AN INVESTIGATION INTO THE ROLE OF HUMAN MESODERM INDUCTION-EARLY RESPONSE 1 (hMI-ER1) IN REGULATING A HISTONE ACETYLTRANSFERASE, A CHROMATIN REMODELING ENZYME

TINA BLACKMORE

NOTE TO USERS

AN INVESTIGATION INTO THE ROLE OF HUMAN MESODERM INDUCTION-EARLY RESPONSE 1 (hMI-ER1) IN REGULATING A HISTONE ACETYLTRANSFERASE, A CHROMATIN REMODELING ENZYME

By

Tina Blackmore

A thesis submitted to the

School of Graduate Studies

in partial fulfillment of the

requirements for the degree of

Master of Science

Division of Basic Medical Science

Faculty of Medicine

Memorial University of Newfoundland

October 2004

St. John's   Newfoundland and Labrador

Library and
Archives Canada

Bibliothèque et
Archives Canada

Published Heritage
Branch

Direction du
Patrimoine de l'édition

395 Wellington Street
Ottawa ON K1A 0N4
Canada

395, rue Wellington
Ottawa ON K1A 0N4
Canada

NOTICE:
The author has granted a non-
exclusive license allowing Library
and Archives Canada to reproduce,
publish, archive, preserve, conserve,
communicate to the public by
telecommunication or on the Internet,
loan, distribute and sell theses
worldwide, for commercial or non-
commercial purposes, in microform,
paper, electronic and/or any other
formats.

AVIS:
L'auteur a accordé une licence non exclusive
permettant à la Bibliothèque et Archives
Canada de reproduire, publier, archiver,
sauvegarder, conserver, transmettre au public
par télécommunication ou par l'Internet, prêter,
distribuer et vendre des thèses partout dans
le monde, à des fins commerciales ou autres,
sur support microforme, papier, électronique
et/ou autres formats.

The author retains copyright
ownership and moral rights in
this thesis. Neither the thesis
nor substantial extracts from it
may be printed or otherwise
reproduced without the author's
permission.

L'auteur conserve la propriété du droit d'auteur
et des droits moraux qui protège cette thèse.
Ni la thèse ni des extraits substantiels de
celle-ci ne doivent être imprimés ou autrement
reproduits sans son autorisation.

In compliance with the Canadian
Privacy Act some supporting
forms may have been removed
from this thesis.

Conformément à la loi canadienne
sur la protection de la vie privée,
quelques formulaires secondaires
ont été enlevés de cette thèse.

While these forms may be included
in the document page count,
their removal does not represent
any loss of content from the
thesis.

Bien que ces formulaires
aient inclus dans la pagination,
il n'y aura aucun contenu manquant.

Canada

Abstract

xmi-er1, *Xenopus mesoderm induction early response 1 gene*, was initially

discovered as a developmentally regulated gene that was transcribed in response to

fibroblast growth factor (FGF). FGF family members are involved in mitogenesis,

differentiation, and angiogenesis (Baird and Klagsbrun, 1991). Additional studies

revealed that XMI-ER1 functioned as a potent transcriptional activator, where the N-

terminal acidic domain was responsible for the activity. The human orthologue of *mi-er1*,

hmi-er1, was also isolated and was shown to be 91% similar to *xmi-er1*. Further analysis

revealed that *hmi-er1* expression levels were upregulated in breast carcinoma cell lines

and tissue, and that it acted as a transcriptional repressor by interaction with a histone

deacetylase, HDAC1, through its ELM2 domain (Paterno *et al.*, 1998; Paterno *et al.*,

2002; Ding *et al.*, 2003). HDACs and histone acetyltransferases (HATs) are enzymes

that play a very important role in modifying histones, altering chromatin, and regulating

transcription.

In this study, we further investigated the role of hMI-ER1 in the regulation of

transcription. We demonstrated that hMI-ER1ß, an isoform of hMI-ER1, inhibited the

HAT activity of the coactivator Creb-binding protein (CBP). hMI-ER1ß physically

interacted with CBP, and the interaction led to the inhibition of CBP HAT activity. The

interaction required a region within aa 1-179 of hMI-ER1, an area containing several

acidic regions. Within the CBP molecule, a region located between aa 1092-2441, which

contains a bromodomain, a C/H2 and C/H3 domain, a HAT domain, and a Q rich

domain, was required for the interaction. The results indicate that hMI-ER1 interacts

with CBP and that it has the potential to play a role in HAT-mediated transcription.

Acknowledgments

Table of Contents

List of Figures

List of Tables

List of Abbreviations

μg	microgram
μl	microlitre
°C	degrees celsius
aa	amino acid
Acetyl-CoA	acetyl-coenzyme A
ACTR	activator of retinoic acid receptor
AD	activation domain
Ada2	adaptor coactivator 2
AML	acute myeloid leukemia
BSA	bovine serum albumin
Br	bromodomain
BRCA1	breast cancer gene 1
C/H-1,-2,-3	cysteine-histidine rich domains
CIAP	calf intestinal alkaline phosphatase
CS	calf serum
CRE	cAMP-response element
CREB	cAMP-response-element-binding protein
CML	chronic myeloid leukemia
CPM	counts per minute
CBP	creb binding protein
DNA	deoxyribonucleic acid
dNTPs	deoxyribonucleotide triphosphates
DTT	dithiothreitol
DBD	DNA binding domain
DMEM	Dulbecco's Modified Eagle's medium
ECL	enhanced chemiluminescence
EDTA	ethyldiamine tetraacetic acid

Egl-27	gene first identified in *Caenorhabditis elegans*
ELM2	EGL27 and MTA1 homology 2
EKLF	erythroid kruppel-like factor
FAT	factor acetyltransferase
FBS	fetal bovine serum
FGF	fibroblast growth factor
FGFR	fibroblast growth factor receptor
FISH	fluorescence in situ hybridization
g	gram
GAL4DBD	pM plasmid containing GAL4 DNA binding domain
GST	glutathione s-transferase
GST-hMI-ER1	glutathione s-transferase tagged hMI-ER1
HAT	histone acetyltransferase domain
HDAC 1	histone deacetylase 1
HEK 293	transformed human embryonic kidney cells
Her2	human epidermal growth factor receptor-type 2
hmi-er1	human mesoderm induction 1 DNA/RNA
hMI-ER1	human mesoderm induction 1 protein
INHAT	inhibitor of acetyltransferases
IP	immunoprecipitation
kDa	kilodalton
KIX	kinase induced interacting domain
LB	Luria broth
Mdm-2	murine double minute
MDS	myeloidysplastic syndrome
mi-er1	mesoderm induction early response 1 DNA/RNA
MOZ	monocytic leukemia zinc finger
M	molar
ml	millitre

mM	millimolar
mRNA	messenger RNA
MTA	metastasis associated protein
ng	nanogram
NCoR	nuclear receptor corepressor
NP-40	Nonidet P-40
NLS	nuclear localization signal
OD	optical density
PBS	phosphate buffered saline
PCAF	p300/CREB-binding protein-associated factor
PCR	polymerase chain reaction
pmoles	picomoles
PMSF	phenylmethylsulfonyl fluroide
PI	protease inhibitors
Q Rich	glutamine-rich domain
RID	receptor binding domain
RT-PCR	reverse transcription-polymerase chain reaction
RNA	ribonucleic acid
RTS	Rubinstein-Taybi syndrome
SANT	SWI3, ADA1, NCoR, TFIIIB
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SH3	src homology 3
SRC-1	steroid receptor coactivator-1
SSB	SDS sample buffer
SWI3	type of SWI/SNF chromatin remodeling complex
TAE	tris-acetate/ EDTA electrophoresis buffer
TBE	tris borate/ EDTA electrophoresis buffer
TCA	trichloroacetic acid
TF	transcription factor

TFIII	transcription factor for RNA polymerase III
tRNA	transfer RNA
WB	Western blot
Xmi-er1	*Xenopus* mesoderm induction early-response 1 DNA/RNA
XMI-ER1	*Xenopus* mesoderm induction early-response 1 protein

Chapter 1: Introduction

1.1   DNA and Chromosomes

Deoxyribonucleic acid (DNA) are molecules that carry the genetic information

necessary for the organization and function of most living cells and conveys this

information from generation to generation. Genes are regions of DNA that are

responsible for a discrete hereditary characteristic, which usually corresponds to a single

RNA or protein.

In eukaryotes, the DNA and genes are packaged into a set of chromosomes

(Figure 1.1). The proteins that bind to DNA to form the chromosomes are known as

histones and nonhistone chromosomal proteins. The complex of both proteins with the

DNA is known as chromatin. Because the uncoiled length of the total human genome

would be approximately 40 inches long, the structure of the chromatin helps to condense

the DNA within a cell. Histones are responsible for the most basic level of chromosome

organization, and are responsible for forming the nucleosome. Histones have a high

proportion of positively charged amino acids (lysine and arginine), and they therefore

bind tightly to the negatively charged DNA. Unfolded chromatin has the appearance of

"beads on a string", with the "string" being the DNA and each "bead" consisting of DNA

wound around a protein core formed from histones (Figure 1.1). Each individual

nucleosome core is made up of a complex of eight histone proteins-two molecules each

of histones H2A, H2B, H3 and H4. A fifth histone, H1, may be attached to the outside of

the bead. In addition to its histone fold, each of the core histones has a long histone

1

short region of
DNA double helix

"beads-on-a-string"
form of chromatin

30-nm chromatin
fiber of
packed nucleosomes

section of
chromosome in an
extended form

condensed section of
metaphase
chromosome
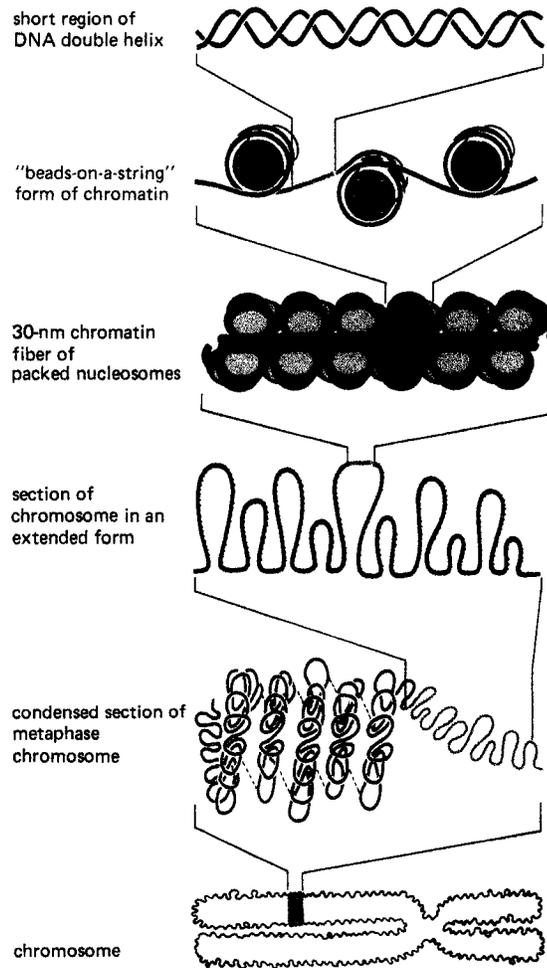
chromosome

Figure 1.1   Levels of chromatin packaging

The above diagram is a schematic illustrating the many levels of chromatin postulated to

give rise to the highly condensed chromosome. Adapted from Bruce *et al.* (1989).

N-terminal amino acid tail, which extends out from the histone core. These histone tails often undergo covalent modifications, which in the end, control the chromatin structure.

The "beaded string" further undergoes higher-order packaging. For instance, with the help of histone H1 the "beaded string" can coil to form a chromatin fiber which consists of a tightly wound coil with 6 nucleosomes per turn. The chromatin fiber further forms loops called looped domains, and in a mitotic chromosome the looped domains fold themselves to further compact the chromosome (Alberts et al., 2002).

1.2 Protein Synthesis: Transcription and Translation

DNA does not directly make a protein within a cell. Ribonucleic acid (RNA) is the bridge between the genetic material and protein synthesis. When a particular protein is needed within a cell, RNA is synthesized through a process known as transcription. The RNA molecules that are copied from these genes are called messenger RNA (mRNA) molecules. Like DNA, RNA is made up of four different types of nucleotide subunits. Overall, there are three eukaryotic RNA polymerases, RNA polymerase I, II, and III. The enzymes called RNA polymerase II transcribe the genes whose RNAs will be translated into protein. In general, these enzymes are responsible for opening and unwinding a small portion of the DNA double helix to expose the bases on each DNA strand. The enzyme is then responsible for linking together the RNA nucleotides complementary to the nucleotide of DNA. Overall, the three key steps in transcription are polymerase binding and initiation, elongation and termination.

RNA polymerases bind to regions of DNA known as promoters. A promoter includes the initiation site, where transcription actually begins, and a large number of nucleotides upstream from the initiation site. RNA polymerases cannot, however, recognize and bind to a promoter by itself. Transcription factors (TF) are proteins that help to position the enzyme correctly on the promoter, help in the unwinding of DNA, and help to release the RNA polymerase from the promoter once transcription has begun. These sequence specific DNA binding proteins must therefore be bound to DNA to form a functional promoter.

As mentioned earlier, DNA is packaged into nucleosomes with further higher order packaging. Therefore, transcription initiation requires transcription activators, mediators and chromatin-modifying proteins. Transcriptional activators bind to specific sequences in DNA and help to recruit RNA polymerase II, and mediators play a role in allowing easy communication between activators and transcription factors. Furthermore, transcription initiation in a cell often requires the presence of chromatin remodeling enzymes which include chromatin remodeling complexes and histone acetylases. Chromatin remodeling complexes play a very important role in remodeling the nucleosome structure. For instance, some proteins within the complex can change the structure of the nucleosomes temporarily so that the DNA becomes less tightly bound to the core histones, permitting the easy access to nucleosomal DNA by other proteins. Often, these proteins are involved in gene expression, DNA replication, and repair. In addition, chromosomal remodeling complexes may also function to re-form nucleosomes when access to DNA is no longer required.

Following the binding of RNA polymerases and initiation of transcription, RNA polymerase II moves along the DNA and the RNA strand is further elongated. As soon as RNA polymerase II has produced approximately 25 nucleotides of RNA, the 5' end of the new RNA molecule is modified by the addition of a special nucleotide, known as "capping". The "cap" helps the cell to distinguish mRNAs from other types of RNA molecules found within the cell. The RNA molecule also undergoes a process of RNA splicing, which involves the removal of noncoding intron sequences. The 3' end is modified by addition of a series of adenylic acid nucleotides immediately after its cleavage. Following the production of the mRNA molecule is the process of translation. Translation is the process of protein synthesis, which occurs under the direction of the mRNA molecule. A protein is built from a series of codons along an mRNA molecule, where a single codon is made up of any three nucleotides. Transfer RNA (tRNA) functions to transfer amino acids from the cytoplasm to a ribosome. The ribosome, in turn, adds each amino acid brought to it to the growing end of the protein. While a protein is being made it coils and folds to form a functional protein with a very specific conformation. Additional steps, such as chemically modification, may be required but the overall process of protein synthesis from DNA is complete (Alberts et al., 2002).

1.3   Histone Acetyltransferases (HATs), Deacetylases and Regulation

Histone acetylation occurs when an acetyl group from acetyl-coenzyme A (acetyl-CoA) is added to the ε-amino group at specific lysine residues within a histone's basic N-terminal tail region. Lysine acetylation, which neutralizes part of a tail region's positive

charge, can weaken the interaction between histones and the negatively charged DNA. The weakened interaction destabilizes the nucleosomes giving transcriptional regulators increased access to the DNA (as reviewed in Sterner and Berger, 2000a).

Histone acetyltransferases (HATs) are the enzymes that function to acetylate the histones. Through their interaction with specific transcription factors they are targeted to specific promoters, where they locally modify histones and regulate transcription (as reviewed in Legube and Trouche, 2003b). Table 1.1 shows a partial list of known and putative HATs. Yeast Gcn5 is the best characterized of all the HATs, both structurally and functionally. Studies have shown that Gcn5 HAT activity directly correlates with cell growth, *in vivo* transcription, and histone acetylation at the Gcn5-dependent promoters (as reviewed in Kuo *et al.*, 1998). *In vitro*, Gcn5 was shown to acetylate histone H3 strongly and H4 weakly (Ornaghi *et al.*, 1999). Gcn5 and p300/CREB-binding protein-associated factor (PCAF) are two closely related HATs found in mammals (as reviewed in Sterner and Berger, 2000b). The function of human Gcn5 is very similar to its role in yeast, in that it was found to carry out transcription regulatory roles (Candau et al., 1996). PCAF studies revealed that it shares the same functional roles as Gcn5 and that it interacts with two other HATs, p300 and Creb Binding Protein (CBP). Studies have shown that PCAF can act as a transcriptional coactivator in a HAT-dependent manner and stimulate transcription when bound either to a promoter-proximal site or at a distant enhancer (Krumm et al., 1998), where distant enhancers are enhancers that can be located a significant distance from promoters.

6

It is important to note that histone acetylation is a reversible process, whereby histone deacetylases (HDACs) are able to remove the acetyl groups from lysine residues. The deacetylation of histones would stabilize the nucleosomes leading to a possible decrease in gene transcription. Both histone acetylation and deacetylation are two regulatory processes that work together to achieve appropriate levels of transcription (Figure 1.2; Braunstein *et al.*, 1996).

HATs and HDACs both play a major role in the control of cell fate and misregulation of these proteins is involved in the development of some human tumors. Abnormal acetylation or deacetylation leads to such diverse disorders as leukemia, epithelial cancers, fragile X syndrome and Rubinstein-Taybi syndrome (as reviewed in Timmermann *et al.*, 2001). Because HAT and HDAC activities are so important, their activity is known to be tightly regulated within a cell. One such mechanism of regulation is to regulate their expression levels. Although not much is known about such regulation, it has recently been shown that *Hdac1* mRNA expression is induced by histone hyperacetylation, suggesting that feedback loops exist (Hauser *et al.*, 2002). Another main mechanism of regulation is through the regulation of the enzymatic activity itself. For instance, the activity of many HATs and HDACs has been shown to be regulated through phosphorylation, whereby phosphorylation can actually stimulate HAT activity. In addition, protein-protein interactions also play a role in regulating HAT activity.

Table 1.1: Summary of known and putative HATs (adapted from Sterner et al., 1999).

HAT	Orthologues found in:
Gcn5	Yeast to Humans
Hat1	Yeast to Humans
PCAF	Humans, mice
Elp3	Yeast
Hpa2	Yeast
Sas2	Yeast
Tip60	Humans
MOZ	Humans
MORF	Humans
MOF	Drosophila
HBO1	Humans
CBP	Various multicellular organisms
p300	Various multicellular organisms
SRC-1	Humans, mice
ACTR	Humans, mice
TIF2	Humans, mice
TFIIIC	Humans

There are many proteins known to interact with HATs, and through their interaction they can stimulate histone acetylation activity (as reviewed in Legube and Trouche, 2003b). For example, transcription factors such as HNF1-α, Sp1, and Zta can stimulate the activity of CBP and p300, two well known histone acetyltransferases (Chen *et al.*, 2001; Soutoglou *et al.*, 2001; Li *et al.*, 2003). In comparison, the transcription factors Msx3, Hox proteins and Twist can interact with HATs and block HAT activity (Hamamori *et al.*, 1999; Mehra-Chaudhary *et al.*, 2001; Shen *et al.*, 2001). Lastly, histone acetylase and deacetylase activity can be regulated by the availability of cofactors such as acetyl-coenzyme A (as reviewed in Legube and Trouche, 2003a).

