The Monocytic Leukemia Zinc Finger Protein MOZ and its Related Factor MORF

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Ce qui a été sera; ce qui s'est fait se fera; il n'y a rien de nouveau sous le soleil. Y a-t-il une chose dont on dise: "Vois, c'est du nouveau?" - cette chose existait déjà aux siècles qui nous ont précédés.

L'Écclésiaste 1, 9-10.

Abstract

Regulation of chromatin structure involves histone modifications such as acetylation. Since 1996, the identification and characterization of histone acetyltransferases have had tremendous impact on our understanding of the molecular mechanisms related to eukaryotic gene regulation and human diseases associated with abnormal chromatin functions. The MYST family of histone acetyltransferases is very interesting because of their various biological functions. In agreement with the correlation between aberrant histone acetylation and cancer, the MYST family proteins MOZ and MORF are linked to leukemogenesis.

Identification and characterization of a gene encoding a novel histone acetyltransferase were the goals of this thesis project. Human MORF gene was cloned and the encoded protein, MORF, was shown to be very similar to MOZ. Biochemical studies demonstrated that both MOZ and MORF possess intrinsic histone acetyltransferase activities. The amino- and carboxy-terminal regions of MOZ and MORF contain transcriptional repression and activation domains, respectively.

Runx2, an osteoblast-specific transcription factor, binds to the activation domains of MOZ and MORF and thus recruits them to the osteocalcin promoter for transcriptional activation. TAZ, a WW-domain transcriptional coactivator of Runx2, potentiates the transcriptional activation of the osteocalcin promoter by MOZ and Runx2. Interestingly, treatment of cells with PMA enhances the synergy between MOZ and TAZ in activating the osteocalcin promoter. Consistent with this, PMA treatment strengthens the interaction of Runx2 with MOZ and TAZ.

This study, therefore, identified the histone acetyltransferase MORF and demonstrated that MOZ and MORF are transcriptional coactivators, thus providing new insights into how histone acetyltransferases are implicated in cell differentiation and leukemogenesis.

Résumé

La structure chromatidienne est régulée par différentes modifications post-traductionnelles des histones, comme l'acétylation. Depuis 1996, l'identification et la caractérisation d'histones acétyltransférases nous ont permis de mieux comprendre la régulation des gènes et les maladies associées au dérèglement de la fonction chromatidienne. La famille MYST d'histones acétyltransférases est très importante, car différentes fonctions biologiques ont été attribuées à ses membres. Une protéine de la famille MYST, MOZ, est une cible importante dans les processus oncogéniques. Des études ont aussi révélé une corrélation entre l'acétylation aberrante des histones et le développement de cancers.

Cette thèse rapporte l'identification et la caractérisation d'une nouvelle histone acétyltransférase: MORF. Le clonage du gène humain MORF que nous avons effectué nous a permis d'observer une ressemblance au gène MOZ. Nos études biochimiques ont aussi démontré que MOZ et MORF sont des histones acétyltransférases. Les extrémités amino et carboxyl-terminales de MOZ et MORF répriment et activent la transcription, respectivement.

Le facteur de la transcription Runx2 joue un rôle crucial dans la différenciation des cellules ostéoblastiques. Nous avons trouvé que les régions activatrices de MOZ et MORF lient Runx2 et augmentent l'activité transcriptionelle de ce dernier au promoteur du gène ostéocalcine. TAZ, un coactivateur de Runx2, stimule davantage l'activité transcriptionelle de MOZ et Runx2 au promoteur ostéocalcine. Il est d'intérêt de noter le fait que la stimulation de cellules avec du PMA augmente la synergie transcriptionelle observée par MOZ, TAZ et Runx2 au promoteur ostéocalcine. De plus, la stimulation

des cellules avec du PMA augmente considérablement l'interaction de Runx2 à MOZ et TAZ.

Ces résultats nous renseignent sur de nouvelles fonctions biologiques attribuées à MOZ et à MORF, et comment ces protéines sont impliquées dans le développement de la leucémie et dans la différentiation ostéoblastique.

Acknowledgements

In a Ph.D. thesis the first thanks usually goes to the research director. For the present thesis, this first place is genuinely deserved by my research director – Dr. Xiang-Jiao Yang. Indeed, I feel indebted to him to have been my scientific mentor; from teaching me the way to resolve the numerous problems and discrepancies which constitute the daily task of a researcher, to assessing and orienting a project in its entirety. This was possible through his exceptional availability, and his care to steadily assist me. His qualities went beyond Science, as he never asked from others what he did not do himself. For all this, I am thankful to you Xiang-Jiao.

I would like to thank Dr. Nathalie Champagne and Miss Siew-Lee Goh for the help they provided me during my experimental work. I highly appreciated their efforts and collaboration which allowed me to obtain important results.

My gratitude is extended to Mr. Nick Bertos, whose expertise with computers made him not only a precious asset for our group, but also an irreplaceable help for the editing and presentation of so many documents, including the present thesis.

Preface

The Guidelines for Thesis preparation issued by The Faculty of Graduate Studies and Research at McGill University reads as follows:

"Candidates have the opinion of including, as part of the thesis, the text of one or more papers submitted, or to be submitted, for publication, or the clearly duplicated text (not the reprints) of one or more published papers. These texts must conform to the "Guidelines for Thesis Preparation" with respect to font size, line spacing and margin sizes and must be bound together as an integral part of the thesis.

The thesis must be more than a collection of manuscripts. All components must be integrated into a cohesive unit with a logical progression from one chapter to the next. In order to ensure that the thesis has continuity, connecting texts that provide logical bridges between the different papers are mandatory.

In addition to the manuscripts, the thesis must include the following: (a) a table of contents, (b) and abstract in English and French, (c) an introduction which clearly states the rational and objectives of the research, (d) a comprehensive review of the literature (in addition to that covered in the introduction to each paper), (e) a final conclusion and summary.

As manuscripts for publication are frequently very concise documents, where appropriate, additional material must be provided (e.g., in appendices) in sufficient detail to allow a clear and precise judgment to be made of the importance and originality of the research reported in the thesis.

In general, when co-authored papers are included in a thesis, the candidate must have made a substantial contribution to all papers included in the thesis. In addition, the candidate is required to make an explicit statement in the thesis as to who contributed to such work and to what extent. This statement should appear in a single section entitled "Contributions of Authors" as a preface to the thesis. The supervisor must attest to the accuracy of this statement at the doctoral oral defense. Since the task of the examiners is made more difficult in these cases, it is in the candidate's interest to clearly specify the responsibilities of all the authors of the coauthored papers. When previously published copyright material is presented in a thesis, the candidate must include signed waivers from the co-authors and publishers and submit these to the Thesis Office with the final deposition, if not submitted previously."

I have chosen to write my thesis according to the guidelines, with two published papers and one to be submitted manuscript. The thesis is organized in seven chapters: (I) Literature review, (II - IV) Manuscripts with its own abstract, introduction, materials and methods, results, and discussion, (V) General discussion, and (VI) contribution to original research.

Publications arising from the work of this thesis

Publications directly related to the thesis project:

1. Champagne N§, Bertos NR§, **Pelletier N§**, Wang AH, Vezmar M, Yang Y, Heng HH, and Yang XJ. (1999) Identification of a human histone acetyltransferase related to monocytic leukemia zinc finger protein. *J.Biol. Chem.* 274: 28528-28536.
§ The authors contributed equally to this work

2. Champagne N, **Pelletier N**, and Yang XJ. (2001) The monocytic leukemia zinc finger protein MOZ is a histone acetyltransferase. *Oncogene* 20: 404-409.

3. **Pelletier N**, Champagne N, Stifani S, and Yang XJ. (2002) MOZ and MORF histone acetyltransferases interact with the Runt-domain transcription factor Runx2. *Oncogene* 21: 2729-2740.

4. **Pelletier N**, Champagne N, Lim H, and Yang XJ. (2003) Expression, purification, and analysis of MOZ and MORF histone acetyltransferases. *Methods* 31:24-32.

5. **Pelletier N**, Goh SL, Miao D, Champagne N, Goltzman D, and Yang XJ. (2003) Signal-dependent regulation of Runx2 transcriptional activity by MOZ and the WW-domain coactivator TAZ. (to be submitted)

6. Biggs JR, Zhang Y, **Pelletier N**, Yang XJ, Kraft AS. (2003) The regulation of AML1c transcriptional activity by phosphorylation. (submitted to Mol. Cell. Biol.)

