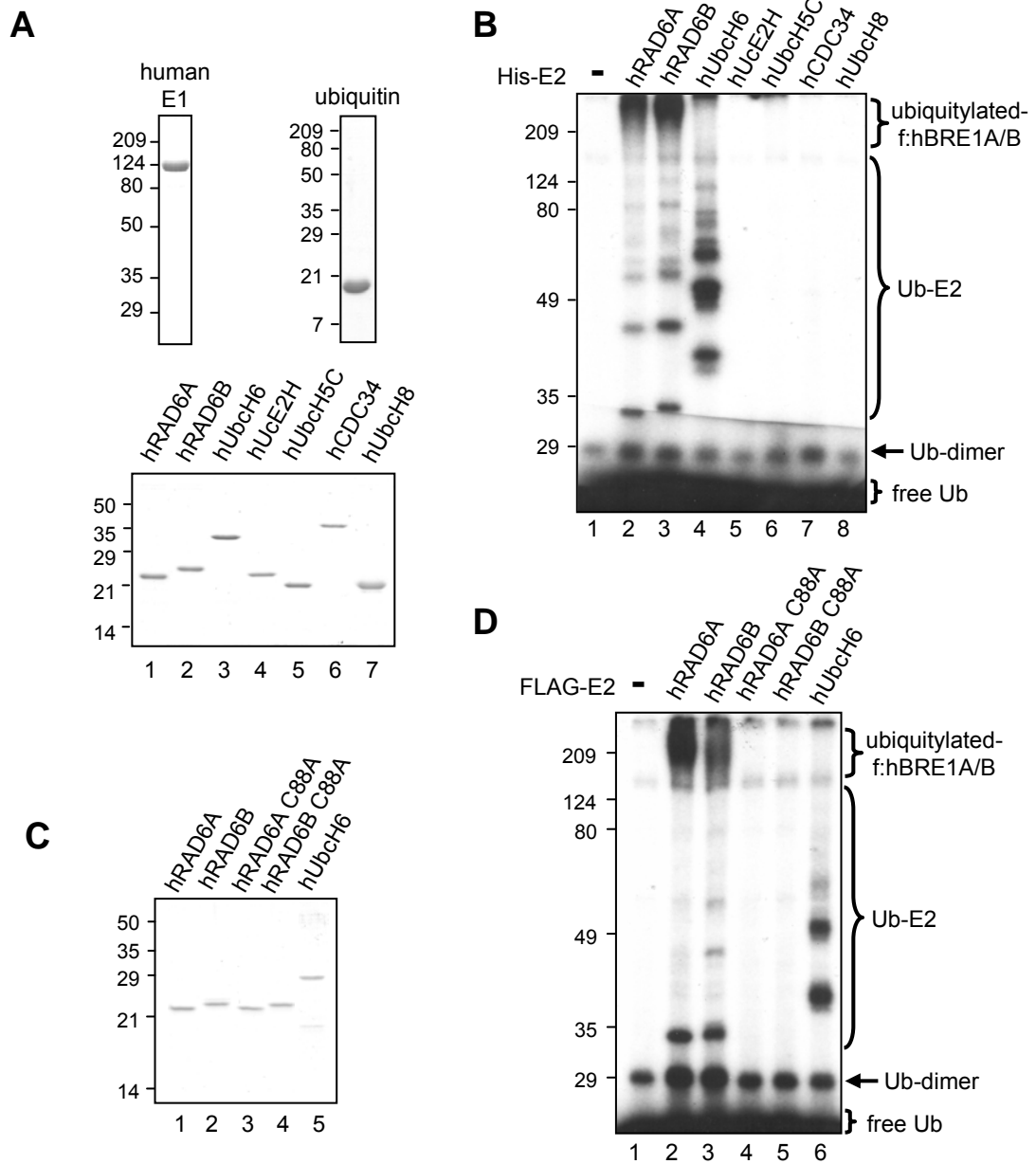
For the *in vitro* ubiquitylation assay, I first purified FLAG-tagged human E1 ubiquitin activating enzyme via a baculovirus expression system and confirmed its enzyme activity through a ubiquitin thioester assay (data not shown) (Hermida-Matsumoto et al., 1996). The recombinant ubiquitin, which can be radio-labeled (Kim et al., 2002), and several His-tagged recombinant E2 enzymes were also purified (Figure 3.2.6A). Among the E2 enzymes tested, and as expected from the protein interaction study, only hRAD6A and hRAD6B could generate the poly-ubiquitylated products that are visualized as larger bands above the hBRE1A/B protein (Figure 3.2.6B lanes 2 and 3). The immunoblot blot analysis confirmed that the lower mobility bands

contain hBRE1A and hBRE1B (data not shown). Although hUbcH6 exhibited ubiquitin conjugating activity, it did not mediate any detectable poly-ubiquitylation of hBRE1A/B (lane 4).

In order to confirm that poly-ubiquitylation of hBRE1A/B is caused by the enzymatic activity of the hRAD6 proteins, I generated catalytically-inactive hRAD6 mutants (hRAD6AC88A and hRAD6BC88A) with C88 changed to alanine (Sung et al., 1990) via baculovirus-mediated expression and affinity purification (Figure 3.2.6C). As shown in Figure 3.2.6D, and in comparison to the strong hBRE1A/B poly-ubiquitylation activity of wild type hRAD6, the hRAD6 C88A mutants and hUbcH6 were inactive.

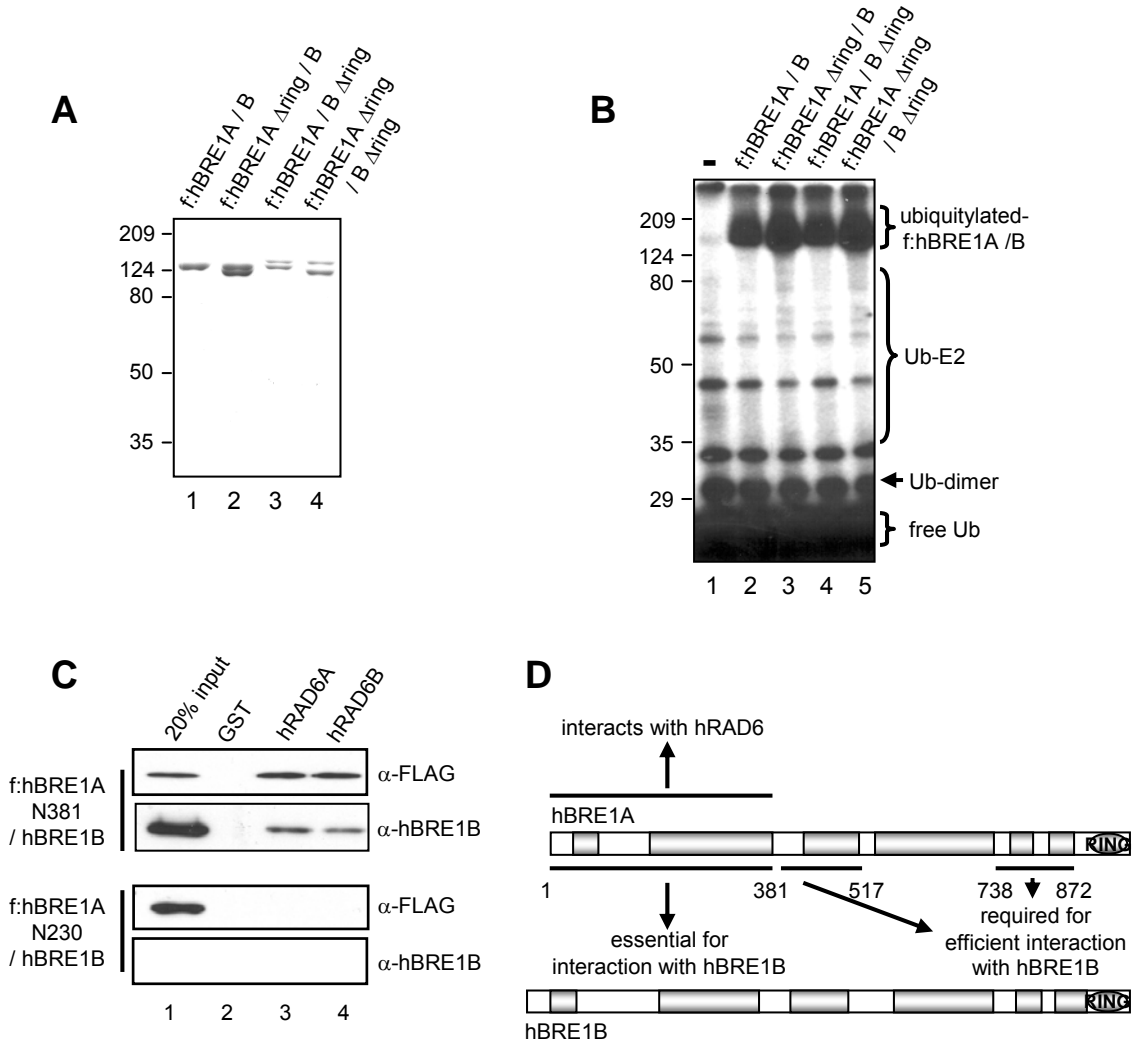
Both hBRE1A and hBRE1B contain a ring-finger domain, one of the signature motifs of an E3 ubiquitin ligase, at their C-termini (Figure 3.2.7D). There is substantial evidence that the ring-finger serves as an E2 binding platform that is essential for protein ubiquitylation activity (Lorick et al., 1999; Zheng et al., 2000). Therefore, it was of interest to determine whether the ring-fingers in hBRE1A/B complex are essential for cognate E2 interaction and protein ubiquitylation activity. To this end, I purified hBRE1A/B mutant complexes with single and/or double ring-finger deletions (Figure 3.2.7A) and tested them in hBRE1A/B auto-ubiquitylation assay. Interestingly, all mutants with single and double ring-finger deletions

Figure 3.2.6 Human RAD6A and RAD6B Specifically Ubiquitylate hBRE1A/B



(A and C) Coomassie blue staining of purified FLAG-tagged human E1 protein via baculovirus expression system and His-tagged ubiquitin and E2 enzymes expressed and purified from bacterial cultures (A) and purified FLAG-tagged E2 enzymes via baculovirus expression system (C). (B and D) Indicated E2 enzymes were applied for hBRE1A/B auto-ubiquitylation assays containing purified FLAG-human E1, FLAG-hBRE1A/B complex and ^{32}P -labelled ubiquitin. Reaction mix were analyzed by SDS-PAGE and autoradiography.

Figure 3.2.7 Human RAD6 Interacts with hBRE1A/B Complex through a Domain Other Than Ring-Finger



(A) Coomassie blue staining of purified FLAG-hBRE1A/B and its mutant complexes in which ring-finger domains are deleted. (B) Indicated hBRE1A/B complexes were applied for E3 auto-ubiquitylation assay with FLAG-hRAD6A and analyzed as described in Figure 3.2.6. (C) Purified FLAG-hBRE1A N381/B complex and FLAG-hBRE1A N230 polypeptide were tested for binding to GST-hRAD6 proteins. Bound proteins were scored by anti-FALG, anti-hBRE1B antibodies as indicated. (D) Schematic presentation of domains in hBRE1A protein which are required for efficient complex formation with hBRE1B and binding to hRAD6.

showed levels of hRAD6-mediated poly-ubiquitylation comparable to that observed with wild type hBRE1A/B, thus suggesting that hRAD6 proteins interact with hBRE1A/B complex through a domain other than the ring-finger motif. Therefore, it was of interest to determine which region in hBRE1A/B is responsible for binding to hRAD6. The approach was simple. Since hBRE1A/B complex formation is essential for interaction with hRAD6 proteins (Figure 3.2.5C and D), I analyzed interactions of truncated hBRE1A/B proteins (Figure 3.2.4B) with GST-fused hRAD6 proteins (Figure 3.2.7C). The FLAG-hBRE1AN381/hBRE1B complex that contains the minimal hBRE1A fragment (residues 1-381) capable of complex formation with hBRE1B showed a strong interaction with the hRAD6 proteins, whereas the FLAG-hBRE1AN230/hBRE1B complex that contains an hBRE1A fragment (1-230) incapable of complex with hBRE1B failed to interact to hRAD6. These observations imply that the hRAD6 binding domain resides in the N-terminal hBRE1A domain that forms a complex with hBRE1B (Figure 3.2.7D).

Taken together, all the data presented above strongly suggest that hRAD6 proteins specifically and functionally interact with hBRE1A/B complex, whereas hUbcH6 does not.

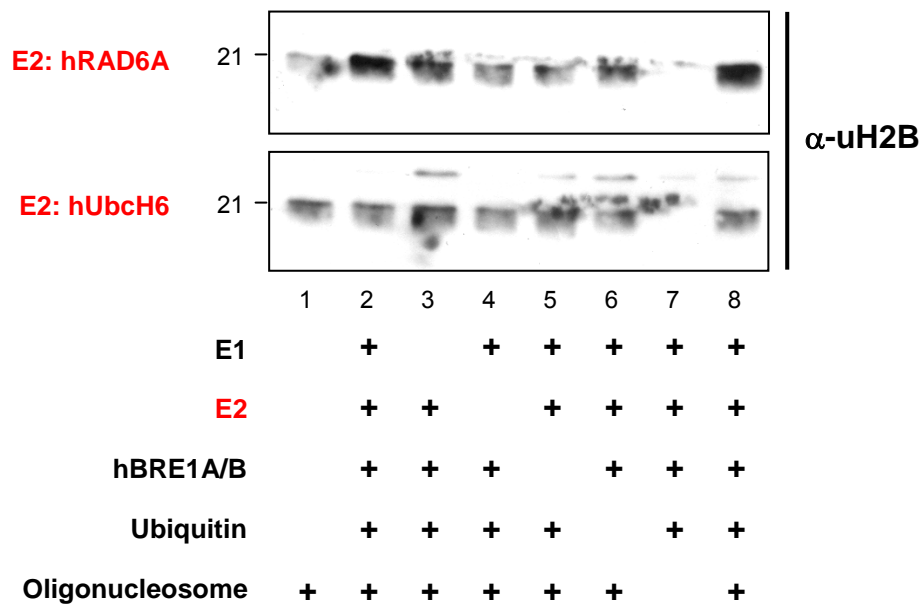
Human RAD6 and hBRE1A/B-Dependent Histone H2B Ubiquitylation *in Vitro*

In order to confirm that hRAD6 proteins, in conjunction with hBRE1A/B complex, are real E2s responsible H2B ubiquitylation, I performed an *in vitro* histone ubiquitylation assay. As shown in Figure 3.2.8, the complete reactions containing hE1, hRAD6A, the hBRE1A/B complex, ubiquitin and oligonucleosomes derived from HeLa cells showed increased level of ubiquitylated H2B at lysine 120 (top panel, lanes 2 and 8), whereas reactions with omissions of any of these components showed no H2B ubiquitylation above the basal level that reflects endogenous ubiquitylated H2B (top panel, compare lanes 1 and lanes 3 to 6). Furthermore, the reactions employing hUbcH6 instead of hRAD6A did not result in any change in the H2B ubiquitylation level (bottom panel), unequivocally indicating that hRAD6 is the cognate E2 for hBRE1A/B complex and responsible for H2B ubiquitylation.

Trimeric hRAD6-hBRE1A/B-hPAF Complexes

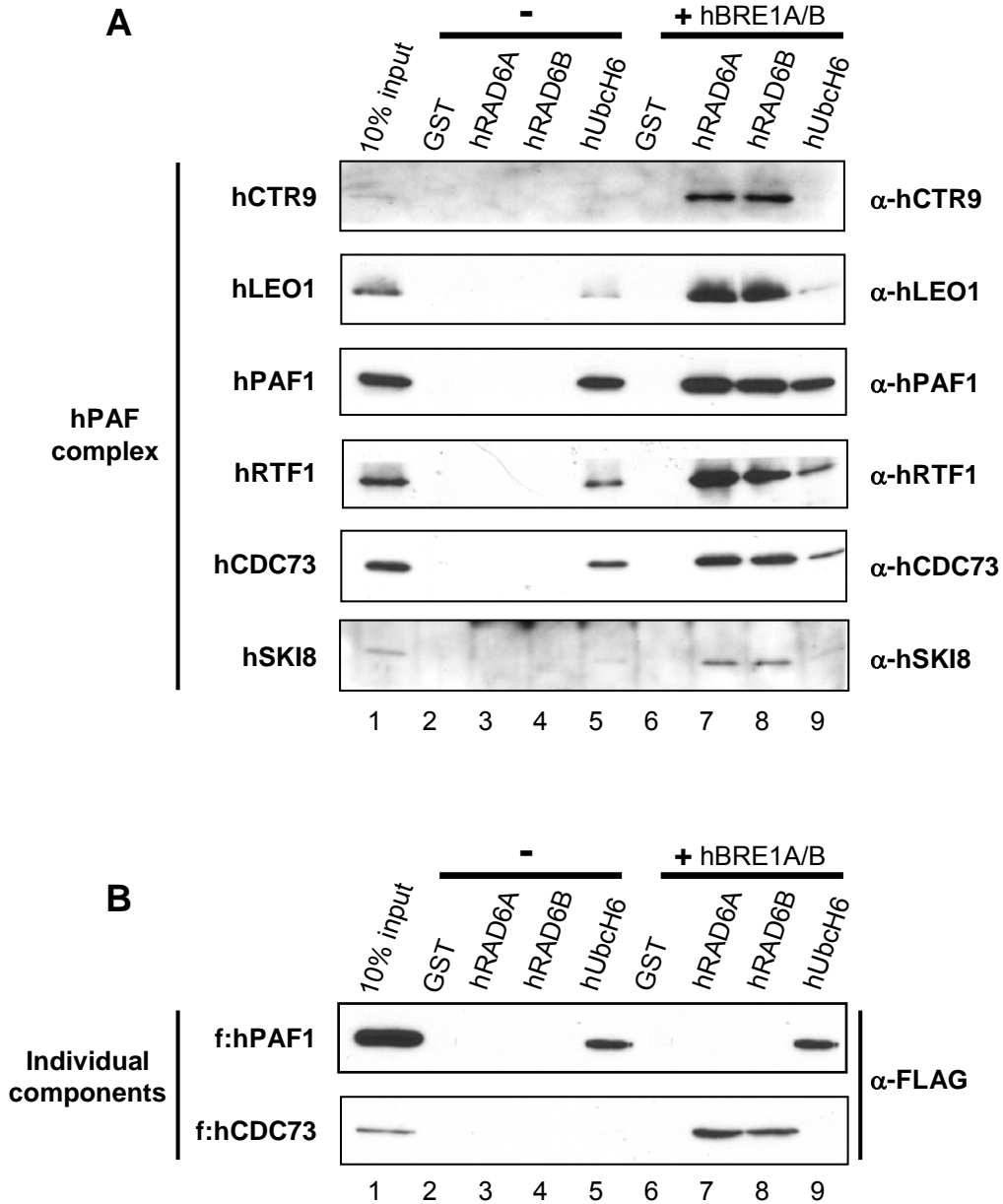
Transcription elongation factor PAF complex is required for H2B ubiquitylation and subsequent H3-K4/K79 methylation both in yeast (Ng et al., 2003b; Ng et al., 2003a; Krogan 2003) and in human (Zhu et al., 2005a). These findings have suggested that H2B ubiquitylation is coupled to ongoing transcription and that there is link between the H2B

Figure 3.2.8 Human RAD6 and BRE1A/B-Dependent H2B K120 Ubiquitylation *in Vitro*



Indicated combinations of purified E1, E2 enzymes, hBRE1A/B, ubiquitin and Hela cell-derived oligonucleosomes were applied to *in vitro* ubiquitylation assay. The ubiquitylated histone H2B at lysine 120 were scored by anti-ubiquitylated H2B immunoblot.