1.4 Creb Binding Protein (CBP) - A Histone Acetyltransferase

CREB Binding Protein (CBP) was originally identified in 1993 through its ability to co-immunoprecipitate with the phosphorylated form of cAMP-response-element-binding protein (CREB) (Chrivia et al., 1993). CREB is a 43 kDA transcription factor that binds to cAMP-response element (CRE) via a leucine zipper motif present at its C terminus. Upon phosphorylation on Ser133, active CREB affects transcription of CRE-dependent genes by interacting with the CREB-binding Protein, CBP. CBP plays a very important role in bridging the CRE/CREB complex to components of the basal transcriptional apparatus and also acts as a transcriptional coactivator. CREB-mediated transcription regulates cellular responses such as neuronal signaling, cell proliferation and apoptosis (as reviewed in Andrisani, 1999).
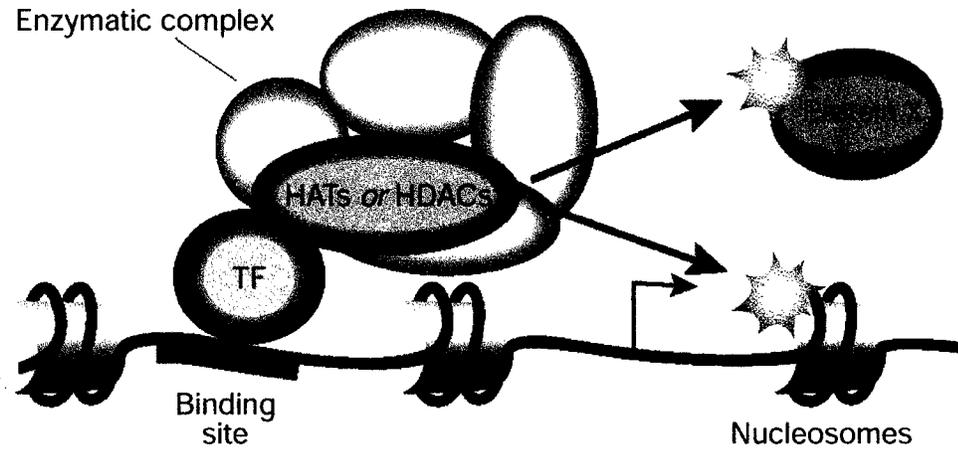
Figure 1.2   A general model of histone acetyltransferase and histone deacetylase action. Through a physical interaction between sequence-specific transcription factor (TF), histone acetyltransferases (HATs) and deacetylases (HDACs) are recruited to their targeted promoters. Both HATs and HDACs can be found within a complex of other histone acetyltransferases or deacetylases, "enzymatic complex", and function to modify chromatin structure. Previous studies also indicate that HATs and HDACs can modify proteins other than histones, as designated by "Protein X" (Adapted from Legube and Trouche, 2003b).

In 1996, CBP was first described to have intrinsic HAT activity (as reviewed in Ogryzko *et al.*, 1996; Bannister and Kouzarides, 1996a). At that time, only PCAF and yeast Gcn5 had been identified as having HAT activity. Unlike other HATs, CBP is able to acetylate all four core histones *in vivo*, whereas Gcn5 and PCAF only acetylate histones H3 and H4 (as reviewed in Sterner and Berger, 2000b). Shortly after, the HAT domain was mapped between residues 1099-1758 and it was therefore this region that acetylated all core histones *in vitro* (Bannister and Kouzarides, 1996b). The overall observations suggested for the first time that CBP was not just an adaptor between DNA-binding factors, transcription-initiation factors, or other described HATs, but that CBP plays a role in transcriptional regulation by histone acetylation and chromatin remodeling. CBP, through its HAT activity, stimulates transcription of specific genes by interacting with numerous promoter-binding transcription factors such as CREB, nuclear hormone receptors, and oncoprotein-related activators such as c-Fos, c-Jun and c-Myb (Bannister and Kouzarides, 1996c; Sterner and Berger, 2000a).

1.4.1 *CBP* Gene and Functional Domains

The human *CBP* locus is located on chromosomal region 16p13.3 and extends approximately 190 Kb (Wydner et al., 1995). When translated, the *CBP* gene yields a protein of 2440 and 2441 amino acid residues in humans and mice, respectively (Chrivia et al., 1993). The CBP protein is evolutionary conserved, sharing 95% identity between human and mouse. Orthologues have also been identified in a number of other organisms, including *Drosophila melanogaster* (fruit fly), *Caenorhabditis elegans*

(nematode worm), and *Arabidopsis thaliana* (self-pollinating weed) (as reviewed in McManus and Hendzel, 2001). The high degree of similarity suggests that CBP plays a very critical role in cellular functions.

The CBP protein resolves to a band of approximately 265kDa on a sodium dodecyl sulphate (SDS) polyacrylamide gel and comprises several different conserved domains. The N terminus is made up of a nuclear receptor binding domain (RID), kinase induced interacting domain (KIX), three cysteine-histidine-rich domains (CH1, CH2, CH3), a bromodomain, two zinc finger motifs, the histone acetyltransferase domain (HAT) and a glutamine Rich (Q Rich) domain (Figure 1.3). The RID, CH1, CH2, CH3 and the KIX domains are thought to be important in mediating protein-protein interactions, since a number of cellular and viral proteins bind to these regions. The bromodomain is an evolutionary conserved domain and found in nearly all histone acetyltransferases. It has been recently discovered to function as an acetyl-lysine binding domain (Dhalluin *et al.*, 1999; Hudson *et al.*, 2000; Jacobson *et al.*, 2000). Overall, it has been shown that CBP associates with at least 45 different molecules including transcription factors, signaling molecules, and nuclear hormone receptors (as reviewed in Giles *et al.*, 1998). As mentioned, CBP also has a HAT domain, found in the central region of the protein. It is this domain that gives CBP its intrinsic histone acetyltransferase activity, labeling CBP as a histone acetyltransferase.
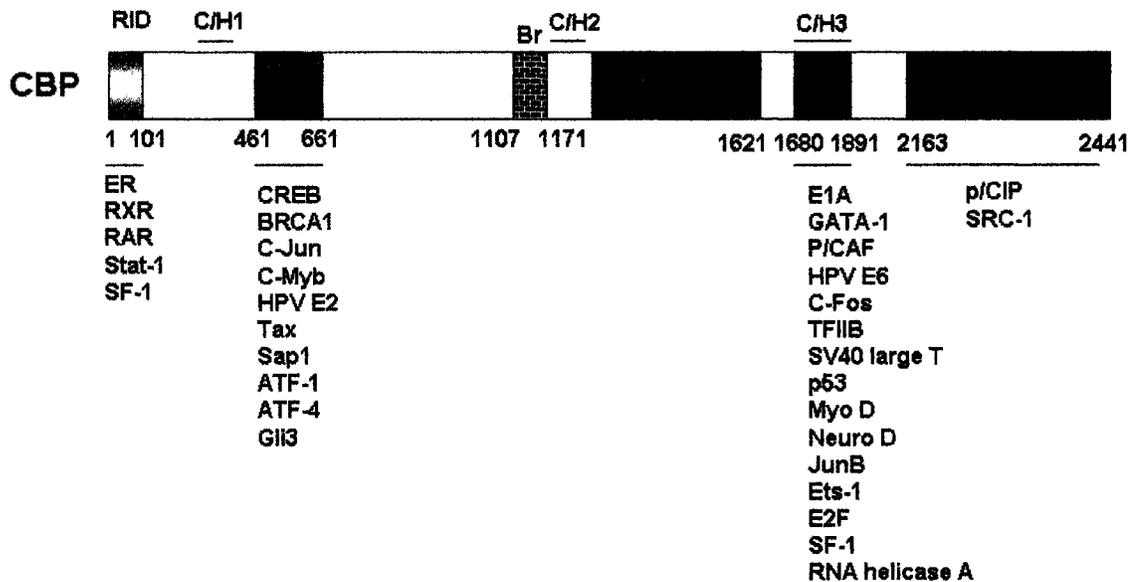
Figure 1.3   Structure of CBP, its functional domains and interacting regions

Schematic representation of mouse CBP, illustrating the amino acid residues 1-2441.

Listed are the previously identified domains as a reference. The domains listed include

the receptor interacting domain (RID), three cysteine or histidine rich domains (C/H-1, -

2, -3), a kinase-induced interacting domain (KIX), a bromodomain (Br), a histone

acetyltransferase domain (HAT), and a glutamine –rich domain (Q Rich). The bars,

located below the diagram, indicate the interacting regions of CBP with the partial list of

CBP-interacting proteins given below. Adapted from Vo and Goodman, 2001.

1.4.2 CBP and Transcriptional Regulation

In addition to the role of CBP as a histone acetyltransferase, CBP may also play a role in transcriptional regulation by acting as a scaffold for the assembly of multiprotein complexes (as reviewed in Chan and La Thangue, 2001). For instance, CBP is often found in association with three additional HATs; PCAF (as reviewed in Ogryzko *et al.*, 1996), SRC-1 (steroid receptor coactivator-1; Yao *et al.*, 1996; Spencer *et al.*, 1997), and ACTR (activator of retinoic acid receptor; Chen *et al.*, 1997). By providing a scaffold for the assembly of transcription cofactors, CBP may act to increase the concentration of these factors in the transcriptional environment (as reviewed in McManus and Hendzel, 2001).

It is also important to note the role of CBP in the acetylation of nonhistone proteins and gene expression. Enzymes that acetylate non-histone proteins are referred to as factor acetyltransferases (FATs). FATs seem to either promote or reduce protein-protein interactions that affect transcriptional activation of specific genes. CBP was first identified as a FAT when it was shown to acetylate the protein p53 (Gu and Roeder, 1997). p53 is a tumor suppressor with most of the critical functions believed to occur through its ability to activate genes involved in the response to DNA damage, such as marine double minute (mdm-2), p21, cyclin G, and bax. Inactivation of p53 function appears to be critical to tumorigenesis in all different types of human cancers (Vogelstein et al., 2000). Studies have demonstrated that p53 interacts with CBP and that the interaction contributes to the transcriptional activation of the p53-responsive mdm-2, p21, and bax promoters (Avantaggiati *et al.*, 1997; Lill *et al.*, 1997; Gu and Roeder, 1997).

Further analyses of p53 and CBP have shown that the acetylation of p53 by CBP

dramatically increases its DNA binding ability (Gu and Roeder, 1997). CBP has also

been shown to acetylate several additional transcriptional regulators, including the

activators GATA-1, and erythroid kruppel-like factor (EKLF) (as reviewed in Imhof *et*

al., 1997; Boyes *et al.*, 1998). Exactly how acetylation affects the transcriptional

properties of these proteins in not yet completely understood (as reviewed in Goodman

and Smolik, 2000).

1.4.3 CBP and disease

The gene encoding CBP, located on 16p13.3, was reported as the causative gene

of Rubinstein-Taybi syndrome (RTS) (Petrij *et al.*, 1995a; Petrij *et al.*, 2000b). RTS is an

autosomal-dominant disease that is present at or before birth, and is characterized by

facial abnormalities, broad thumbs and toes, short stature and mental

retardation. The first English description of RTS occurred in 1963 (Rubinstein and Taybi,

1963), and included additional characteristic features such as growth retardation,

microcephaly (small brain at birth), and keloid formation (formation of a sharply

elevated, irregular-shaped, enlarging scar). 1 out of 300 patients institutionalized for

mental retardation is diagnosed with RTS, suggesting that the disease is fairly common

(as reviewed in Goodman and Smolik, 2000). The underlying cause of RTS was shown to

be microdeletions and chromosomal breakpoints within the CBP locus. Most RTS

individuals are heterozygous for the mutations in *CBP*, suggesting that a full complement

of the CBP gene is critical for normal development (Petrij *et al.*, 1995b). Recent evidence

suggests that it may be the loss of the HAT activity of CBP that may cause RTS. Sequence analysis revealed that 100% of the individuals with mutations of *CBP* have either a protein truncating mutation that lacks all or part of the HAT domain or a missense mutation from arginine to proline. Further *in vitro* studies have shown that the truncated CBPs analyzed from patients in the study, as well as the missense mutation *CBP*, labeled CBP ^{R1379P}, do not have HAT activity. Because HAT activity of CBP is required for transcriptional activity of CREB (Korzus *et al.*, 1998;Kurokawa *et al.*, 1998), additional *in vitro* assays were performed. Using microinjection experiments the transcriptional potency of CBP ^{R1379P} was measured. The results show that CBP ^{R1379P} was unable to transactivate CREB (Murata et al., 2001).

Patients with RTS have also been shown to have an increased predisposition for cancer (as reviewed in Miller and Rubinstein, 1995).The precise incidence of tumors in RTS is not known, but estimates have been in the range of 5% (as reviewed in Goodman and Smolik, 2000). Unfortunately, the recognition that RTS is due to mutations in *CBP* has not been very useful clinically, as only 3-25% of patients have deletions large enough to be detected by fluorescence in situ hybridization (FISH) or the protein truncation test (Taine *et al.*, 1998; Petrij *et al.*, 2000a).

As direct evidence that the loss of one copy of the mammalian CBP gene affects pattern formation, *CBP* heterozygous and *CBP*-deficient mutant mice were generated by gene targeting. Consistent with RTS patients, mice lacking one functional copy of the CBP allele exhibited some of the skeletal abnormalities as seen with RTS patients. CBP

was further found to be essential for mouse embryonic development in that mice lacking two functional CBP alleles die 8-10 days post conception (Tanaka et al., 1997).

1.4.4 CBP and Cancer

Since chromatin remodeling enzymes, such as CBP, play an essential role in transcription regulation, it is not surprising that they have been linked to cancer. The involvement of the CBP gene has been implicated in a number of hematological malignancies, including acute myeloid leukemia (AML), chronic myeloid leukemia (CML) and myeloidysplastic syndrome (MDS) (McManus and Hendzel, 2001). AML, CML, and MDS affect the white blood cells which increase in the bone marrow, replace the normal blood cells, and spread throughout the body. It was initially discovered that the CBP gene was involved in leukemia-associated chromosomal translocations. Translocations are chromosomal abnormalities that occur when chromosomes break and the fragments rejoin to other chromosomes. The process brings two previously unlinked segments of the genome together. The translocations found in hematological malignancies involving CBP generally result in fusion products that preserve most of the CBP molecule, suggesting that the disease mechanism does not necessarily involve the loss of function of the histone acetyltransferase activity as seen in RTS patients (Blobel et al., 1998). It is possible that CBP has an altered function through fusion to another molecule. For example, AML-leukemic blast cells (immature white blood cells) containing a *CBP* translocation, have the CBP gene fused to the Monocytic Leukemia Zinc Finger (MOZ) gene. The fusion results in a small deletion of the N-terminal 266

17

amino acids of CBP leaving the rest of the molecule intact (Borrow *et al.*, 1996; Korzus *et al.*, 1998; Kurokawa *et al.*, 1998).

A study conducted by Kung et al., 2000 further strengthens the role of dysfunctional CBP protein in hematological malignancies. Mice engineered to contain a null mutation in one CBP allele developed a variety of hematological abnormalities, including extramedullary myelopoiesis (formation of tissue resembling bone marrow, outside of the bone marrow), splenomegaly (enlargement of the spleen), and lymph node hyperplasia (increased number of cells in lymph nodes). With advancing age, the mice developed a very high incidence of hematological malignancies, such as lymphocytic leukemia. Overall, nearly 40% of the *CBP* heterozygotes either developed tumors or harbored tumorigenic cells (Kung et al., 2000).

1.5   MI-ER1

Mesoderm Induction Early Response 1 gene, or *mi_er1*, was initially discovered as a developmentally regulated gene whose expression levels were increased during mesoderm induction by fibroblast growth factors (FGF) in *Xenopus laevis* (Paterno et al., 1997). In vertebrates, FGFs belong to a 22 member family known to bind to heparin and heparan sulphate, and share a highly conserved 140 amino acid central core (as reviewed in Ornitz and Itoh, 2001). It has been shown that many members of the FGF family are involved in mitogenesis, differentiation, and angiogenesis (Baird and Klagsbrun, 1991). It has also been previously shown that deregulated FGF signaling pathways are involved in tumor development and progression (as reviewed in Powers *et al.*, 2000). FGF activity

has also been implicated in mesoderm induction and formation in *Xenopus* embryonic tissue (Slack et al., 1987). During mesoderm induction, FGF binds to FGF receptors that, upon tyrosine phosphorylation, form a signaling complex by recruiting signaling molecules (Gillespie et al., 1992). The binding of the intracellular substrates then leads to the activation of various signal transduction pathways. In a signal transduction pathway, the end result is the transcription of target genes. The first genes to be transcribed are referred to as the immediate-early or early-response genes. Many of the known early response genes are known as transcription factors, in that they play an important role in initiating or repressing the transcription of other genes.

In efforts to further explain the molecular mechanisms of FGF signal transduction, a PCR-based differential display method was used to identify and characterize genes that are expressed early during the cellular response to FGF. The results revealed a novel *Xenopus* gene, known as *Xmi-er1*, which demonstrated an increase in steady state levels upon stimulation with FGF. The PCR product was then used to obtain a 2.3 kilobase pair cDNA, with the open reading frame predicted to encode a protein of 493 amino acids. In order to further investigate the possibility that *Xmi-er1* is an immediate-early gene, the FGF-induced increase was measured in the presence and absence of cycloheximide. Cycloheximide, which is a protein synthesis inhibitor, did not prevent the increase in XMI-ER1 expression levels. Taken together the results suggest that the transcription of the gene is very rapid and is not dependent on de *novo* protein synthesis, furthering supporting the idea that *Xmi-er1* is an immediate early gene. Additional analysis also revealed that the *Xmi-er1* sequence contained two putative

nuclear localization signals, as well as stretches of acidic residues. Immuno-

histochemistry and transient transfections in HEK 3T3 cells revealed that the XMI-ER1

protein is targeted to the nucleus and the N-terminus functions as a potent transactivator

(Paterno et al., 1997).

Subsequent studies on this gene led to the discovery of a human orthologue of *mi-er1* (Paterno *et al.*, 1998). Amino acid comparisons of both the Xenopus and human *mi-er1* (*hmi-er1*) isoforms showed a 91% sequence similarity overall, with 100% similarity in the common internal domains.

Because FGFs play a role in the pathology of human cancers, the expression levels of hMI-ER1 in various tissues and cell lines were investigated. Reverse transcription-polymerase chain reaction (RT-PCR) revealed that *hmi-er1* expression was upregulated in breast carcinoma cell lines and breast tumors, while remaining undetectable in normal breast cell lines and breast tissue. The results suggest that hMI-ER1 plays a role in the neoplastic state of human breast carcinoma (Paterno et al., 1998).

With the evidence that *hmi-er1* associates with the neoplastic state of human breast carcinoma and the potential role of *mi-er1* as a transcription activator in the regulation of embryonic development, it was of importance to examine the human *mi-er1* gene further. Human *mi-er1* cDNAs were initially isolated, cloned and characterized using polymerase chain reaction techniques. It was initially shown that the *hmi-er1* gene was a single copy gene located at position 1p31.2, spans 63 Kb and consists of 17 exons in total (Figure 1.4). Sequence analysis of the variant forms of *hmi-er1* cDNAs further

GENE

A

ML MAE PAS i PAS ii PAS iii

β α

1A 2A 3A 1B 4 ... ... 14 15 16

B

Transcripts

alternate 5' ends alternate 3' ends

N1 *biii*

N2 *bii*

N3 *bi*

a

— 500bp

■ Exon

— Intron

▨ Facultative Intron

□ α or β C-terminal coding region

ML, MAE- alternate AUG starts

PASi, ii, iii- alternate poly A signals

N1, N2, N3- alternate N-termini
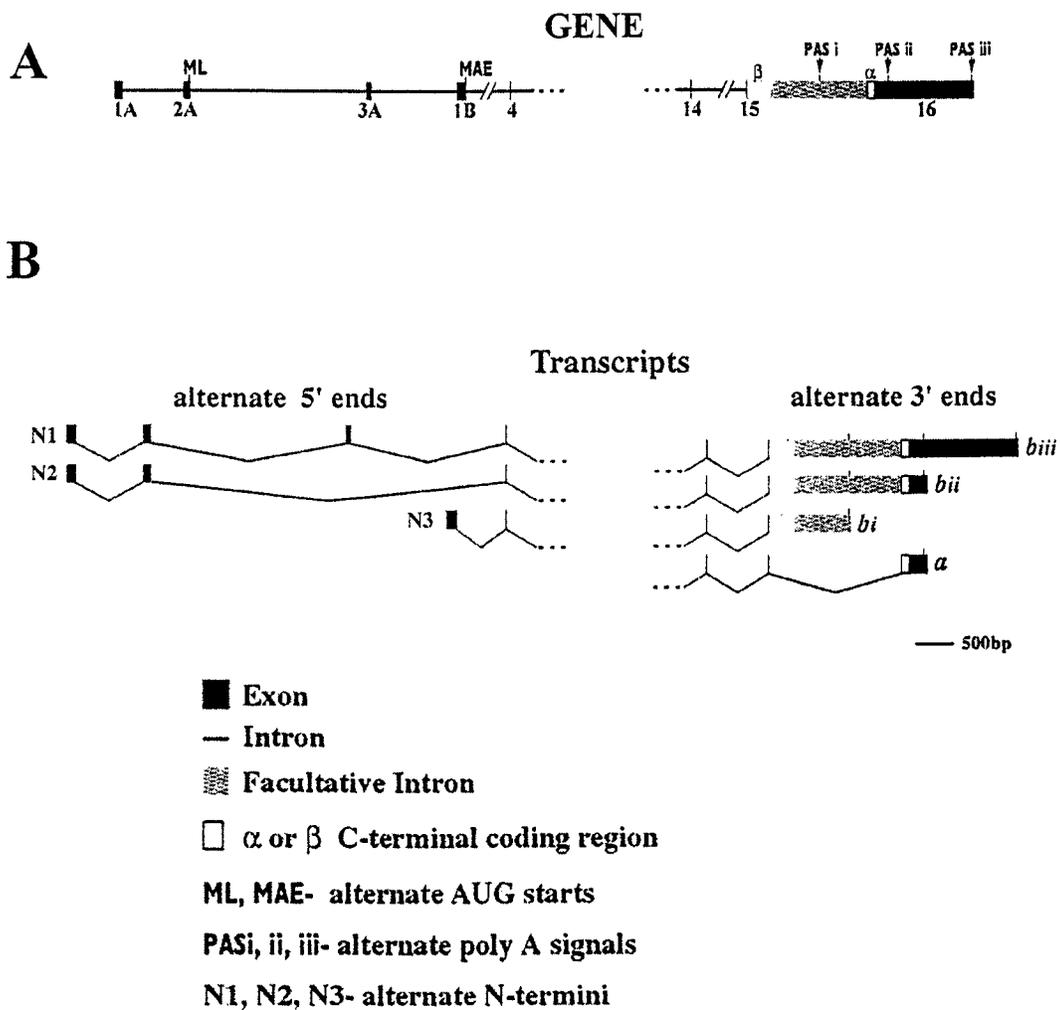
Figure 1.4   Structure of the human *mi-er1* gene and splice variants. The 17 exons arē shown in black, the introns as horizontal lines, the gray area represents the facultative intron, and the white area represents the position of α and ß carboxy-terminal coding regions. (A) The exon/intron organization of the hmi-er1 gene is shown, where the exons are numbered below the schematic. (B) Schematic illustrating three distinct 5' ends , referred to as N1, N2, N3. Also shown is the variation in the 3' end generated from alternative splicing, alternate promoter usage and/ or PAS. Reproduced from Paterno et al., 2002.

revealed 3 possible 5' ends, referred to as N1, N2 and N3 (Figure 1.4B). The 3 distinct 5'

ends were shown to result from alternate promoter usage or alternate inclusion of exon

3A. The N1 5' end results from the inclusion of exons 1A, 2A, and 3A which gives rise

to the MLKMCIRCLCLIGLQTVCGLFSCQITQ amino acid sequence. N2 results from

the usage of exon 1A and 2A only, resulting in a ML-amino acid sequence. The usage of

exon 1B alone gives rise to the N3 5' end, which consists of the MAE-N-terminal amino

acid sequence.