Other publications:

1. Wang AH, Bertos NR, Vezmar M, **Pelletier N**, Crosato M, Heng HH, Th'ng J, Han J, and Yang XJ. (1999) HDAC4, a human histone deacetylase related to yeast HDA1, is a transcriptional corepressor. *Mol. Cell. Biol.* 19: 7816-7827.

2. Fernandes I, Bastien Y, Wai T, Nygard K, Lin R, Cormier O, Lee HS, Eng F, Bertos NR, **Pelletier N**, Mader S, Han VKM, Yang XJ, White JH. (2003) Ligand-dependent nuclear receptor corepressor LCoR functions by histone deacetylase-dependent and-independent mechanisms. *Mol. Cell* 11:139-150.

Contributions of Authors

1. Identification of a human histone acetyltransferase related to monocytic leukemia zinc finger protein.

Bertos NR:
He performed some HAT assays.

Champagne N:
She did reporter gene assays and cloned several domains of MORF.

Heng HH:
He performed fluorescence in situ hybridization (FISH) assay.

Wang AH and Yang Y:
They constructed several mutants of MORF.

2. MOZ and MORF histone acetyltransferases interact with the Runt-domain transcription factor Runx2.

Champagne N:
She performed some of the reporter gene assays.

3. Signal-dependent regulation of Runx2 transcriptional activity by MOZ and the WW-domain coactivator TAZ.

Champagne N:
She initially identified the synergistic activity between MOZ, TAZ and Cbfa1. Also, she cloned different MOZ domains in vectors required for reporter gene assays and immunoprecipitations.

Goh SL:
She performed some of the reporter assays.

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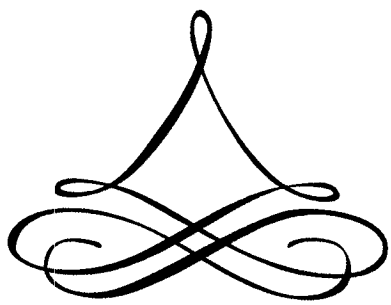
CHAPTER I

LITERATURE REVIEW



1. Chromatin Structure

An eukaryotic cell contains different compartments with specialized functions, one of which is the nucleus occupying about 10% of the total cell volume. The nucleus is delimited by a nuclear envelope formed by two concentric membranes. These membranes are punctured at intervals by nuclear pores that permit the shuttling of molecules to and from the cytoplasm. The nucleus contains the genetic information distributed into 24 pairs of chromosomes which are themselves composed of very long DNA molecules and associated proteins involved in the structure of the chromosomes or in the transmission of the genetic information to the next cell generation. The DNA molecules contain all the information to make thousands of different proteins and RNA molecules. Each cell type expresses only a subset of its genes and thus, different types of cells arise.

Chromosomes are composed of two types of domains, euchromatin and heterochromatin. Euchromatic domains are accessible to DNA binding factors (e.g. transcription factors) and are transcriptionally active portions of the genome. Heterochromatic domains, in contrast, are generally inaccessible to transcription factors and are transcriptionally silent. Large blocks of heterochromatin surround functional chromosome structures such as centromeres and telomeres.

Eukaryotic cells contain from 10 million to 100 billion DNA base pairs in a nucleus just a few microns in diameter. The DNA molecules that comprise the human genome would span almost 2 meters in length if they were laid end to end (Alberts et al., 1998). Nuclear DNA is not an open structure, but is highly packaged by the histone proteins into a hierarchical structure called the chromatin (Figure 1). The folding of

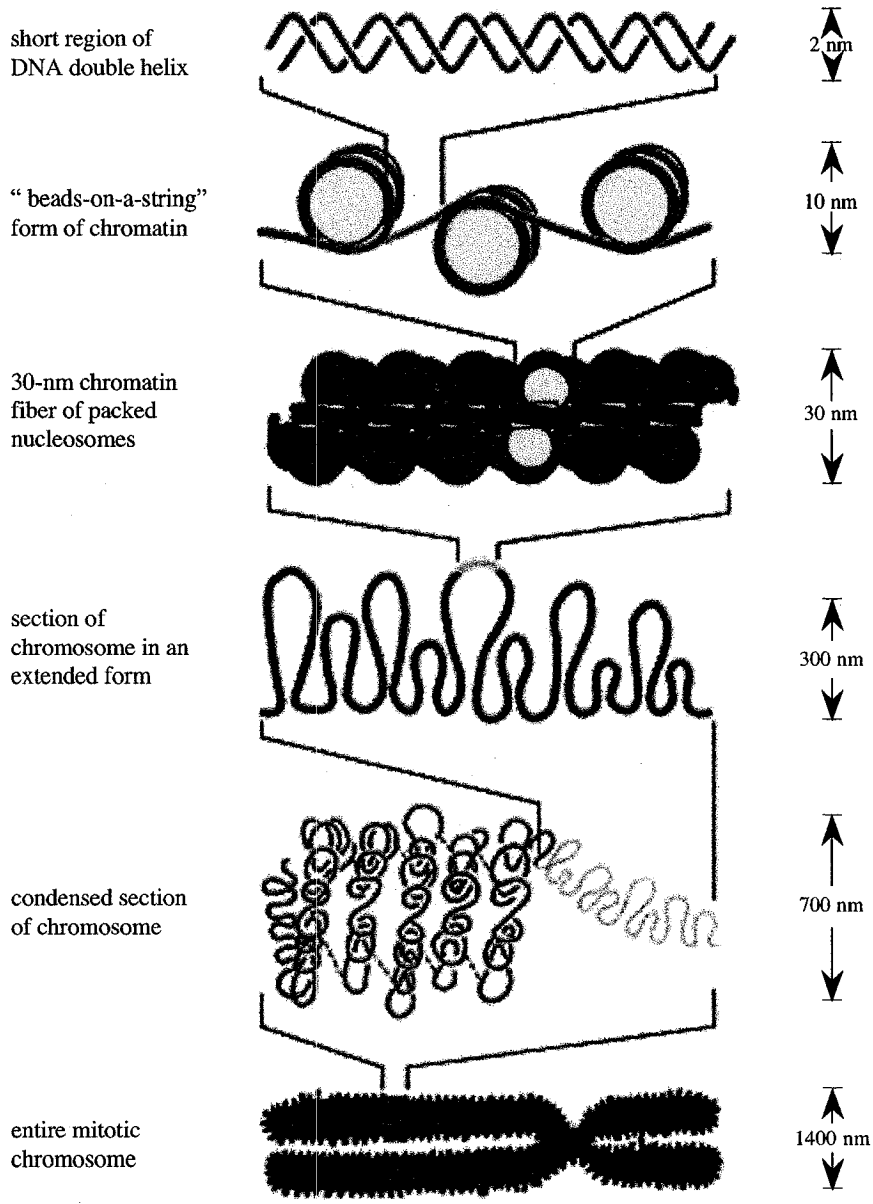
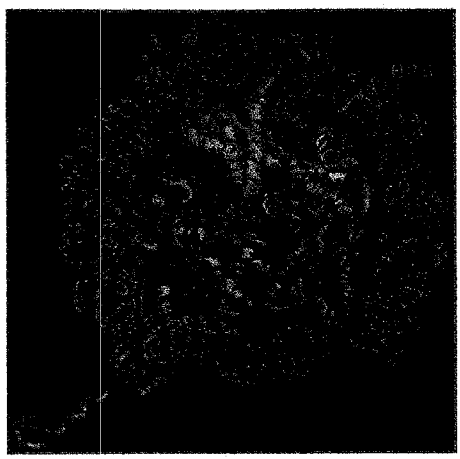


Figure 1. Levels of chromatin packing. This schematic drawing shows some of the chromatin packing thought to give rise to the highly condensed mitotic chromosome. Adapted from *Alberts et al., 1998, Essential Cell Biology, Garland Publishing Inc.*

naked DNA into nucleosomes is the best understood level of packing. During interphase, the chromatin is maintained in a state that is more compact than a simple extended chain of nucleosomes called "beads-on-a-string". Often this chromatin is visible by electron microscopy as dispersed or clustered fibers called the 30 nm chromatin fiber (Harrington, 1985). By stabilizing nucleosomes, the linker histone H1 plays an important role in maintaining the 30 nm fiber structure (Shen, 1995; Thoma, 1979). When a cell enters mitosis, the chromatin fiber undergoes additional levels of compaction, yielding the chromosome structure (Adolph, 1981). This highly organized chromatin packaging permits the DNA in chromatin to be compacted over 10,000 fold compared to its unfolded form. Also, the degree of chromatin folding directly influences cellular functions such as transcription, replication, recombination and repair.