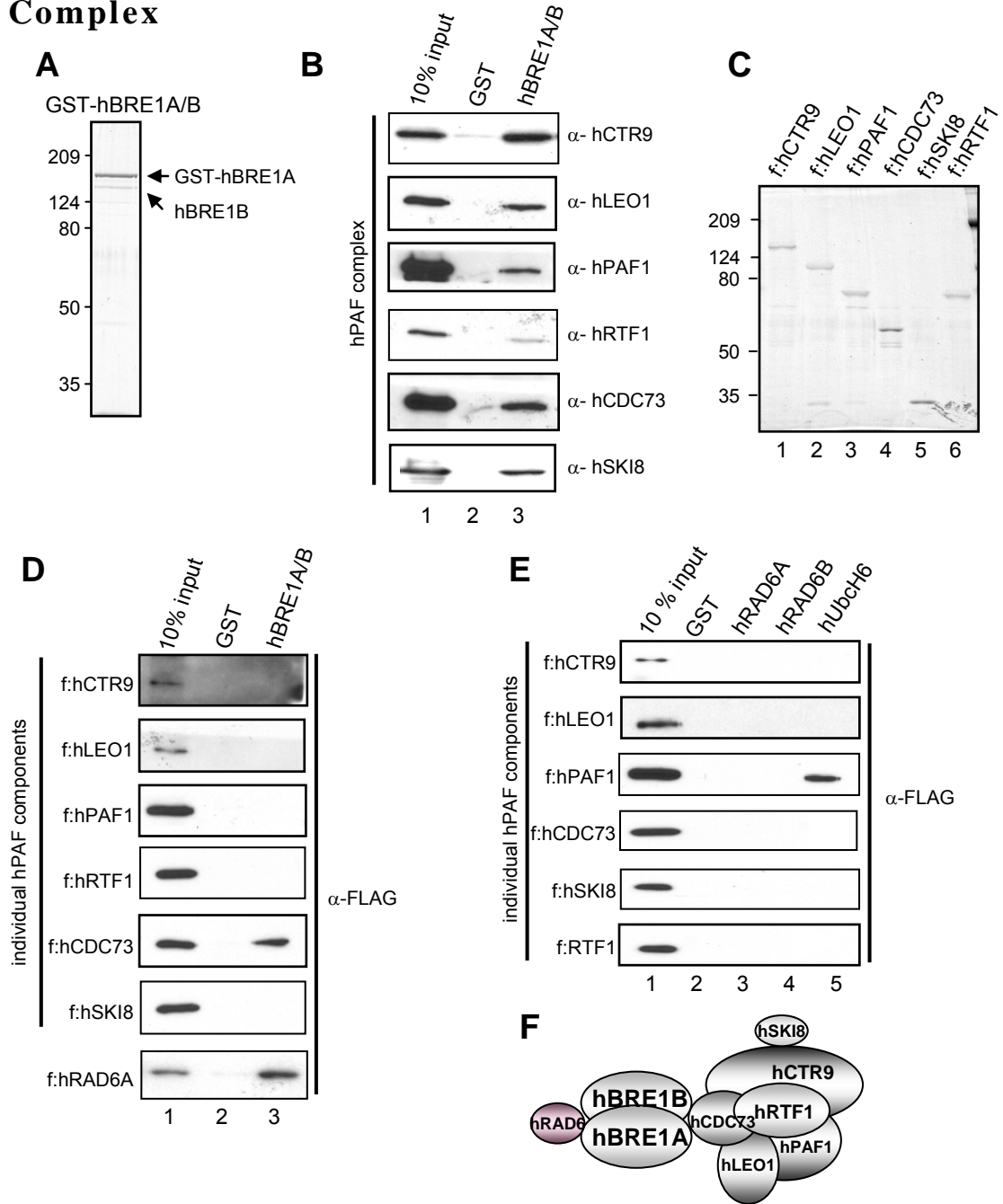
Figure 3.2.9 Trimeric Complex Formation of hRAD6-hBRE1A/B-hPAF: hBRE1A/B Enables hRAD6 to Interact with hPAF Complex



(A and B) Reconstituted hPAF complex (A) and individual hPAF components, FLAG-tagged hPAF1 and hCDC73, (B) were tested for binding to GST or GST-E2 ubiquitin conjugating enzymes in the presence and in the absence of hBRE1A/B, and bound proteins were analyzed by immunoblots with indicated antibodies.

ubiquitylation machinery and PAF complex. To explore this possibility, I first examined interactions of the purified hPAF complex (see Chapter 4 for preparation of recombinant hPAF complex via baculovirus expression system) with GST-E2 fusion proteins (Figure 3.2.9A). Whereas hUbcH6 showed a direct interaction with the hPAF complex, hRAD6 proteins showed no interactions (lanes 3-5). Interestingly, the further addition of hBRE1A/B complex in the same binding assay effected a strong binding of hRAD6 proteins to the hPAF complex (lanes 7 and 8), whereas the direct interaction between hUbcH6 and hPAF complex was not altered (compare lanes 5 and 9). These results are consistent with two possibilities; (i) an interaction of hRAD6 proteins with hBRE1A/B complex generates new binding surface that interacts directly with the hPAF complex and (ii) the hBRE1A/B complex directly interacts with the hPAF complex and mediates an indirect interaction between hRAD6 proteins and hPAF complex. To test the second possibility, I examined interactions of the purified hPAF complex with GST-hBRE1A/B (Figure 3.2.10A). Importantly, as shown in Figure 3.2.10B, the hBRE1A/B complex was shown to directly interact with the hPAF complex (Figure 3.2.10B). Next, I tested the ability of individual components of the hPAF complex to bind to a GST-hBRE1A/B complex (Figure 3.2.10C). Among the 6 subunits tested, hCDC73 was identified as the sole binding partner for hBRE1A/B (Figure 3.2.10D). To confirm that hCDC73 is responsible for hBRE1A/B-

Figure 3.2.10 Human BRE1A/B Directly Binds to hPAF Complex



(A and C) Coomassie blue staining of purified GST-hBRE1A/B (A) and FLAG-tagged individual components of hPAF complex (C) prepared via baculovirus expression system. (B), (D and E) The reconstituted hPAF complex (B) and individual components in hPAF complex (D and E) were tested for binding to GST or GST-hBRE1A/B (B and D) and GST or GST-E2 ubiquitin conjugating enzymes (E). Bound proteins were analyzed by immunoblots with indicated antibodies. (F) Schematic presentation of hRAD6-hBRE1A/B-hPAF trimeric complex.

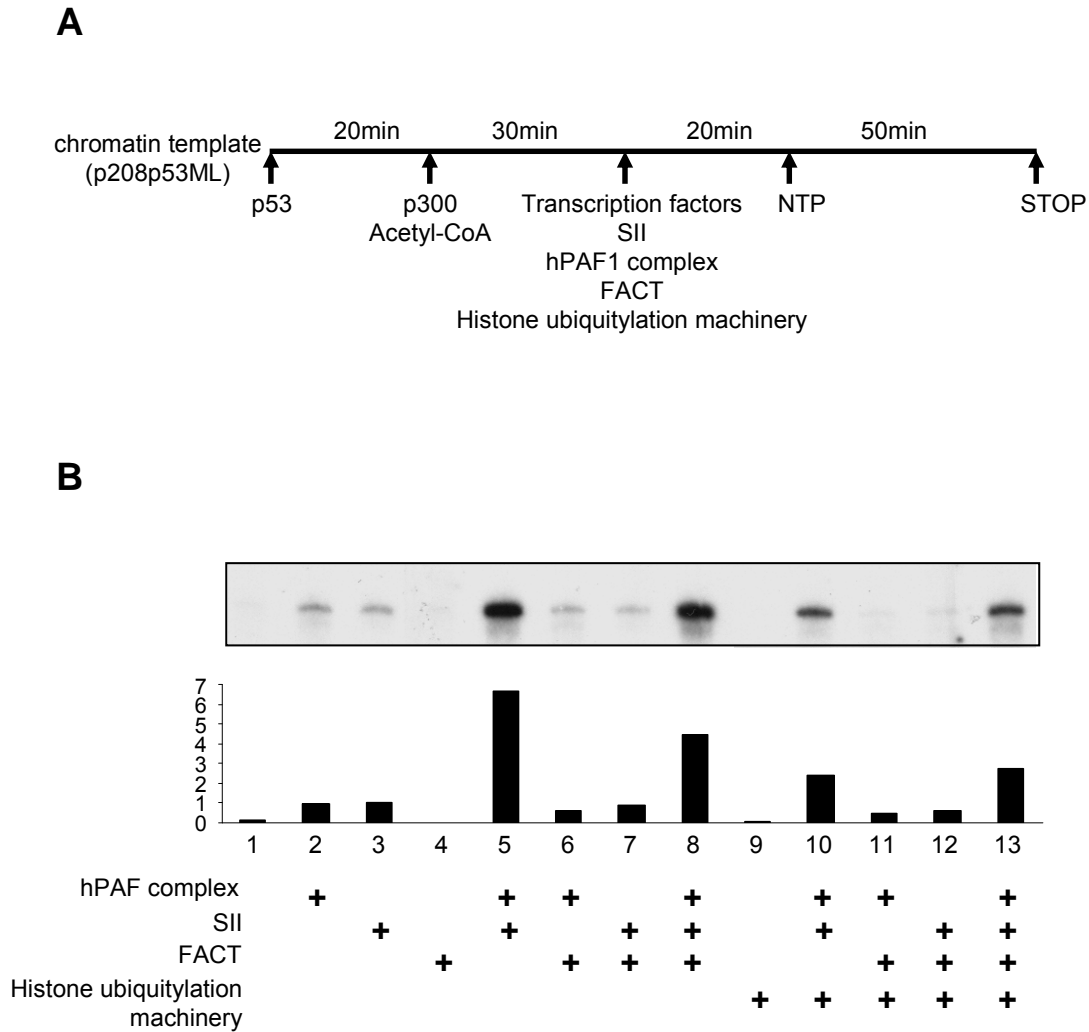
mediated hPAF complex association with hRAD6, the binding assay described in Figure 3.2.9A was repeated with FLAG-hCDC73. Addition of the hBRE1A/B complex in a binding reaction resulted in binding of hRAD6 proteins to hCDC73 (Figure 3.2.9B, bottom panel, lanes 7 and 8), thus confirming the formation of a trimeric hRAD6-hBRE1A/B-hPAF complex and, importantly, that hBRE1A/B serves as a bridge between hRAD6 and the hPAF complex (Figure 3.2.10F). In relation to the observed direct interaction of hUbcH6 with hPAF complex (Figure 3.2.9A), the hPAF1 component of the hPAF complex was shown to be responsible for direct binding to hUbcH6 (Figure 3.2.10E).

Of note, the Reinberg group also proposed a trimeric association model for a hBRE1A/B-hUbcH6-hPAF complex (Zhu et al., 2005b). However, the presented data rules out this model since there is no direct interaction between hBRE1A/B and hUbcH6. The fact that hUbcH6 directly interacts with hPAF complex (Figures 3.2.9 and 3.2.10E) leaves open possibility of a role for hUbcH6 in transcription, but my data strongly suggest that hUbcH6 is not involved in transcription as part of the H2B ubiquitylation machinery.

Effect of Histone H2B Ubiquitylation Factors on Transcription with Purified Factors *in Vitro*

My previous RNAi-coupled transient transfection experiments revealed that endogenous hRAD6 and hBRE1A/B proteins are required for optimal activator-dependent transcription and thus suggest that H2B ubiquitylation is associated with active gene transcription *in vivo*. To investigate the detailed molecular mechanisms for the role of H2B ubiquitylation in transcription, I examined the effect of H2B ubiquitylation factors in a transcription assay with purified factors from a chromatin template (See Chapter 4 for details about establishment of *in vitro* transcription assay with purified factors). From the notion that the H2B ubiquitylation machinery works with transcription elongation factor PAF complex and is linked to ongoing transcription, H2B ubiquitylation factors (including hE1, hRAD6A, hBRE1A/B, and ubiquitin) and the hPAF complex were added to reactions with purified transcription factors (Figure 3.2.11A). Related, I recently found that the purified hPAF complex has strong synergistic effect with SII on transcription elongation (Figure 3.2.11B, lanes 1-3 and 5) (see Chapter 4 for details). Interestingly, albeit surprisingly, addition of the H2B ubiquitylation factors resulted in decrease in transcription (compare lanes 5 and 10). This repression is caused by protein ubiquitylation since addition of the unrelated hUbcH6 instead of hRAD6A did not alter the transcription level (data not

Figure 3.2.11 Addition of H2B Ubiquitylation Factors Decreases p53-Dependent Transcription with Purified Factors



(A) Schematic representation of the in vitro transcription assays indicating the order in which the reagents were added. (B) The in vitro transcription assay with purified transcription factors including histone H2B ubiquitylation factors. Purified p53 was added in all reactions. Transcription factors include TFIIA, B, E, D, F, H, PC4, mediator and pol II. Histone ubiquitylation machinery includes hE1, hRAD6, hBRE1A/B and ubiquitin. The relative transcription levels were scored at the bottom.

shown). H2B ubiquitylation factors alone did not show any effect on transcription (compare lanes 1 and 9).

In addition, I also tested effects of the H2B ubiquitylation factors in the presence of transcription elongation factor FACT. However, our well established *in vitro* transcription system has failed to show any effect of FACT on transcription from chromatin templates (lanes 4 and 6-8), thus precluding a clear test the effect of H2B ubiquitylation factors on FACT-dependent transcription. These results are completely contradictory to the Reinberg group's recent report describing cooperative and stimulatory functions of H2B ubiquitylation and elongation factor FACT in transcription from chromatin templates (Pavri et al., 2006).

3.3 DISCUSSION

Systematic studies of the mechanism and the role(s) of H2B ubiquitylation in transcription in mammalian cells have been precluded by the failure to identify a cognate E2 enzyme for hBRE1A/B. Although the Reinberg group has reported hUbch6 as an E2 for H2B ubiquitylation (Zhu et al., 2005b), my present study provides solid strong evidence indicating that hRAD6 proteins, rather than hUbch6, work as specific E2s responsible for H2B ubiquitylation in human cells. Studies in which hRAD6 proteins were depleted (by RNAi) have documented a role for hRAD6 in H2B ubiquitylation, subsequent H3

methylation and efficient activator-dependent transcription. Most importantly, I showed that hRAD6 proteins specifically ubiquitylate H2B within a nucleosomal substrate *in vitro* via a functional interaction with hBRE1A/B complex. I also verified that the H2B ubiquitylation machinery interacts with hPAF complex, thus explaining how H2B ubiquitylation is linked to transcription. Although several control experiments remained to be performed, my *in vitro* transcription study suggests that H2B ubiquitylation alone is not sufficient to stimulate transcription. These studies report the identification of bona-fide factors involved in H2B ubiquitylation and molecular mechanism of how they work together and how they participate in transcription in mammalian cells.

Human BRE1A/B Complex as a Functional E3 Ubiquitin Ligase

Successful reconstitution of the hBRE1A/B complex in my study confirmed that two different ring-finger E3s are involved in H2B ubiquitylation in mammalian cells. Since the apparent mass of native hBRE1A/B complex is around 600KDa (Zhu et al., 2005b), it is thought that 2 copies of hBRE1A/B heterodimer form a functional complex. Based on similar sequences of hBRE1A and hBRE1B and an identification of the region in hBRE1A that is responsible for its interaction with hBRE1B, it is proposed that hBRE1A and hBRE1B are tethered through interactions of their N-terminal domains. In view of the function of a single BRE1 in H2B ubiquitylation in yeast, it was

surprising to find a joint requirement for two BRE1 paralogues in human cells. However, very recent studies from *Schizosaccaromyces pombe* also reported that both SpBRE1A and SpBRE1B are absolutely required for H2B ubiquitylation (Tanny et al., 2007; Zofall and Grewal 2007). Moreover, I have found that the yeast BRE1 actually exists as a homodimeric complex (data not shown). These results are consistent with the universal function of a dimeric E3 in RAD6 mediated H2B ubiquitylation, although it is not clear why some species utilize a heterodimeric complex.

The complex catalyzing H2AK119 ubiquitylation also contains multi-E3 ligases, Ring1A, Ring1B and Bmi-1 (Wang et al., 2004). Further studies have reported that Ring1B is the catalytic subunit in the complex and that Ring1A and Bmi-1 stimulate Ring1B activity *in vitro* (Cao et al., 2005), although both Ring1A and Ring1B contribute to H2A ubiquitylation *in vivo* (de Napoles et al., 2004). Thus, it becomes of interest to determine which ring-finger E3s in the hBRE1A/B complex are the catalytic enzyme(s), which will soon be answered through the *in vitro* ubiquitylation assay employing wild type and ring-finger mutant hBRE1/B complexes.