In addition to the variation found within the 5' end of *hmi-er1*, there is also

variation found at the C terminal, or 3' end, of the gene as well. With the usage of

alternative splicing or alternate polyadenylation signals (PAS) four 3' ends, a, bi, bii, and

biii are produced (Figure 1.4B). The α C-terminus results from the *a* 3' end portion of the

transcript and encodes a 23 amino acid domain, while the bi, bii, and biii 3' portions are

predicted to produces the same 102 amino acid domain known as the ß C-terminus. The

difference in the amino acid sequences would suggest that both the α and ß isoforms have

two distinct functions. With the 3 distinct N termini (N1, N2 and N3), in combination

with the 2 possible C termini, it is predicted that 6 distinct hMI-ER1 proteins are

produced: N1α, N1ß, N2α, N2ß, N3α and N3ß (Paterno et al., 2002).

By comparing both hMI-ER1 and XMI-ER1 isoforms with other known proteins,

a number of conserved functional domains were identified. The common internal

sequence contains a highly acidic residue region, an ELM2 domain, and a signature

SANT domain. As well, there is a proline rich motif, a LXXLL motif and a functional

22

nuclear localization signal (NLS), all of which are later described in full detail (Figure 1.5).

1.5.1 The Acidic Activation Domain

At the N terminus of hMI-ER1 there are known to be four stretches of highly acidic regions encompassing amino acids 18–172, suggesting that hMI-ER1 contains an acidic activation domain. Acidic amino acids are amino acids whose side chains can carry a negative charge at neutral pH, and include aspartic and glutamic acids. Acidic activation domains were first described as being present in the yeast GAL4 protein (Ma and Ptashne, 1987). GAL4 is a transcriptional activator of the genes GAL1 and GAL10. Both genes are required for galactose catabolism in the yeast Saccharomyces cerevisiae. Galactose catabolism is the process of breaking down galactose into a simpler component, called aldohexose galacto-hexose. Ma and Ptashne (1987) initially assayed the ability of various deletion mutants of GAL4 to activate transcription in yeast. The results revealed that the two regions of GAL4 (residues 148-196 and 768-881), which are rich in acidic residues, play a very important role in transcriptional activation. In addition, further studies have also shown that acidic amino acids are indeed crucial for transcriptional activation. For example, Blair et al (1994) have shown that the potent C-terminal activation domain of the protein RelA contains several acidic activation domains, and that the RelA activation domain is dependent on the acidic activator sequences.
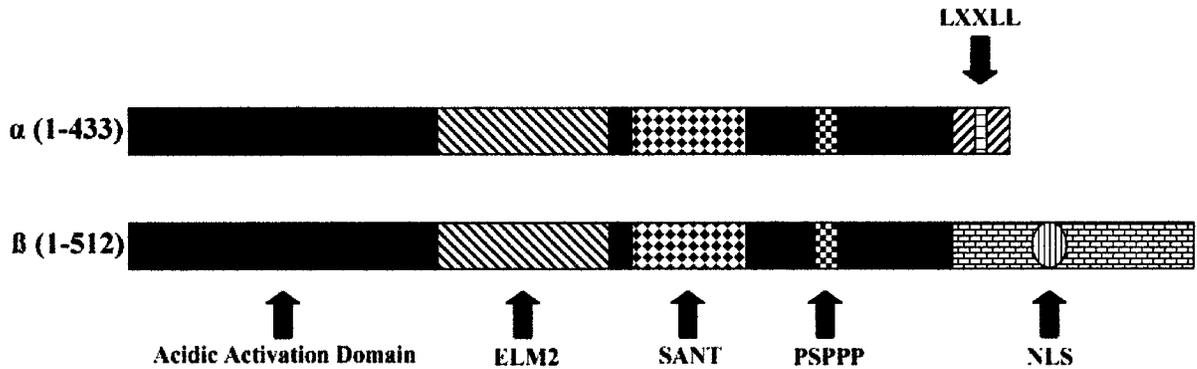
Figure 1.5   Functional protein domains in both hMI-ER1 isoforms.

hMI-ER1α and hMI-ER1ß consists of 433 and 512 amino acids respectively, as noted left

of the schematic. At the N terminus, both hMI-ER1 isoforms contain four regions rich in

acidic amino acids labeled as the acidic activation domain (18-172), an ELM2 domain

(179-283), a signature SANT domain (287-332) and a PXXP motif C terminal to the

SANT domain. As seen in the diagram, hMI-ER1α is distinct from the hMI-ER1ß

isoform at the C Termini. In addition, the LXXLL motif is found within the C-terminal

region of hMI-ER1α, whereas the NLS is found within the C terminal region of hMI-

ER1ß.

The stretches of highly acidic regions found in the N terminus of MI-ER1, characteristic of acidic activation domains, were first studied in Xenopus and the transactivation potentials were tested. The results revealed that although full length XMI-ER1 did not activate transcription, the deletion construct harboring the first 3 acidic amino acid stretches (aa 1-98) stimulated transcription 80-fold (Paterno et al., 1997). It was therefore shown that XMI-ER1 contains an acidic activation domain, and that it has the potential to function in activating transcription.

As previously mentioned XMI-ER1 and hMI-ER1 isoforms share 91% sequence similarity, therefore suggesting that hMI-ER1 contains a putative acidic activation domain and may play a role in transcriptional activation.

1.5.2 The ELM-2 Domain

The ELM-2 Domain is found between amino acids 179-283 in both of the hMI-ER1 protein isoforms. ELM-2 gets its name from the EGL-27 and MTA1 homology domain 2, and was initially identified in a C. *elegans* protein known as EGL-27, as well as in the MTA1 protein found in humans (Solari et al., 1999). The EGL-27 protein has been shown to play a fundamental role in developmental patterning. Like the MTA1 protein, it is thought that EGL-27 plays a very important role in transcriptional regulation by regulating histone acetylation. The MTA1 protein is a component of a protein complex with histone deacetylase and nucleosome remodeling activity and is therefore thought to play a role in repressing gene transcription. Interestingly, elevated expression levels of the human *mta1* gene occur in metastatic carcinomas (as reviewed in Nicolson

et al., 2003). It has recently been shown that hMI-ER1 plays a role in repressing

transcription by recruiting HDAC1, a histone deacetylase, via its ELM2 domain (Ding et

al., 2003).

1.5.3 The SANT Domain

The SANT domain, found C-terminal to the ELM2 domain and between amino

acids 288-332 in hMI-ER1, was initially found in the proteins SWI3, Ada2, N-CoR and

TFIIIB, from which the name is derived. SWI3 and Ada2 are involved in transcriptional

activation complexes, TFIIIB is found in RNA polymerase initiation complexes, while N-

CoR is involved in regulating nuclear hormone activity. Sequence alignment and

secondary structure studies revealed a great deal of similarity between the SANT domain

and the DNA binding domain (DBD) of myb related proteins. For instance, the Myb-

DBD consists of tandem repeats of three α helices arranged in a helix-turn-helix motif

while the predicted secondary structure suggests the presence of three α helices in the

SANT domain. The overall analysis suggests that the tertiary structure of the SANT and

Myb-DBD are similar, and that SANT domain may be involved in transcriptional

regulation via DNA binding and protein-protein interactions (as reviewed in Aasland *et

al.*, 1996). The SANT domain is also found in the protein SMRT (silencing mediator for

retinoid and thyroid receptors), which has been shown to play a major role in

transcriptional and regulatory complexes. The SANT domains of both SMRT and N-

CoR interact with and activate histone deacetylases (HDACs) thereby inhibiting

acetylation of histones and further repressing transcription (Guenther et al., 2001).

Recently, it has been shown that the SANT domain of CoREST, a corepressor to the REST transcription factor, is responsible for interacting with HDAC (You et al., 2001). Similarly, the SANT domain has been shown to interact with Gcn5, the first histone acetyltransferase to be identified, and later found to be a subunit of the transcriptional regulatory complex SAGA in yeast. Further analysis revealed that effective histone acetylation by Gcn5 requires the SANT domain of Ada2, further strengthens the idea that the SANT domain plays a role in transcriptional regulation (Sterner et al., 2002). In addition, Ding et al., (2004) showed that the SANT domain of hMI-ER1 is important for binding Sp1 and regulating its function.

1.5.4 The Proline-Rich Region

MI-ER1 also has a proline-rich motif (PXXP, where P represents the amino acid proline, and X represents any other amino acid), C terminal to its SANT domain. Proline-rich motifs are regions that can bind to the Src homology 3 (SH3) domains (Alexandropoulos et al., 1995). SH3 domains are one of the best characterized protein interacting modules that mediates protein-protein interactions and control signaling within the cell. By binding to proline- rich motifs, SH3 domains play critical roles in organizing protein complexes within the cell, bringing substrates to enzymes, and regulating enzymatic activity (Cohen et al., 1995).

The fact that MI-ER1 contains a proline-rich motif suggests that MI-ER1 may possibly function as a SH3 recognition motif. Furthermore, the presence of a PXXP

amino acid sequence suggests that MI-ER1 may function in transcriptional regulation via protein-protein interactions.

It has recently been shown that the overexpression of XMI-ER1 during *Xenopus* embryonic development induces abnormalities involving truncations of the anteroposterior axis. A mutation in the first proline of the proline rich motif (^{365}P) completely eliminated the effects seen (Teplitsky et al., 2003). The results demonstrate that the critical residue for XMI-ER1 developmental effects is the amino acid ^{365}P, found within the proline-rich region.

1.5.5 LXXLL Motif

Found in the C terminus of hMI-ER1α is an LXXLL motif, in which L represents leucine and X represents any amino acid. The LXXLL motifs can be found in many different nuclear hormone receptor co-activators and corepressors and have been shown to be required for the binding of the transcriptional regulators to the nuclear hormone receptors.

Recently, an 8 amino acid sequence spanning positions -2 to +6, relative to the primary conserved leucine residue, has been defined as the minimal "core" LXXLL motif. In addition, it has been shown that differences in the minimal core region affect the selectivity and affinity of the transcription co-regulators to the nuclear hormone receptors. For instance, LXXLL motifs that contain a hydrophobic residue at the -1 position and a non-hydrophobic residue at position +2 display a high affinity for steroid and retinoid receptors (Heery *et al.*, 1997). hMI-ER1α contains the minimal core LXXLL

motif with a hydrophobic residue at the positions -1 and +2. Unlike the hMI-ER1α

isoform, there are no LXXLL motifs present in the hMI-ER1ß.

1.6   Research Goals

The presence of a SANT domain in hMI-ER1 suggests that hMI-ER1 might

interact with HATs, as seen with the SANT domain of Ada2 (Paterno *et al.*, 1998; Sterner

et al., 2002), and may play an additional role in chromatin remodeling and transcription.

Given that only the hMI-ER1ß isoform contains a functional NLS and is localized in the

nucleus, my goal was to determine if hMI-ER1ß interacts with CBP, to determine the

domains responsible for any interaction, and to determine its effect on histone

acetylation.

Objective 1: Identification of CBP as a hMI-ER1ß interacting protein.

hMI-ER1ß contains a SANT domain which is also found in many known

transcriptional regulators, such as Ada2. Recent studies have shown that the SANT

domain of Ada2 is responsible for the interaction with Gcn5, a well known histone

acetyltransferase. Therefore, my first objective was to determine if there was an

interaction between hMI-ER1ß and CBP *in vitro* and *in vivo*. With the evidence that CBP

plays a very important role in transcription, as well as previous studies that indicate hMI-

ER1 levels are increased in breast tumor cells and tissues (Paterno *et al.*, 1998),

identifying the interaction between the two proteins are of extreme importance. Initially,

GST pull downs were completed with GST-hMI-ER1ß and ^{35}S-labelled CBP to

determine if an interaction occurs *in vitro*. To further determine if an interaction occurs *in vivo*, cotranfections of both hMI-ER1ß and CBP plasmids were performed in HEK 293 cells.

Objective 2: Identification of the CBP domains responsible for the interaction.

In an attempt to further characterize the domains of CBP required for the interaction with hMI-ER1ß, further *in vitro* and *in vivo* experiments were conducted. CBP deletion constructs were made and GST pull down assays were performed using GST-hMI-ER1ß and ^{35}S-labelled CBP Br-Q and CBP RID-Br deletion constructs. In addition, cotransfections of both hMI-ER1ß and CBP deletion constructs were performed in HEK 293 cells.

Objective 3: Identification of the region of hMI-ER1ß responsible for the interaction with CBP.

In addition to characterizing the region of CBP responsible for the interaction with hMI-ER1ß, another goal was to identify the region of hMI-ER1ß responsible for the interaction with CBP. GST-hMI-ER1ß deletion constructs were cloned that contained different regions of the acidic amino acid regions, and solely the ELM2 domain. Other deletion constructs previously made include those which harbored the SANT domain or the SANT domain including the C terminus. GST pull down assays were performed using the GST-hMI-ER1 deletion constructs and ^{35}S-labelled CBP Br-Q protein.

Objective 4: Determination of the effects of hMI-ER1ß on histone acetylation and possible mechanisms of action.

With the recent evidence that the SANT domain of Ada2 affects the histone acetylation activity of Gcn5, the next goal was to investigate whether hMI-ER1ß could also affect CBPs ability to acetylate histones. Histone acetyltransferase assays were performed *in vitro* with hMI-ER1ß and CBP proteins expressed in HEK 293 cells. In addition, HAT assays were also carried out using the CBP Br-Q and hMI-ER1ß protein expressed in these cells.

HAT activity may be regulated by altering substrate recognition and/or the enzymatic activity of histone acetyltransferases. To determine the mechanism by which hMI-ER1ß inhibits histone acetylation, further HAT assays were carried out to eliminate the possibilities that hMI-ER1ß functions by histone "masking" or by selectively binding to inactive CBP.

Chapter 2: Materials and Methods

All the vectors used in this project are listed in Table 2.1. The description of each

vector is also included.

Table 2.1: List of the plasmids used, a brief description, and the tags they include.

Plasmids Used	Construct Description
pGEX-4T-1	Glutathione S-transferase gene fusion vector used in the expression, detection and purification of GST fusion proteins in bacteria (Pharmacia, Biotech).
pCS3 + MT	Mammalian expression vector with 6 N-terminal myc tags. Myc epitopes contains amino acid residues MEQKLISEEDLNE and recognized by anti-myc antibody. A kind gift from David Turner.
pCMV-Tag2	Mammalian expression vector with 1 N-terminal flag tag. Flag epitope contains amino acid residues DYKDDDDK and is recognized by anti-flag antibody (Stratagene).
pRC/RSV	Mammalian expression vector with enhancer-promoter sequences from the Rous sarcoma virus long terminal repeat (RSV LTR) (Invitrogen, Inc.). A kind gift from Roland Kwok
pCDNA3	Mammalian expression vector (Invitrogen, Inc.)
pCR2.1	Bacterial expression vector used for TA cloning (Invitrogen, Inc.)
pCR3.1	Mammalian expression vector, used for TA cloning (Invitrogen, Inc.)

2.1 Plasmids and constructs

2.1.1 Generation of flag tagged CBP fusion constructs

2.1.1.1 PCR

The pRc/RSV vector, containing the complete coding sequence of full length mouse CBP [accession number NM_ P45481] with an N-terminal Bluescript KS polylinker and a C terminal HA tag, was a kind gift from Dr. Roland Kwok. The entire coding sequence was excised with HindIII and NotI restriction enzymes (Invitrogen, Inc., Burlington, Ontario, Canada) and ligated into the HindIII and NotI site of the pcDNA3 vector (Invitrogen, Inc). For all CBP constructs designed, full length CBP with the Bluescript KS polylinker and a HA tag in pcDNA3 was used. The polymerase chain reaction (PCR) was used to amplify DNA sequences *in vitro* for the production of CBP RID-Br, and CBP Br-Q constructs.

For the generation of CBP RID-Br and CBP Br-Q constructs, different regions of CBP were amplified by PCR with CBP specific primers containing BamHI 5' and 3' recognition sites (ggatcc underlined in primer sequence, Table 2.2) to facilitate cloning. The PCR master mix was prepared as per the Platinum Taq DNA polymerase manufacturer's instructions (Invitrogen, Inc). In general, 1X high fidelity PCR buffer was mixed with 0.2mM of each dNTP (Invitrogen, Inc.), 2mM MgSO₄ (Invitrogen, Inc.) and deionized water. Platinum Taq high fidelity polymerase (Invitrogen, Inc.) was then added to the tube. The contents of the tubes were mixed, centrifuged, and incubated in a thermal cycler at 94°C for 5 minutes to denature the template and activate the enzyme. 30 cycles of the PCR amplification steps were performed as follows: 94°C for 30 seconds for

denaturation, 55°C for 30 seconds for primer annealing, 68°C for 4 minutes for primer

extension, 1 cycle of 72°C for 10 minutes and 30°C for 1 second. Finally, the PCR

products were treated with *Taq* polymerase (1.0 units; Invitrogen, Inc) at 72°C for 10

minutes to create a TA-overhang for use in Topo cloning (Invitrogen, Inc). Upon

completion, the samples were analyzed by 1% Tris borate/ EDTA (TBE) gel

electrophoresis and visualized by ethidium bromide staining. 1% TBE agarose gels were

made up of 1% agarose (Invitrogen, Inc) in 1.0X TBE buffer (Table 2.3).

Table 2.2: PCR primer pairs used for preparing pCMV-Tag2B-CBP constructs.

Construct	Forward Primer	Reverse Primer
CBP RID-Br aa 1-1096	5'-GG<u>GGATCC</u>ATGGCCGA GAACTTGCTGGACG -3'	5'-CG<u>GGATCC</u>CTACATAAGTGC CTGGCGTAGCTCCTCG -3'
CBP Br-Q aa 1094-2441	5'-GG<u>GGATCC</u>GCACTTATG CCAACTCTAGAAG -3'	5'-CC<u>GGATCC</u>CTACAAACC CTCCACAAACTTTT -3'

The BamHI recognition sites are underlined.

Table 2.3: Components of TBE

	10X Stock	1X Stock
Tris Base (Fisher)	108g	10.8g
Boric Acid (Fisher)	55g	5.5g
EDTA (0.5M, pH 8.0) Fisher	40ml	4ml
dH_2O	Up to 1L	Up to 1L

2.1.1.2 Ligation of PCR Products into pCR 2.1

Immediately after the treatment with Taq polymerase, the PCR products were ligated into pCR2.1-Topo according to the manufacturer's protocol (Topo TA cloning, Invitrogen, Inc). Briefly, 1μl of PCR product was added to 1μl 1M NaCl, 0.5μl of pCR2.1-Topo (Invitrogen, Inc) and 3.5μl of deionized water. All samples were mixed gently, placed at room temperature for 5 minutes and then placed on ice. Transformations were then performed using One Shot competent cells provided with the TA Cloning kit (Invitrogen, Inc). The transformation involved adding 2μl of the Topo Cloning reaction into a vial of One Shot chemically competent E. Coli. The mixture was gently mixed and placed on ice for 5 minutes. The cells were then heat shocked at 42°C for 30 seconds and immediately transferred to ice for 2 minutes. 250μl SOC medium (Invitrogen, Inc.) was added to the sample and then shaken at 37°C for 1 hour at 225 rpm. 20 and 50μl of the reaction were plated on pre-warmed luria-broth (LB) + ampicillin agar plates (5g peptone, 2.5g yeast extract, 5g NaCl, 7.5g Agar, 500mL dH_20; autoclaved; 50 μg/ml ampicillin) and grown overnight at 37°C.