The fundamental repeating unit of the chromatin is the nucleosome (Finch, 1977; Kornberg, 1974). The nucleosome core particle, linker DNA and histone H1 make up the complete nucleosome. The nucleosome core particle consists of 146 base pairs of DNA wrapped almost twice around an octamer of histones (Luger et al., 1997; Wolffe and Hayes, 1999). Histone proteins fall into five classes: H1, H2A, H2B, H3 and H4, which are derive from their amino acid composition and sequence (Johns, 1967). Histones are extremely conserved during evolution, suggesting they play an extremely vital role in DNA packaging. Each nucleosome core contains two copies of each histone H2A, H2B, H3 and H4. The crystal structure of the nucleosome has given tremendous details on the three-dimensional structures of each histone in a nucleosomal context (Luger et al., 1997) (Figure 2A). Each core histone contains two separate functional domains, a histone fold domain sufficient for both histone-histone and histone-DNA contacts within the

A



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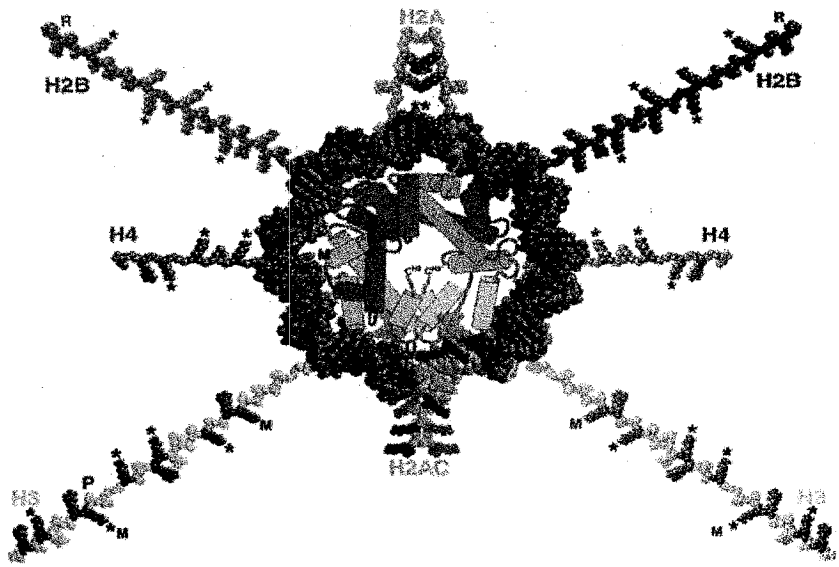

Figure 2. The nucleosome structure.

(A) **Crystal structure of the nucleosome core particle.** Adapted from *Luger et al., 1997, Nature 389:251-260.*

(B) **Model of a nucleosome and post-translational modifications within the histone tail domains.** Each nucleosome consists of 146 bp of DNA and 8 histones: two copies of each H2A, H2B, H3 and H4. Sites of acetylation on lysines are indicated by an asterisk. Also, sites of methylation (M), phosphorylation (P), ribosylation (R) and ubiquitination (U) are also indicated. Adapted from *Wolffe & Hayes, 1999, Nucl. Acids Res. 27:711-720.*

nucleosome, and the amino-terminal tail domain that is subject to a wide variety of covalent modifications that can directly influence the degree of compaction of the DNA (Figure 2B). These histone tails appear to emanate from the nucleosome and are positioned to associate with linker DNA residing between nucleosome or with adjacent nucleosome. Post-translational modifications of histone tails such as acetylation, methylation, phosphorylation, ADP-ribosylation and ubiquitination influence chromatin folding.

2. Regulation of the chromatin structure

It has become clear that chromatin not only packages DNA, but also regulates DNA accessibility through covalent modifications. The cell uses sophisticated chromatin-remodeling factors to open up the tight DNA-chromatin structure locally at sites of transcription, replication, recombination and repair. The level of chromatin compaction determines if a gene is active for expression. Open chromatin permits the access of transcription factors to their *cis*-regulatory elements, while condensed chromatin causes inaccessibility for the transcription factors to bind DNA. Histone variants, like H2A.Z, are also very important in keeping chromatin open (Meneghini et al., 2003). Modification of the chromatin structure occurs by two mechanisms. The first one involves ATP-hydrolyzing enzymes that can remodel the nucleosomal patterning along the chromatin fiber (Becker, 2002b). The second mechanism includes a set of enzymes that are able to modify histone covalently at specific residues located mainly in their amino-terminal and carboxy-terminal domains (Berger, 2002). Such modifications

include acetylation, methylation, phosphorylation, ADP-ribosylation and ubiquitination. Other then histone modifications, DNA methylation also plays an important role in chromatin structure. Methylated cytosines bind MeCP2 (methyl-CpG-binding domain protein) proteins that recruit histone modifying enzymes like histone deacetylases and methylases (Bird, 2002; Fuks et al., 2003).

2.1 ATP-dependent chromatin remodeling

ATP-dependent chromatin remodeling factors identified so far are multisubunit complexes that contain an ATPase subunit, which belongs to the Swi2/Snf2 ATPase superfamily (Eisen, 1995). Based on the sequence similarity of their ATPase subunit, there are four different subfamilies of ATP-dependent chromatin remodeling complexes: SWI/SNF, ISWI, CHD and INO80 (Figure 3A). ATP-dependent chromatin remodeling complexes use the energy of ATP hydrolysis to transiently disrupt the histone-DNA interactions (Becker, 2002b). Their action increases the accessibility of nucleosomal DNA to transcription factors by relocating histone octamers to adjacent DNA segments (sliding), and/or by displacing histone octamers to a different DNA segment (Becker, 2002a). In addition to their similarity to DNA helicase, the ATPase subunits of ATP-dependent chromatin remodeling complexes contain other conserved motifs: the bromodomain in the SWI/SNF (switching/sucrose non-fermenting) subfamily, the SANT (swi3, ada2, N-CoR and TFIIIB) domain in the ISWI subfamily, and a chromodomain and a putative DNA-binding domain in the CHD subfamily (Figure 3A). These motifs provide information about how ATP-dependent chromatin remodeling complexes are targeted to different chromatin regions. For example, the bromodomain that is present in