Identification of hRAD6 Proteins as Cognate E2 Ubiquitin Conjugating Enzymes for hBRE1A/B Complex

RAD6 proteins are highly conserved not only at the amino acid sequence level but also in functional aspect (Koken et al., 1991; Prakash et al., 1993). The function of RAD6 as an E2 in H2B ubiquitylation is well established in yeast (Robzyk et al., 2000), in *Schizosaccaromyces pombe* (Tanny et al., 2007; Zofall and Grewal 2007) and in *C. elegans* (Crowe and Candido, 2004), although all these organisms have clear homologues of the hUbcH6 protein (Figure 1.3B) that was claimed by the Reinberg group to be E2 for H2B ubiquitylation in mammalian cells. Strikingly, my current analysis also indicates that hRAD6 proteins functions as E2s for H2B ubiquitylation in human cells. In support of my observations, a recent report has showed that exposure of mammalian cells to nickel results in the disappearance of ubiquitylated H2B concomitant with a decrease in RAD6 but not hUbcH6 (Karaczyn et al., 2006).

As shown for hBRE1A in my previous report (Kim et al., 2005), depletion of endogenous hRAD6 proteins (by RNAi) affects histone modifications and impairs activator-dependent transcription. This implies that hRAD6 and hBRE1A/B are linked and act physiologically in same pathway. In addition, protein interaction analyses have found that hRAD6 directly binds to the hBRE1A/B complex by ring-finger-independent manner. Although there is substantial evidence for interactions between ring-fingers and E2s (Zheng et

al., 2000), several reports have shown that the role of the ring-finger is not merely to recruit E2s to the vicinity of proteins to be ubiquitylated. For example, both Ubr1 and RAD18 E3s bind to their cognate E2 RAD6 through regions outside ring-finger domains while mutations in their ring-fingers abolish substrate ubiquitylation activity (Xie and Varshavsky, 1999; Bailly et al., 1997). It is very important to note that RAD6 is involved in all known examples, including those presented here. This implies that RAD6 is a non-canonical enzyme that does not interact with E3 through the ring-finger motif, and further strengthens the idea that the ring-finger has a role distinct from providing binding surface to E2 in H2B ubiquitylation. It is also interesting to note that hRAD6 interacts with the hBRE1A/B complex, but not with hBRE1A or hBRE1B alone. Surprisingly, this appears to be a unique characteristic of RAD6 since an earlier study found that RAD6 binds to RAD18 homodimer and RAD5/RAD18 heterodimer DNA repair complexes (Ulrich and Jentsch, 2000).

I also documented that hRAD6 is indirectly associated with the hPAF complex through its direct interaction with the hBRE1A/B complex. This trimeric association explains why H2B ubiquitylation is dependent on the PAF complex and ongoing transcription. The direct interaction between hBRE1A/B and the hPAF complex fits the yeast model in which deletion of yBRE1 or the RTF1 component of the yPAF complex completely abolishes

RAD6 association with RNA polymerase II and chromatin (Wood et al., 2003a; Xiao et al., 2005). In relation to this, my CHIP analysis showed that hRAD6 is recruited to mdm2 promoter region upon ectopic p53 expression (Figure 2.2.9B).

Consistent with the finding that hUbcH6 colocalizes with RNA polymerase II at transcriptionally active genes *in vivo* (Zhu et al., 2005b), I also found that hUbcH6 directly binds to hPAF complex through its hPAF1 component. Therefore, it is still possible that hUbcH6 participates in active transcription, but through an unknown role and not as an E2 for H2B ubiquitylation.

Role of Histone H2B Ubiquitylation in Transcription

Several lines of evidence suggest that H2B ubiquitylation is associated with actively transcribed genes *in vivo*. My previous and present studies have also found that there is a correlation between the H2B ubiquitylation level and activator-dependent transcription. But the important question as to whether H2B ubiquitylation itself can enhance the transcription has been unresolved. To answer this question, I performed *in vitro* transcription from a chromatin template with highly purified factors. Although several control experiments remained to be executed, my initial experiments have shown that the addition of H2B ubiquitylation factors actually represses transcription. However, this result should not be over-interpreted to indicate that H2B

ubiquitylation causes transcriptional repression in the cells. Thus, it must be realized that my defined transcription system lacks many other essential positive effector molecules for efficient transcription. One of these could be, for example, a hypothetical ubiquitylated H2B-binding factor that affects downstream events (histone modification, chromatin remodeling or some RNA polymerase II function), although no protein has been reported to have such activity. In addition, and possibly related, both the H2B ubiquitylation and deubiquitylation activities are required for efficient transcription in yeast, implying a dynamic histone ubiquitylation state during transcription (Zhang, 2003). If this were also the case in my *in vitro* transcription assay, persistent histone ubiquitylation due to lack of deubiquitylation machinery might restrict the overall level of transcription in my assay system.

If H2B ubiquitylation serves as a precursor for downstream events that positively affect transcription, rather than enhancing transcription by itself, what might be the candidate positive effectors? It is well established that H2B ubiquitylation is a prerequisite for H3 methylation although detailed mechanisms are unknown. In related to effect of H3-K4 methylation on transcription, human H3-K4 methyltransferase MLL-1 complex has been shown to coactivate activator-dependent transcription of a nucleosomal template in a nuclear extract based assay (Dou et al., 2005). More interestingly, several recent studies have proposed elaborate mechanisms for

how H3-K4 methylation affects transcription. Trimethylated H3-K4 is recognized by PHD fingers in chromatin remodeling complex NURF and in the Yng1 protein in the NuA3 HAT complex (Wysocka et al., 2006; Haitao et al., 2006; Taverna et al., 2006). These two activities, i.e. ATP-dependent chromatin remodeling by remodeling complex and chemical modifications of nucleosomes by histone-modifying enzymes, are well documented to cause both nucleosome disruption and repositioning (Vignali et al., 2000; Strahl and Allis, 2000). These studies raise interesting questions regarding the mechanism by which the histone ubiquitylation machinery communicates with other histone modification, chromatin remodeling and transcription machineries for efficient transcription in mammalian cells. The present report sets the stage for analyses of these questions.

CHAPTER 4

*Synergistic Effect of Two Distinct
Transcription Elongation Factors on
Transcription from a Chromatin
Template in Vitro*

4.1 PREFACE: WHY MANY TRANSCRIPTION ELONGATION FACTORS IN THE CELLS?

Inside the cell, eukaryotic DNA is wrapped a histone core octamers composed of an (H3-H4)₂ tetramer and two H2A-H2B dimmers to generate a structural unit called the nucleosome. Apart from inhibitory effect on transcription initiation, reflecting a block in pre-initiation complex formation, nucleosomes also serve as barrier to a transcribing RNA polymerase II by restricting free elongation. (Knezetic and Luse 1986; Lorch et al., 1987; Workman and Roeder 1987), thus suggesting the presence of accessory factors to help RNA polymerase II to overcome nucleosome barrier. Through the genetic and biochemical approaches, several factors have been identified as putative transcription elongation factors. These include SII (TFIIS), TFIIF, DSIF, Elongin, ELL, NELF, PAF complex and histone chaperone FACT (Shilatifard et al., 2003). Direct function and detailed mechanism of action of these factors in transcription elongation factors are just beginning to be understood (Sims et al., 2003). Recent efforts have shown that histone chaperone FACT (Orphanides et al., 1998) and elongation factor SII (Guermah et al., 2006) help RNA polymerase to transverse nucleosomal structure and facilitate productive transcription from chromatin template *in vitro*.

Beyond these factors, in related to histone ubiquitylation study, the PAF complex is of my special interest because it is required for H2B ubiquitylation and subsequent H3-K4 and H3-K79 methylation in yeast. Although several

groups have reported purification of the human PAF complex (Yart et al., 2005; Rozenblatt-Rosen et al., 2005; Zhu et al., 2005), there has been no direct evidence to show its function as transcription elongation factor.

The presence of multiple elongation factors in cells suggests their potential cooperativities as well as possible redundancies, as expected, recent genomic and proteomic experiments have revealed genetic and physical interactions between distinct elongation factors (Arndt and Kane, 2003). However, there has been no direct evidence to show their cooperative roles in transcription *in vitro*. During the course of an H2B ubiquitylation study, I have found, for the first time, that hPAF complex strongly interacts with SII and that these two distinct elongation factors function synergistically in a transcription from chromatin template.

4.2 RESULTS

Reconstitution of the Human PAF Complex

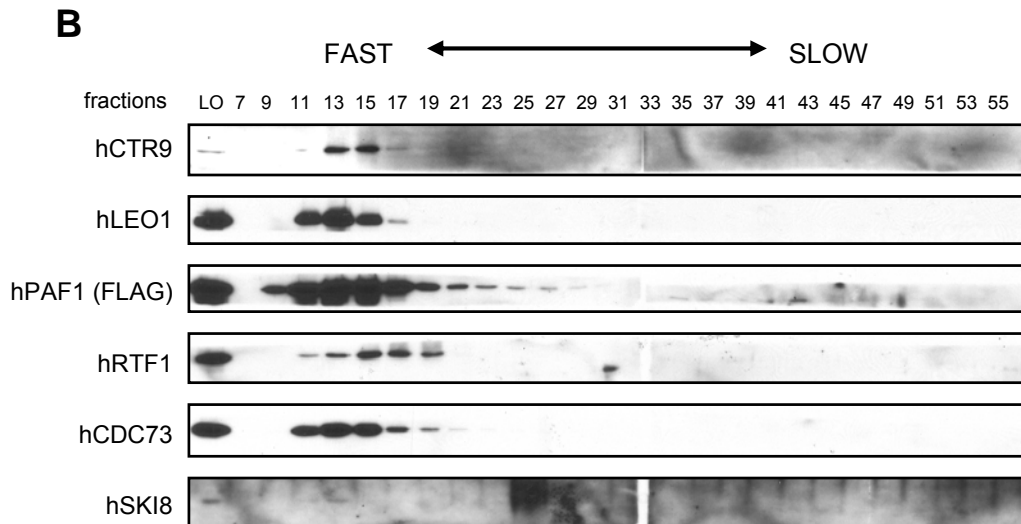
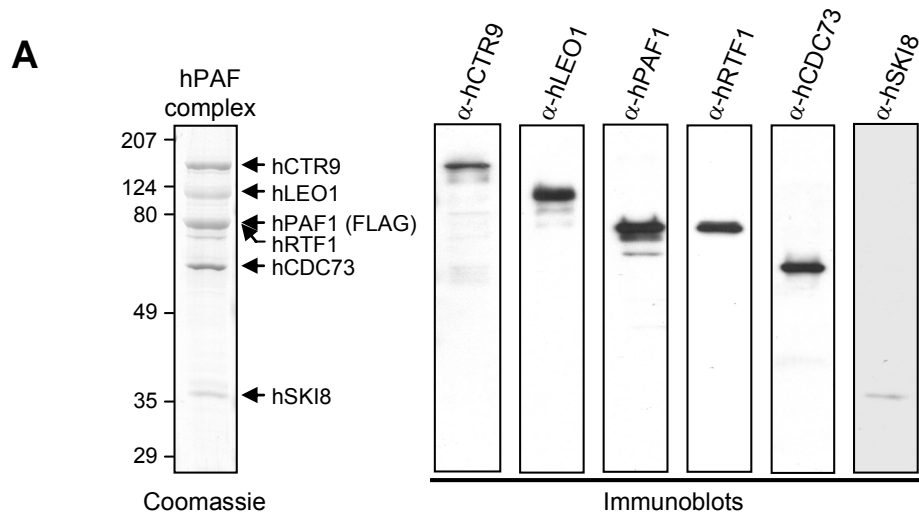
The yeast PAF complex minimally contains CTR9, LEO1, RTF1, PAF1 and CDC73 (Mueller et al., 2002). A recent affinity purification study of the human PAF complex has identified a new subunit, SKI8, in hPAF complex (Zhu et al., 2005a). For a systematic analysis of the function of each component in the hPAF complex and to provide a convenient source of purified complex, the hPAF complex was reconstituted in insect cells by

coinfection with recombinant baculoviruses that individually express each of these components: hCTR9, hLEO1, FLAG-hPAF1, hRTF1, hCDC73 and hSKI8. Although several studies showed that hPAF complex, purified from cell extracts, is devoid of hRTF1 (Yart et al., 2005; Rozenblatt-Rosen et al., 2005; Zhu et al., 2005), I included hRTF1 baculovirus in coinfection to test whether it could be incorporated into the reconstituted complex. FLAG-hPAF1 associated proteins were purified by affinity chromatography using M2-agarose followed by Superose-6 gel filtration to separate free FLAG-hPAF1 protein from the stable complex. The resulting purified complex (Figure 4.2.1A, left panel) was analyzed to confirm the presence of all components of the expected size by immunoblot (right panel). The protein elution profiles from a gel filtration chromatography showed that the six-components of the complex coeluted in the same peak fractions (Figure 4.2.1B) (Apparent molecular weight of the complex will be tested). The hRTF1 component peaked 2 fractions behind the core complex, but clearly was not present as free form. This indicates that it also associates, but perhaps less tighter, with the main complex.

Structural Organization of Human PAF Complex

Having reconstituted the hPAF complex, I tested the contribution of each component to the structural organization of the complex. Based on reports

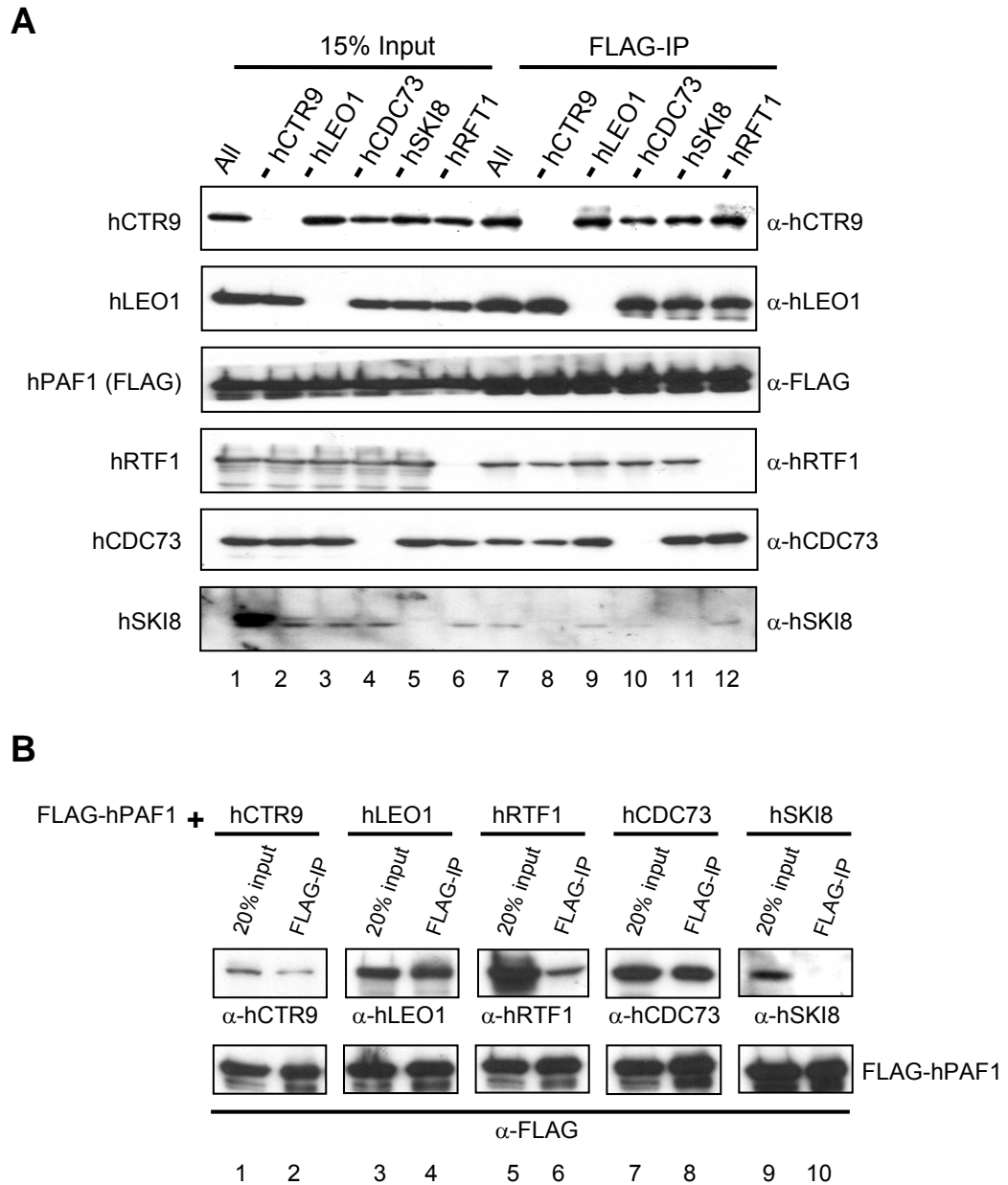
Figure 4.2.1 Reconstitution of Human PAF Complex via Baculovirus Expression System



(A) Coomassie blue staining (left panel) and immunoblot analysis with indicated antibodies (right panel) of reconstituted hPAF complex eluted from M2 agarose beads after FLAG-tagged hPAF1 purification. (B) Immunoblot analysis of hPAF complex components after Superose 6 gel filtration purification.

that a deletion of the yPAF1 subunit severely affects the abundance of other components in yPAF complex (Mueller et al., 2004) and shows obvious transcription elongation defects (Costa and Arndt 2000; Betz et al., 2002), I speculated that the PAF1 subunit functions, minimally, as a scaffold protein in the PAF complex. To test this idea, insect cells were coinfecting with baculoviruses expressing FLAG-hPAF1 and combinations of other untagged subunits of the hPAF1 complex, and the resultant complexes were isolated by affinity purification on M2-agarose. Immunoblot analysis confirmed that all proteins were expressed efficiently and that omissions of any subunit do not affect the abundance of other proteins (Figure 4.2.2A, lanes 1-6). This is as expected because all individual subunits are expressed under the control of same strong promoter (the polyhedrin promoter). Immunoblot analysis of purified complexes revealed the physical association relationship of each component with hPAF1. Individual omissions of hLEO1, hCDC73, hSKI8 or hRTF1 subunit did not affect the efficiency of association of other components with hPAF1 (compare lane 7 and lanes 9-12). However, omission of hCTR9 completely abolished hSKI8 in the purified complex (sixth panel, lane 8), indicating that the physical association of hSKI8 with the hPAF complex is dependent on hCTR9. These data indicate that hPAF1 interacts with all other subunits directly or indirectly and that it plays a role as a scaffold molecule in the hPAF complex.