After transformation, colonies of competent E. Coli containing desired plasmids were inoculated in LB medium (5g peptone, 2.5g yeast extract, 5g NaCl, 500ml dH_20; autoclaved) containing 50 μg/ml ampicillin, and to grow overnight at 37°C, shaking at 225 rpm.

2.1.1.3 Plasmid Isolation

DNA purification using the Plasmid Mini Kit (Promega, Ottawa, Ontario, Canada) was carried out as per the manufacture's instructions. To determine the concentration of the isolated plasmid DNA, agarose gel electrophoresis and spectrophotometry were performed as described in 2.1.1.2.

2.1.1.4 Digestion and purification of inserts from pCR2.1 and pcDNA3

To isolate the CBP RID-Br and CBP Br-Q inserts, the pCR2.1 vectors containing the inserts were digested with the BamHI restriction enzyme (Invitrogen, Inc.). Likewise, CBP full length was obtained by initially removing it from pcDNA3 following the same protocol. The pCMV Tag 2B vector (Stratagene, La Jolla, California, U.S.A.) used as the ligating vector for all CBP constructs was also digested with BamHI. The total amount of DNA used in each case was 10μg, and the final volume was made up with deionized water. The samples were mixed briefly and incubated at 37°C for 1 hour as directed. After 1 hour, the pCMVTag2B vector was treated with calf intestinal alkaline phosphatase (CIAP; Invitrogen, Inc.) for 10 minutes at 37°C. CIAP catalyzes the hydrolysis of 5' phosphate groups from DNA, RNA and both ribo-and deoxyribonucleoside triphosphates. The digested inserts and vector were loaded on a 1% Tris-acetate/ EDTA (TAE) gel. 1% TAE agarose gels were made up of 1% agarose (Invitrogen, Inc) in 1.0X TAE buffer (Table 2.4).

The gel was visualized under low UV light and the area containing desired DNA was removed. Ultrafree DNA purification filters (Millipore Corporation, Billerica,

Table 2.4: Components of TAE

	50X Stock	1X Stock
Tris Base (Fisher)	242g	4.84g
Glacial Acetic Acid (Fisher)	57.1ml	1.14ml
$Na_2EDTA.dH_2O$	37.2g	0.744g
dH_2O	Up to 1L	Up to 1L

Massachusetts, U.S.A.) were then used to purify the isolated DNA, as per manufacturer's instructions.

2.1.1.5 Ligation of CBP, CBP RID-Br and CBP Br-Q into pCMV-Tag2B.

The CBP, CBP Rid-Br and CBP Br-Q inserts were ligated into the pCMV-Tag 2B vector (Stratagene) using T4 DNA ligase HC, according to the manufacturer's protocol (Invitrogen, Inc). 5X ligase reaction buffer (Invitrogen, Inc.), 10mM rATP (Promega), and deionized water were added. The mixture was placed at 14°C overnight. Later, 2μl of the ligation mix was transformed into XL1-Blue supercompetent cells (Stratagene). The transformed XL1-Blue cells were plated on LB + 30 μg/mL kanamycin and incubated overnight at 37°C at 225 rpm.

PCR was performed to identify positive transformants. For the CBP/ pCMV Tag2B, CBP RID- Br/pCMV Tag2B and CBP Br-Q/ pCMV Tag2B constructs, a reverse vector primer (T7) made specific for the T7 promoter region of the pCMV Tag2B vector was used. In addition, a specific primer made for the 5' region of each construct was used. The forward primer (CBP 3) was used as an internal primer for both CBP and CBP Br-Q, and CBP 1 was used as a 5' primer for CBP RID-Br (Table 2.5). The PCR master mix was prepared as per the Taq DNA polymerase manufacturer's instructions (Invitrogen, Inc). In general, 1X PCR buffer was mixed with 0.8M dNTP mixture (Invitrogen), 2mM $MgCL_2$ (Invitrogen, Inc.), 0.2mM of each set of the appropriate primers mentioned above, deionized water, and Taq DNA polymerase. The tubes were first placed in a thermal cycler at 94°C for 5 minutes. 30 cycles of PCR amplification

steps were performed as follows: 94°C for 1 minute, 55°C for 1 minute, and 72°C for 1 minute. 1 cycle of 72°C for 10 minutes and 30°C for 1 second was then performed. The samples were run on a 1% TBE agarose gel and visualized under ultraviolet light. The positive transformants were used to inoculate LB + kanamycin and shaken at 225 rpm overnight. The next day the plasmids were isolated using the mini-prep kit, as described in 2.1.1.3. All sequences were verified by DNA sequencing.

2.1.1.6 DNA Sequencing

All ligated segments of the CBP constructs were sequenced using the United States Biochemical (USB, Cleveland, Ohio, U.S.A.) sequenase version 2.0 DNA sequencing kit. In general, 2 µl of 0.2N NaOH/ 0.2N EDTA was added to 5 µg of the previously isolated plasmid DNA. The mixture was then incubated at 37°C for 20 minutes. After the incubation, the mixture was precipitated with 2µl 3M sodium acetate (NaOAc, pH 5.2) and 60µl of 95% ethanol. The mixture was placed at -70°C for 15 minutes, and centrifuged at full speed for 20 minutes. The supernatant was discarded and the pellet was washed with 70% ethanol, dried in a dessicator and finally resuspended in 7µl water.

The second step of DNA sequencing involves the annealing of the appropriate primers to the DNA samples. This step involves the addition of sequencing reaction buffer and the corresponding primer to each DNA sample. To sequence all the CBP constructs, a 5' forward pCMV-Tag2B vector primer (T3) and a reverse primer (T7) were used (Table 2.5). The components were then mixed and heated at 65°C in a beaker for 2

Table 2.5: Primers used to analyze and sequence CBP/ pCMV-Tag2B constructs

Primer Name	Forward or Reverse Primer	Primer Sequence
T7	Reverse	5'-GGTACCTAATACGACTCACTATAGGG-3'
CBP 1	Forward	5'-GGGGATCCATGGCCGAGAACTTGCTGGACG-3'
CBP 3	Forward	5'-GGGGATCCGCACTTATGCCAACTCTAGAAG-3'
T3	Forward	5'-GCTCGAAATTAACCCTCACTAAAG-3'

minutes, slowly cooled to <35°C during 15-30 minutes, centrifuged briefly and chilled on ice.

Next, the annealed primer-DNA was mixed with 0.1M dithiothreitol (DTT), diluted labeling mix/ dGTP, ^{35}S-dATP (NEN Technologies, Zaventem, Belgium), enzyme dilution buffer, and sequenase, and then placed at room temperature for 5 minutes. 3.5µl of the labeled mix was then added to the four nucleoside phosphates (ddATP, ddGTP, ddCTG, ddTTP) that were previously heated for 3 minutes at 37°C. The reaction mixture was heated for an additional 5 minutes at 37°C. 4µl of the stop solution was then added to stop the reaction. The samples were heated at 75°C for 2 minutes and loaded on a polyacrylamide sequencing gel.

6% polyacrylamide sequencing gels were prepared using the following components: 100 ml of a previously prepared sequencing mix [containing 48g Urea (Fisher, Nepean, Ontario, Canada), 15ml 40% bis acrylamide (BioRad, Mississauga, Ontario,Canada), 10ml 10X TBE and 35ml water], 440 µl of 10% ammonium persulfate and 44µl TEMED (BioRad). Before loading, the prepared DNA sequencing gel was first warmed up to about 45°C. 4µl of each pre-warmed sample was loaded per well. The gel was run at 60V for approximately 2 hours and was fixed with 10% glacial acetic acid/ 10% methanol (Fisher), exposed onto film overnight and then developed.

Once the DNA sequence was verified, the previously mini-preped samples were transformed into XL1-Blue supercompetent cells as previously described. To isolate plasmid DNA from the cultured bacterial cells, the HiSpeed Plasmid Midi Kit (Qiagen,

Mississauga, Ontario, Canada) was used. The concentration of the isolated DNA was determined by agarose gel electrophoresis and spectrophotometry.

2.1.2 CS3MT and CS3MT-*hmi-er1* ß

CS3+MT vectors (a kind gift from David Turner, University of Michigan) were engineered to contain full length *hmi-er1β* (Ding *et al.*, 2003). The entire coding sequence of *hmi-er1β* (accession number AF515447) was amplified using specific 5' and 3' primers as described in Ding *et al.*, 2003. The PCR fragments were then inserted into the *Bgl*II site of CS3+MT plasmid.

2.1.3 GST-*hmi-er1β* full length and GST-*hmi-er1β* deletion constructs

GST-*hmi-er1β* and the *hmi-er1β* deletion mutants fused to GST, except for the deletion mutant *hmi-er1β* aa 1-179 described in 2.1.4, were constructed by Z. Ding in our laboratory (Ding *et al.*, 2003).

2.1.4 Generation of pGEX-4T-1-*hmi-er1β* aa 1-179

2.1.4.1 PCR

To produce pGEX-4T-1-*hmi-er1* aa 1-179, the CS3-MT- *hmi-er1β* plasmid was used. The fragment encoding the amino acid residues 1-179 was amplified using a forward hmi-er1 primer made specifically for the 5' region of *hmi-er1* (Her34K) and a reverse primer specific for the 3' region of *hmi-er1* aa 1-179 sequence (mi-her1T100; Table 2.6). The samples were initially incubated at 94°C for 2 minutes to denature the

template and activate the enzyme. 30 cycles of PCR amplification steps were performed

as follows: 94°C for 30 seconds for denaturation, 55°C for 30 seconds for primer

annealing, and 68°C for 36 seconds for primer extension. 1 cycle of 72°C for 10 minutes

and 30°C for 1 second was then performed. Finally, the PCR products were treated with

Taq polymerase (1.0 units; Invitrogen, Inc) at 72°C for 10 minutes to create a TA-

overhang for use in Topo cloning (Invitrogen, Inc). 10µl of each amplification product

was analyzed by 1% TBE agarose gel electrophoresis and visualized by ethidium

bromide staining.

2.1.4.1 Ligation of PCR Products into pCR 3.1

The PCR products were ligated into pCR 3.1 according to the manufacturer's

protocol. All components provided by the TA cloning kit (TA cloning, Invitrogen, Inc)

were used. 12.7 ng of the PCR product added to the following: 10X ligation buffer, pCR

3.1 vector (30ng/ µl), T4 DNA ligase (4units/ µl) and deionized water. The mixture was

incubated overnight at 15°C. The next day, transformations were performed using

XL1Blue-supercompetent cells (Stratagene). The entire reaction was later plated on LB +

ampicillin (50 µg/ml), inverted and incubated overnight at 37°C.

↝ The next step was to screen the bacterial colonies for the uptake of the ligated

DNA. A 3' reverse pCR 3.1 vector primer (pCR 3.1) and a 5' forward primer (Her34K)

were used (Table 2.6). The PCR master mix was made up as described in section 2.1.1.6

and the samples were initially incubated at 94°C for 3 minutes. 30 cycles of PCR

amplification steps were performed as follows: 94°C for 45 seconds, 55°C for 30 seconds,

Table 2.6: PCR primers used to construct pCR 3.1-*hmi-erlβ* aa 1-179 and pGEX-4T-1-*hmi-erlβ* aa 1-179.

Primer Name	Reverse or Forward Primer	Primer Sequence
pCR 3.1	Reverse	5' CTGATCAGCGGGTTTAAACGGG-3'
Her34K	Forward	5'CACCATGGCGGAGCCATCTGTTGAATC-3'
pGEX 1.1	Forward	5'GGGCTGGCAAGCCACGTTTGGT-3'
mi-her1T100	Reverse	5'GCCCTACCAGTCTTCTGATGGAAT-3'

and 72°C for 1 minute and 30 seconds. 1 cycle of 72°C for 10 minutes and 30°C for 1

second was then performed. The samples were run on a 1% TBE agarose gel and

visualized under ultraviolet light. Colonies that tested positive were used to inoculate LB

+ ampicillin and allowed to shake at 225 rpm, overnight. The next day the plasmids were

isolated using the mini-prep kit, described in 2.1.14, and then prepared for restriction

enzyme digestion.

2.1.4.2 Digestion and purification of insert from pCR 3.1

To isolate the *hmi-er1* aa1-179 insert, both the pCR3.1 vector containing the

insert and the pGEX 4T-1 vector (Pharmacia, Biotech, Piscataway, New Jersey, U.S.A)

were digested with the EcoRI restriction enzyme (Invitrogen, Inc.) as previously

described.

2.1.4.3 Ligation of hmi-er1 1-179 into pGEX-4T-1

After purification, the insert was ligated into the pGEX-4T-1 vector, transformed,

and screened for positive colonies. In all incidences the same protocol was followed as in

section 2.1.1.3, except ampicillin was added to the LB medium instead of kanamycin. As

well, the primers used to screen for positive colonies included a forward primer specific

for the 5' region of pGEX-4T-1 (pGEX 1.1) and a reverse primer (mi-her1T100) specific

for the 3' region of *hmi-er1* aa 1-179 sequence (Table 2.6).

Following colony screening, the plasmid was isolated using the mini-prep kit. To confirm the nucleotide sequences of the isolated plasmid DNA, DNA sequencing was performed, as in section 2.1.4.4.

2.2 *In vitro* Transcription-Translation

In order to perform GST pull down assays, *in vitro* translations were performed using the TnT® Couple Reticulocyte Lysate System (Promega). The following reaction components, provided in the kit, were mixed for a 50 µl reaction : 25µl TnT® Rabbit Reticulocyte Lysate , 2 µl TnT Reaction Buffer, 1 µl of the appropriate TnT® RNA Polymerase (SP6, T3 or T7), 1 µl amino acid mixture, minus methionine, and 1 µl of ribonuclease inhibitor [RNA Guard (27-0815-01); Amersham Biosciences]. 66.6 µCi of [^{35}S] methionine and DEPC water was then added to make up to a final volume of 50µl master mix. This master mix was equally distributed to all samples with the corresponding plasmid DNA (1µg). The samples were analyzed using tricholoroacetic acid (TCA) precipitation assays and then stored at -70°C for future use.

After completion of the *in vitro* transcription-translation reaction, TCA precipitation assays were conducted in order to analyze the results of the translation. TCA precipitation assays provide a numerical value for the amount of protein made based on the amount of [^{35}S]-methionine incorporated into the protein. To perform this assay 2 µl of the reaction was removed and bleached with 1M NaOH/2% H_2O_2, vortexed briefly and incubated at 37°C for 10 minutes. 900 µl of ice-cold 25% TCA/2% casamino acids (Merck) was then added to precipitate the translated product. The mixture was placed on

ice for 30 minutes. The precipitate was then collected on a filter paper (Fisher) as

follows: 1 ml of each sample was filtered using a vacuum flask and then washed with 5%

TCA. Approximately 3mls of 95% ethanol was added to dry the filter paper. To

determine $[^{35}S]$ methionine incorporation, the filter was placed in 5ml Biodegradable

Counting Scintillant (Amersham) and analyzed in a Beckman S3801 Scintillation

Counter.

2.3 GST Pull Down Assays

2.3.1 Preparation of Glutathione Sepharose 4B Matrix

Glutathione Sepharose 4B matrix (Pharmacia Biotech) was prepared as per

manufacturer's instructions. Approximately 1.33mls of the original 75% slurry was

removed and transferred to a 15ml conical tube (Fisher). The matrix was centrifuged at

500 x g for 5 minutes, and the supernatant was carefully removed. The matrix was then

washed with 10ml of cold 1X phosphate buffered saline (PBS), mixed, and centrifuged

again at 500 x g for 5 minutes. After the supernatant was removed, 1ml of 1X PBS was

added which resulted in a 50% slurry. The beads were mixed well before use and stored

with 0.02% azide at 4°C.

2.3.2 GST Fusion Protein Production

To produce GST fusion protein, 10ng of the pGEX-4T1 plasmid was added to

100μl of BL21 Codon Plus RP competent E. coli cells (Stratagene). The sample was

placed on ice for 30 minutes and then heat shocked for 45 seconds at 42°C. 250μl of LB

was added to the reaction mixture and shaken at 37°C for 1 hour. After shaking, all of the

reaction mixture was plated on LB ampicillin plates and stored at 37°C overnight. The

following day, one colony from each plate was allowed to grow in 5mL of LB medium

with 50µl/ml ampicillin, while shaking overnight at 37°C. The next day, 2mls of the

overnight culture was added to 250ml LB medium with 50µg/ml ampicillin and allowed

to grow while shaking at 37°C for approximately 3.5 hours. The optical density (OD) of

the culture was measured at a wavelength of 600nm in a spectrophotometer (Beckman,

Du-64). The culture was allowed to grow until it reached an OD of 0.6-0.8 after which

25µl of 1M IPTG (isopropyl-ß-D-thiogalactopyranoside; Invitrogen, Inc) was added and

shaken at 37°C for 4 hours. The culture was poured into a 250ml Nalgene polypropylene

bottle and centrifuged at 4000 rpm for 15 minutes in a Sorval centrifuge. All the liquid

was drained and the pellet was resuspended in 5ml ice cold 1X PBS (8.0g NaCl, 0.2g

KCl, 0.24g $KH_2HPO_4.7H_2O$, 1L H_2O; autoclaved) + 25µl 0.2M Phenylmethylsulfonyl

Fluoride (PMSF; Sigma, Oakville, Ontario, Canada) and 50µl 100X protease inhibitors

[PI; 10mg aprotinin (Sigma), 10mg leupeptin (Sigma), and 50mg nor-P-Tosyl-L-Lysine

Chloromethyl Ketone (Sigma) in the total volume of 10ml dH_2O]. The solution was

placed into a 15ml conical tube whereby the cells were lysed by mild sonication for 1

minute using a sonicator (Virsonic Cell Disrupter, 16-850). Following sonication, 500µl

of 10% Triton X-100 (Sigma) was added to the mixture. The mixture was then

transferred to a 30ml Corex glass tube and centrifuged at 6000 rpm in a Sorvall RC-SB

centrifuge for 20 minutes. The supernatant was removed from the corex tubes and placed

at -70°C. GST fusion protein expression levels and purity was checked by SDS-polyacrylamide gel electrophoresis (PAGE).

2.3.3 GST Pull Down Assays

For each GST pull down assay, 50µl of GST Sepharose 4B beads was used for each individual reaction. The beads were then washed twice with 500µl of GST pull down buffer [20mM Tris-HCl, pH 7.5, 150 mM NaCl, 1mM EDTA, 10% Glycerol, 0.2% Nonidet P-40 (NP-40), 1X Protease Inhibitor (PI), 0.5% Bovine Serum Albumin (BSA)]. After subsequent washes, beads were suspended in GST pull down buffer (minus BSA) with the indicated GST fusion protein, and rotated for 1 hour at 4°C. Following incubation, unbound proteins were removed from the beads by washing the samples five times with 500µl of GST pull down buffer and resuspended in 500µl GST pull down buffer (minus BSA). Once resuspended, 100,000 counts per minute (cpm) of the appropriate ^{35}S labeled *in-vitro* transcription and translation proteins (Flag tagged CBP, Flag tagged CBP Br-Q, and Flag tagged CBP Rid-Br) were added. With the addition of the ^{35}S labeled protein, the samples were further rotated at 4°C for 2 hours. Following the rotation, the beads were washed three times with 500µl GST pull down buffer (minus BSA), twice with 500µl of GST pull down buffer (minus NP40; interferes with SDS-PAGE) and twice with 150mM NaCl. After washing, 30µl of 2X SSB (SDS sample buffer) was added to each sample and the sample run on a 8% SDS-PAGE at 30mA for approximately 1hour and 20 minutes. Input lanes contained 1/10 volume of the indicated TNT used for each reaction. The gel was then fixed in a fixative solution (45ml Methanol

(Fisher), 10ml Glacial Acetic Acid (Fisher), 45ml dH2O) for 15 minutes, shaking at room temperature. The gel was then destained (20ml Methanol (Fisher), 6ml Glacial Acetic Acid (Fisher), 74ml dH2O), shaking at room temperature for 15 minutes. Finally, the gel was placed in Amplify solution (Amersham) for 15 minutes, dried and visualized using autoradiography.