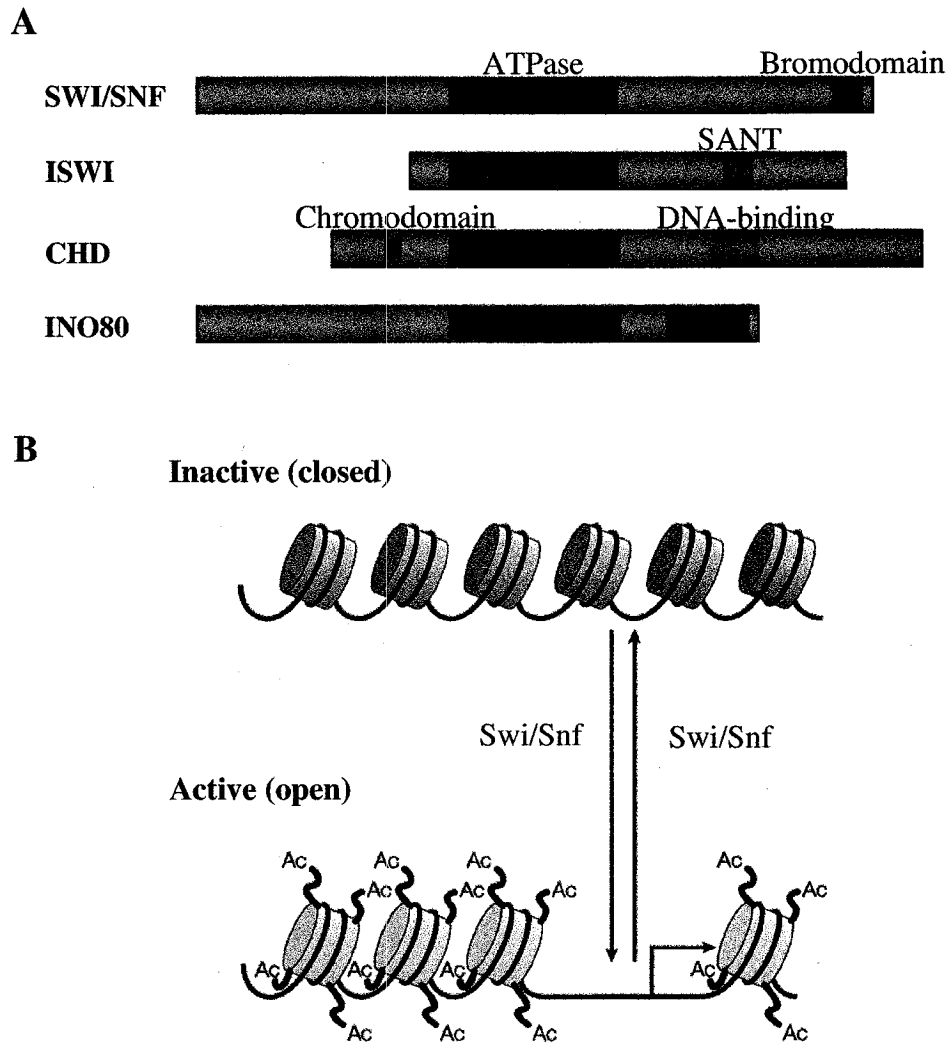

Figure 3. ATP-dependent chromatin-remodeling factors.

(A) **Structures of ATPase subunits.** Different motifs found in ATPase subunits are shown; ATPase domains (red), bromodomain (purple), SANT (blue), chromodomain (gray) and a putative DNA-binding domain (green).

(B) **The *in vivo* roles of ATP-dependent chromatin-remodeling factors.** ATP-dependent chromatin-remodeling factors have been proposed to be involved in the formation of both active and inactive chromatin structures *in vivo*. Adapted from *Tsukiyama, 2002, Nat. Rev. Mol. Cell Biol. 3:422-429.*

a large number of transcriptional regulatory complexes, such as the SWI/SNF ATPase subunit Swi2p, was shown to interact with acetylated histones (Dhalluin et al., 1999; Winston and Allis, 1999).

It has been shown that transcriptional activators directly recruit ATP-dependent chromatin remodeling factors to target genes to activate transcription. In the case of the yeast SWI/SNF complex, the purified complex has been shown to directly interact with a variety of transcription activators, including yeast GCN4, SWI5 and GAL4-VP16 (Krebs, 2000; Natarajan et al., 1999; Neely et al., 1999; Yudkovsky et al., 1999). The direct recruitment of chromatin remodeling complexes by gene-specific transcription activators is not unique to the yeast enzymes. For examples, the human SWI/SNF complex interacts with the transcription factors c-Myc, C/EBPβ, MyoD and EKLF (Cheng, 1999; De La Serna, 2001; Kadam, 2000; Kowenz-Leutz, 1999).

Interestingly, there is growing evidence to support a role for ATP-dependent remodeling complexes in the repression of transcription (Tyler and Kadonaga, 1999). For example, the CHD subfamily can be found in complexes containing histone deacetylase activities (Vignali et al., 2000). In mammalian cells, it was shown that the Sin3 co-repressor and histone deacetylase proteins co-purified with the SWI/SNF complex (Sif et al., 2001). One possible mechanism to explain the role of SWI/SNF complex in both transcriptional activation and repression might be that this complex alters the nucleosome position and/or the nucleosome structure to either increase or decrease accessibility of the DNA to either activators or repressors (Figure 3B) (Tsukiyama, 2002).

2.2 Post-translational modifications of histones

By using the energy from ATP hydrolysis, ATP-dependent chromatin remodeling complex modulates chromatin structure in a noncovalent manner. The chromatin structure can also be regulated by covalent modifications of histones. The amino-terminal and carboxy-terminal tails of each core histone are subject to post-translational modifications such as acetylation, methylation, phosphorylation, ADP-ribosylation and ubiquitination. These covalent modifications on the amino-terminal tails function as master on/off swiches that determine whether a gene is active or not. Such modifications play a major role in regulating transcription, replication, recombination and repair.

2.2.1 Histone acetylation

Among the well-known covalent modifications of core histones, the acetylation of lysine residues has attracted the most attention and has been correlated to transcriptional activation (Allfrey et al., 1964; Grunstein, 1997a; Workman and Kingston, 1998). Acetylation of lysine residues is a reversible reaction that is controlled by the opposed actions of histone acetyltransferase (HAT) and histone deacetylase (HDAC) enzymes (Figure 4A). HATs catalyse the transfer of the acetyl moiety from acetyl-coenzyme A to the ε–amino groups of specific lysine residues within the amino-terminal tails of histones, whereas HDACs reverse the process by removing the acetyl group from acetyl lysine residues. The mechanisms by which histone acetylation affects chromatin structure are not yet clear but different models have been proposed and studied. The first model suggests that acetylation of histone tails reduces their positive charge and their affinity for DNA, which overall destabilizes the nucleosome structure (Figure 4B)

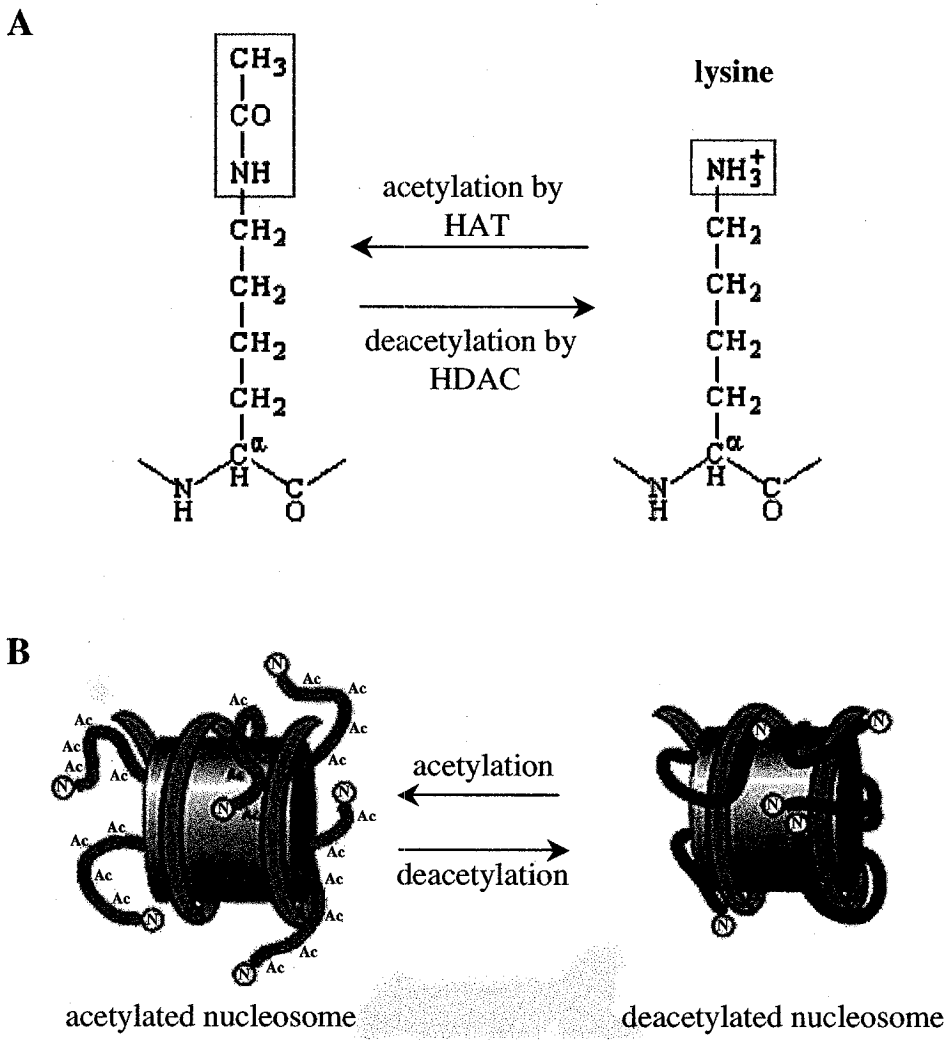

Figure 4. Histone acetylation.

(A) **Acetylation and deacetylation of the lysine residue.** Histone acetylation is catalysed by histone acetyltransferase and histone deacetylation is catalysed by histone deacetylase.

(B) **Consequences of acetylating histone in a nucleosomal context.** Acetylation of the lysine residues at the amino terminal region of histone proteins removes positive charges, thereby reducing the affinity between histones and DNA.