Figure 4.2.2 Determination of the Human PAF Complex Architecture: FLAG-hPAF1 basis

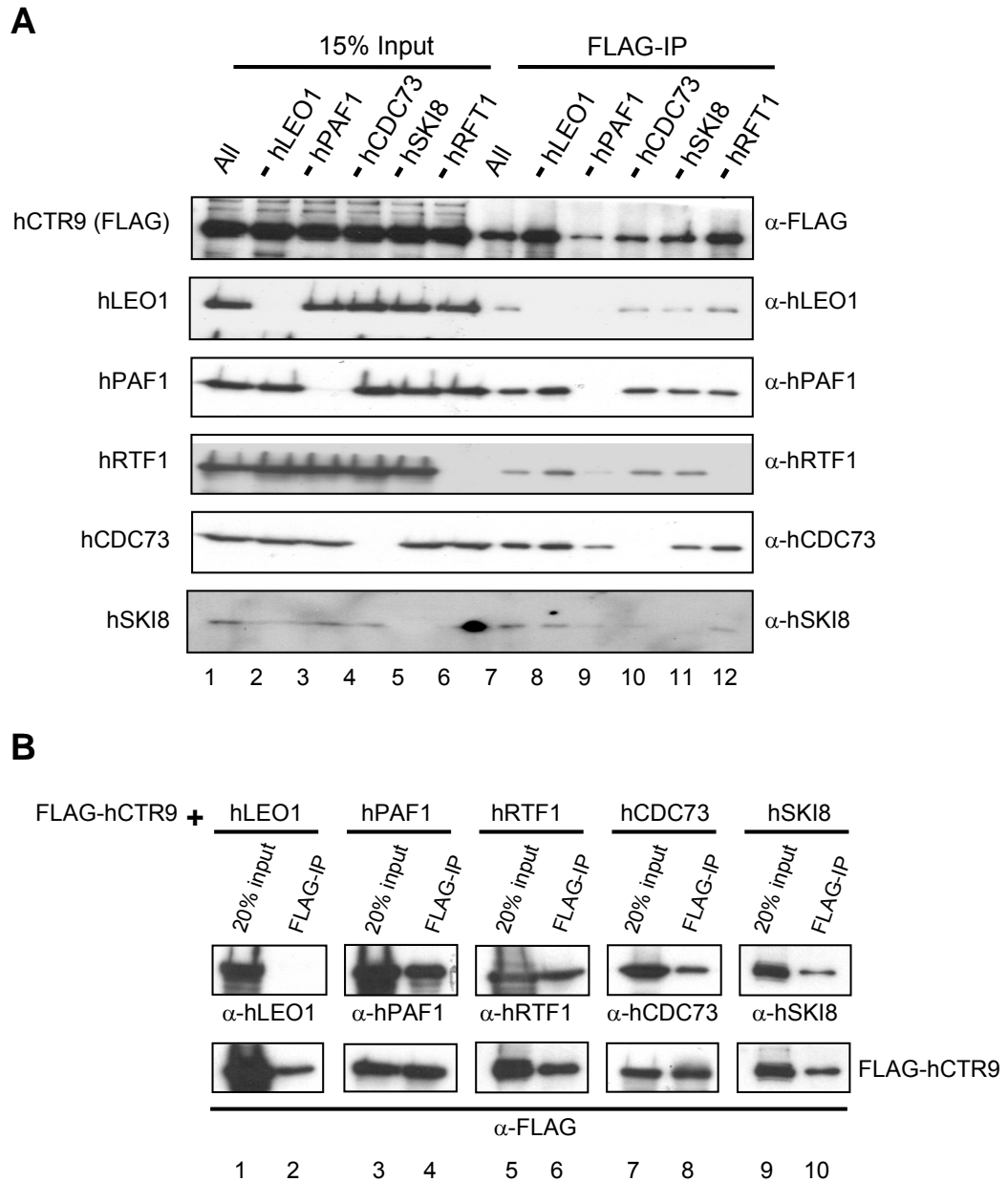


(A) Immunoblot analysis of FLAG-purified complexes from baculovirus co-infected insect cells. Complete (all) or omitted baculoviruses used for co-infection are indicated at the top. (B) Immunoblot analysis of protein interactions between FLAG-hPAF1 and indicated components of hPAF complex after co-infection and purification.

To examine whether hPAF1 interacts with other subunits directly or indirectly, insect cells were coinfecting with pairs of baculoviruses expressing FLAG-hPAF1 and one other subunit. Affinity purification analyses showed that hPAF1 directly interact with all subunits (Figure 4.2.2B, lanes 1-8) other than hSKI8 (lanes 9-10). These data confirmed that hSKI8 is included in the hPAF complex through the interaction with hCTR9. Interestingly, hRTF1 successfully co-purified in all of the different complexes (Figure 4.2.2A, fourth panel). It also interacted directly with hPAF1, although the affinity appeared less than for other subunits (Figure 4.2.2B, lanes 5-6). These data further indicate that hRTF1 clearly associates with core hPAF complex. Direct and indirect protein interactions established by this study are summarized in Figure 4.2.5A.

Using the same general approach, I carried out a similar study with FLAG-hCTR9 instead of FLAG-hPAF1. A complete set of baculovirus coinfections also resulted in successful co-purification of all components including hRTF1 (Figure 4.2.3A, lane 7). Remarkably, omission of hPAF1 decreased the amounts of all other subunits, with hLEO1 showing the most severe reduction (compare lanes 7 and 9). This result indicates that the absence of hPAF1 affects the stability and efficiency of complex formation and thus confirming again the role of hPAF1 as a scaffold molecule in hPAF complex. However, the absence of hLEO1, hCDC73, hSKI8 or hRTF1 alone had little

Figure 4.2.3 Determination of the Human PAF Complex Architecture: FLAG-hCTR9 basis



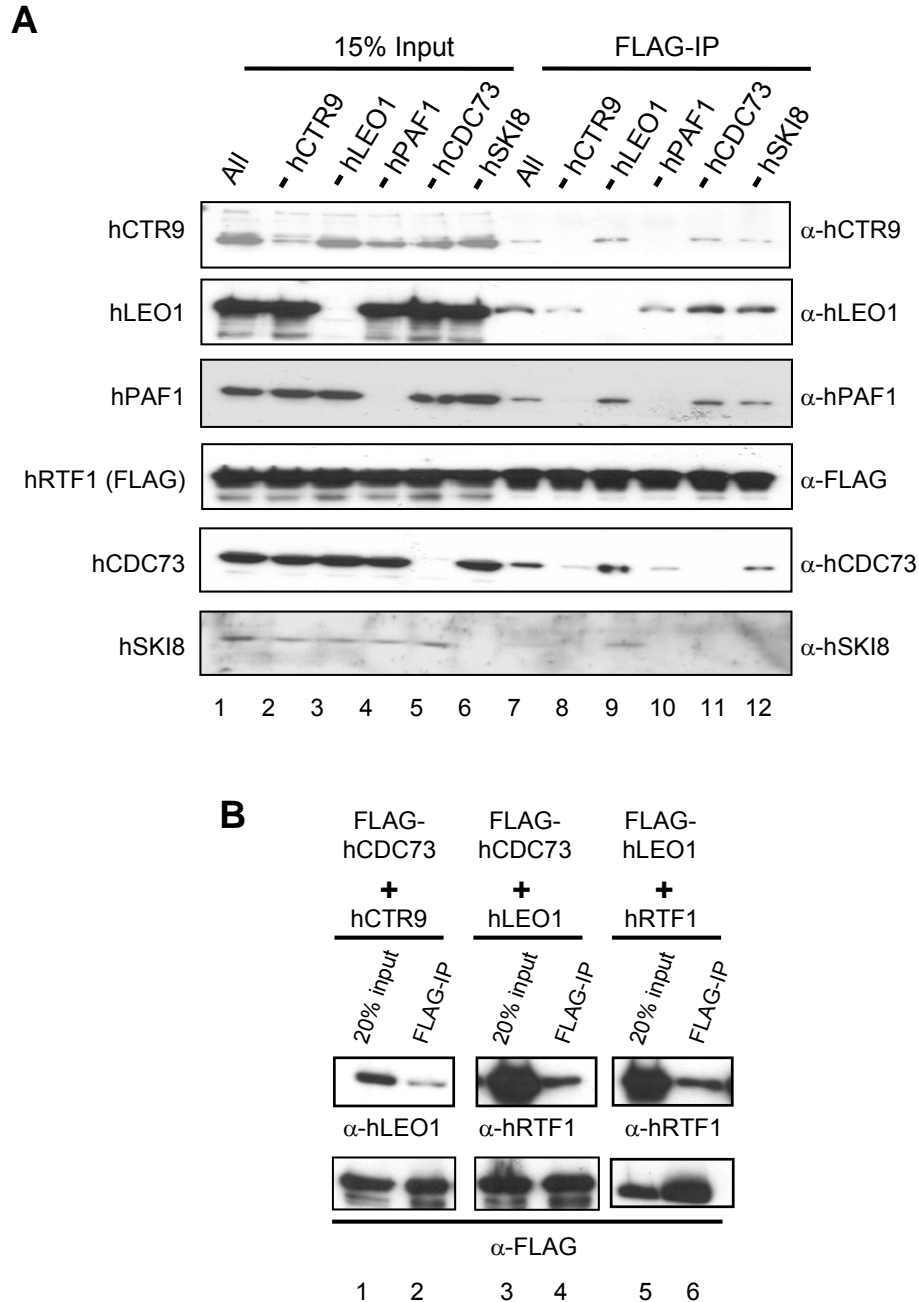
(A) Immunoblot analysis of FLAG-purified complexes from baculoviruses co-infected insect cells. Complete (all) or omitted baculoviruses used for co-infection are indicated at the top. (B) Immunoblot analysis of protein interactions between FLAG-hCTR9 and indicated components of hPAF complex after co-infection and purification.

effect on the overall composition of other subunits (lanes 8 and 10-12). In a pair-wise interaction studies (Figure 4.2.3B), hCTR9 was shown to efficiently interact directly with all other subunits (lanes 3-10) except hLEO1 (lanes 1-2). These data indicate that the association of hLEO1 to hCTR9 is achieved by indirect manner through other subunits. The information regarding direct and indirect protein interactions obtained from this study is also depicted in Figure 4.2.5A.

Finally, I repeated experiments with FLAG-hRTF1. This approach is of special interest because of the controversy regarding hRTF1 association with hPAF complex (Yart et al., 2005; Rozenblatt-Rosen et al., 2005; Zhu et al., 2005). Interestingly, in a complete set of baculovirus coinfections, all the components were successfully co-purified with FLAG-hRTF1 (Figure 4.2.4A, lane 7). In this set of experiments, omission not only of hPAF1 and but also of hCTR9 affected the overall composition of hPAF complex (lanes 8 and 10). The absence of hLEO1, hCDC73 or hSKI8 had no effect on other subunits (lanes 9, 11 and 12). Several direct protein interaction studies (as shown in Figure 4.2.4B), also

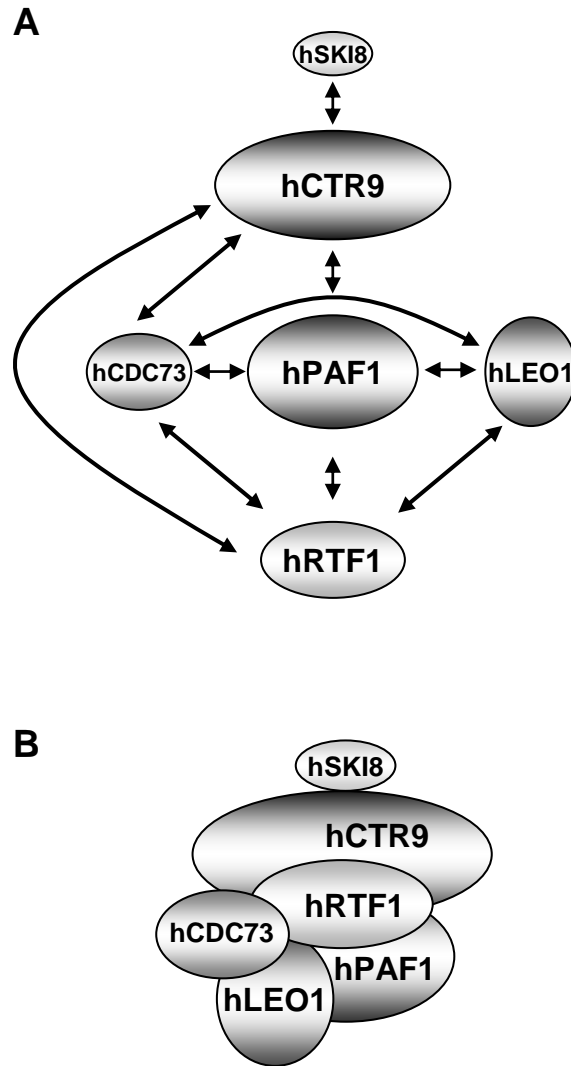
The combined information from all the presented results led me to propose a model for the structural organization of the hPAF complex (Figure 4.2.5B). In the hPAF complex, hPAF1 play a role as a key scaffold protein, and hCTR9

Figure 4.2.4 Determination of the Human PAF Complex Architecture: FLAG-hRTF1 basis



(A) Immunoblot analysis of FLAG-purified complexes from baculoviruses co-infected insect cells. Complete (all) or omitted baculoviruses used for co-infection are indicated at the top. (B) Immunoblot analysis of protein interactions between indicated components of hPAF complex after co-infection and purification.

Figure 4.2.5 Proposed Structural Organization of the Human PAF complex



(A) Interactions between the components in hPAF complex. The arrows indicate the confirmed direct interactions. (B) Schematic representation of reconstituted hPAF complex deduced from interaction studies.

also affects the overall protein stability. This physical model fits quite well to a model from a functional study in yeast. Thus it was reported that the loss either of PAF1 or of CTR9 results in nearly identical severe phenotypes related to many different cellular processes (Betz et al., 2002). In summary, each of four components, hCTR9, hLEO1, hPAF1 and hRTF1, directly interacts with other three components. hLEO1 associates with complex by interacting with hPAF1, hRTF1 and hCDC73, but not by hCTR9. The association of hSKI8 is totally dependent on hCTR9.

Establishment of an Activator-Dependent *in Vitro* Transcription with Purified Factors from a Chromatin Template

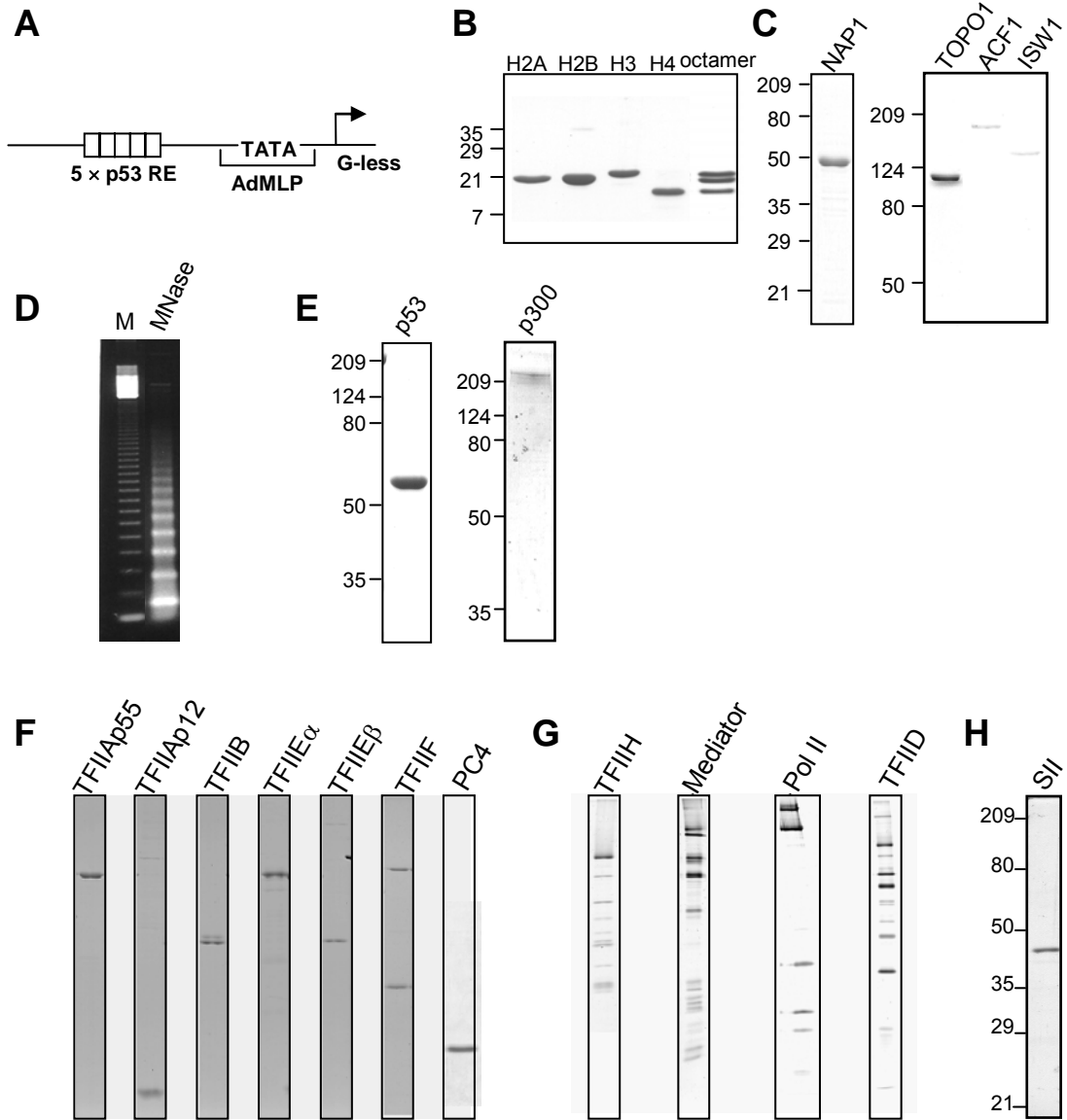
In order to investigate the function of transcription elongation factors in transcription from a chromatin template *in vitro*, a chromatin assembly system involving a pGADD45₅ML array DNA template (An et al., 2004), recombinant histones purified via *E. coli* (Luger et al., 1997) and recombinant chromatin assembly factors (Ito et al., 1999) has been established. The pGADD45₅ML array template (5.4 kb) contains a 700bp promoter/transcription region (comprised of five tandem p53 binding sites from GADD45 gene, the adenovirus major late core promoter and G-less cassette) flanked on either side by five 208 bp repeats of a nucleosome-positioning sequence from the sea urchin 5S rRNA gene (Figure 4.2.6A). The individual *Xenopus* histones were expressed in and purified from bacteria,

and then assembled into histone octamers (Figure 4.2.6B). Chromatin templates were assembled with purified recombinant histone octamers using purified recombinant *Drosophila* ACF1, ISW1, and mouse NAP1 proteins (Figure 4.2.6C).