2.4   Cell Culture

Transformed human embryonic kidney cells (HEK 293), obtained from American Tissue Culture Collection, were cultured in Dulbecco's Modified Eagle's medium (DMEM; Hyclone) with the addition of 7.5% Calf Serum (CS; Invitrogen, Inc.) and 2.5% Fetal Bovine Serum (FBS; Invitrogen, Inc.). The cells were cultured in a 37°C incubator with 10% CO_2.

2.5   Transfections

18 hours prior to transfection, approximately 5.0×10^5 HEK 293 cells/well were seeded. The following plasmids were used in co-transfections experiments: pCMV Tag2B *CBP*, pCMV Tag2B *CBP Br-Q*, pCMV Tag2B *CBP Rid-Br*, CS3MT, and CS3MT-*hMI-ER1β*. Cells were cotransfected with 1.5μg of plasmid DNA by Lipofectamine Plus reagent (Invitrogen, Inc.) according to supplier's protocol. Briefly, 1.5 μg of plasmid DNA was incubated with 6 μl Lipofectamine and 6 μl Plus reagent in the total of 200 μl serum-free medium at room temperature for 30 min. The mixture was added to cells previously seeded in 6-well plates containing 800 μl of serum-free medium

and incubated at 37°C for 4h, following which serum-free medium was replaced by

DMEM + 7.5% CS + 2.5% FBS medium.

2.6 Protein Extracts and Immunoprecipitation

Total cellular extracts were analyzed to ensure successful transfections.

Approximately 48 hours after transfection, the transfected cells were washed with 2ml

1X PBS. 200 μl of 1.5X SSB (without bromophenol blue) was added to each well

containing the transfected cells. The cells were passed through a syringe approximately

15-20 times. After centrifugation at 16,000 x g, 4°C for 5 minutes, the supernatant was

removed, placed in a new 1.5ml tube, and placed at -20°C until ready for use.

The samples ready for immunoprecipitation (IP) were washed with 2ml 1X PBS.

1ml of 1X Triton Cocktail (10mM Tris pH 7.5, 1% Triton X 100, 10mM EDTA, 0.02%

Sodium Azide, dH$_2$0) mixed with 100X PI was then added to the samples. The cell

lysates were collected as previously described. Either the anti-flag monoclonal antibody

(1:750; F-3165, Sigma) or the anti-myc monoclonal antibody (1:100; 9E10,

Developmental Hybridoma Bank) was added to the cell lysate and incubated overnight

while rotating at 4°C.

Following the overnight incubation, 50μl of a 50% slurry of protein G beads

(Amersham) was added to the tubes and rotated for an hour at 4°C. Following the

incubation the beads were washed twice with 1ml 1X Triton Cocktail + 1X PI, and three

times with 1ml 150mM NaCl. Beads were then resuspended in 35μl 2X SSB containing

bromophenol blue. The samples were boiled for 4 minutes, vortexed, centrifuged briefly

and loaded on an 8% SDS-PAGE. Gels were run for approximately 1h20min to allow full separation of protein components. The gels were agitated in three washes of 1X transfer buffer [200ml 5X Stock buffer (60.54g Tris, 288.4g glycine, 3L dH$_2$0) + 200ml methanol, 600ml dH$_2$0] for 15 minutes. Proteins were then transferred onto a Hybond-ECL nitrocellulose membrane (Amersham) for 2 hours at 60 V. The membrane was then blocked for 2 hours at room temperature in 5% skim milk/ 1X TBS-T (20mM Tris pH 7.6, 137mM NaCl, 0.1% Tween-20, dH$_2$0).

2.7   Western blotting

Western blot (WB) analysis was performed by incubating the membrane overnight with either the anti-flag antibody (1:2000) or anti-myc monoclonal antibody (1:100) in 5% skim milk/1X TBS-T at 4°C. The next day the membrane was washed with 1X TBS-T for 1 hour at room temperature, followed by incubation in sheep anti-mouse conjugated to Horseradish Peroxidase (1:3000) in 5% skim milk/1X TBS-T for 1 hour at room temperature. Finally, the membrane was washed 4-5 times with 1X TBS-T for 1 hour at room temperature and then analyzed using the ECL Plus Detection Kit (Amersham). The membrane was exposed to ECL Hyperfilm (Amersham).

2.8   Histone Acetyltransferase Assays

Histone acetyltransferase assay was used to detect *in vitro* HAT activity. HEK 293 cells were first transfected with the appropriate construct(s). After 48 hours, the transfected cells were lysed with 1X HAT assay IP buffer (50mM Tris HCl pH 7.5,

53

150mM NaCl, 10% glycerol, 1% Triton X-100, dH$_2$0), followed by protein extraction and immunoprecipitation as described in section 2.6. The samples were incubated with protein G beads and washed three times with 1ml 1X triton cocktail (10mM Tris HCl pH 7.5, 1% triton, 10mM EDTA, 0.02% azide, dH$_2$0), and twice with 1ml 1X HAT assay buffer (50mM Tris HCl pH 7.5, 10% glycerol, 1mM DTT, 300nM TSA, dH$_2$0). 30µl of HAT master mix [1µg/µl histone biotinylated H4 peptide (Upstate Biotechnology), [1-^{14}C] Acetyl-coenzyme A (Amersham, 10µCi), dH$_2$0, 300nM Trichostatin A (TSA), 2X HAT assay buffer] was then prepared and added to each sample. The samples were incubated for 45 minutes in a waterbath at 30ºC. The HAT master mix was also added to the appropriate TNT samples as a control.

After the incubation, the samples were centrifuged for 30 seconds and the supernatant containing acetylated histones was isolated. The remaining mixture was washed again with 70 µl 1X HAT assay buffer and pooled with the previously isolated supernatant, to ensure that the maximum amount of acetylated histone samples was obtained. 20 µl of prepared streptavidin beads (Pierce) was added to each sample and rotated for 30 minutes at 4ºC. The samples were then washed twice with 1ml RIPA buffer (50mM Tris pH 7.5, 150mM NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% SDS, 1mM EDTA, dH$_2$0) and once with 1X HAT assay buffer, followed by resuspension in 100µl of 1X HAT assay buffer. Finally, 5mls of biodegradable counting scintillant (Amersham) was added and the samples were analyzed by a scintillation counter (Beckman S3801).

Chapter 3: Results

To simplify the description of the results, the names of all constructs used are abbreviated. They are listed in Table 3.1 below.

Table 3.1: A list of all constructs used and their abbreviated name.

Constructs Used	Abbreviated Name:
pGEX-4T-1- *hmi-er1β*	GST ß
pGEX-4T-1	GST
pCMV-Tag2B	flag
pCMV-Tag2B-*CBP*	CBP
pCMV-Tag2B- *CBP Rid-Br*	Rid-Br
pCMV-Tag2B- *CBP Br-Q*	Br-Q
pCS3 + MT- *hmi-er1β*	ß ·
pCS3 + MT	myc
pGEX-4T-1- *hmi-er1* aa 1-283	1-283
pGEX-4T-1- *hmi-er1* aa 164-283	164-283
pGEX-4T-1- *hmi-er1* aa 287-512	287-512
pGEX-4T-1- *hmi-er1* aa 1-179	1-179

3.1: hMI-ER1ß interacts with CBP *in vitro*

To determine if hMI-ER1ß interacts with Creb Binding Protein (CBP), GST pull down assays were performed using ^{35}S labeled *in vitro* translated CBP protein (100,000 cpm) and GST-hMI-ER1ß fusion protein (18 picomoles). ^{35}S labeled *in vitro* translated CBP protein (100,000 cpm) and GST (18 picomoles) alone were tested and used as a control to ensure the interaction seen was not a result of CBP interacting with GST. Figure 3.1 is a representative autoradiograph obtained from one of the two independent experiments performed. As shown in Figure 3.1, the ^{35}S labeled *in vitro* translated CBP protein interacted with GST-hMI-ER1ß but not with GST alone. These results demonstrate that hMI-ER1ß interacts with CBP *in vitro*.

3.2: The amino acids of hMI-ER1ß required for interaction with CBP are located in the region 1-179, a region rich in acidic amino acid residues

Given that hMI-ER1ß interacts with CBP *in vitro*, it was important to determine the region of hMI-ER1ß responsible for the interaction. The complete amino acid sequence of hMI-ER1ß shows the presence of an acidic activation domain, an ELM2 domain and a SANT domain (Figure 3.2A). In order to investigate which domain is required for the interaction, the following GST-hMI-ER1 deletion constructs were used in GST pull down assays: the acidic activation domain (aa 1-179), the ELM2 domain (aa 164-283), the acidic activation and ELM2 domain (aa 1-283), and the SANT domain and ß-C terminus (aa 287-512) (Figure 3.2A).

Figure 3.1: hMI-ER1ß interacts with CBP *in vitro*

GST-pull down assays were performed by incubating ^{35}S labeled *in vitro* translated CBP

protein (100,000 cpm) with either 18 picomoles of GST alone (lane 2) or with 18

picomoles of GST-hMI-ER1ß (GST ß; lane 3). The Input TNT lane represents 5% of the

input of ^{35}S labeled CBP protein used in each pull down assay (lane 1) and shows the

proper position of CBP (265kDa). Proteins were resolved by SDS- PAGE and visualized

by autoradiography. Results were obtained from two independent experiments and shown

is a representative autoradiograph. The positions of CBP and a molecular weight marker

are indicated.
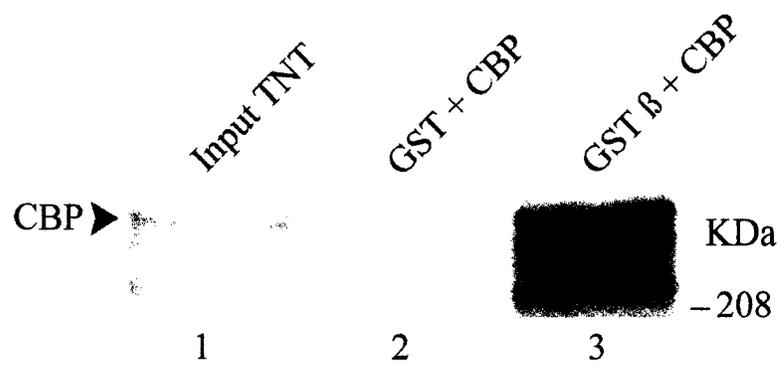
Figure 3.2: The amino acids of hMI-ER1ß required for interaction with CBP are located in the region 1-179, a region rich in acidic amino acid residues.

(A)The schematic illustrates the deletion mutants of hMI-ER1 fused to GST and used in the GST pull down assays shown in (B). A scaled representation of hMI-ER1 and its domains are shown. The amino acid numbers encoding each construct is shown on the left, and a key for the various hMI-ER1 domains is located below the diagram.

(B) GST pull down assays were performed by incubating *in vitro* translated ^{35}S labeled CBP protein (100,000 cpm) with approximately 18 picomoles of one of the following hMI-ER1 deletion constructs fused to GST; 1-283 (lane 1), 1-179 (lane 2), 164-283 (lane 3), 287-512 (lane 4), and GST alone (lane 5). The Input TNT lane represents 5% of the input of ^{35}S labeled CBP protein used in each pull down assay (lane 6). Proteins were resolved by autoradiography. Shown is a representative autoradiograph with the position of CBP indicated by the arrowhead.

(A)
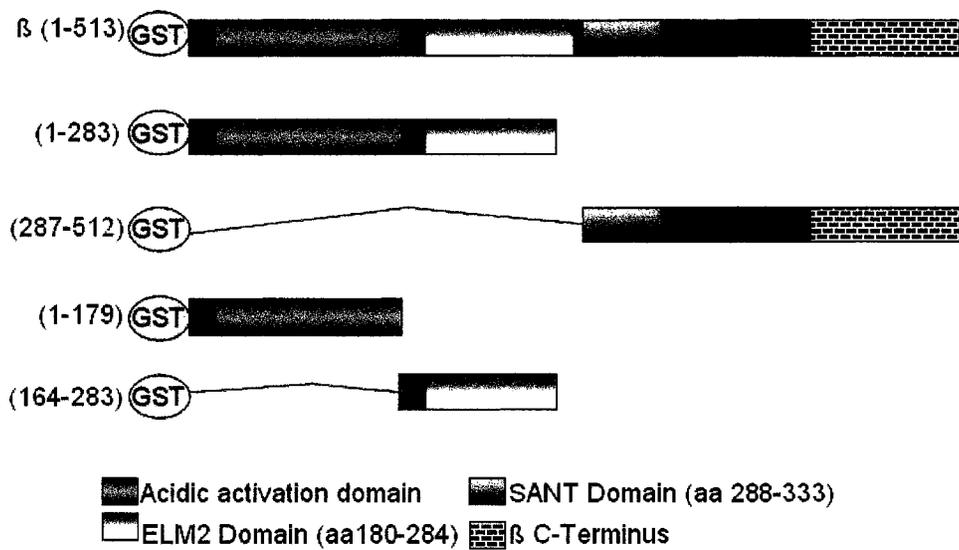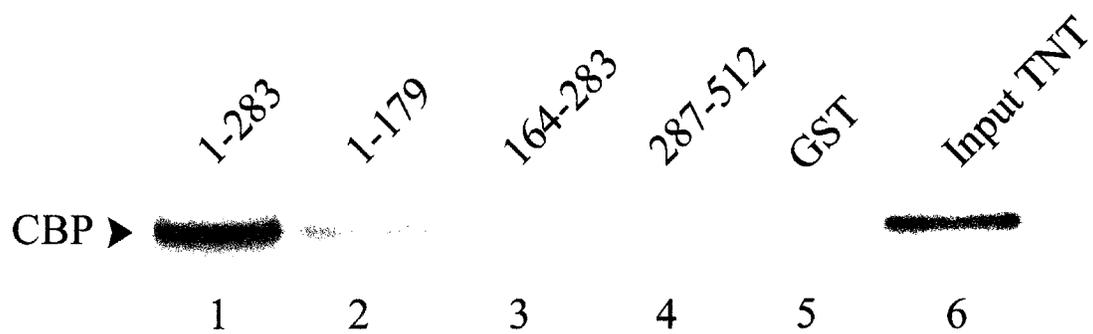
- β (1-513) GST ▨ Acidic activation domain | ELM2 | ▨ SANT | ▨ β C-Terminus
- (1-283) GST ▨ Acidic activation domain | ELM2
- (287-512) GST ⎯⎯⎯ ▨ SANT | ▨ β C-Terminus
- (1-179) GST ▨ Acidic activation domain
- (164-283) GST ⎯⎯⎯ ▨ | ELM2

▨ Acidic activation domain   ▨ SANT Domain (aa 288-333)

☐ ELM2 Domain (aa180-284)   ▨ β C-Terminus

(B)

	1-283	1-179	164-283	287-512	GST	Input TNT
CBP ▶	▬	░				▬
	1	2	3	4	5	6

GST pull down assays were performed using ^{35}S labeled *in vitro* translated CBP protein (100,000 cpm) and 18 picomoles of each of the GST-hMI-ER1ß deletion constructs. As a control, the ability of ^{35}S labeled *in vitro* translated CBP protein to interact with GST alone was tested. Figure 3.2B is a representative autoradiograph from two independent experiments and shows a strong interaction between CBP and the hMI-ER1ß deletion construct containing the acidic activation domain and ELM2 domain (aa 1-283). No interaction was observed between CBP and the deletion construct harboring the SANT domain and ß-C terminus (aa 287-512) or with the deletion construct harboring solely the ELM2 domain (164-283). Further analysis revealed that the hMI-ER1ß deletion construct that is made up of amino acids 1-179 is responsible for the interaction with CBP *in vitro*. Overall, the results indicate that the region of hMI-ER1ß rich in acidic amino acids is responsible for the interaction with CBP.

3.3: hMI-ER1ß interacts with CBP, in HEK 293 cells, and inhibits histone acetylation

In addition to testing the ability of hMI-ER1ß to physically associate with CBP *in vitro*, it was important to test whether a similar interaction occurs *in vivo*. This was done using a flag-tagged CBP (CBP) and myc-tagged hMI-ER1ß (ß). Transformed human embryonic kidney cell lines (HEK 293) were transiently cotransfected with *myc + CBP, ß + flag*, or *ß + CBP*. Expression of *ß* was verified for each experiment by WB analysis, of whole cell extracts, using an anti-myc antibody. We attempted to examine the expression of *CBP*, using an anti-flag antibody, however the signal on the WB was weak (data not

shown). CBP has a molecular weight of 265 kDa and is therefore a very large protein. It is known that high-molecular weight proteins have a low transfer efficiency, which results in low levels of detection on Western blots (as reviewed in Kurien and Scofield, 2003).

To examine the interaction between CBP and hMI-ER1ß, cell extracts were subjected to IP with anti-flag antibody, followed by Western blotting with anti-myc. As shown in Figure 3.3A, no interaction occurred in the controls: cells transfected with ß and flag and cells transfected with *CBP* and *myc*. However, the results revealed that ß coimmunoprecipitated with CBP in HEK 293 cells, suggesting that hMI-ER1ß interacts with CBP *in vivo*.

Since hMI-ER1ß interacts with CBP, a histone acetyltransferase, it was important to determine whether hMI-ER1ß had an effect on CBP activity. To determine the effects of hMI-ER1ß on histone acetylation by CBP, HEK 293 cells were transiently cotransfected with either *CBP + ß* or *CBP* alone. Non-transfected, *CBP + myc* and *ß + flag*-transfected cells were used as controls. Cell extracts were subjected to IP with either an anti-myc or anti-flag antibody. HAT assays were then performed using HAT assay buffer containing Trichostatin A (TSA). TSA was included because it was shown that hMI-ER1 interacts with histone deacetylase 1 (HDAC 1) and therefore I wanted to inhibit any HDAC activity associated with hMI-ER1 since HDAC activity might interfere with our ability to detect HAT activity.

As shown in Figure 3.3B, the control samples from the non-transfected, *myc +* *CBP*, and *flag + ß*-transfected cells contained very little HAT activity. The results also

Figure 3.3: hMI-ER1ß interacts with CBP *in vivo* and inhibits histone acetylation.

HEK 293 cells were transiently cotransfected with either *myc* + *CBP*, *flag* + *ß*, or *CBP* +

ß plasmids. 0.75µg of each plasmid was used in the cotransfections. Additional controls

consisted of mock-transfected 293 cells, as well as cells transfected with *CBP* alone, for

use in the HAT assay. Cells were grown in DMEM rich medium (see Material and

Methods) for 48 hours. **(A)** Cell lysates were prepared and added directly to sample

buffer (lanes 1 and 5) or subjected to IP with anti-flag antibody (1:750; lanes 2-4). WB

analysis was performed using anti-myc antibody (1:100). Results were obtained from

three independent experiments and shown is a representative WB, with the position of

hMI-ER1ß indicated by the arrowhead. **(B)** Additional cell lysates were subjected to IP

with anti-flag antibody (1:750) or with anti-myc (1:100) and assayed for HAT activity.

The histogram shows the average values and standard deviations obtained from the three

experiments conducted.

(A)

WB: anti-myc

Flag IP:	−	+	+	+	−
	myc+CBP		ß+flag	ß+CBP	ß+flag

◄ hMI-ER1ß

  1   2   3   4   5

(B)

Plasmid: /   CBP   ß+CBP   ß+flag   myc+CBP

IP: anti-flag   anti-myc

(Y-axis: HAT Activity (cpm), scale 0 to 2500)
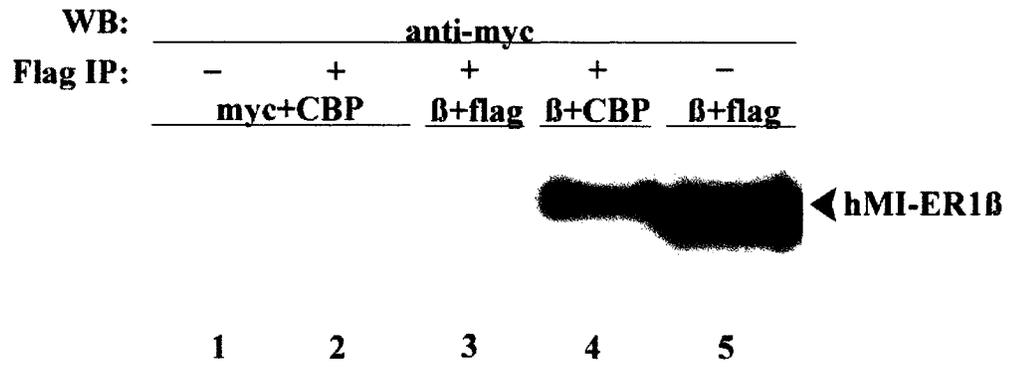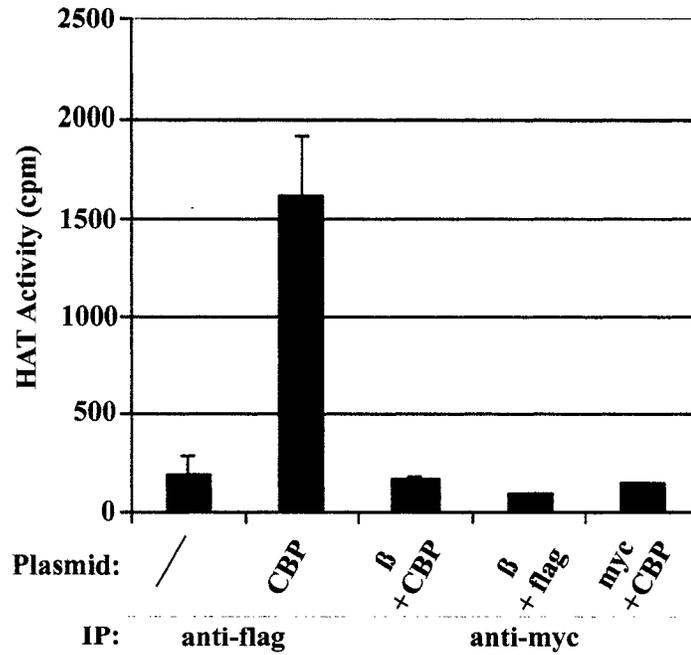
revealed that the CBP sample, in the absence of ß, contained significant HAT activity.