(Ausio and van Holde, 1986; Simpson, 1978). Interestingly, this destabilization of the nucleosomal structure has been shown to increase the *in vitro* binding of transcription factors to DNA contained within the nucleosome, which was otherwise suppressed by the histone tails (Lee et al., 1993; Vettese-Dadey et al., 1996). An alternative model is that acetylation of histone might be very important in the packaging of the nucleosomal array into the 30nm chromatin fiber leading to a more open and permissive chromatin environment for transcription (Garcia-Ramirez et al., 1995; Tse et al., 1998). Another function of acetylated histone tails is to act as direct interaction sites for regulatory protein complexes. The unacetylated tails of histones H3 and H4 have been shown to bind the yeast Sir3p/Sir4p and Tup1p transcriptional repressors (Edmondson et al., 1996; Hecht et al., 1995), the *Drosophila* polycomb repressor (Breiling et al., 1999), and the mammalian transducin-like enhancer of split repressor (Palaparti et al., 1997). An interesting finding revealed that the bromodomains can bind acetylated lysine residues within histone tails and these motifs are found in a number of transcriptional regulatory complexes, including the TAFII250 subunit of TFIID, the Gcn5p subunit of SAGA, and the Swi2p subunit of SWI/SNF (Dhalluin et al., 1999; Hassan et al., 2002; Ornaghi et al., 1999; Winston and Allis, 1999). Thus, the unacetylated tails of histone may provide interaction sites for transcriptional repressors, while the acetylated tails may provide interaction sites for activating complexes.

2.2.2 Histone phosphorylation

Eukaryotic cells possess mechanisms for condensing and decondensing chromatin structure. Chromatin condensation is observed during mitosis, whereas chromatin

relaxing is observed during interphase and is necessary for transcription, replication, recombination and repair. The phosphorylation of histones H1 and H3 tails has been associated with both condensed and relaxed chromatin.

During mitosis, it has been observed that Ser10 and more recently Ser28 of histone H3 are highly phosphorylated on condensed chromosomes (Goto et al., 1999; Hendzel et al., 1997; Wei et al., 1998; Wei et al., 1999). Mitotic phosphorylation of histone H3 Ser10 is mediated by members of the Aurora kinase family such as the Ipl1 kinase in yeast and nematodes (Hsu et al., 2000), Aurora B in *Drosophila* (Giet and Glover, 2001) and Aurora A and B kinases in mammals (Crosio et al., 2002). Aurora B is also important in mitotic Ser28 phosphorylation (Goto et al., 2002). Different models have been proposed to explain the role of histone H3 phosphorylation during mitosis. The first model is based on the hypothesis that phosphorylation of histone H3 tail affects histone H3/DNA interactions (Sauve et al., 1999). UV crosslinking experiments indicated that, in interphase, the histone H3 tail is bound to DNA, but this interaction is reduced in mitosis when all histone H3 are phosphorylated (Sauve et al., 1999). Because the phosphorylation of histone H3 at Ser10 weakens histone tail/DNA interactions, this favors DNA-polyamine binding. Polyamines neutralize the negative charge of DNA, thus decreasing repulsion between nucleosomes and allowing the formation of condensed chromatin. The second model is based on the idea that condensation factors are recruited to the chromosomes through direct interactions with phosphorylated histone H3 tails (Cheung et al., 2000a; Wei et al., 1999).

During interphase, histone H3 phosphorylation is associated with transcriptional activation of immediate-early genes, such as c-fos and c-jun (Chadee et al., 1999; Cheung

et al., 2000b; Clayton et al., 2000). Recently, it was demonstrated that MSK1/2 kinases mediated the mitogen and stress-induced phosphorylation of histone H3 at Ser10 and Ser28 in mouse embryonic fibroblasts (Soloaga et al., 2003). In yeast, the Snf1 kinase has been identified as an H3 kinase responsible for the inducible histone H3 phosphorylation observed at the activated INO1 gene in response to inositol deprivation (Lo et al., 2001). Two reports have revealed a new function of IKK-α kinase in response to extracellular cytokine proteins such as TNFα (Anest et al., 2003; Yamamoto et al., 2003). The authors have demonstrated a nuclear accumulation of IKK-α and its recruitment to promoter regions regulated by NF-κB after cytokine exposure. Interestingly, IKK-α can promote phosphorylation of histone H3 at Ser10 *in vitro* and the subsequent acetylation of histone H3 at Lys14. These results demonstrated that IKK-α could be the kinase that regulates the cytokine-induced phosphorylation of histone H3.

Interestingly, it seems that histone H3 phosphorylation at Ser10 is dependent on other post-translational modifications. For example, acetylation of Lys9 or Lys14 influences Ser10 phosphorylation. The histone H3 tail is a better substrate for Ser10 kinase when Lys9 or Lys14 is acetylated but not when Lys9 is methylated (Clements et al., 2003; Rea et al., 2000). Also, peptides that mimic the histone H3 tail and carry a phospho-Ser10 are better substrates for histone acetyltransferases that target Lys14 (Cheung et al., 2000b; Lo et al., 2000).

Histone H1 is viewed as a general repressor of transcription because it promotes higher order structures in which potential DNA binding sites are rendered inaccessible. Histone H1 plays an important role in maintaining the 30 nm fiber structure and at metaphase, chromatin compaction has been correlated with its phosphorylation and

dephosphorylation (Allan et al., 1986; Roth and Allis, 1992; Thoma, 1979). Phosphorylation of histone H1 by cdk2 kinase at the MMTV promoter was demonstrated to be associated with glucocorticoid receptor-mediated chromatin remodeling (Bhattacharjee et al., 2001). Further evidence has confirmed that histone H1 is specifically phosphorylated and depleted from the MMTV promoter during activation (Bhattacharjee et al., 2001; Koop et al., 2003).

2.2.3 Histone methylation

Five years ago, the identification and characterization of histone methyltransferases (HMTs) opened a new chapter in the studies of gene regulation (Chen et al., 1999; Rea et al., 2000). Histone methylation is catalysed by histone lysine methyltransferases (K-HMT) and histone arginine methyltransferases (R-HMT). All known HMTs utilize S-adenosylmethionine as the methyl group donor. To date, there are no reliable reports of a histone demethylase, so it seems that histone methylation is a relatively stable chromatin mark that can only be lost by successive rounds of DNA replication or by replication-independent histone replacement. Alternatively, there may be demethylases yet to be identified.

Histone lysine methylation occurs on lysines 4, 9, 27, 36 and 79 in H3, and on position 20 in H4 (Figure 5) (Lachner et al., 2003). Methylation of histones can have multiple effects on chromatin function, depending on the specificity of the lysine residue and the level of modification (either mono-, di-, or tri-methylation) (Table 1) (Jaskelioff and Peterson, 2003). For instance, H3-K4 and H3-K9 di-methylation, and H3-K27 tri-methylation are both associated with gene silencing and heterochromatin formation

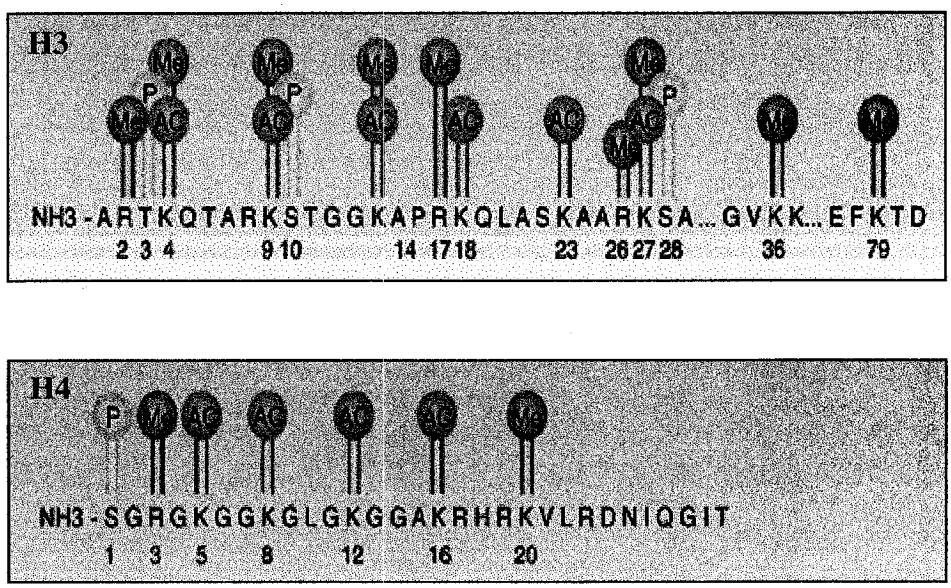

Figure 5. Potential sites of post-translational modifications of histone H3 and H4 tails. Many modification patterns have been linked to biological outcomes (see Table 1). AC, acetylation; Me, methylation; P, phosphorylation. Adapted from *Jaskelioff & Peterson, 2003, Nat. Cell Biol. 5:395-399.*