Analysis of the assembled chromatin by micrococcal nuclease digestion revealed a 200 bp ladder of DNA intermediates (Figure 4.2.6D), indicating successful chromatin assembly. An *in vitro* transcription assay that involved preincubation of the assembled chromatin template with acetyl-CoA, recombinant p53 and p300 (Figure 4.2.7E), and subsequent incubation in a HeLa nuclear extract showed successful activator (p53)- and coactivator (p300)-dependent transcription (data not shown).

Although useful for many purposes, the transcription assay with HeLa nuclear extract is not suitable for studying the function of transcription elongation factors because these extracts contain all the factors facilitating transcription elongation on chromatin template. Therefore, I employed a transcription assay system that was reconstituted with highly purified factors shown previously to be necessary and sufficient for optimal activator-dependent transcription from DNA templates (Guermah et al., 2001). The TFIIA (p55 and p12 subunits), TFIIB, TFIIE (α and β subunits), and TFIIF (RAP30 and RAP74) components of the general transcription machinery, as

Figure 4.2.6 Analysis of Purified Factors Required for *in Vitro* Chromatin Assembly and Transcription



(A) Schematic representation of the p53 binding sites adjacent to the Adel promoter in the pal array template. (B and C) Analysis of recombinant histones, reconstituted histone octamer (B), NAP1, TOPO1, ACF1 and ISW1 by Coomassie blue staining (C). (D) MNase analysis of *in vitro* assembled chromatin. (E-H) Analysis of purified p53 and p300 (E), recombinant factors (F), affinity purified factors (G) and SII (H) by Coomassie blue staining (E, F and H) and silver staining (G), respectively.

well as the general coactivator PC4, were expressed in and purified from bacteria (Figure 4.2.6F). The multi-subunit TFIID, TFIIH, and RNA polymerase II components of the general transcription machinery, as well as the TRAP/Mediator coactivator complex, were purified from cell lines expressing FLAG-epitope-tagged subunits by combinations of conventional chromatography and affinity purification (Figure 4.2.6G). Transcription elongation factor SII was expressed in and purified from bacteria (Figure 4.2.6H).

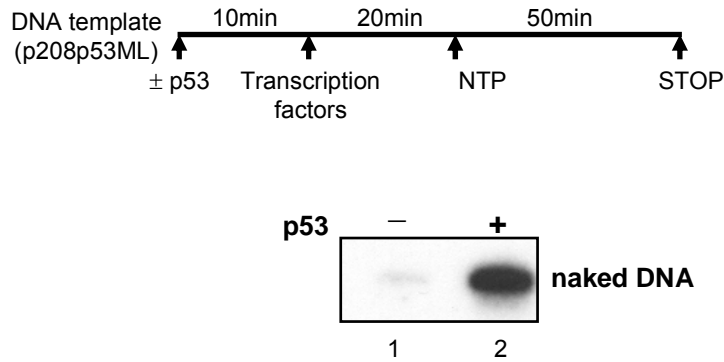
A transcription assay from a naked DNA template with purified factors showed clear and efficient p53-dependent transcription (Figure 4.2.7A), thus indicating that all purified factors are functional and suitable for *in vitro* transcription. More importantly, transcription of a recombinant chromatin template in this assay, but with inclusion of elongation factor SII (Guermah et al., 2006), clearly showed p53- and SII-dependent transcription (Figure 4.2.7B), thus demonstrating the successful establishment of a chromatin assembly and *in vitro* transcription system with purified factors.

Human PAF Complex Facilitates Transcription from a Chromatin Template

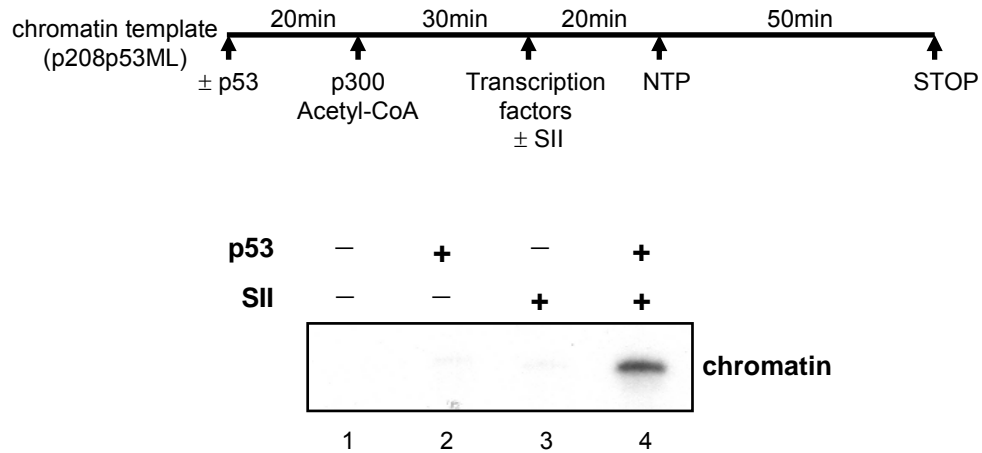
Having a reconstituted hPAF complex and a defined reconstituted system that effects transcription from a chromatin template, I tested the function of

Figure 4.2.7 Establishment of an *in Vitro* Transcription Assay System with Purified Factors

A



B

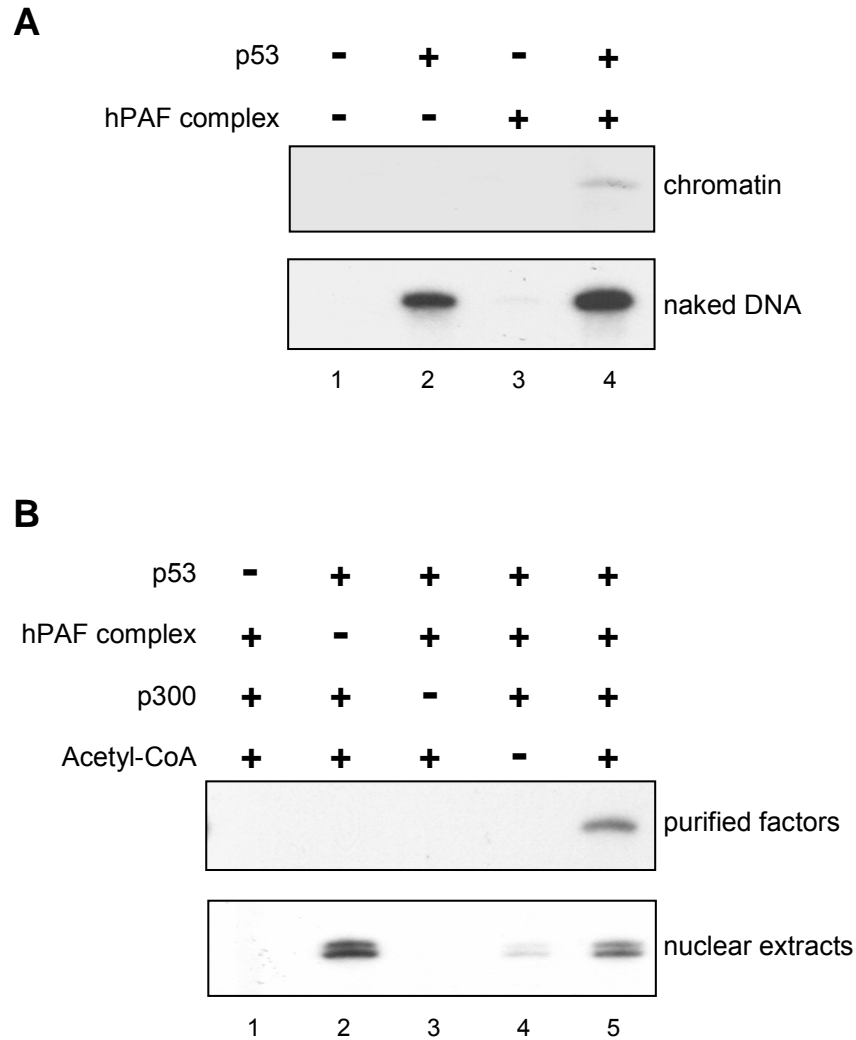


(A and B) An *in vitro* transcription assay with purified factors of a naked DNA template (A) and of a chromatin template (B). Schematic representations of the *in vitro* transcription assays were depicted on top. Transcription factors include TFIIA, B, E, D, F, H, PC4, mediator and pol II.

the hPAF complex in this system. Surprisingly, the hPAF1 complex effected a low level of p53-dependent transcription from a chromatin template in this assay in the absence of SII (Figure 4.2.8A, upper panel). A transcription assay from a corresponding DNA template showed that the hPAF complex also enhances (moderately) p53-dependent transcription (Figure 4.2.8B, bottom panel, compare lanes 2 and 4), thus strongly suggesting that hPAF complex somehow bolsters the transcribing activity of RNA polymerase II. The observed effect of the hPAF complex appears not to be caused by contamination of the complex with SII during purification of the hPAF complex, since an immunoblot with anti-SII failed to reveal any SII in purified hPAF complex (data not shown).

Our previous characterization of transcription elongation factor SII showed synergistic function of SII and p300 in activator-dependent transcription and effect of both factors on elongation (Guermah et al., 2006). The hPAF complex-facilitated transcription from chromatin template is also dependent on activator, p300 and acetyl-CoA (Figure 4.2.8B, top panel). However, a comparable experiment with HeLa nuclear extract in place of the purified transcription factors revealed activator-dependent transcription in the absence of ectopic hPAF complex and a moderate inhibitory effect of added hPAF complex (Figure 4.2.8B bottom panel, compare lanes 2 and 5). This observation suggests that there are redundant transcription elongation

Figure 4.2.8 Human PAF Complex-Facilitated Transcription of a Chromatin Template Depends upon Activator (p53), p300 and Acetyl-CoA



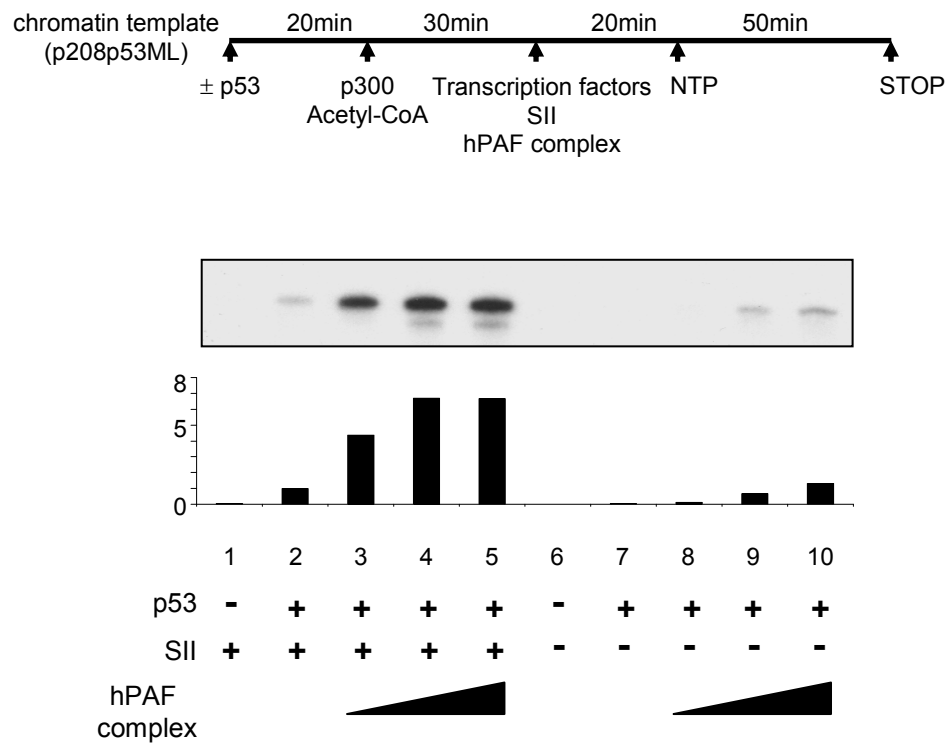
Characterization of hPAF complex function (A) Activator-dependent transcription from chromatin template and naked DNA template as indicated. (B) Activator, p300 and acetyl-CoA-dependent transcription with purified transcription factors and Hela nuclear extracts as indicated.

factors in HeLa nuclear extract (likely including endogenous hPAF complex) and that an excess dose of (ectopic) elongation factors may inhibit (possibly by squelching) transcription. The acetyl-CoA-independent transcription might be due to the presence of residual amount of acetyl-CoA in the HeLa nuclear extracts (lane 4).

Synergistic Effect of SII and the hPAF Complex on p53-dependent Transcription of a Chromatin Template with Purified Factors

Cells contain several putative transcription elongation factors, although there is only a little evidence to show their biochemical function. Interestingly, several genetic and physical interaction studies in yeast have indicated cooperative functions of elongation factors in transcription (Arndt and Kane, 2003). Having previously documented effects of transcription elongation factor SII on transcription from chromatin template (Guermah et al., 2006), I tested the possibility of synergism between SII and the hPAF complex. The activator-dependent transcription activity observed with SII was significantly enhanced by addition of the hPAF1 complex (Figure 4.2.9, lane 1-3) in a dose-dependent manner, and the synergistic effect was saturated at a higher dose of hPAF1 complex (lanes 2-5). The hPAF complex alone also showed a dose-dependent enhancement of transcription (lanes 6-10).

Figure 4.2.9 Synergistic Effect of SII and the hPAF Complex on p53-dependent Transcription of a Chromatin Template with Purified Factors



An *in vitro* transcription assay with purified factors including transcription elongation factors SII and hPAF complex of a chromatin template. Schematic representations of the *in vitro* transcription assays were depicted on top. Transcription factors include TFIIA, B, E, D, F, H, PC4, mediator and pol II. The relative transcription levels were scored at the bottom.

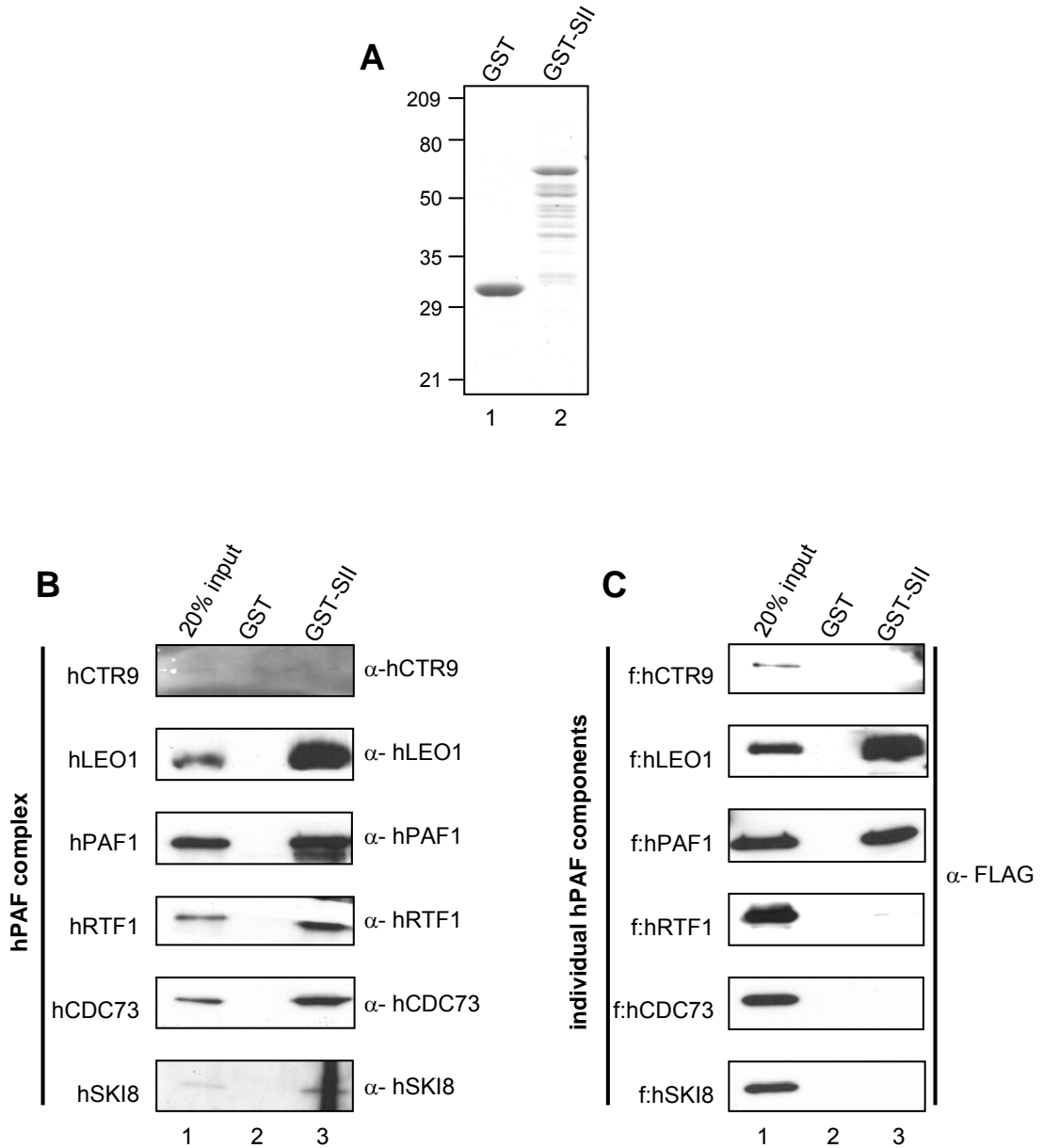
The Human PAF Complex Directly Interact with SII

Given that the hPAF complex and SII show a strong synergistic effect on activator-dependent transcription, I first examined interactions of the purified hPAF complex with GST-SII fusion proteins (Figure 4.2.10A). Interestingly, the hPAF complex showed a direct interaction with SII (Figure 4.2.10B). Next, I examined which subunit(s) in the hPAF complex is responsible for direct interaction with SII. To this end, individual FLAG-tagged subunits of the hPAF complex (Figure 3.2.10C) were tested for interactions with GST-SII. As shown in Figure 4.2.10C, the hLEO1 and hPAF1 subunits were shown to directly and strongly interact with SII.