However, the activity was reduced to control levels in the presence of ß. Taken together,

the results show that hMI-ER1ß interacts with CBP *in vivo* and suggests that this

interaction inhibits CBP acetylase activity.

3.4: The CBP Br-Q region (aa 1092-2441) that harbors the HAT domain is responsible for the interaction with hMI-ER1ß *in vitro*.

To determine which region of the CBP protein was responsible for the interaction

with hMI-ER1ß, flag tagged deletion mutants were constructed. The schematic

illustration of the CBP constructs, as shown in Figure 3.4A, illustrates that CBP Br-Q

deletion construct (aa 1092-2441) is made up of the bromodomain, the HAT domain, two

cysteine-histidine rich domains and a Q rich domain. The CBP Rid-Br construct (aa 1-

1095), however, is made up of the receptor interacting domain, a cysteine-histidine rich

domain, and the kinase induced interacting domain.

GST pull down assays were performed with ^{35}S labeled *in vitro* translated Rid-Br

and Br-Q protein and GST-ß fusion proteins. As controls, the ability of the deletion

constructs to interact with GST alone was also tested. Figure 3.4B shows the results of a

representative GST pull down assay. As shown, the ^{35}S labeled *in vitro* translated Br-Q

protein did interact with GST-ß and not with GST alone. However, the ^{35}S labeled *in

vitro* translated Rid-Br did not interact with GST-ß. The results reveal that hMI-ER1ß

interacts with Br-Q *in vitro*.

Figure 3.4: The CBP Br-Q region (aa 1092-2441), that harbors the HAT domain, is responsible for the interaction with hMI-ER1ß *in vitro*.

(A) The schematic illustrates the deletion mutants of CBP fused to a flag tag and used in GST pull down assays. A scaled diagram representing CBP and its deletion mutants together with the number of amino acids encoding each construct is shown. 'RID': receptor interacting domain; 'KIX': kinase induced interacting domain; 'C/H-1,-2,-2': cysteine-histidine rich domains; 'Bromodomain': acetyl-lysine domain; 'HAT': histone acetyltransferase domain; 'Q Rich': glutamine-rich domain. (B) GST pull down assays were performed by incubating *in vitro* translated ^{35}S labeled Rid-Br and Br-Q protein (100,000 cpm) with GST alone (12.3 picomoles)(lanes 2 & 4) or with GST-ß (ß; 12.3 picomoles; lanes 1 & 3). The Br-Q TNT and Rid-Br TNT (lanes 5 & 6) represent 5% and 10% of the input of ^{35}S labeled protein used in each pull down assay respectively. Proteins were resolved by autoradiography. Shown is a representative autoradiograph with the position of Rid-Br and Br-Q indicated.
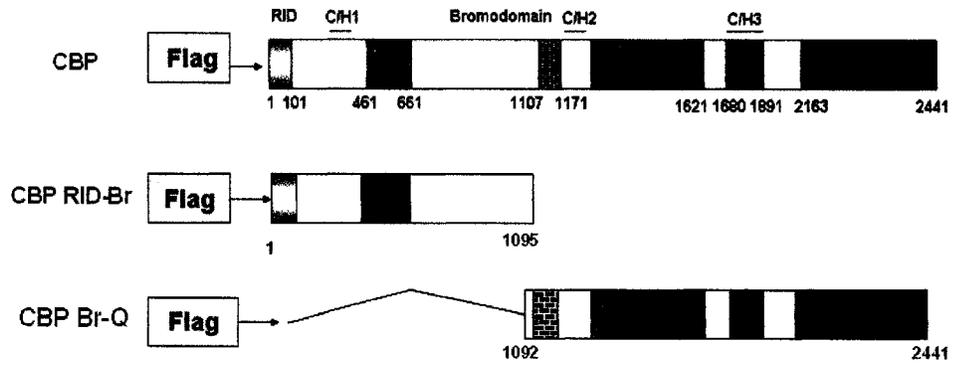
(A)

(B)
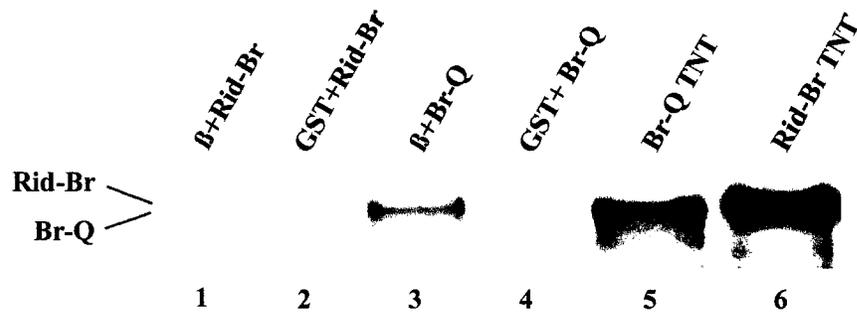
3.5: hMI-ER1ß interacts with CBP Br-Q *in vivo* and inhibits histone acetylation

In addition to testing the ability of hMI-ER1ß to physically associate with CBP

Br-Q *in vitro*, it was important to test whether the interaction could also be seen *in vivo*.

In vivo analysis involved the transient cotransfections with *Br-Q* + *ß*. Non-transfected,

CBP + *myc*, *CBP* + *ß*, and *flag* + *ß*-transfected HEK 293 cells were used as controls.

Expression of ß was verified by performing IP and Western blotting with an anti-myc

antibody. Cell extracts were also subjected to IP with anti-flag antibody, followed by

Western blotting with anti-myc. As shown in Fig. 3.5A, no interaction was found

between ß and flag. Similarly, an interaction did not occur between CBP and myc. The

results also revealed that ß interacted with Br-Q *in vivo*, similar to the interaction found

between ß and CBP.

Since hMI-ER1ß interacts with Br-Q, which harbors the HAT domain, it was

important to determine if hMI-ER1ß could inhibit its ability to acetylate histones. For this

purpose, *in vitro* HAT assays were performed to measure the HAT activity of Br-Q in the

presence and absence of ß. HEK 293 cells were transfected with *Br-Q*, *ß* + *Br-Q*, *Br-Q* +

myc or *ß* + *flag* plasmids. Mock transfected cells were used as an additional control. Cell

extracts were subjected to IP with either an anti-myc or anti-flag antibody and assayed for

HAT activity. As shown in Figure 3.5B, the control samples from the non-transfected,

and *myc* + *Br-Q*- transfected cells contained very little HAT activity. The results also

revealed that Br-Q, in the absence of ß, contains significant HAT activity. However, the

activity was reduced to control levels in the presence of ß. Taken together, the overall

results show that hMI-ER1ß interacts with Br-Q *in vivo* and inhibits histone acetylation.

Figure 3.5: hMI-ER1ß interacts with CBP Br-Q *in vivo* and inhibits histone acetylation. HEK 293 cells were transiently cotransfected with *myc* + *CBP*, ß + CBP, *flag* + ß, or *Br-Q* + *ß* plasmids. **(A)** Cell lysates were prepared 48 hours after transfection and subjected to IP with anti-flag antibody (flag; 1:750; lanes 1, 3-6) or with anti-myc antibody (myc; 1:100; lane 2). WB analysis was performed using anti-myc antibody (1:1000). Results were obtained from three independent experiments and shown is a representative WB, with the position of hMI-ER1ß indicated by the arrowhead. **(B)** Additional cell lysates were subjected to IP with anti-flag antibody (1:750) or with anti-myc antibody (1:100) and further examined for HAT activity. The histogram shows the average values and standard deviations obtained from the experiments conducted.
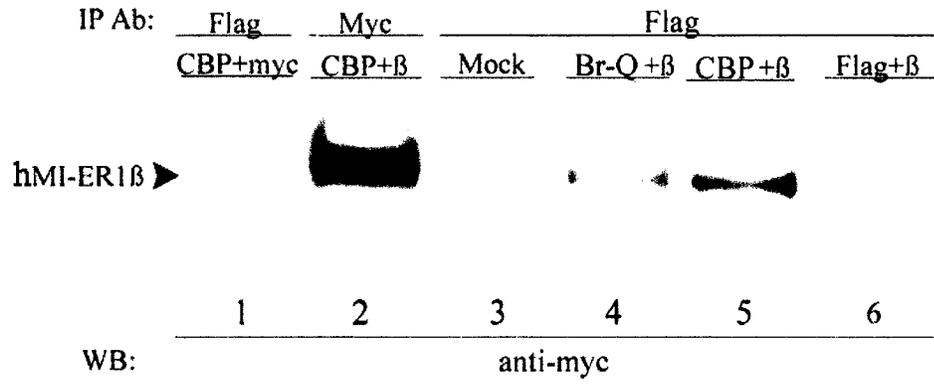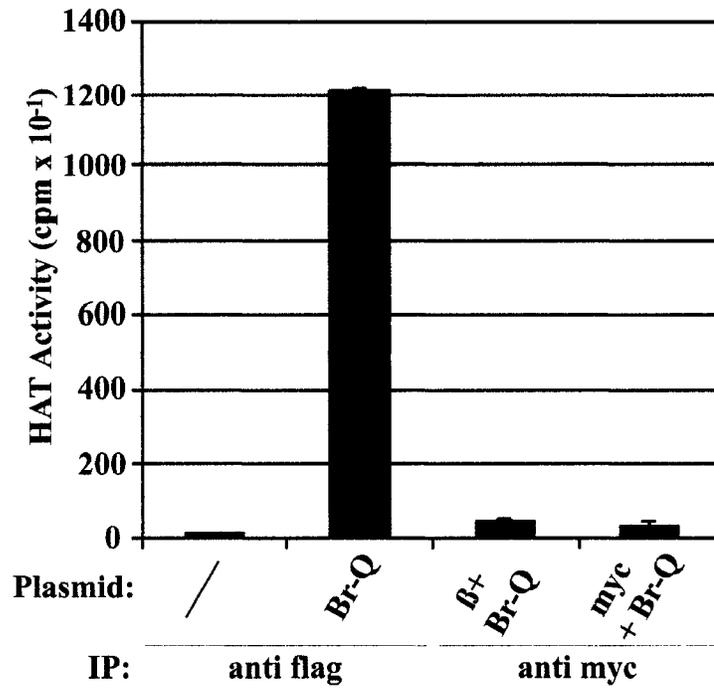
(A)

IP Ab:	Flag	Myc			Flag		
	CBP+myc	CBP+ß	Mock	Br-Q +ß	CBP+ß	Flag+ß

hMI-ER1ß ▶

1   2   3   4   5   6

WB: anti-myc

(B)

HAT Activity (cpm x 10⁻¹)

Plasmid: /   Br-Q   ß+ Br-Q   myc + Br-Q

IP: anti flag   anti myc

3.6: Inhibition of histone acetylation in vitro by hMI-ER1ß is not the result of competition for histone binding.

There are many different ways in which hMI-ER1ß could be affecting histone acetylation. For instance, hMI-ER1ß could bind to histones and prevent them from being acetyltransferase substrates. As well, it is also possible that the results obtained are a result of hMI-ER1ß selectively binding to inactive CBP. Finally, it is also possible that hMI-ER1ß is inhibiting histone acetylation by affecting the conformation of CBP. Such a conformational change could alter the enzymatic activity of CBP and prevent histone acetylation.

Preliminary studies in our laboratory have demonstrated that hMI-ER1 binds to unacetylated histone H4 *in vitro* (Ding et al., unpublished data), initially suggesting a possible mechanism of HAT inhibition. Such a mechanism of HAT inhibition is not uncommon. For instance, it has been recently shown that a cellular complex termed INHAT (inhibitor of acetyltransferases) binds to histones and inhibits p300, CBP and PCAF mediated histone acetylation (Seo *et al.*, 2001), a mechanism known as histone masking. In general "histone masking" refers to the ability of a protein/s to bind to histones and mask them from being acetyltransferase substrates. Therefore, to further investigate the ability of hMI-ER1ß to inhibit histone acetylation, it was important to determine if hMI-ER1ß competes for histone binding.

To test whether hMI-ER1ß inhibits histone acetylation by competing for histone binding, HEK 293 cells were transiently cotransfected with the following plasmids: *myc + Br-Q, ß + Br-Q,* or *ß + flag.* Cell extracts were subjected to IP with anti-flag or with

anti-myc antibody and HAT assays were performed. The ability of Br-Q to acetylate histones, in the presence and absence of ß, was used in each experiment as positive and negative controls respectively. Additional samples immunoprecipitated for Br-Q were incubated with HAT master mix that had been preincubated with ß for 45 minutes at 37°C, and HAT assays were then performed. HAT master mix preincubated with myc or preincubated HAT master mix alone was added to immunoprecipitated Br-Q and used as a control.

If hMI-ER1ß competes for histone binding, few histones would remain in the supernatant and very little HAT activity should be recovered when incubated with Br-Q. However, if HMI-ER1ß does not compete for histone binding, the histones would remain in the supernatant and similar HAT activity should be detected as with the control (Br-Q incubated with HAT master mix preincubated with mix or preincubated alone).

Figure 3.6A shows that as expected, myc does not contain significant amounts of HAT activity and that the ability of Br-Q to acetylate histones was dramatically reduced in the presence of ß. In addition, Figure 3.6A shows that the Br-Q sample incubated with HAT master mix preincubated with immunoprecipitated ß for 45 minutes at 37°C contains similar HAT activity to the controls (Br-Q incubated with HAT master mix preincubated with myc or preincubated alone). These results suggest that inhibition of histone acetylation by hMI-ER1ß, *in vitro*, is not the result of competition for histone binding.

Figure 3.6: Inhibition of histone acetylation *in vitro* by hMI-ER1ß is not the result of competition for histone binding. HEK 293 Cells were transiently cotransfected with a total of 1.5μg of *myc + Br-Q, ß + Br-Q,* or *ß + flag* plasmids. Cell extracts were prepared 48 hours after transfection. **(A)** Cell lysates were further subjected to IP with anti-flag antibody (IP-flag; 1:750; lanes 3, 4, 5) or with anti-myc antibody (IP-myc; 1:100; lanes 1 & 2). HAT assays were performed as previously described and the samples were assayed for HAT activity (samples 1&2). Additional samples were incubated with HAT master mix preincubated with immunoprecipitated ß (MMPreß; lane 3), preincubated alone (MMPre; lane 4) or preincubated with immunoprecipitated myc (MMPremyc; lane 5). The histogram shows the average values and standard deviations from three independent experiments. Cells were also cotransfected with *Br-Q + myc* and the lysates subjected to IP with anti-flag. HAT assays were then performed on the samples and the overall results were normalized by the Br-Q activity to account for variance between experiments. **(B)** Additional cell lysates were added directly to sample buffer (lanes 1-5). WB analysis was performed using anti-flag antibody (anti-flag; 1:2000; lanes 1&2) or anti-myc antibody (anti-myc; 1:1000; lanes 3-5). Results were obtained from three independent experiments and shown is a representative WB, with the position of ß and Br-Q indicated by the arrowheads.

For these experiments, the expression of *ß* and *Br-Q* in transfected HEK 293 cells was verified by WB using an anti-flag or anti-myc antibody. As shown in Figure 3.6B, both proteins were significantly expressed in all samples tested for HAT activity.

(A)

Plasmid:	ß-myc + Br-Q	myc + Br-Q	myc + Br-Q	myc + Br-Q	myc + Br-Q
	1	2	3	4	5
IP-Flag:	-	-	+	+	+
IP-Myc:	+	+	-	-	-
MMPreß:	-	-	+	-	-
MMPre:	-	-	-	+	-
MMPremyc:	-	-	-	-	+

(B)
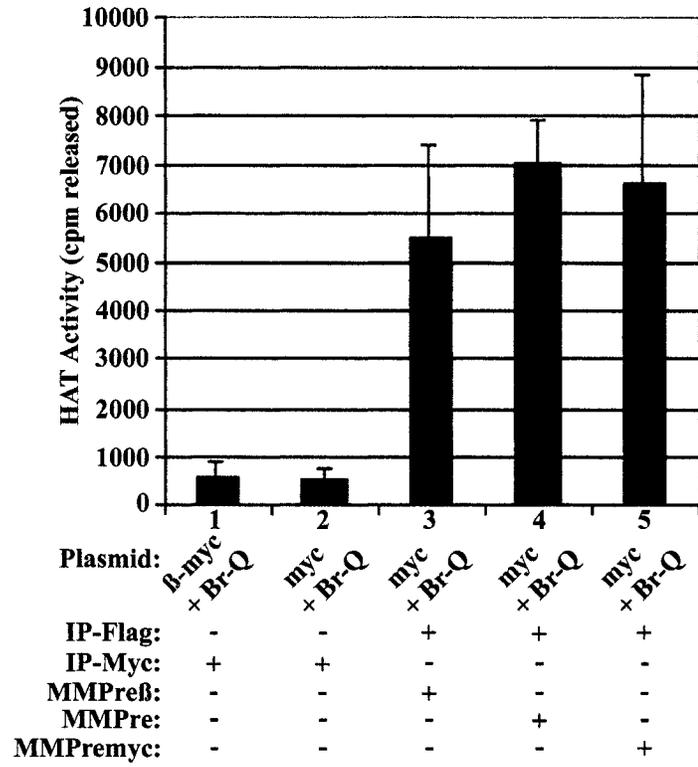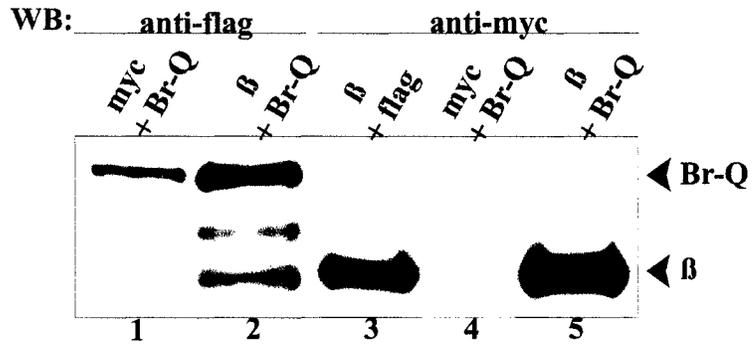
3.7: hMI-ER1β does not selectively bind to inactive CBP

As mentioned, it is also possible that the results obtained are a result of hMI-ER1β selectively binding to inactive CBP. The function of CBP, like most proteins, can be regulated by activating or inactivating its enzymatic activity. For instance, phosphorylation of CBP by cyclin E/Cdk2 increases HAT activity *in vitro* (Ait-Si-Ali *et al.*, 1998). As well, the protein E1A has been recently shown to inactivate CBP by binding to the C/H3 region and repressing its HAT activity (Chakravarti *et al.*, 1999).

HEK 293 cells were transiently cotransfected with *myc* + *Br-Q*, or β + *Br-Q* plasmids. Cell extracts were immunoprecipitated with an anti-myc or anti-flag antibody and assayed for HAT activity. To determine whether or not hMI-ER1β selectively binds to inactive CBP the supernatant was also recovered from both samples, immunoprecipitated with an anti-flag antibody, and assayed for HAT activity. If hMI-ER1β only binds to inactive Br-Q, all of the active Br-Q would remain in the supernatant. Therefore, one would expect to recover almost all of the same HAT activity as with Br-Q alone. However, if hMI-ER1β selectively binds to active Br-Q, all of the inactive Br-Q would remain in the supernatant and one would expect to recover very little HAT activity. Finally, if hMI-ER1β binds to both inactive and active Br-Q one would expect to recover less HAT activity as with Br-Q alone.