Site	*Enzyme*	*Function*
Histone H3		
K4-Me	SET7/SET9 (*Hs*)	Transcriptional activation
	Set1 (*Sc*)	Gene silencing
K9-Ac	SRC1 (*Mm*)	Transcriptional activation
K9-Me	SUV39H1 (*Hs*)	Gene silencing
S10-P	MSK1/2 (*Mm*) & Aurora B (*Sc*)	Mitotic chromosome condensation & Transcriptional activation of I.E. genes
K14-Ac	p300 & PCAF (*Hs*)	Transcriptional activation
R17-Me	CARM1 (*Hs*)	Transcriptional activation
R26-Me	CARM1 (*Hs*)	Transcriptional activation
K27-Me	EZH2 (*Hs*)	Transcriptional repression
S28-P	Aurora B (*Hs*)	Mitotic chromosome condensation
Histone H4		
R3-Me	PRMT1	Transcriptional activation
K20-Me	SET7/SET8 (*Hs*)	Gene silencing

Table 1. Roles of histone H3 and H4 modifications. Species abbreviations: *Hs*, *Homo sapiens*; *Mm*, *Mus musculus*; *Sc*, *Saccharomyces cerevisiae*.

(Briggs et al., 2001; Cao et al., 2002; Fischle et al., 2003; Lachner et al., 2001), whereas H3-K4 tri-methylation is associated with active chromatin (Bernstein et al., 2002; Santos-Rosa et al., 2002). Thus, it is clear that the specificity of lysine methylation, as well as the number of methyl groups attached to a particular lysine, can have distinct consequences on gene regulation.

There are four families of K-HMTs: SET1 (Su(var), E(z), Trithorax 1), SET2, SUV39 (suppressor of position effect variegation 3-9), and the RIZ (retinoblastoma-interacting zinc finger) family (Kouzarides, 2002). The SET domain, a conserved signature motif that is crucial for catalytic activity, is present in all four families (Jenuwein, 2001).

Methylation of H3-K9 has recently attracted a great deal of experimental attention, mainly because of its association with gene silencing. The mammalian Suv39h enzymes and their *S. pombe* homologue, Clr4, were the first K-HMT identified (Rea et al., 2000). The *Drosophila* Su(var)3-9 and the *S. pombe* Clr4 K-HMTs catalyse the methylation of H3-K9 (Nakayama et al., 2001; Schotta et al., 2002). This modification of histone H3 creates a high-affinity binding site for the chromodomain present in the heterochromatin protein 1 (HP1) (Bannister et al., 2001). HP1 protein is a transcriptional repressor and was found to co-localize and interact with the K-HMT Suv39h at heterochromatic sites (Agaard et al., 1999). In yeast, it has been shown that the recruitment of HP1 to centromeric heterochromatin by Clr4 is necessary for gene silencing (Nakayama et al., 2001). Dosage compensation in female mammals involves the inactivation of one X chromosome (Avner and Heard, 2001). H3-K9 methylation is

associated with the inactive chromosome (Heard et al., 2001). This is another example of the importance of H3-K9 methylation in gene silencing.

Like acetylation, histone arginine methylation has been associated with transcriptional activation (Table 1) (Roth et al., 2001; Stallcup, 2001). Methylation of arginines occurs within the tails of histone H3 (R2, R17, R26) and H4 (R3) (Figure 5). Arginines can be either mono- or di-methylated (which can be asymmetric or symmetric). The arginine histone methyltransferase (R-HMT) family does not contain a SET domain, but has a highly conserved S-adenosyl methionine (SAM) binding site.

There are five known R-HMTs possessing highly conserved catalytic domains. PRMT1, PRMT3 and PRMT4/CARM1 enzymes catalyse the formation of asymmetric dimethylated arginine, whereas PRMT5 catalyses symmetric dimethylation (McBride and Silver, 2001). The PRMT2 protein has not yet been established as an enzyme but a recent study has demonstrated that PRMT2 is capable of binding S-adenosylmethionine (Qi et al., 2002). Post-translational modification of histones by R-HMTs has recently been implicated in a variety of cellular processes including nuclear receptor transcriptional regulation (McBride and Silver, 2001). PRMT1 is the first R-HMT identified and was shown to interact with the nuclear receptor co-activator SRC-2 and enhance nuclear receptor transactivation function possibly by methylating H4-R3 (Koh et al., 2001; Strahl et al., 2001; Wang et al., 2001). By yeast two-hybrid analysis, PRMT2 and PRMT4 were shown to interact specifically with the estrogen receptor alpha (ERα) and with the co-activator SRC-2, respectively (Chen et al., 1999; Qi et al., 2002). PRMT4 can methylate H3-R17 and H3-R26, and this methyltransferase activity is essential for its ability to act as a coactivator (Chen et al., 1999). Indeed, H3-R17

methylation is found to be enriched on hormone-responsive promoters after induction (Ma et al., 2001). Interestingly, PRMT4 acts synergistically with other co-activators, such as the acetyltransferases p300 and P/CAF, to stimulate transcription by nuclear receptors (Koh et al., 2002). This raises the possibility of a cross-talk between histone methylation and acetylation to stimulate transcription (Daujat et al., 2002).

2.2.4 Histone ubiquitination

While many functional and structural studies have focused on modifications such as acetylation, phosphorylation and methylation, few have focused their attention on ubiquitination. Histones H3, H1, H2A and H2B were shown to be ubiquitinated *in vivo* (Chen et al., 1998b; Nickel et al., 1989; Nickel and Davie, 1989; Pham and Sauer, 2000; Zhang, 2003). The most studied and the most ubiquitinated histones are H2A and H2B. Both histones are reversibly ubiquitinated at highly conserved lysines within their carboxy-terminal tails. In *S. cerevisiae*, H2B-K123 is a substrate for the Rad6 ubiquitin ligase (Robzyk et al., 2000). TAFII250, which is a subunit of the TFIID complex, has been shown to possess histone H1 ubiquitination activity (Pham and Sauer, 2000). This modification is critical to mitotic and meiotic growth. Experiments conducted so far have not clearly demonstrated the involvement of histone ubiquitination in gene regulation and chromatin structure (Kleinschmidt and Martinson, 1981). Different models have been proposed to explain how histone ubiquitination affects transcription (Zhang, 2003). First, histone ubiquitination may affect higher-order chromatin folding, thereby resulting in greater access of DNA to the transcription machinery. Second, histone ubiquitination could operate as a tagging mechanism for recognition by chromatin remodeling

complexes that could disrupt the chromatin structure. The third possibility is that histone ubiquitination affects transcription through its impact on other histone modifications, such as acetylation and methylation (Dover et al., 2002; Seigneurin-Berny et al., 2001; Sun and Allis, 2002).

2.2.5 Histone ADP-ribosylation

In the past decade, poly(ADP-ribosyl)ation has gained much more interest because of its newly discovered roles in DNA repair, DNA-damage signaling, genomic stability and transcriptional regulation (Burkle, 2001; Pleschke et al., 2000). Poly(ADP-ribose) polymerase-1 (PARP-1) is a nuclear chromatin-associated enzyme that catalyses the transfer of the ADP-ribose moiety of NAD to a variety of nuclear proteins (D'Amours et al., 1999). Poly(ADP-ribosyl)ation affects chromatin compaction and also has an impact on gene expression. Transcriptionally active regions of the chromatin are associated with PARP activity (Mullins et al., 1997). Indeed, poly(ADP-ribosyl)ated nucleosomes were associated with actively transcribed chromatin (Hough and Smulson, 1984). Poly(ADP-ribosyl)ation of chromatin proteins, such as histones, play a role in chromatin decondensation. Histones H1 and H2B are the main histones being poly(ADP-ribosyl)ated *in vivo* (Adamietz and Rudolph, 1984). Another piece of evidence demonstrating the importance of PARP-1 in gene regulation is that in PARP-$1^{-/-}$ mice, the expression levels of histone acetyltransferases p300, CBP and PCAF were reduced, suggesting that PARP-1 is required for the proper expression of particular histone acetyltransferases (Ota et al., 2003). Genetic studies with *Drosophila melanogaster* showed that PARP is an essential gene required to organize chromatin throughout the life

cycle (Tulin et al., 2002). Recently it was shown that PARP is required to produce normal-sized puffs, an expanded chromatin state (Tulin and Spradling, 2003). Such local loosening may facilitate transcription. This finding suggests that poly(ADP-ribosyl)ation affects chromatin structure and facilitates transcription.