The Human LEO1 and PAF1 Subunits are Required for Synergistic Effects of the hPAF complex and SII on Transcription

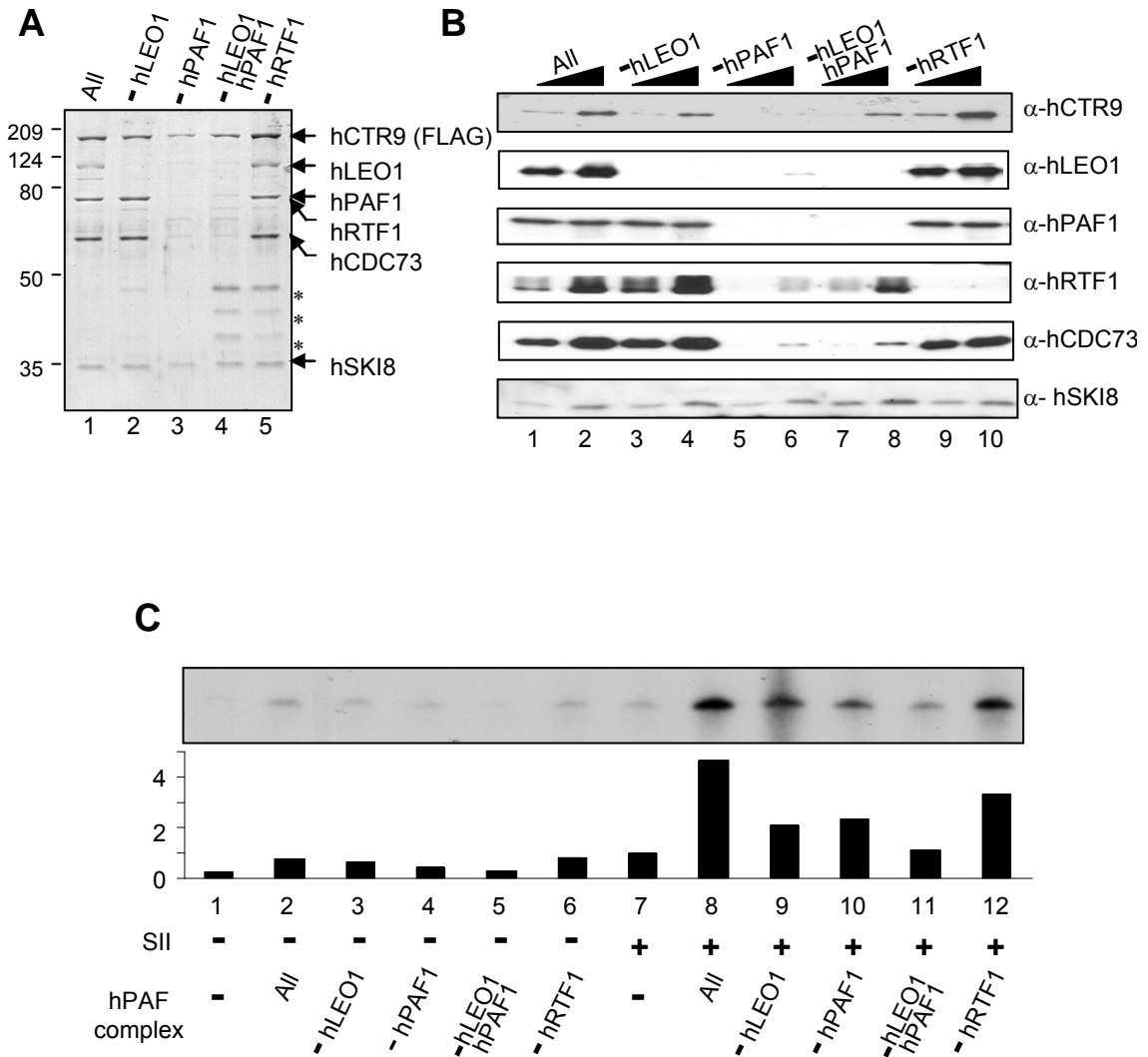
In order to verify that a direct interaction between the hPAF complex and SII is essential for the observed synergistic effect on transcription, several different hPAF complexes were prepared. Insect cells were coinfecting with baculoviruses expressing FLAG-hCTR9 and combinations of other untagged hPAF complex subunits, and corresponding complexes were isolated by affinity purification using M2-agarose (Figure 4.2.11A). The integrity of each subunit in different complexes was examined by immunoblot. Protein loading was normalized to equal amounts of hSKI8. Consistent with the previous determination of the structural organization of the hPAF complex (Figure

Figure 4.2.10 Direct Interaction between SII and the hPAF Complex



(A) Purified GST and GST- tagged SII proteins were analyzed by SDS/PAGE and Coomassie blue staining. (B and C) Purified hPAF complex (B) and individual components of hPAF complex (C) were tested for binding to GST-SII proteins, and bound proteins were analyzed by immunoblots with indicated antibodies.

Figure 4.2.11 The hLEO1 and hPAF1 Subunits are Required for Interaction with SII and Synergistic Effect on Transcription



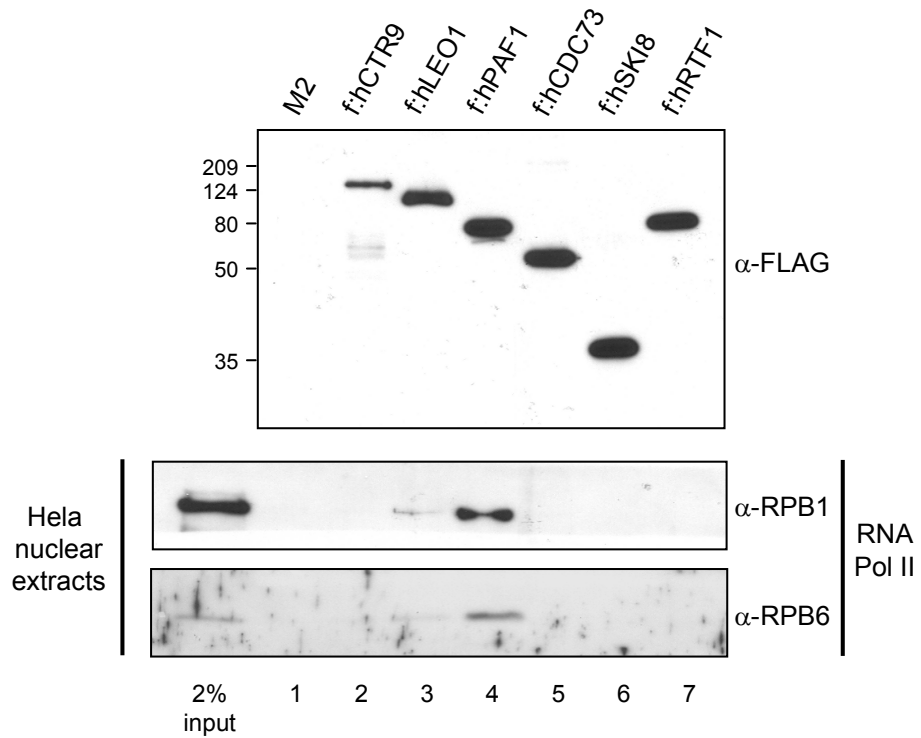
(A) Coomassie blue staining of FLAG-purified complexes from baculoviruses co-infected insect cells. Complete (all) or omitted baculoviruses used for co-infection are indicated at the top. (B) The integrity of each component in hPAF complexes was tested by immunoblot analysis with indicated antibodies. The loadings were normalized by hSKI8. (C) *In vitro* transcription assay with purified factors including indicated hPAF complex of a chromatin template. The relative transcription levels were scored at the bottom.

4.2.3), the omission of hLEO1 and hRTF1 did not affect the integrity of other components (Figure 4.2.11B, lanes 3-4 and 9-10), whereas omission of hPAF1 decreased the amounts of the other subunits (lanes 5-8). These different hPAF complexes were then tested for the synergistic effect with SII in transcription. Like the FLAG-hPAF1-derived hPAF complex, the FLAG-hCTR9-derived hPAF complex also showed a significant synergistic effect with SII (Figure 4.2.11C, compare lanes 7 and 8), suggesting that epitope tagging of different subunits in the hPAF complex has deleterious effect in its transcription activity. In contrast, exclusion of either hLEO1 or hPAF1 led to about 50% reductions in effects on transcription compared to that of the intact hPAF complex (compare lanes 8 and 9-10). The simultaneous omission of hPAF1 and hLEO1 dropped the level of transcription to that observed with SII alone (compare lanes 7 and 11). These data unequivocally indicate that the synergistic effect of hPAF complex and SII is achieved by their physical interaction via the hLEO1 and hPAF1 subunits in hPAF1 complex. In addition, the omission of hRTF1 led to little decrease in synergistic effect (lane 12), suggesting that hRTF1 is not critical component for the synergistic function with SII, but leaving open possibility that it may contribute somehow to some functional aspect of the hPAF complex.

Human PAF Complex Interacts with RNA Polymerase II via hLEO1 and hPAF1 Subunits

The multi-subunit PAF complex was initially identified as an RNA polymerase II-associated factor in yeast (Wade et al., 1996; Shi et al., 1996). The ability of the hPAF complex to facilitate transcription from chromatin templates led us to ask whether the mechanism involved a hPAF complex interaction with RNA polymerase II. To this end, M2-agarose beads were first coupled to equal amounts of individual hPAF complex subunits and then were incubated in HeLa nuclear extracts. An anti-FLAG immunoblot scored the coupling efficiency of bait proteins (Figure 4.2.12, top panel). The lower yield for the FLAG-hCTR9 containing reaction might be due less efficient coupling to M2-agarose and/or poor protein transfer during immunoblot procedure because of its large molecular weight compared to other proteins. Co-immunoprecipitated proteins were analyzed with antibodies directed against subunits of RNA polymerase II. This experiment revealed that hLEO1 and hPAF1 subunits, presumably directly, interact with RNA polymerase II (Figure 4.2.12, bottom panel). hPAF1 showed a stronger interaction than hLEO1 (compare lanes 3 and 4). In a functional assay, the hPAF complex lacking hLEO1 showed little reduction in SII-independent transcription compared to intact complex (Figure 4.2.11C, compare lanes 2 and 3), whereas an hPAF complex lacking hPAF1 showed a more severe reduction (compare lanes 2 and 4). Finally hPAF complex missing both

Figure 4.2.12 Human PAF Complex Interacts with RNA Polymerase II via hLEO1 and hPAF1 Components



HeLa nuclear extracts were incubated with M2 agarose previously coupled with FLAG-tagged hPAF components and bound proteins were analyzed by immunoblots with indicated antibodies.

hLEO1 and hPAF1 led to complete loss of its SII-independent chromatin transcription enabling activity (lane 5). These results indicate that the hPAF complex functions as a transcription elongation factor through a direct interaction with RNA polymerase II that is mediated by hLEO1 and hPAF1. In addition, it is interesting that hLEO1 and hPAF1 subunits were also responsible for interaction with SII.

Human PAF Complex Directly Interacts with Transcriptional Activator

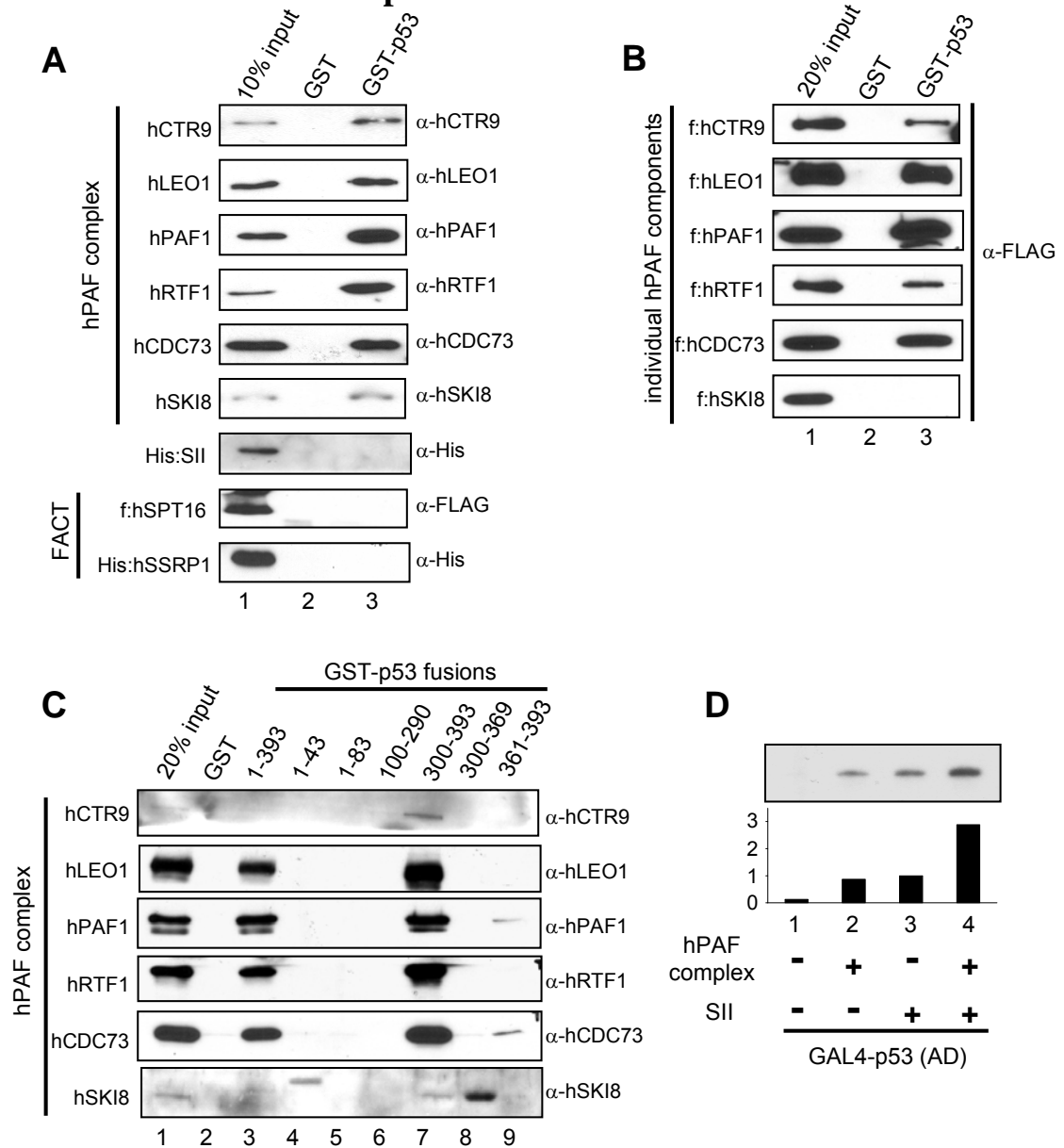
Besides its role in transcription elongation, the PAF complex was initially implicated in transcription initiation. The PAF complex originally was identified as a collection of proteins associated with the unphosphorylated form of RNA polymerase II (Wade et al., 1996) and PAF complex subunits have been found at promoter (Pokholok et al., 2002; Mueller et al., 2004), with significant increases upon gene activation (Pavri et al., 2006). Genetic studies in yeast also have suggested key roles for the PAF complex in transcript site selection (Stolinski et al., 1997) and in communication with DNA-binding factors (Betz et al., 2002).

To test the possibility that the hPAF complex is recruited to the promoter region through the direct interaction with DNA-binding activators, I examined the interactions of purified hPAF complex and GST-p53 fusion

proteins. The hPAF complex was shown to interact efficiently with p53. This is a unique characteristic of the hPAF complex since transcription elongation factor SII and histone chaperone/elongation factor FACT do not bind to p53 (Figure 4.2.13A). Next, I tested which component(s) in hPAF complex is responsible for binding to p53. To this end, individual FLAG-tagged proteins in the hPAF complex (Figure 3.2.10C) were tested for binding to GST-p53 fusion proteins. Interestingly, all subunits in the hPAF complex other than hSKI8 strongly interacted with p53. These observations led us to more detailed protein interaction study. To determine the region(s) in p53 responsible binding to the hPAF complex, I tested for hPAF complex binding to GST-fused fragments of p53 (Figure 2.2.8A, top panel). The hPAF complex showed an exclusive direct interaction with C-terminal 300-393 region (Figure 4.2.13C, lane 7) and a weak interaction with the extreme C-terminal 361-393 regions of p53 (lane 9).