As shown in Figure 3.7A, myc does not contain any HAT activity similar to the results seen with Br-Q in the presence of β. The results also show that the supernatant from the Br-Q sample preincubated with β contained approximately half of the HAT activity as compared to the control (supernatant IP for Br-Q preincubated with myc), and

contained approximately 50 times more HAT activity than Br-Q in the presence of ß. The

results reveal that hMI-ER1ß does not selectively bind to inactive CBP *in vitro.*

For these experiments, the expression of ß and Br-Q in transfected HEK 293 cells

was verified by WB using an anti-flag or anti-myc antibody. As shown in Figure 3.7B,

both proteins were expressed in all samples tested for HAT activity.

Figure 3.7: hMI-ER1ß does not selectively bind to inactive CBP. HEK 293 Cells

were transiently cotransfected with a total of 1.5µg of either *ß* + *Br-Q*, or *myc* + *Br-Q*

plasmids, with cell extracts prepared 48 hours after transfection. **(A)** Cell lysates were

subjected to IP with anti-myc antibody (anti-myc; 1:100) and the immunoprecipitated

pellet (IP-Pellet) was assayed for HAT activity (lanes 2&4). The supernatant from each

samples was immunoprecipitated with anti-flag antibody (IP-Sup) and then assayed for

HAT activity (lanes 1&3). The histogram shows the average values and standard

deviations from three independent experiments. **(B)** Additional cell lysates were added

directly to sample buffer (lanes 1-4). WB analysis was performed using anti-flag

antibody (anti-flag; 1:2000; lanes 1&2) or anti-myc antibody (anti-myc; 1:1000; lanes

3&4). Results were obtained from three independent experiments and shown is a

representative WB, with the position of ß and Br-Q indicated.

(A)

Plasmid:	ß +Br-Q		myc +Br-Q	
IP-Sup:	+	−	+	−
IP-Pellet:	−	+	−	+
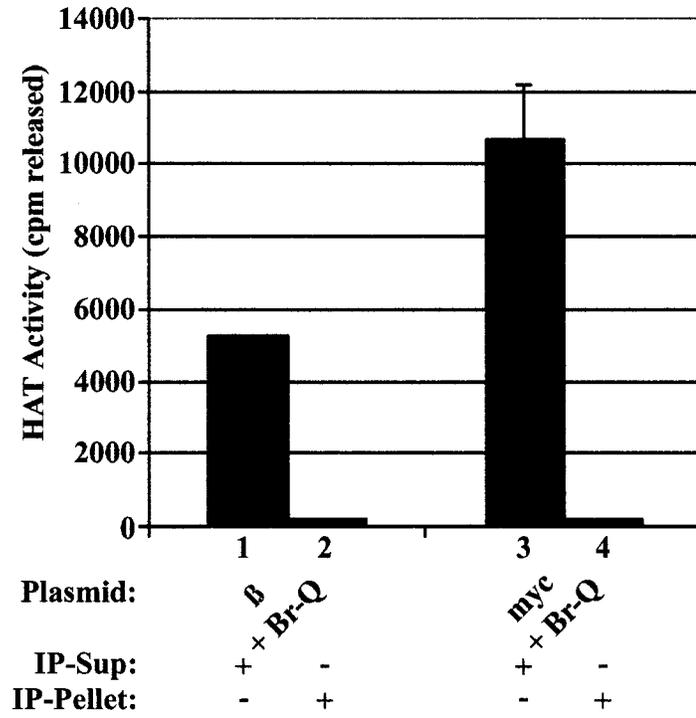
(B)
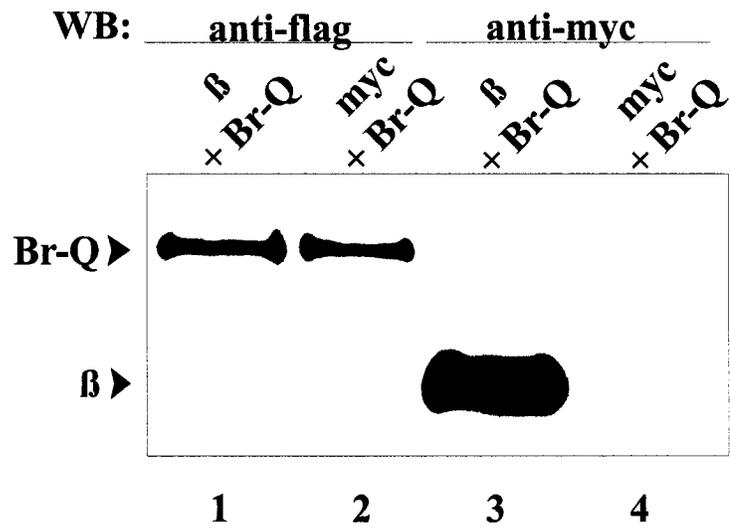
Chapter 4: Discussion

The deregulation of gene expression plays a very important role in the development of cancer. The altered patterns of gene expression are often the result of the inappropriate expression of a specific transcriptional activator or repressor (Kirmizis and Farham, 2004). For instance, the BRCA1 protein is a transcriptional regulator that is found to be mutated in approximately 8% of breast cancer cells. A mutation in BRCA1 increases the transcription of target genes by ERα, estrogen receptor α, thereby promoting proliferation of abnormal cells (Zheng *et al.,* 2001). Proto-oncogenes, or genes that have cancer causing potential, also play a very important role in breast cancer. For example, Her2, human epidermal growth factor receptor-type 2, is one proto-oncogene found to be overexpressed in 10-40% human breast cancer. An excess of Her2 protein signals for cells to divide, multiply and grow at a faster rate than normal cells, thus contributing to the occurrence and progression of cancer (Kurebayashi., 2001).

Previous studies have revealed that *hmi-er1* expression was upregulated in breast carcinoma cell lines and breast tissue, suggesting that *hmi-er1* is associated with the neoplastic state of human breast carcinoma (Paterno *et al.,* 1998). Recent work has also shown that hMI-ER1 can function as a transcriptional repressor by recruiting histone deacetylase, HDAC1, to its ELM2 domain. The results suggest a connection exists between *hmi-er*1 upregulation in breast cancer and its role in transcriptional regulation. The role of hMI-ER1 in transcriptional regulation is therefore very important, and further investigation is needed.

As mentioned, hMI-ER1 consists of many evolutionary conserved domains, such as the acidic activation domain, the ELM2 domain and the SANT domain. Although a list of interacting proteins has not been fully established, the presence of these domains suggests that there are potentially many possible interacting proteins. For example, the SANT domain within Ada2 has been shown to interact with the histone acetyltransferase, Gcn5. It was also shown that the interaction is needed for effective histone acetylation by Gcn5 (Sterner et al., 2002). Because hMI-ER1 contains a SANT domain, similar to that of Ada2, it was initially hypothesized that hMI-ER1 would also interact with a histone acetyltransferase, particularly CBP. An interaction between the two proteins is of interest because CBP plays a very important role in transcriptional regulation. The regulation of gene transcription is very complex in that it involves DNA-binding transcription factors, chromatin remodeling enzymes such as CBP, p300, PCAF, as well as other cofactors such as SRC and p/CIP (Perissi et al., 1999a). The histone acetyltransferase function of CBP, as well as other histone acetylases, has proven critical for transcription function (Chrivia et al., 1993), through its ability to modify chromatin structure, suggesting that gene regulation is tightly regulated. In addition, CBP can play an indirect role in transcriptional regulation by acetylating DNA binding transcription factors, such as p53 and GATA-1. Upon acetylation, these proteins have been shown to have increased DNA binding potential (Gu and Roeder, 1997; Boyes et al., 1998).

When compared to other histone acetyltransferases, CBP plays a critical role in a wide variety of cellular processes, including cell cycle control, differentiation and apoptosis. Mutations in CBP are associated with certain cancers and other human disease

processes. As a global coactivator in higher eukaryotes, CBP is one of the most potent and versatile of the histone acetyltransferases (Sterner et al., 2000).

As expected, an interaction between hMI-ER1ß and CBP was originally discovered in vitro, by performing GST pull down assays (Figure 3.1). However, unlike originally hypothesized, the SANT domain of hMI-ER1ß was not responsible for the interaction between the two proteins in vitro (Figure 3.7) Instead, a series of GST pull down assays demonstrated that it was the region found at the N-terminus of hMI-ER1, encompassing amino acids 1-179 containing the four stretches of highly acidic regions that is responsible. As mentioned, it was known that acidic amino acids play a very important role in transcriptional activation and the deletion construct harboring the first 3 acidic amino acid stretches (aa 1-98) of XMI-ER1 stimulated transcription 80 fold (Paterno et al., 1997). The finding that it is the region encompassing amino acids 1-179 that was responsible for the interaction, suggests that it may also be the region responsible for HAT inhibition as well. The involvement of acidic residues in inhibition of HAT activity is not uncommon. It was recently shown that the major HAT inhibitory domain of pp32 resides within a region of the acidic C-terminal residues (Seo et al., 2002). In addition, similar results were found for the Set/Taf-Iß and TAF-Iα proteins (Seo et al., 2001). It would be of interest to develop further constructs containing various deletions of the four stretches of acidic amino acids found at the N-terminus of hMI-ER1 for use in future GST pull down assays. The results would allow for the identification of the amino acids responsible for the interaction. Further HAT assays could be conducted using the deletion constructs to determine if the region for the interaction is also the

HAT inhibitory function. Overall, the results would map the HAT interacting and inhibiting domain(s) of hMI-ER1.

To further confirm that an interaction occurs between hMI-ER1ß and CBP, *in vivo* assays were conducted. HEK 293 cells were co-transfected with various combinations of *hMI-ER1ß* and *CBP* and cell extracts immunoprecipiated with an antibody for CBP. Western blotting was performed with an antibody for hMI-ER1ß and the results showed an interaction occurred between hMI-ER1ß and CBP (Figure 3.2). To further confirm that an interaction is not an artifact of overexpression, endogenous experiments were performed. However, problems were encountered trying to detect the CBP or hMI-ER1 protein (data not shown). It is well known that very large proteins, such as CBP, do not transfer very well during immunoblotting, which results in low levels of detection (as reviewed in Kurien and Scofield, 2003). Attempts were made to increase transfer efficiency by transferring for a longer period of time and lowering the percentage of gels used. However, the CBP protein could not be clearly detected. It is also possible that both proteins do interact within a cell however a better antibody or increased transfer efficiency is required for the detection. Therefore, future studies would include further testing for an endogenous interaction between CBP and hMI-ER1.

As mentioned, CBP mutations and inactivity is often associated with disease and *hMI-ER1* is upregulated in breast carcinoma. It was therefore important to further investigate the role of hMI-ER1 with respect to transcriptional regulation. *In vitro* HAT assays were performed on CBP and hMI-ER1ß protein co-expressed in HEK 293 cells. It was found that hMI-ER1 inhibits the ability of CBP to acetylate histones (Figure 3.3),

75

and therefore may function to repress transcription through such a mechanism. The concept that a protein can bind to CBP and repress transcription is not a novel idea. For instance, the Kaposi's sarcoma-associated herpesvirus K8 protein, p34SEI-1, the adenovirus E1A, RSK2, and PU.1 are all proteins that have been recently shown to repress the HAT activity of CBP (Chakravarti *et al.*, 1999; Merienne *et al.*, 2001; Hwang *et al.*, 2001; Hong *et al.*, 2002; Hirose *et al.*, 2003).

How CBP is regulated within a cell is still not fully understood. However, many studies have been conducted to determine if a protein regulates the HAT activity of CBP by either altering histone/ substrate recognition or the enzymatic activity of CBP. For instance, it has recently been shown that a multiprotein cellular complex, INHAT (inhibitor of acetyltransferases), associates with histones *in vivo* and blocks them from serving as acetylase substrates, suggesting a critical role for histone binding in HAT inhibition (Seo *et al.*, 2002). Therefore, the next goal was to determine if hMI-ER1 competes for histone binding blocking them from serving as acetylase substrates. Further HAT Assays were conducted and the results revealed that the inhibition of histone acetylation by hMI-ER1ß *in vitro* is not the result of competition for histone binding (Figure 3.5).

It is also possible that hMI-ER1ß selectively binds to inactive CBP. HAT assays were carried out to determine if hMI-ER1ß interacts with inactive CBP, active CBP, or both. The results obtained suggest that hMI-ER1ß binds to both inactive and active CBP, and with binding, hMI-ER1 functions to repress the HAT activity of previously active CBP (Figure 3.6).

76

Although the exact mechanisms of histone acetyltransferase inhibition have not yet been fully determined, many studies suggest that an interaction with CBP could induce conformational changes. Such a conformational change could alter the enzymatic activity of CBP. For instance, the HAT domain is adjacent to the C/H3 region of CBP, and it is often this region that corresponds to a variety of protein binding sites. The RSK-2, E1A, and KSHV proteins all inhibit acetyltransferase activity of CBP by binding to the C/H3 domain (Perissi *et al.*, 1999b;Merienne *et al.*, 2001;Hwang *et al.*, 2001). Structural studies, such as structural proteomics, could be conducted in the future to determine whether or not the conformation of CBP changes in the presence and absence of hMI-ER1. Structural proteomics is a recent development to help determine three dimensional protein structures and has a major application in the area of oncology (Jung and Lew, 2004).

It has also been shown that the adenoviral E1A oncoprotein represses transcription by binding to the C/H3 region of CBP thereby displacing the PCAF and p/CIP proteins form the complex (Chakravarti *et al.*, 1999). CBP is also found in an enzymatic complex with a variety of other histone acetylases, therefore the results suggest another possible mechanism of HAT inhibition upon protein binding.

In an attempt to further map the CBP domain/s involved in the physical interaction with hMI-ER1, CBP deletion mutants were made and used in pull down assays. The results reveal that the domain responsible for the interaction *in vitro* and *in vivo* is found between amino acids 1092-2441 which harbors the HAT, bromodomain, C/H2, C/H3, and Q-rich domains (Figures 3.4 & 3.5). In addition, no interaction was

found between the region encompassing amino acids 1-1095, which harbors the receptor interacting domain, the C/H1, and kinase induced interacting domain (Figure 3.4). In the future it would be of interest to further map the domain responsible by constructing CBP deletion constructs harboring each individual domain and performing GST pull down assays and co-transfections.

With the results obtained it is possible that hMI-ER1 could play a role in repressing transcription by interacting with CBP and inhibiting acetylation of histones. It has been demonstrated that hMI-ER1 binds to HDAC1 and functions to repress transcription. However the regulation of gene transcription, although extensively studied, has not been fully established. It is known that the acetylation state of histones is reversibly regulated by HAT and HDAC, and an imbalance between the two leads to abnormal behavior of the cell in morphology, cell cycle, differentiation and carcinogenesis (as reviewed in Kim *et al.*, 2003). Numerous studies also suggest that HDAC and HAT activity may not act independently and that their activity may be linked to one another (Perez-Martin and Johnson, 1998;Wittschieben *et al.*, 1999;Vogelauer *et al.*, 2000). For example, the proteins YY1 and Sp1 interact with both HAT and HDAC, thereby acquiring an activator or repressor function depending on the promoter context and other factors (Cress and Seto, 2000). In general, the results suggest that hMI-ER1 may function to repress transcription by regulating the activity of both HAT and HDAC. A regulation of both enzymes might ensure continuous transcriptional repression, preventing a switch to a transcriptionally active state.

Future studies are needed to more precisely determine the effect that hMI-ER1 has on HAT-mediated gene transcription. It is well documented that CBP plays a role in the development of a variety of diseases and that *hmi-er1* is upregulated in breast carcinoma cell lines and tissues, therefore a further understanding of the interaction between both proteins is needed. The results obtained in the present study indicate that hMI-ER1 plays a role in the functioning of CBP and therefore could have a huge impact on the development of breast cancer and other diseases where CBP is important in chromatin remodeling and gene expression.

One future goal is to further identify the region of CBP responsible for the interaction with hMI-ER1. Identifying the exact region might suggest a mechanism for HAT inhibition. To do this, GST pull down assays could be performed, using various deletions of the CBP Br-Q construct, to determine the interacting region *in vitro*. In addition, the deletion constructs could be used in a series of co-transfection assays with *hmi-er1* to determine the region responsible *in vivo*.

Although an interaction between CBP and hMI-ER1 was found in HEK 293 cells, it is possible that the results obtained are an artifact of the over-expression of both proteins. Therefore, further endogenous testing is required to support the findings thus far. In addition, experiments could be conducted to co-localize CBP and hMI-ER1 in the cell. To do this, immunocytochemistry and laser scanning confocal microscopy would be useful in an attempt to support the evidence for endogenous interaction. It would be possible that the interaction seen, as well as the effects of hMI-ER1 on CBP function, is

HEK 293 cell specific. In the future, it would be of interest to perform co-transfection, endogenous, and HAT assays on a variety of cell lines.

The demonstration that hMI-ER1 repressed HAT activity suggests that hMI-ER1 may play a role in HAT-mediated transcription. It has been shown that a Gal4-DNA binding domain fusion of CBP activates transcription in a HAT dependent manner when transfected with a TK-Luc reporter gene sensitive to CBP (Martinez-Balbas *et al.*, 1998;Seo *et al.*, 2001). Co-transfection of the Gal4-CBP and various *hmi-er1* constructs could be performed and the expression of Gal4 responsive reporter gene could be analyzed. The results would suggest whether or not hMI-ER1 has a regulatory role in HAT-mediated transcription.

It would also be of interest to determine if hMI-ER1 interacts with other histone acetyltransferases *in vivo*. For instance, Chakravarti et al. have recently shown that the adenovirus E1A interacts with CBP, p300, and PCAF and represses their HAT activity. It has been shown that E1A represses transcription by binding to the C/H3 region of both CBP and p300 thereby displacing PCAF and p/CIP proteins from the complex. In addition, the binding of E1A to the N terminus of PCAF may further repress transcription, since p300 and CBP also binds to the N terminus of PCAF (Chakravarti et al, 1999). Therefore, similar to the E1A protein, hMI-ER1 might also interact with such HATs as PCAF, p300, and Gcn5 and possibly inhibit their HAT activity.

It has also been demonstrated that hMI-ER1 inhibits the ability of CBP to acetylate the histone H4 peptide *in vitro*. Additional HAT assays could be employed using other individual histone subunits, such as histone H2A, H2B, and H3. It is possible

that hMI-ER1 may not have the same effect on acetylation of the other histones mentioned. Lastly, it would be of interest to purify chromatin (DNA wrapped around histones H2A, H2B, H3 and H4) from cells, to act as a natural template in HAT assays in the presence and absence of hMI-ER1. From this, the histones can be separated on a gel and the acetylation state of each specific histone determined. With the histones in a more natural state, the effects of hMI-ER1 on each specific histone can be precisely determined.

In conclusion, the purpose of this study was to determine if hMI-ER1ß could interact with CBP. If an interaction occurred, we wanted to determine the region of both CBP and hMI-ER1ß responsible and the effects of hMI-ER1 on CBP function.

The results clearly showed that hMI-ER1 does interact with CBP *in vitro* and *in vivo*, and it is a region found within the acidic activation domain of hMI-ER1 that is responsible. As well, further analysis revealed that hMI-ER1 functions to repress the HAT activity of CBP. Overall, the results suggest that hMI-ER1 may play a role in HAT-mediated transcription.

Reference List

Aasland,R., Stewart,A.F., Gibson,T. (1996). The SANT domain: a putative DNA-binding domain in the SWI-SNF and ADA complexes, the transcriptional co-repressor N-CoR and TFIIIB. Trends Biochem.Sci. *21*, 87-88.

Ait-Si-Ali,S., Ramirez,S., Barre,F.X., Dkhissi,F., Magnaghi-Jaulin,L., Girault,J.A., Robin,P., Knibiehler,M., Pritchard,L.L., Ducommun,B., Trouche,D., Harel-Bellan,A. (1998). Histone acetyltransferase activity of CBP is controlled by cycle-dependent kinases and oncoprotein E1A. Nature *396*, 184-186.

Alexandropoulos,K., Cheng,G., Baltimore,D. (1995). Proline-rich sequences that bind to Src homology 3 domains with individual specificities. Proc.Natl.Acad.Sci.U.S.A *92*, 3110-3114.

Andrisani,O.M. (1999). CREB-mediated transcriptional control. Crit Rev.Eukaryot.Gene Expr. *9*, 19-32.

Avantaggiati,M.L., Ogryzko,V., Gardner,K., Giordano,A., Levine,A.S., Kelly,K. (1997). Recruitment of p300/CBP in p53-dependent signal pathways. Cell *89*, 1175-1184.

Baird,A., Klagsbrun,M. (1991). The fibroblast growth factor family. Cancer Cells *3*, 239-243.

Bannister,A.J., Kouzarides,T. (1996a). The CBP co-activator is a histone acetyltransferase. Nature *384*, 641-643.