2.2.6 Histone Sumoylation

Small ubiquitin-related modifiers (SUMO) are members of the ubiquitin-like protein family (Johnson and Gupta, 2001). Enzymes that covalently attach SUMO to target proteins are similar to the ones involved in ubiquitination. Several proteins have been found to be sumoylated, but unlike ubiquitination, sumoylation has not been linked to protein degradation (Nathan et al., 2003). Different functions have been associated with sumoylation, such as the regulation of protein-protein interaction, subcellular localization, inhibition of ubiquitin-mediated degradation and stimulation of transcriptional activity. In addition to all these biological functions, sumoylation has been correlated with gene repression (Verger et al., 2003). Recently, it was demonstrated that histone H4 is sumoylated both *in vitro* and *in vivo* (Shiio and Eisenman, 2003). The biological function of histone H4 sumoylation has been associated with transcriptional repression because of the recruitment of the heterochromatin protein HP1γ and the histone deacetylase HDAC1 (Shiio and Eisenman, 2003). This interesting finding suggests that sumoylation is another post-translational modification of histones and appears to govern chromatin structure and function to mediate gene repression and silencing.

2.3 The histone code hypothesis

Every cell from a multicellular organism carries the same set of genes. The defining patterns of gene expression are put in place and stabilized by epigenetic mechanisms during cellular differentiation. This gene expression pattern often persists through many cell generations and has been termed cellular memory or epigenetic code (Turner, 2003). In the recent years, it has become clear that chromatin is a central component of gene regulation and a carrier of epigenetic information (Turner, 2002). This information resides in the histone tail domains that are subject to different post-translational modifications leading to specific cellular activities. It has been suggested that a single or a combination of histone tail modifications form a code specifying chromatin structure and patterns of gene expression (Turner, 2002). To add further complexity, DNA methylation, in conjunction with the histone code, is very important for maintaining the epigenetic code (Turner, 2002). The pattern of histone modifications controls the association of specific proteins with chromatin leading directly or indirectly to changes in the functional state of the underlying DNA.

The histone code hypothesis relies on there being two groups of biochemical activities (Figure 6). The first group contains enzymes that "write" the code either by adding or removing modifications at specific target sites in the histone tail. Interestingly, multiple modifications can occur simultaneously within a single histone tail, and one modification can modulate another to mediate a variety of events. For example, H3-S10 phosphorylation stimulates H3-K9 and H3-K14 acetylation, leading to gene activation (Cheung et al., 2000b; Thomson et al., 2001; Zhang and Reinberg, 2001). Methylation of H4-R3 by PMRT1 facilitates the subsequent acetylation of H4-K8 and H4-K12 by p300,

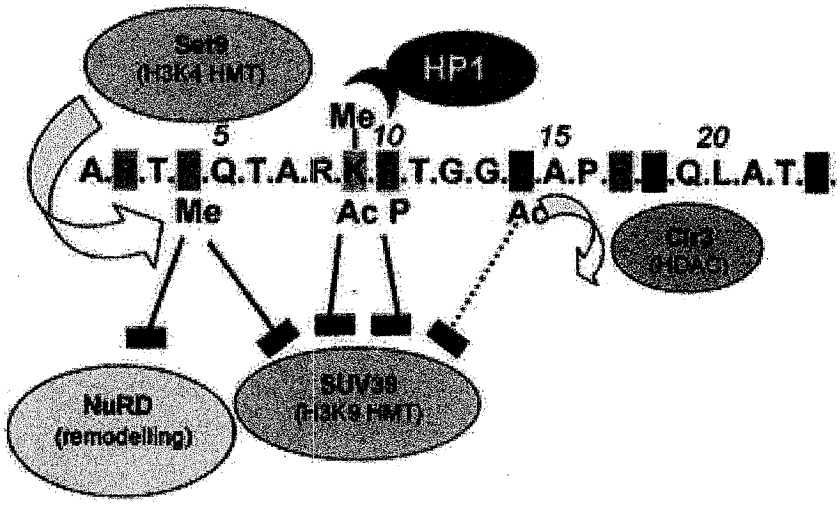

Figure 6. The histone code hypothesis. The combination of post-translational modifications of the histone H3 tail constitute a code that defines transcriptional states. The code is set by chromatin remodeling enzymes and read by nonhistone proteins that bind specifically to modify histone. Lysines (K) and arginines (R) that can be methylated are shown in blue, lysines that can be acetylated are in red, and the serine (S) that can be phosphorylated is in green. Lysine 9 can either be acetylated or methylated and is blocked in violet. Methylation of K4 by Set9 blocks chromatin remodeling/deacetylation by NuRD complex and methylation of K9 by SUV39. Methylation of K9 by SUV39 is also prevented when K9 is acetylated, S10 is phosphorylated or K14 is acetylated. The methylation of K9 is favored when the K9 and K14 are deacetylated by HDAC. Adapted from *Turner, 2002, Cell 111:285-291.*

and consequently, activates transcription (Wang et al., 2001). Methylation of H3-K4 by Set9 blocks chromatin remodeling/deacetylation and methylation of H3-K9 by SUV39H (Nishioka et al., 2002) (Figure 6). Interestingly, modification of one histone can affect that of another present on the same nucleosome. Ubiquitination of H2B-K123 by Rad6 ubiquitin-conjugating enzyme regulates H3-K4 methylation and transcriptional silencing in yeast (Sun and Allis, 2002). The second group of biochemical activities is composed of proteins that "read" the code and mediate subsequent functional effects. Several protein modules are capable of interacting with specifically modified histone tails. The bromodomains, found in chromatin modifying complexes, have been shown to interact with acetylated lysine residues (Dhalluin et al., 1999; Hassan et al., 2002; Jacobson et al., 2000). The chromodomain of HP1 binds specifically to H3-K9 methylated by the K-HMT SUV39H enzyme (Bannister et al., 2001; Lachner et al., 2001). Recently, the chromodomain of the drosophila polycomb protein was shown to bind H3-K26 methylated and to be essential for maintaining the silencing of homeotic genes during development (Min et al., 2003).

3. Histone deacetylases and acetyltransferases

3.1 Histone deacetylases

Structural changes in chromatin play an important role in the control of gene expression and are governed by complexes that remodel chromatin and by enzymes that post-translationally modify histone. All core histones are reversibly acetylated at multiple sites within their amino-terminal tails. Hyperacetylated histones are generally

found in transcriptionally active genes and hypoacetylated histones in silent regions, such as heterochromatin. The level of histone acetylation is regulated by competing activities between histone acetyltransferases and histone deacetylases. So far, eighteen human genes encoding HDACs have been identified. These can be classified into three distinct families based on their sequence homology to three *Saccharomyces cerevisiae* histone deacetylases. The class I HDACs (HDAC1, 2, 3, and 8) are most closely related to the yeast transcriptional regulator Rpd3 (reduced potassium dependency 3) (Buggy et al., 2000; Dangond et al., 1998; Taunton et al., 1996). Class II HDACs share domains similarity to the yeast deacetylase Hda1 (histone deacetylase 1) and can be subdivided into two subclasses, class IIa (HDAC4, 5, 7, 9 and its splice variant MITR (MEF2 interacting transcriptional repressor) (Fischle et al., 2001; Sparrow et al., 1999; Wang et al., 1999; Wu et al., 2001; Zhou et al., 2001) and class IIb (HDAC6 and 10) (Kao et al., 2002; Verdel et al., 2000). Finally, the class III HDACs (SIRT1, 2, 3, 4, 5, 6 and 7) were identified on the basis of sequence similarity with Sir2 (silencing information regulator 2), a yeast transcriptional repressor that requires the cofactor NAD^+ for its deacetylase activity (Landry et al., 2000; Smith et al., 2000b). Recently, a new member of the HDAC family, HDAC11, has been identified, (Gao et al., 2002). No classification of HDAC11 could be made because its overall sequence similarity to class I/II and III is too low.