Next, I questioned whether a direct interaction of the hPAF complex with p53 is essential for its activity on transcription enabling activity from chromatin template. The p53 C-terminal deletion mutant is functionally inactive as a transcription activator since it abolishes the functional tetramer formation of p53 (data not shown). Therefore, as an alternative way, I employed GAL4-fused p53 transcription activation domain (GAL4-p53AD) (Suzuki-Yagawa et al., 1997) which is active in transcriptional activation and lacks C-terminal

Figure 4.2.13 Human PAF Complex Strongly Interacts with C-terminus of p53



(A-C) Purified hPAF complex (A and C) and individual components of hPAF complex (B) were tested for binding to GST-tagged full length p53 (A and B) and to GST-tagged partial fragments of p53 (C). Bound proteins were analyzed by immunoblots with indicated antibodies. (D) GAL4-p53(AD: activation domain)-dependent *in vitro* transcription from pG₅ML based chromatin template with purified factors. pG₅ML template contains 5-copies of GAL4-binding sites in front of AdML promoter and subsequent G-less cassette. The relative transcription levels were scored at the bottom.

part of p53. If the direct interaction of hPAF complex with C-terminus of p53 is essential for its transcription elongation activity, it is expected that the hPAF complex is not able to facilitate transcription from chromatin template with GAL4-p53AD. However, in an *in vitro* transcription assay, the hPAF complex still showed significant activity in transcription (Figure 4.2.13D, lane 2) as well as synergistic function with SII (lane 4). These data suggest that a physical interaction of the hPAF complex with a transcriptional activator is not essential for transcription elongation activity of hPAF complex. However, the quite strong binding property of the hPAF complex to p53 leaves open possibility of a mechanism in which the hPAF complex is recruited to the promoter region through direct interactions with natural activators (including intact p53) in cells.

Cooperative Binding of SII and the hPAF Complex to RNA Polymerase II

To elucidate the mechanism underlying synergistic effect of the hPAF complex and SII on transcription, I tested the possibility of cooperative binding of these factors to RNA polymerase II. GST-fused SII, previously immobilized to beads, was incubated with HeLa nuclear extracts in the presence and in the absence of purified hPAF complex (Figure 4.2.14A). As expected from previous reports, SII showed an association with RNA polymerase II (compare lanes 2 and 3) and, interestingly, addition of the

purified hPAF complex significantly enhanced the binding efficiency (compare lanes 3 and 5). In a reciprocal approach, the hPAF1 complex (purified via FLAG-epitope on hPAF1 subunit) was immobilized on M2-agarose beads and then incubated with HeLa nuclear extracts (Figure 4.2.14B). RNA polymerase II bound selectively to M2 agarose-immobilized hPAF complex, relative to M2 alone (compare lanes 2 and 3), and the binding efficiency was greatly increased by addition of purified SII (compare lanes 3 and 5).

To verify that the observed cooperative binding of transcription elongation factors to RNA polymerase II is mediated by direct protein interactions, the experiment in Figure 4.2.14A was repeated with purified RNA polymerase II instead of HeLa nuclear extracts. Consistent with previous experiments, SII showed significantly enhanced binding to purified RNA polymerase II in the presence of the purified hPAF complex (Figure 4.2.14C). Taken together, these data confirm a direct interaction between transcription elongation factors and RNA polymerase II *in vitro* and, further indicate cooperative binding of two distinct transcription elongation factors to RNA polymerase II.

4.3 DISCUSSION

Successful reconstitution of the hPAF complex via the baculovirus expression system enabled us to perform a systematic study of the structural

organization transcriptional functions of the hPAF complex. Interestingly, I found that the hPAF complex stimulates transcription on a chromatin template in a manner that is dependent upon p300 and acetyl-CoA but independent of H2B ubiquitylation and H3-K4 methylation. Of special note, this newly described transcription elongation activity of the hPAF complex was greatly enhanced by the elongation factor SII. From a mechanistic view, I also found that the hPAF complex strongly interacts with SII and that they bind cooperatively to RNA polymerase II. By identifying the hPAF complex as functional transcription elongation factor that acts cooperatively with another elongation factor, this study expands our knowledge of the transcription elongation process and open a new view of 'cross-talk' between elongation factors.

Reconstitution of a Functional Human PAF Complex

Using the baculovirus/Sf9 cell expression system, I have been able to generate a complete hPAF complex that markedly enhances activator- and p300-dependent transcription from a chromatin template. This complex also shows a modest, but significant, effect on transcription from a DNA template. This reflects a novel intrinsic activity of the PAF complex that is clearly independent of previously described (indirect) effects of the PAF complex through its requirement for H2B ubiquitylation and subsequent H3-K4 trimethylation. Through the systematic deletion of specific subunits during

reconstitution, I have been able to determine the role of specific subunits in both the structural organization and function of the hPAF complex. With respect to structural organization of the complex, and consistent with studies of the yeast PAF complex (Mueller et al., 2004), the hPAF1 and hCTR9 subunits were identified as scaffold proteins within the complex. Beyond this key function, I was also able to deduce a number of other subunit interactions as summarized in Figure 4.2.5A. I also found that human-specific subunit SKI8 is tethered to the complex through exclusive binding to hCTR9 (Figure 4.2.5). Although there could be unidentified factors associated with the hPAF complex in human cells, the obvious activity in transcription indicates the successful reconstitution of a functional hPAF complex with a minimal set of core components.

The yeast PAF complex contains 5 subunits, CTR9, LEO1, RTF1, PAF1 and CDC73 (Mueller et al., 2002). yRTF1 plays pivotal roles in some PAF complex functions in yeast. yRTF1 deletion leads complete decrease in H2B ubiquitylation and subsequent H3 methylation, whereas deletion of other subunits showed less effect (Xiao et al., 2005). Related to this, yRTF1 was found to be required for association of RAD6-BRE1 to RNA polymerase and thus for H2B ubiquitylation at coding regions (Xiao et al., 2005). In addition, recent study in *Drosophila* showed that RTF1, while not found in the *Drosophila* PAF complex, is also required for H3-K4 trimethylation, implying

that function of RTF1 in histone modification is conserved from yeast to higher organism (Tenney et al., 2006). *Drosophila* RTF1 colocalizes broadly with actively transcribing, phosphorylated RNA polymerase II in a pattern very similar to that of PAF1 and CDC73 (Adelman et al., 2006). These data suggest that RTF1 functions with the PAF complex *in vivo*. Nonetheless, other than histone modifications, loss of yRTF1 causes relatively subtle phenotypes in yeast compared to the loss of other subunit such as yCTR9 and yPAF1 (Porter et al., 2005). Furthermore, *in vitro* transcription of a naked DNA template in nuclear extracts prepared from RTF1 deletion strains caused little change compared to that from WT strain (Rondon et al., 2004).

My reconstitution study revealed that hRTF1 can associate with the hPAF complex (Figure 4.2.1), and importantly, that all other subunits co-purify with an ectopically expressed FLAG-hRTF1 (Figure 4.2.4). Given the previous failure to observe hRTF1 in hPAF complex isolated from human cells by other methods in other labs (Yart et al., 2005; Rozenblatt-Rosen et al., 2005), these data suggest that hRTF1 is associated with the hPAF complex in cells, but might be easily dissociated during biochemical purification procedures. In my H2B ubiquitylation- and H3-K4 methylation-independent transcription assays, hRTF1 was shown to be dispensable for activity of the hPAF complex (Figure 4.2.11). In conjunction with the above described observation, these data suggest an idea that RTF1 plays a more important

role in H2B ubiquitylation and less in the newly described transcription activity of the hPAF complex. A recent study in human cells also found that hCTR9 and hSKI8 are required for H2B ubiquitylation and H3-K4 methylation (Zhu et al., 2005b). This remains an interesting question that discuss further speculation, therefore, whether hRTF1 is involved in H2B ubiquitylation in human cells might be interesting question to be answered soon.

Cooperative Function of Human PAF Complex and SII in Transcription

My present study, for the first time, reports the synergistic effect of two distinct elongation factors on transcription form chromatin template (Figure 4.2.9) As a mechanistic explanation, I show that hPAF complex directly binds to SII (Figure 4.2.10) and results in their cooperative interaction to RNA polymerase II (Figure 4.2.14). This observation is consistent with accumulated data showing genetic and physical interaction between transcription elongation factors. For example, the yeast PAF complex shows genetic interaction with TFIIIS (SII), DSIF, FACT and Spt6 (Costa and Arndt 2000; Squazzo et al., 2002) and physical interactions with DSIF, FACT and Chd1 (Squazzo et al., 2002; Simic et al., 2003). TFIIIS shows an over broader range of genetic interactions with DSIF, FACT, the PAF complex, Spt6, Elongator and Rad26 (Hartzog et al., 2002, Lee et al., 2001) and physical

interaction with DSIF (Lindstrom et al., 2003). Importantly, my observation provides the first report of a direct physical interaction between SII and PAF complex. The presence of multiple elongation factors and a complex array of genetic/physical interactions strongly suggest a network of sophisticated communications and cooperative functions in transcriptional control in eukaryotic cells.

Mechanism of PAF Complex Function in Transcription

It was found that where the hPAF complex and elongation factor SII can markedly stimulate activator- and p300/acetyl-CoA-dependent transcription from chromatin template, they can also act synergistically to effect synthesis both short (12 bp and 21 bp) and long (1.3 kb) transcripts (data not shown). Interestingly, however, it was also found that the hPAF complex alone shows stronger activity on short transcript than does SII, and only negligible activity on long transcripts (data not shown). Therefore, I speculate that the hPAF complex preferentially functions at a very early stage of transcription elongation (or possibly even at initiation) and that SII participation strengthens and maintains activity through later stage of transcription. Related to this, the hPAF complex, but not SII, was shown to interact with the promoter DNA binding activator p53 (Figure 4.2.13). More interestingly, detailed recruitment studies of various elongation factors have shown that, upon galactose induction, yPAF complex subunits yPAF1 and yCDC73

accumulate both at promoter and coding regions whereas level of TFIIS (SII) is unaltered at the promoter region and increased within the coding region (Pokholok et al., 2002). These observations led to a mechanistic model involving separate roles of distinct elongation factors at different stage of transcription elongation.

It has been proposed that elongation factor SII acts by rescuing RNA polymerase II from backtracking and thus reactivating arrested RNA polymerase II for subsequent transcription through pause/arrest sites that include nucleosome (Knezetic and Luse 1986; Lorch et al., 1987; Workman and Roeder 1987; Kireeva et al., 2005). Elongation factor FACT functions, through its action as a histone chaperone, in both disassembly and reassembly of H2A/H2B dimers during transcription (Belotserkovskaya et al., 2003). However, I currently have no information regarding the mechanism involved in the intrinsic (histone ubiquitylation and methylation-independent) activity of transcription elongation factor hPAF complex. Given the multi-subunit composition of the hPAF complex, it is plausible that different subunits have different roles (e.g. for directly stimulating the elongation function of RNA polymerase II and for interactions with other transcription components that include activator, the H2B ubiquitylation and H3-K4/K79 methylation machineries and other elongation factors). The present observation sets the stage for subsequent studies of detailed functional

aspects of the hPAF complex and its communication with the transcription machinery.

CHAPTER 5

Conclusions and Perspectives

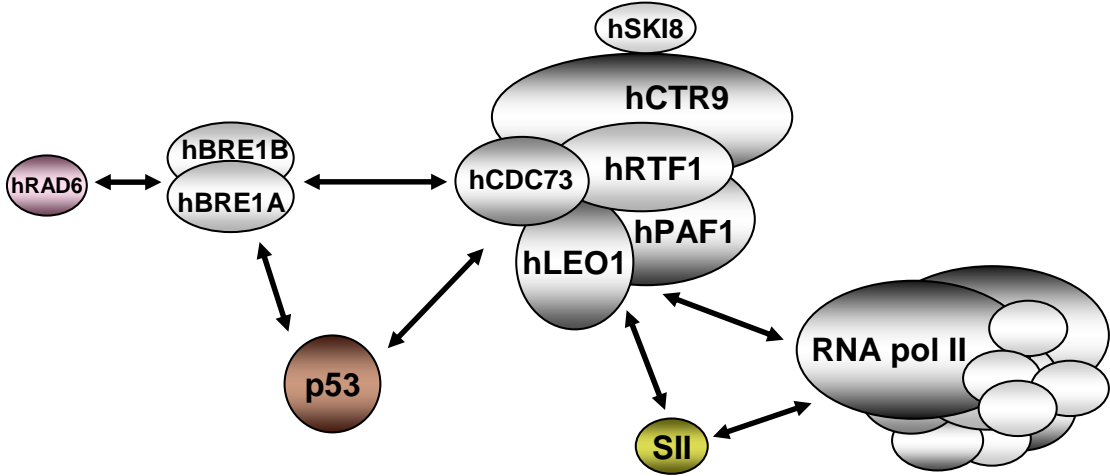
Network of Interactions of H2B Ubiquitylation Related Factors

H2B ubiquitylation is enriched in both the promoter regions and the 5' of coding regions in transcriptionally active genes and linked to ongoing transcription by participating in transcription elongation process (Osley, 2004). In this regard, through the extensive protein interaction studies, my study has identified a network of interactions between transcription factors that include both H2B ubiquitylation factors and transcription elongation factors (Figure 5.1).

First, it was found that E3 ubiquitin ligases hBRE1A and hBRE1B form a heterodimeric complex through their N-terminal domains (Figure 3.2.4). Complex formation is essential for H2B ubiquitylation activity (Figure 3.2.8) since only the dimer form can functionally interact with its cognate E2 ubiquitin conjugating enzyme (Figure 3.2.5). Although the Reinberg lab previously reported that hUbcH6 functions as an E2 for hBRE1A/B (Zhu et al., 2005), my protein interaction studies and functional assays unequivocally indicate that hRAD6 is an E2 enzyme for H2B ubiquitylation.

In relation to mechanisms for hBRE1A/B recruitment and function, data presented here show both *in vivo* and direct *in vitro* interactions of hBRE1A/B with the C-terminal region in p53 (Figure 2.2.8). Hence, it is

Figure 5.1 Cross-Talk between Transcription Factors



Protein interactions identified in this study were depicted by arrows. See text for details.

likely that DNA-bound activators are involved directly in hBRE1A/B recruitment. Consistent with this possibility, p53-enhanced accumulation of hBRE1A on p53 target gene has been demonstrated in this study (Figure 2.2.9). My observations and conclusions are strengthened by the report that yPAF complex is dispensable for yBRE1 recruitment to the promoter but is absolutely required for recruitment to the coding region (Xiao et al., 2005). Hence, it is thought that the histone ubiquitylation machinery associates with the promoter and with RNA polymerase II through separate processes-- i.e. that yBRE1/yRAD6 is recruited to the promoter via an activator and then transferred to the transcription machinery via the PAF complex. My observations have also documented how H2B ubiquitylation factors communicate with transcription machinery. It was found that hBRE1A/B directly interacts with the hPAF complex through the hCDC73 subunit (Figure 3.2.10), thus indicating how hRAD6-hBRE1A/B complex travels with RNA polymerase II. The trimeric complex formation model of hRAD6-hBRE1A/B-hPAF (Figure 3.2.9) (not hBRE1A/B-hUbch6-hPAF claimed by the Reinberg group) is supported by recent yeast studies, which showed that RAD6 association with the promoter/coding regions and with RNA polymerase II is absolutely dependent on the presence of BRE1 (Wood et al., 2003a; Xiao et al., 2005).

I also found that the hPAF complex strongly binds to C-terminus of transcription activator p53. It is interesting that 5 out of 6 subunits in hPAF complex appear to be involved in this direct interaction (Figure 4.2.13). This binding property is unique to the hPAF complex since transcription elongation factor SII and histone chaperone FACT do not bind to p53. Although it was also found that direct interaction of the hPAF complex with p53 is dispensable for at least some of the intrinsic transcription elongation activity of the hPAF complex *in vitro* (Figure 4.2.13D), my data still opens the possibility of a secondary mechanism involving hPAF complex recruitment to the promoter region through direct activator interaction in cells.

During the course of studying the role of the hPAF complex in H2B ubiquitylation, and interestingly, I found that the hPAF complex has an intrinsic ability, independent of any potential effect through its role in H2B ubiquitylation and H3-K4/K79 methylation, to enhance transcription of a chromatin template *in vitro* (Figure 4.2.8). Based on the notion of direct interactions between transcription elongation factors and RNA polymerase II, the hLEO1 and hPAF subunits of the hPAF complex were identified as direct interaction partners with RNA polymerase II (Figure 4.2.12) and found to be indispensable for the transcription elongation function of hPAF complex (Figure 4.2.11). I also found that the activity of hPAF complex was significantly enhanced by another transcription elongation factor SII (Figure

4.2.9). This synergistic effect is achieved by direct interaction between SII and the hPAF complex via hLEO1 and hPAF1 subunits (Figure 4.2.10). The cooperative binding of these two distinct transcription elongation factors to RNA polymerase II helps to elucidate the mechanism of their synergistic effect on transcription (Figure 4.2.14).

The information about the protein interactions identified in this study provide insight into the molecular mechanism involved in the function and cross-talk of transcription factors, including both H2B ubiquitylation factors and transcription elongation factors, in the complex transcription process in mammalian cells.