Blobel,G.A., Nakajima,T., Eckner,R., Montminy,M., Orkin,S.H. (1998). CREB-binding protein cooperates with transcription factor GATA-1 and is required for erythroid differentiation. Proc.Natl.Acad.Sci.U.S.A *95*, 2061-2066.

Borrow,J., Stanton,V.P., Jr., Andresen,J.M., Becher,R., Behm,F.G., Chaganti,R.S., Civin,C.I., Disteche,C., Dube,I., Frischauf,A.M., Horsman,D., Mitelman,F., Volinia,S., Watmore,A.E., Housman,D.E. (1996). The translocation t(8;16)(p11;p13) of acute myeloid leukaemia fuses a putative acetyltransferase to the CREB-binding protein. Nat.Genet. *14*, 33-41.

Boyes,J., Byfield,P., Nakatani,Y., Ogryzko,V. (1998). Regulation of activity of the transcription factor GATA-1 by acetylation. Nature *396*, 594-598.

Braunstein,M., Sobel,R.E., Allis,C.D., Turner,B.M., Broach,J.R. (1996). Efficient transcriptional silencing in Saccharomyces cerevisiae requires a heterochromatin histone acetylation pattern. Mol.Cell Biol. *16*, 4349-4356.

Candau,R., Moore,P.A., Wang,L., Barlev,N., Ying,C.Y., Rosen,C.A., Berger,S.L. (1996). Identification of human proteins functionally conserved with the yeast putative adaptors ADA2 and GCN5. Mol.Cell Biol. *16*, 593-602.

Chakravarti,D., Ogryzko,V., Kao,H.Y., Nash,A., Chen,H., Nakatani,Y., Evans,R.M. (1999). A viral mechanism for inhibition of p300 and PCAF acetyltransferase activity. Cell *96*, 393-403.

Chan,H.M., La Thangue,N.B. (2001). p300/CBP proteins: HATs for transcriptional bridges and scaffolds. J.Cell Sci. *114*, 2363-2373.

Chen,C.J., Deng,Z., Kim,A.Y., Blobel,G.A., Lieberman,P.M. (2001). Stimulation of CREB binding protein nucleosomal histone acetyltransferase activity by a class of transcriptional activators. Mol.Cell Biol. *21*, 476-487.

Chen,H., Lin,R.J., Schiltz,R.L., Chakravarti,D., Nash,A., Nagy,L., Privalsky,M.L., Nakatani,Y., Evans,R.M. (1997). Nuclear receptor coactivator ACTR is a novel histone acetyltransferase and forms a multimeric activation complex with P/CAF and CBP/p300. Cell *90*, 569-580.

Chrivia,J.C., Kwok,R.P., Lamb,N., Hagiwara,M., Montminy,M.R., Goodman,R.H. (1993). Phosphorylated CREB binds specifically to the nuclear protein CBP. Nature *365*, 855-859.

Cohen,G.B., Ren,R., Baltimore,D. (1995). Modular binding domains in signal transduction proteins. Cell *80*, 237-248.

Cress,W.D., Seto,E. (2000). Histone deacetylases, transcriptional control, and cancer. J.Cell Physiol *184*, 1-16.

Dhalluin,C., Carlson,J.E., Zeng,L., He,C., Aggarwal,A.K., Zhou,M.M. (1999). Structure and ligand of a histone acetyltransferase bromodomain. Nature *399*, 491-496.

Ding,Z., Gillespie,L.L., Paterno,G.D. (2003). Human MI-ER1 alpha and beta function as transcriptional repressors by recruitment of histone deacetylase 1 to their conserved ELM2 domain. Mol.Cell Biol. *23*, 250-258.

Giles,R.H., Peters,D.J., Breuning,M.H. (1998). Conjunction dysfunction: CBP/p300 in human disease. Trends Genet. *14*, 178-183.

Gillespie,L.L., Paterno,G.D., Mahadevan,L.C., Slack,J.M. (1992). Intracellular signalling pathways involved in mesoderm induction by FGF. Mech.Dev. *38*, 99-107.

Goodman,R.H., Smolik,S. (2000). CBP/p300 in cell growth, transformation, and development. Genes Dev. *14*, 1553-1577.

Gu,W., Roeder,R.G. (1997). Activation of p53 sequence-specific DNA binding by acetylation of the p53 C-terminal domain. Cell *90*, 595-606.

Guenther,M.G., Barak,O., Lazar,M.A. (2001). The SMRT and N-CoR corepressors are activating cofactors for histone deacetylase 3. Mol.Cell Biol. *21*, 6091-6101.

Hamamori,Y., Sartorelli,V., Ogryzko,V., Puri,P.L., Wu,H.Y., Wang,J.Y., Nakatani,Y., Kedes,L. (1999). Regulation of histone acetyltransferases p300 and PCAF by the bHLH protein twist and adenoviral oncoprotein E1A. Cell *96*, 405-413.

Hauser,C., Schuettengruber,B., Bartl,S., Lagger,G., Seiser,C. (2002). Activation of the mouse histone deacetylase 1 gene by cooperative histone phosphorylation and acetylation. Mol.Cell Biol. *22*, 7820-7830.

Heery,D.M., Kalkhoven,E., Hoare,S., Parker,M.G. (1997). A signature motif in transcriptional co-activators mediates binding to nuclear receptors. Nature *387*, 733-736.

Hirose,T., Fujii,R., Nakamura,H., Aratani,S., Fujita,H., Nakazawa,M., Nakamura,K., Nishioka,K., Nakajima,T. (2003). Regulation of CREB-mediated transcription by association of CDK4 binding protein p34SEI-1 with CBP. Int.J.Mol.Med. *11*, 705-712.

Hong,W., Kim,A.Y., Ky,S., Rakowski,C., Seo,S.B., Chakravarti,D., Atchison,M., Blobel,G.A. (2002). Inhibition of CBP-mediated protein acetylation by the Ets family oncoprotein PU.1. Mol.Cell Biol. *22*, 3729-3743.

Hudson,B.P., Martinez-Yamout,M.A., Dyson,H.J., Wright,P.E. (2000). Solution structure and acetyl-lysine binding activity of the GCN5 bromodomain. J.Mol.Biol. *304*, 355-370.

Hwang,S., Gwack,Y., Byun,H., Lim,C., Choe,J. (2001). The Kaposi's sarcoma-associated herpesvirus K8 protein interacts with CREB-binding protein (CBP) and represses CBP-mediated transcription. J.Virol. *75*, 9509-9516.

Imhof,A., Yang,X.J., Ogryzko,V.V., Nakatani,Y., Wolffe,A.P., Ge,H. (1997). Acetylation of general transcription factors by histone acetyltransferases. Curr.Biol. *7*, 689-692.

Jacobson,R.H., Ladurner,A.G., King,D.S., Tjian,R. (2000). Structure and function of a human TAFII250 double bromodomain module. Science *288*, 1422-1425.

Jung,J.W., Lew,W. (2004). Structure-based functional discovery of proteins: structural proteomics. J Biochem Mol Biol *37*, 28-34.

Kim,D.H., Kim,M., Kwon,H.J. (2003). Histone deacetylase in carcinogenesis and its inhibitors as anti-cancer agents. J.Biochem.Mol.Biol. *36*, 110-119.

Kirmizis,A.,Fraham,P.J. (2004). Genomic approaches that aid in the identification of transcription factor target genes.Exp Biol Med. 229 (8), 705-21.

Korzus,E., Torchia,J., Rose,D.W., Xu,L., Kurokawa,R., McInerney,E.M., Mullen,T.M., Glass,C.K., Rosenfeld,M.G. (1998). Transcription factor-specific requirements for coactivators and their acetyltransferase functions. Science *279*, 703-707.

Krumm,A., Madisen,L., Yang,X.J., Goodman,R., Nakatani,Y., Groudine,M. (1998). Long-distance transcriptional enhancement by the histone acetyltransferase PCAF. Proc.Natl.Acad.Sci.U.S.A *95*, 13501-13506.

Kung,A.L., Rebel,V.I., Bronson,R.T., Ch'ng,L.E., Sieff,C.A., Livingston,D.M., Yao,T.P. (2000). Gene dose-dependent control of hematopoiesis and hematologic tumor suppression by CBP. Genes Dev. *14*, 272-277.

Kuo,M.H., Zhou,J., Jambeck,P., Churchill,M.E., Allis,C.D. (1998). Histone acetyltransferase activity of yeast Gcn5p is required for the activation of target genes in vivo. Genes Dev. *12*, 627-639.

Kurebayashi,J. (2001). Biological and clinical significance of HER2 overexpression in breast cancer.Breast Cancer. *8*, 45-51.

Kurien,B.T., Scofield,R.H. (2003). Protein blotting: a review. J.Immunol.Methods *274*, 1-15.

Kurokawa,R., Kalafus,D., Ogliastro,M.H., Kioussi,C., Xu,L., Torchia,J., Rosenfeld,M.G., Glass,C.K. (1998). Differential use of CREB binding protein-coactivator complexes. Science *279*, 700-703.

Legube,G., Trouche,D. (2003a). Identification of a larger form of the histone acetyl transferase Tip60. Gene *310*, 161-168.

Legube,G., Trouche,D. (2003b). Regulating histone acetyltransferases and deacetylases. EMBO Rep. *4*, 944-947.

Li,Q.J., Yang,S.H., Maeda,Y., Sladek,F.M., Sharrocks,A.D., Martins-Green,M. (2003). MAP kinase phosphorylation-dependent activation of Elk-1 leads to activation of the co-activator p300. EMBO J. *22*, 281-291.

Lill,N.L., Grossman,S.R., Ginsberg,D., DeCaprio,J., Livingston,D.M. (1997). Binding and modulation of p53 by p300/CBP coactivators. Nature *387*, 823-827.

Ma,J., Ptashne,M. (1987). Deletion analysis of GAL4 defines two transcriptional activating segments. Cell *48*, 847-853.

Martinez-Balbas,M.A., Bannister,A.J., Martin,K., Haus-Seuffert,P., Meisterernst,M., Kouzarides,T. (1998). The acetyltransferase activity of CBP stimulates transcription. EMBO J. *17*, 2886-2893.

McManus,K.J., Hendzel,M.J. (2001). CBP, a transcriptional coactivator and acetyltransferase. Biochem.Cell Biol. *79*, 253-266.

Mehra-Chaudhary,R., Matsui,H., Raghow,R. (2001). Msx3 protein recruits histone deacetylase to down-regulate the Msx1 promoter. Biochem.J. *353*, 13-22.

Merienne,K., Pannetier,S., Harel-Bellan,A., Sassone-Corsi,P. (2001). Mitogen-regulated RSK2-CBP interaction controls their kinase and acetylase activities. Mol.Cell Biol. *21*, 7089-7096.

Miller,R.W., Rubinstein,J.H. (1995). Tumors in Rubinstein-Taybi syndrome. Am.J.Med.Genet. *56*, 112-115.

Murata,T., Kurokawa,R., Krones,A., Tatsumi,K., Ishii,M., Taki,T., Masuno,M., Ohashi,H., Yanagisawa,M., Rosenfeld,M.G., Glass,C.K., Hayashi,Y. (2001). Defect of histone acetyltransferase activity of the nuclear transcriptional coactivator CBP in Rubinstein-Taybi syndrome. Hum.Mol.Genet. *10*, 1071-1076.

Nicolson,G.L., Nawa,A., Toh,Y., Taniguchi,S., Nishimori,K., Moustafa,A. (2003). Tumor metastasis-associated human MTA1 gene and its MTA1 protein product: role in epithelial cancer cell invasion, proliferation and nuclear regulation. Clin.Exp.Metastasis *20*, 19-24.

Ogryzko,V.V., Schiltz,R.L., Russanova,V., Howard,B.H., Nakatani,Y. (1996). The transcriptional coactivators p300 and CBP are histone acetyltransferases. Cell *87*, 953-959.

Ornaghi,P., Ballario,P., Lena,A.M., Gonzalez,A., Filetici,P. (1999). The bromodomain of Gcn5p interacts in vitro with specific residues in the N terminus of histone H4. J.Mol.Biol. *287*, 1-7.

Ornitz,D.M., Itoh,N. (2001). Fibroblast growth factors. Genome Biol. *2*, REVIEWS3005.

Paterno,G.D., Ding,Z., Lew,Y.Y., Nash,G.W., Mercer,F.C., Gillespie,L.L. (2002). Genomic organization of the human mi-er1 gene and characterization of alternatively spliced isoforms: regulated use of a facultative intron determines subcellular localization. Gene *295*, 79-88.

Paterno,G.D., Li,Y., Luchman,H.A., Ryan,P.J., Gillespie,L.L. (1997). cDNA cloning of a novel, developmentally regulated immediate early gene activated by fibroblast growth factor and encoding a nuclear protein. J.Biol.Chem. *272*, 25591-25595.

Paterno,G.D., Mercer,F.C., Chayter,J.J., Yang,X., Robb,J.D., Gillespie,L.L. (1998). Molecular cloning of human er1 cDNA and its differential expression in breast tumours and tumour-derived cell lines. Gene *222*, 77-82.

Perez-Martin,J., Johnson,A.D. (1998). Mutations in chromatin components suppress a defect of Gcn5 protein in Saccharomyces cerevisiae. Mol.Cell Biol. *18*, 1049-1054.

Perissi,V., Dasen,J.S., Kurokawa,R., Wang,Z., Korzus,E., Rose,D.W., Glass,C.K., Rosenfeld,M.G. (1999a). Factor-specific modulation of CREB-binding protein acetyltransferase activity. Proc.Natl.Acad.Sci.U.S.A *96*, 3652-3657.

Perissi,V., Dasen,J.S., Kurokawa,R., Wang,Z., Korzus,E., Rose,D.W., Glass,C.K., Rosenfeld,M.G. (1999b). Factor-specific modulation of CREB-binding protein acetyltransferase activity. Proc.Natl.Acad.Sci.U.S.A *96*, 3652-3657.

Petrij,F., Dauwerse,H.G., Blough,R.I., Giles,R.H., van der Smagt,J.J., Wallerstein,R., Maaswinkel-Mooy,P.D., van Karnebeek,C.D., van Ommen,G.J., van Haeringen,A., Rubinstein,J.H., Saal,H.M., Hennekam,R.C., Peters,D.J., Breuning,M.H. (2000a). Diagnostic analysis of the Rubinstein-Taybi syndrome: five cosmids should be used for microdeletion detection and low number of protein truncating mutations. J.Med.Genet. *37*, 168-176.

Petrij,F., Dauwerse,H.G., Blough,R.I., Giles,R.H., van der Smagt,J.J., Wallerstein,R., Maaswinkel-Mooy,P.D., van Karnebeek,C.D., van Ommen,G.J., van Haeringen,A., Rubinstein,J.H., Saal,H.M., Hennekam,R.C., Peters,D.J., Breuning,M.H. (2000b). Diagnostic analysis of the Rubinstein-Taybi syndrome: five cosmids should be used for microdeletion detection and low number of protein truncating mutations. J.Med.Genet. *37*, 168-176.

Petrij,F., Giles,R.H., Dauwerse,H.G., Saris,J.J., Hennekam,R.C., Masuno,M., Tommerup,N., van Ommen,G.J., Goodman,R.H., Peters,D.J., . (1995a). Rubinstein-Taybi syndrome caused by mutations in the transcriptional co-activator CBP. Nature *376*, 348-351.

Petrij,F., Giles,R.H., Dauwerse,H.G., Saris,J.J., Hennekam,R.C., Masuno,M., Tommerup,N., van Ommen,G.J., Goodman,R.H., Peters,D.J., . (1995b). Rubinstein-Taybi syndrome caused by mutations in the transcriptional co-activator CBP. Nature *376*, 348-351.

Powers,C.J., McLeskey,S.W., Wellstein,A. (2000). Fibroblast growth factors, their receptors and signaling. Endocr.Relat Cancer *7*, 165-197.

Rubinstein,J.H., TAYBI,H. (1963). Broad thumbs and toes and facial abnormalities. A possible mental retardation syndrome. Am.J.Dis.Child *105*, 588-608.

Seo,S.B., Macfarlan,T., McNamara,P., Hong,R., Mukai,Y., Heo,S., Chakravarti,D. (2002). Regulation of histone acetylation and transcription by nuclear protein pp32, a subunit of the INHAT complex. J.Biol.Chem. *277*, 14005-14010.

Seo,S.B., McNamara,P., Heo,S., Turner,A., Lane,W.S., Chakravarti,D. (2001). Regulation of histone acetylation and transcription by INHAT, a human cellular complex containing the set oncoprotein. Cell *104*, 119-130.

Shen,W.F., Krishnan,K., Lawrence,H.J., Largman,C. (2001). The HOX homeodomain proteins block CBP histone acetyltransferase activity. Mol.Cell Biol. *21*, 7509-7522.

Slack,J.M., Darlington,B.G., Heath,J.K., Godsave,S.F. (1987). Mesoderm induction in early Xenopus embryos by heparin-binding growth factors. Nature *326*, 197-200.

Solari,F., Bateman,A., Ahringer,J. (1999). The Caenorhabditis elegans genes egl-27 and egr-1 are similar to MTA1, a member of a chromatin regulatory complex, and are redundantly required for embryonic patterning. Development *126*, 2483-2494.

Soutoglou,E., Viollet,B., Vaxillaire,M., Yaniv,M., Pontoglio,M., Talianidis,I. (2001). Transcription factor-dependent regulation of CBP and P/CAF histone acetyltransferase activity. EMBO J. *20*, 1984-1992.

Spencer,T.E., Jenster,G., Burcin,M.M., Allis,C.D., Zhou,J., Mizzen,C.A., McKenna,N.J., Onate,S.A., Tsai,S.Y., Tsai,M.J., O'Malley,B.W. (1997). Steroid receptor coactivator-1 is a histone acetyltransferase. Nature *389*, 194-198.

Sterner,D.E., Berger,S.L. (2000a). Acetylation of histones and transcription-related factors. Microbiol.Mol.Biol.Rev. *64*, 435-459.

Sterner,D.E., Wang,X., Bloom,M.H., Simon,G.M., Berger,S.L. (2002). The SANT domain of Ada2 is required for normal acetylation of histones by the yeast SAGA complex. J.Biol.Chem. *277*, 8178-8186.

Taine,L., Goizet,C., Wen,Z.Q., Petrij,F., Breuning,M.H., Ayme,S., Saura,R., Arveiler,B., Lacombe,D. (1998). Submicroscopic deletion of chromosome 16p13.3 in patients with Rubinstein-Taybi syndrome. Am.J.Med.Genet. *78*, 267-270.

Tanaka,Y., Naruse,I., Maekawa,T., Masuya,H., Shiroishi,T., Ishii,S. (1997). Abnormal skeletal patterning in embryos lacking a single Cbp allele: a partial similarity with Rubinstein-Taybi syndrome. Proc.Natl.Acad.Sci.U.S.A *94*, 10215-10220.

Teplitsky,Y., Paterno,G.D., Gillespie,L.L. (2003). Proline365 is a critical residue for the activity of XMI-ER1 in Xenopus embryonic development. Biochem.Biophys.Res.Commun. *308*, 679-683.

Timmermann,S., Lehrmann,H., Polesskaya,A., Harel-Bellan,A. (2001). Histone acetylation and disease. Cell Mol.Life Sci. *58*, 728-736.

Vogelauer,M., Wu,J., Suka,N., Grunstein,M. (2000). Global histone acetylation and deacetylation in yeast. Nature *408*, 495-498.

Vogelstein,B., Lane,D., Levine,A.J. (2000). Surfing the p53 network. Nature *408*, 307-310.

Wittschieben,B.O., Otero,G., de Bizemont,T., Fellows,J., Erdjument-Bromage,H., Ohba,R., Li,Y., Allis,C.D., Tempst,P., Svejstrup,J.Q. (1999). A novel histone acetyltransferase is an integral subunit of elongating RNA polymerase II holoenzyme. Mol.Cell *4*, 123-128.

Wydner,K.L., Bhattacharya,S., Eckner,R., Lawrence,J.B., Livingston,D.M. (1995). Localization of human CREB-binding protein gene (CREBBP) to 16p13.2-p13.3 by fluorescence in situ hybridization. Genomics *30*, 395-396.

Yao,T.P., Ku,G., Zhou,N., Scully,R., Livingston,D.M. (1996). The nuclear hormone receptor coactivator SRC-1 is a specific target of p300. Proc.Natl.Acad.Sci.U.S.A *93*, 10626-10631.

You,A., Tong,J.K., Grozinger,C.M., Schreiber,S.L. (2001). CoREST is an integral component of the Co. Proc.Natl.Acad.Sci.U.S.A *98*, 1454-1458.

Zheng,L., Annab,L.A., Afshari, C.A., Lee,W.H., Boyer, T.G. (2001). BRCA1 mediates ligand-independent transcriptional repression of the estrogen receptor. Proc.Natl.Acad.Sci.U.S.A *98*, 9587-9592.