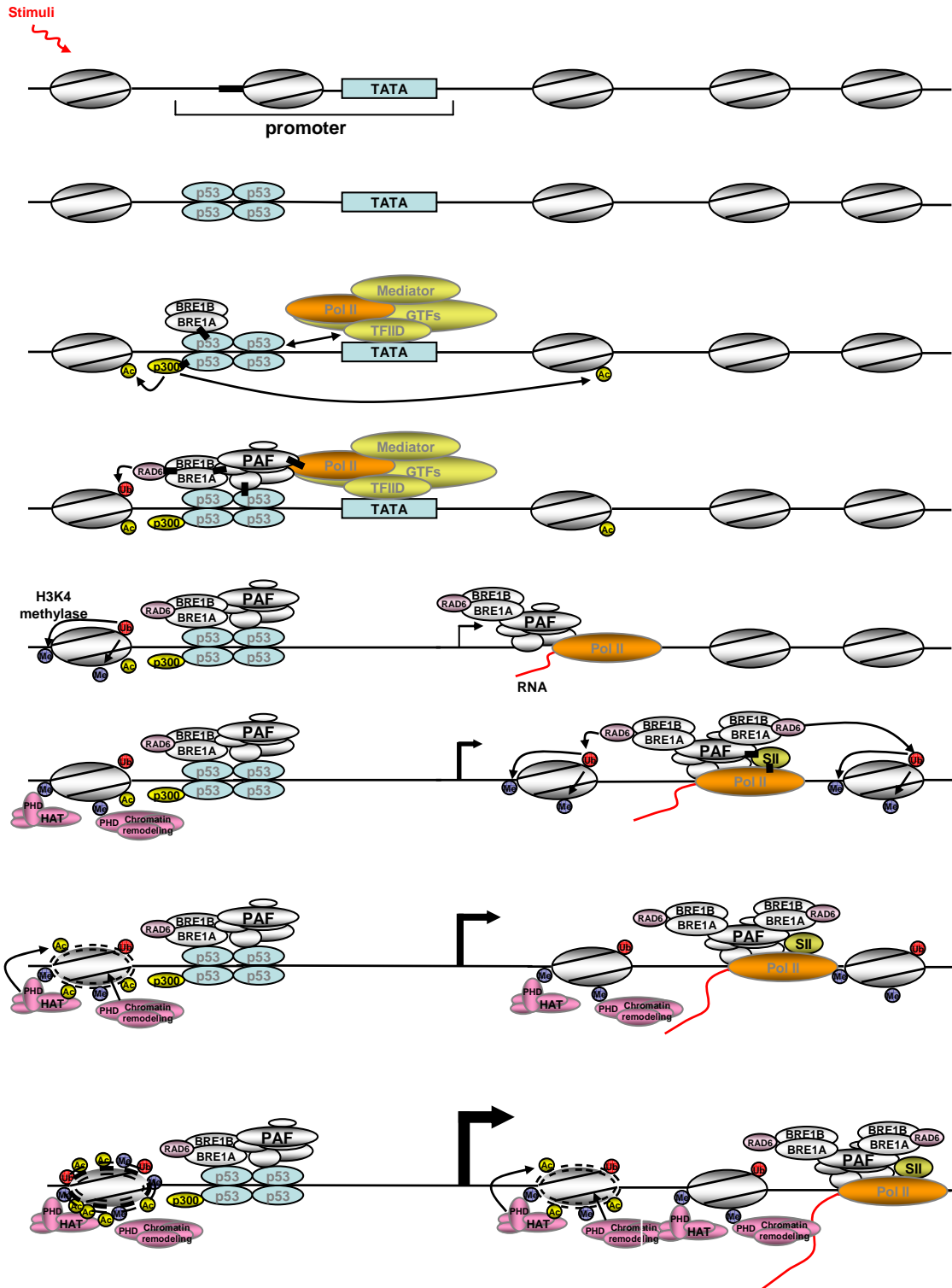
Mechanistic Model for the Role of Histone Modifications and Transcription Elongation Factors in Transcriptional Control in Mammalian Cells

I previously examined the function of coactivators and resulting histone modifications such as acetylation and methylation in p53-dependent transcription (An et al., 2004). Through the extensive protein interaction and functional assays performed in this study, I suggest that H2B specific E3 ubiquitin ligase hBRE1A/B also functions, through direct activator interactions, as a transcriptional coactivator. This observation, along with my finding that the hPAF complex links H2B ubiquitylation to the transcription

elongation process, leads to a mechanistic model for how an activator-bound transcriptional coactivator communicates with the transcription machinery in mammalian cells (Figure 5.2).

Genotoxic stress activates p53 to bind to its responsive elements and DNA-bound p53 and, through a mechanism that is not yet clear, stimulates the assembly of transcription pre-initiation complex on the TATA box containing core promoter. Concomitantly or sequentially, p53, through direct interactions, first recruits coactivators such as the histone acetyltransferase p300 and other histone modifying factors, the BRE1A/B complex and transcription elongation factor PAF complex. p300, following recruitment through interaction with N-terminus of p53 (An et al., 2004), acetylates nucleosomal histones around the promoter region. Following BRE1A/B recruitment by p53, RAD6 is recruited to the promoter region through direct interactions with BRE1A/B and ubiquitylates H2B at lysine 120. Although it has been found that PAF complex is required for global H2B ubiquitylation, it is interesting question whether PAF complex is indispensable for H2B ubiquitylation around promoter region. However, it appears that the PAF complex can be recruited to the promoter through alternative and potentially cooperative mechanisms, since it was shown to interact with BRE1A/B and RNA polymerase II. Upon transcription initiation, RNA polymerase II passes

Figure 5.2 Mechanistic Model for the Role of Histone Modifications and Transcription Elongation Factors in Transcription from Chromatin



See text for details.

early encountered nucleosomes with the assistance of PAF complex. The PAF complex may transfer (carry along) the BRE1A/B-RAD6 H2B ubiquitylation machinery from the promoter region and/or recruit free ubiquitylation components during transcription elongation and thus effect in H2B ubiquitylation at the coding region. Although the PAF complex is thought to facilitate transcription elongation through its role in H2B ubiquitylation and H3-K4/K79 methylation, it also exhibits an intrinsic transcriptional stimulating activity that is independent of, but may nonetheless contribute to, H2B ubiquitylation and H3-K4/K79 methylation events. This intrinsic transcriptional stimulatory activity of the PAF complex is markedly enhanced by transcription elongation factor SII, which binds cooperatively with the PAF complex to RNA polymerase II. However, at least in the *in vitro* assays, H2B ubiquitylation itself around the promoter and coding region has no effect on transcription and persistent H2B ubiquitylation may even hamper efficient transcription.

Although more careful consideration and further efforts are necessary, recent findings suggest a mechanism(s) for how H2B ubiquitylation may regulate gene activation. One mechanism may involve ubiquitylated H2B-binding molecules and/or enzymes that affect downstream histone modification and/or chromatin remodeling events. In relation to this, it is well established that H2B ubiquitylation is a prerequisite for H3-K4 and H3-K79 methylation

(Fischle et al., 2003). Although the mechanism of how ubiquitylated H2B might be recognized by H3-K4 histone methyltransferase and how it directs histone methylation is not known, trimethylated H3-K4 can be recognized by PHD finger containing complexes such as chromatin remodeling and histone acetyltransferase complexes. These two activities, i.e. ATP-dependent chromatin remodeling and covalent histone modifications may facilitate transcription factors access to target genes and, thus, more efficient transcription by RNA polymerase II.

Perspectives

Several other key experiments also remain to be done. Since the hBRE1A/B complex contains two ring-finger motifs, it is an interesting question as to which ring-finger(s) is responsible for its E3 activity. Whether the requirement for the hPAF complex in efficient H2B ubiquitylation is linked to ongoing transcription is also an intriguing unresolved. The observed reduction in transcription upon addition of H2B ubiquitylation factors should be examined and confirmed by testing catalytically-inactive ubiquitylation enzymes and chromatin assembled with H2B mutant (H2BK120R) that cannot be ubiquitylated. Beyond global effects, gene-specific and region-specific (promoter versus coding regions) changes in histone modifications following reduction of endogenous H2B ubiquitylation related factors must also be analyzed.

Many questions remain regarding the mechanism of H2B ubiquitylation and subsequent H3 methylation, and their roles in transcription, in mammalian cells. For example, it is still unclear as to which or the several known H3-K4 methylase(s) in mammalian cells is responsible for H2B ubiquitylation-directed H3-K4 methylation. And we still have no idea about the mechanism by which H2B ubiquitylation directs H3-K4 and H3-K79 methylation. Understanding the functional links between H2B ubiquitylation, H3-K4 methylation and chromatin remodeling and/or transcription complexes that recognize methylated H3-K4 residues, and consequent effects on transcriptional control in mammalian cells, will serve as intriguing research topics for many years.

CHAPTER 6

Materials and Methods

cDNAs, Plasmids, and Mutagenesis

The cDNAs for hE1 (Genebank accession number: M58028), hBRE1A (NM_019592), hBRE1B(BC018647), hRAD6A (M74524), hRAD6B (M74525), hUbcH6 (X92963), hUbcE2H (Z29328), hUbcH5a (BC005980), hUbcH5C (U39318), hCDC34 (NM_004359), hUbcH8 (AF031141), hUBE2E2 (NM_152653), ubiquitin (M26880), hCTR9 (BC058914), hLEO1 (BC018147), hPAF1 (AJ401156), hRTF1 (BC015052), hCDC73 (NM_024529) and hSKI8 (AK024754) were obtained from ATCC and subcloned into appropriate plasmids. Human histone cDNAs from bacterial expression vectors were modified to add N-terminal FLAG-epitopes and subcloned into pCDNA3. Yeast cDNAs for yRAD6, yBRE1 were amplified from a yeast genomic DNA (Clontech), and then inserted into pCDNA3. Serial deletion mutants were generated by introducing stop codons by site-directed mutagenesis (Stratagene). Vectors for luciferase reporter plasmids were described (An et al., 2004).

Protein Expression and Purification

For the affinity purification of recombinant proteins and complexes via baculovirus expression system, cDNAs were subcloned in pFASTBAC1 vector and baculoviruses were generated according to the manufacturer's instruction (Invitrogen). Sf9 cells were infected with baculoviruses and resulting cell extracts were subjected to standard purification procedures.

Infected sf9 cells were collected, resuspended in lysis buffer (20 mM Tris-HCl (pH 7.9), 500 mM NaCl, 4 mM MgCl₂, 0.4 mM EDTA, 20% glycerol, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 2 mM dithiothreitol (DTT)), and disrupted with a Dounce homogenizer. After removal of cell debris by centrifugation, the supernatant was adjusted to 300 mM NaCl (by dilution with 20 mM Tris-HCl and 10% glycerol) and 0.1% NP-40 and incubated with M2-agarose beads (Sigma). Beads with the bound proteins were washed extensively with wash buffer (20 mM Tris-HCl (pH 7.9), 150 mM NaCl, 2 mM MgCl₂, 0.2 mM EDTA, 15% glycerol, 0.1% NP-40, 1 mM PMSF, and 1 mM DTT) and proteins were eluted with FLAG peptide. For GST-tagged proteins, cDNAs were subcloned into pGEX4T-1, expressed in *E. coli*, and purified on glutathione-sepharose 4B beads (Amersham).

Nuclear extracts (NE) were prepared as described (Dignam et al., 1983). TFIID, TFIIH, and the Mediator complex were purified from cell lines expressing FLAG-TBP, FLAG-ERCC3, and FLAG-TRAP220/Med1 (AB1), respectively, on phosphocellulose (Whatman P11), DEAE-cellulose (DE52), and anti-FLAG M2 agarose antibody columns (Guermah et al., 2001). RNA polymerase II was purified from nuclear pellets of a HeLa cell line expressing the FLAG-RPB9 subunit, essentially as described (Guermah et al., 2001) but with addition of an M2 agarose affinity step at the end. TFIIA subunits (p55 and p12), TFIIB, and TFIIIE subunits (α and β) were expressed as FLAG-

tagged proteins in *E. coli* and purified on M2 agarose. TFIIF subunits (RAP30 and RAP74) were expressed as histidine-tagged proteins in *E. coli* and purified on Ni-NTA. TFIIA and TFIIF were reconstituted from individually purified components following denaturation and renaturation (Guermah et al., 2001). FLAG-p53 and FLAG-GAL4p53(AD) were expressed in bacteria and purified on M2-agarose according to standard procedures. FLAG-p300 was expressed in Sf9 cells and purified as described previously (Kraus and Kadonaga, 1998).

Antibodies

A histidine-tagged hBRE1A fragment (residues 1-101), hBRE1B fragment (1-70) and full-length hRAD6B (1-152) were expressed in bacteria, affinity-purified and used as an antigen to produce polyclonal antibodies (Covance). The resulting antiserum was purified by immuno-affinity chromatography on a cognate antigen column according to manufacturer's procedure (Pierce). The following antibodies were obtained commercially: anti-acetyl H3, anti-mono-methyl H3-K4 and anti-di-methyl H3--K4 (Upstate), anti-H3, anti-tri-methyl H3-K4, anti-mono-methyl H3-K79 and anti-di-methyl H3-K79 (Abcam), anti-p53, anti-HA and anti-GAPDH (Santa Cruz Biotechnology), anti-FLAG and anti-FLAG-HRP (Sigma).

Protein Interaction Assays

For GST-pull down assays, 4 μg of GST-fused proteins and 200 ng of purified factors were mixed with glutathione-sepharose 4B beads in 20 mM Tris-Cl (pH 7.5), 150 mM KCl, 0.2 mM EDTA, 20 % glycerol, 0.05 % NP-40, 0.5 mg/ml BSA and 0.5 mM phenylmethylsulfonyl fluoride (PMSF). After 3 hr at 4°C, the beads were washed and bound proteins were eluted and analyzed by immunoblot. For the assay in Figure 2.2.2B, 1 μg of FLAG-hBRE1 protein and 5 μg of purified histones were mixed with M2-agarose under the same conditions used for GST-pull down assays. Bound proteins were visualized by Coomassie blue staining. For the coimmunoprecipitation assays, cells were transfected with 2 μg of each expression plasmid by calcium phosphate precipitation, as indicated. After 48 hr, cells were lysed in buffer containing 50 mM Hepes (pH 7.5), 150 mM NaCl, 1 mM EDTA, 2.5 mM EGTA, 0.1 % Tween 20, and 1 mM PMSF. FLAG-tagged proteins were captured by incubation of cell extracts with M2-agarose and eluted with SDS sample loading buffer. Bound proteins were analyzed by immunoblot.

Immunofluorescence (IF) Microscopy

HeLa cells were transfected with HA-hBRE1 and HA-hRad6B expression vectors with Lipofectamine (Invitrogen) and cell lines that stably express HA-hBRE1 or HA-hRad6B were established. Immunofluorescence staining was performed as described (Hake et al., 2005).

***In Vivo* Histone Ubiquitylation Assays**

Approximately 5×10^5 293T cells were transfected with combinations of 2 μg FLAG-histone, 50 ng HA-ubiquitin, and 4 μg histone ubiquitylation-related protein expression plasmids by calcium phosphate precipitation. After 48 hr, cells were washed twice with PBS and lysed in 700 μl of denaturing immunoprecipitation (IP) buffer (20 mM Tris-Cl (pH 7.5), 50 mM NaCl, 2.5 % sodium dodecyl sulfate, 2.5 % sodium deoxycholate, 2.5 % NP-40 and 0.5 mM PMSF). After brief sonication, cell extracts were supplemented with 20 μl (bead volume) of M2-agarose. Following 3 hr incubation at 4°C, beads were washed with denaturing IP buffer, and precipitated proteins were recovered by boiling with SDS-sample loading buffer and analyzed by immunoblot using anti-FLAG-HRP (Sigma).

Transient Transfection Assays

For the luciferase assays, about 10^4 H1299 cells or 2.5×10^4 GAL4-293T cells (Kim et al., 2005) were transfected with expression vectors, as indicated, using Fugene-6 (Roche Molecular Biochemical). Cells were harvested at 44 hr and analyzed for luciferase activity (Promega).

RT-PCR Assays

About 2.5×10^5 H1299 cells were transfected with combinations of p53 and hBRE1A expression vectors using Fugene 6. After 2 days, total RNAs were

prepared using Trizol (Invitrogen), and RT-PCR (Invitrogen) and real time PCR analyses were performed.

Chromatin Immunoprecipitation (ChIP) Assays

About 2×10^6 H1299 cells were transfected with 10 μg of empty or p53 expression vector and incubated for 2 days (Figure 2.2.9B) and about 10^6 U2OS cells were treated with 10 ng/ml of actinomycin D for 5 hrs (Figure 2.2.10C). ChIP assays were performed according to the manufacturer's instruction (Upstate). Primers used for PCR were from the mdm2 p53-responsive element region: 5' primer, 5'-AGG TGC CTG TCG GGT CAC TAG TGT G-3'; 3' primer, 5'-GAG AAA AAG TGG CGT GCG TCC GTG CC-3'.

RNA Interference

Oligonucleotides for RNAi experiments were from Dharmacon. For the RNAi-coupled histone modification and ChIP analyses, about 4×10^4 293T cells were treated with siRNA duplex using oligofectamine (Invitrogen) according to the manufacturer's instruction. After 3 days, total cells extracts were prepared and subjected to immunoblots. For the RNAi-coupled transient transfection assay, about 10^4 H1299 cells were transfected with siRNA and, after 24 h, second round transfections for luciferase assays were performed. Cells were harvested at 44 h after second round transfections and analyzed for luciferase activity.

***In Vitro* E3 Auto-Ubiquitylation Assays**

Conjugation of proteins was monitored in a reconstituted cell-free system essentially as described previously (Breitschopf et al., 1998). A reaction containing 1.3 µg of ³²P-labelled ubiquitin, 100 ng of E1, and 0.6 µg of E2s and 150 ng of E3s was incubated at 37 °C for 1 h. The proteins were resolved in 10 % SDS-PAGE gels and ubiquitin-conjugated products were scored by auto-radiography.

***In Vitro* Histone Ubiquitylation Assays**

5 µg of HeLa cell-derived oligonucleosomes were incubated with recombinant proteins in a 20-µl reaction containing 50 mM Tris-HCl (pH 7.9), 5 mM MgCl₂, 2 mM NaF, 0.4 mM dithiothreitol, 4 mM ATP, 0.1 µg of recombinant hE1, 0.6 µg of recombinant E2 ubiquitin conjugating enzyme, 150 ng of purified hBRE1A/B, 2.5 µg of recombinant ubiquitin (Sigma). After incubation at 37 °C for 1 h, reactions were terminated by addition of SDS-PAGE loading buffer. Proteins were resolved in 15% SDS-PAGE gels and blotted with anti-uH2B antibody.

Chromatin Assembly

Recombinant histone octamers were prepared as described (An et al., 2002). FLAG-Acf1, FLAG-ISWI and FLAG-Topo1 were expressed in Sf9 cells and

purified using M2 agarose (An et al., 2002). Histidine-tagged NAP1 (Kundu et al., 2000) was expressed in bacteria and purified on Ni-NTA and Q Sepharose columns. Chromatin assembly was on pGADD45₅ML and pG₅ML plasmids essentially as described (Ito et al., 1999).

***In Vitro* Transcription**

Transcription assays with the reconstituted system (GTFs, RNA polymerase II, PC4, and Mediator) were performed as described (Guermah et al., 2001 and Kundu et al., 2000). Reconstituted chromatin templates (40 ng DNA) or equivalent amounts of histone-free DNA were incubated with activators for binding, followed by a p300 acetylation step in the presence of 2 μ M acetyl-CoA (AcCoA). H2B ubiquitylation factors, the purified hPAF complex and recombinant SII then were added, followed by the general transcription machinery described above.

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