3.1.1 Class I histone deacetylases

Class I HDACs have been well studied. They all have ubiquitous tissue expression and, except for HDAC3, which is found both in the nucleus and in the cytoplasm, localization of class I HDACs is nuclear (Yang et al., 2002). HDACs are

usually embedded in large multimolecular complexes required for their enzymatic activities (Guenther et al., 2001). Three protein complexes have been characterized that contain both HDAC1 and HDAC2: Sin3 (Hassig et al., 1997), NuRD (nucleosome remodeling and deacetylating) (Tong et al., 1998) and Co-REST (corepressor to the transcription factor REST) (Humphrey et al., 2001). HDAC3 appears to be functionally distinct from HDAC1 and HDAC2. Biochemical studies have demonstrated that HDAC3 is a subunit of stable complexes containing nuclear receptor corepressors SMRT (silencing mediator for retinoic acid and thyroid hormone receptors) and N-CoR (nuclear receptor co-repressor) (Guenther et al., 2000; Li et al., 2000). Both SMRT and N-CoR function as cofactors for HDAC3 enzymatic activity (Guenther et al., 2001). It is now well known that class I HDACs are recruited to promoters by transcription factors for transcriptional repression or gene silencing (Luo et al., 1998; Yang et al., 1996).

3.1.2 Class IIa histone deacetylases

Whereas most class I HDACs are ubiquitously expressed, the class IIa HDACs are expressed in a restricted number of cell types. HDAC4, HDAC5 and HDAC9 show highest expression in heart, skeletal muscle and brain (Grozinger et al., 1999; Wang et al., 1999; Zhou et al., 2001), whereas highest expression of HDAC7 is in heart and lung tissues (Fischle et al., 2001). All class IIa HDACs shuttle between the nucleus and the cytoplasm (Dressel et al., 2001; Grozinger and Schreiber, 2000; Kao et al., 2002; McKinsey et al., 2000a; Miska et al., 1999; Wang et al., 2000; Zhao et al., 2001). This nucleocytoplasmic shuttling is mediated by their association with 14-3-3 proteins, which mask their nuclear localization signal and regulate their subcellular localization

(Grozinger and Schreiber, 2000; Kao et al., 2002; Wang et al., 2000). Class IIa HDACs are also found in large multisubunit complexes associated with the corepressors CtBP (E1A C-terminal binding protein), SMRT, N-CoR and B-CoR (Bcl-6-interacting co-repressor) (Huang et al., 2000; Kao et al., 2000; Lemercier et al., 2002; Zhang et al., 2001). Class IIa HDACs are recruited to specific promoters through their interaction with the MEF2 (myocyte enhancer-binding factor 2) transcription factors family (Wang and Yang, 2001). The MEF2 family of transcription factors is one of the major targets of class IIa HDACs and are important regulators in myogenesis (Black and Olson, 1998). Class IIa HDACs inhibit myogenesis by binding to MEF2 at several promoters critical for the muscle differentiation program (Lu et al., 2000; McKinsey et al., 2001). Activation of the CaMK (Ca^{2+}/calmodulin dependent protein kinase) signaling pathway overcomes the HDAC-mediated repression of muscle-specific genes expression and induces the myogenic program (Lu et al., 2000; McKinsey et al., 2000b). Phosphorylation of class IIa HDACs by CaMK triggers their dissociation with MEF2, promotes their association with 14-3-3 proteins, and finally, their transport to the cytoplasm. These observations suggest that class IIa HDACs are the master group of regulators of myocyte development. Bound to MEF2 proteins, they play a critical role in repressing myogenic genes until the appropriate myogenic differentiation signal is delivered.

3.1.3 Class IIb histone deacetylases

Class IIb HDACs are characterized by duplicated HDAC domains. This duplication is partial in the case of HDAC10 in which the carboxy-terminal catalytic

domain lacks the active pocket residues required for enzymatic activity (Kao et al., 2002; Tong et al., 2002). Class IIb HDACs show some degree of tissue-specific gene expression. HDAC6 is predominantly expressed in testis (Seigneurin-Berny et al., 2001; Verdel and Khochbin, 1999), and HDAC10 is expressed in liver, spleen and kidney (Fischer et al., 2002; Kao et al., 2002; Tong et al., 2002). Analysis of the catalytic activity of the two separate HDAC domains of HDAC6 by site-directed mutagenesis suggested that the two domains might function independently (Grozinger et al., 1999); however, separation of the two domains results in loss of enzymatic activity (W. Fischle and E. Verdin, unpublished). HDAC6 shuttles between the nucleus and the cytoplasm. In the absence of a stimulus, HDAC6 is localized to the cytoplasm, but cell-cycle arrest is associated with the partial translocation of the protein to the nucleus (Verdel et al., 2000). HDAC10 is primarily cytoplasmic but shows significant nuclear staining in several cell lines (Fischer et al., 2002; Guardiola and Yao, 2002; Tong et al., 2002). Interestingly, HDAC6 was shown to co-localize with and deacetylate α-tubulin, thus playing a major role in microtubule dynamics and functions (Hubbert et al., 2002; Matsuyama et al., 2002).

3.1.4 Class III histone deacetylases

Class III HDACs are composed of seven members of SIRT. These sirtuins (Sir2-like proteins) deacetylate core histones in an NAD^+-dependent manner (Imai et al., 2000). The best-characterized sirtuin is yeast Sir2, which is important for mediating silencing of telomeres, mating-type loci and ribosomal DNA loci (Gartenberg, 2000). Both Sir2 and SIRT1 are localized to the nucleus. SIRT1 has been shown to deacetylate p53 and

attenuate its transcriptional activity (Vaziri et al., 2001). Overall, the cellular function of mammalian sirtuins has not been so well studied.

3.2 Histone acetyltransferases

Four decades ago it was proposed that acetylation of histones within chromatin is correlated with gene regulation (Allfrey et al., 1964). A major breakthrough in understanding the mechanism of histone acetylation came with the cloning of a histone acetyltransferase (HAT) enzyme from *Tetrahymena thermophila* as a homologue of the previously identified Gcn5 transcriptional coactivator from yeast (Brownell et al., 1996). This finding suggested that targeting HATs by transcriptional activators is responsible for enrichment of acetylated histones in active chromatin. In general, HAT activity has been grouped into two general classes based upon nuclear (type A) or cytoplasmic (type B) origin. Type B HATs have been linked to histone deposition during replication and type A HATs linked to transcription (Brownell and Allis, 1996). The primary targets of HAT enzyme are the ε-amino groups of specific lysine residues on the amino-terminal tails of the histone proteins (Wolffe, 1998). Two theories on how histone acetylation might facilitate transcription have been proposed. The first one predicts that acetylation affects transcription by neutralizing the positive charge of histones, which weakens histone-DNA and internucleosomal contacts, reducing chromatin compaction (Workman and Kingston, 1998). This allows transcriptional activators to access DNA more easily. The second theory proposed that covalent modification of histones provides an epigenetic marker for gene expression (Strahl and Allis, 2000). For example, acetyl-lysines on

histone tails provide recognition sites for factors involved in either activation or repression of transcription.

The HAT enzymes are divided into five families, including the GNAT (Gcn5 N-related acetyltransferase) family, the MYST (MOZ, Ybf2/Sas3, Sas2 and Tip60)-related HATs, p300/CBP HATs, the general transcription factor TAFII250 family, and the nuclear hormone-related HATs (Table 2). Sequence analysis of these HATs revealed that they show high sequence similarity within families but poor sequence similarity between families (Kuo and Allis, 1998). Since my thesis focuses on two MYST members, I will discuss this family in detail.

3.2.1 The MYST family

MYST family members are involved in a wide range of cellular functions (Table 2 and Figure 7A) (Utley and Cote, 2003). The MYST domain, or the catalytic domain, is a region of homology well conserved among all family members. This region includes the acetyl-CoA binding motif as well as a C_2HC zinc finger that is very important for HAT activity (Akhtar and Becker, 2001; Takechi and Nakayama, 1999; Yan et al., 2000). The divergence within the amino- and carboxy-terminal regions of the MYST family members may contribute to their substrate specificity (Yan et al., 2000). Well characterized yeast, drosophila and human MYST proteins are described below.

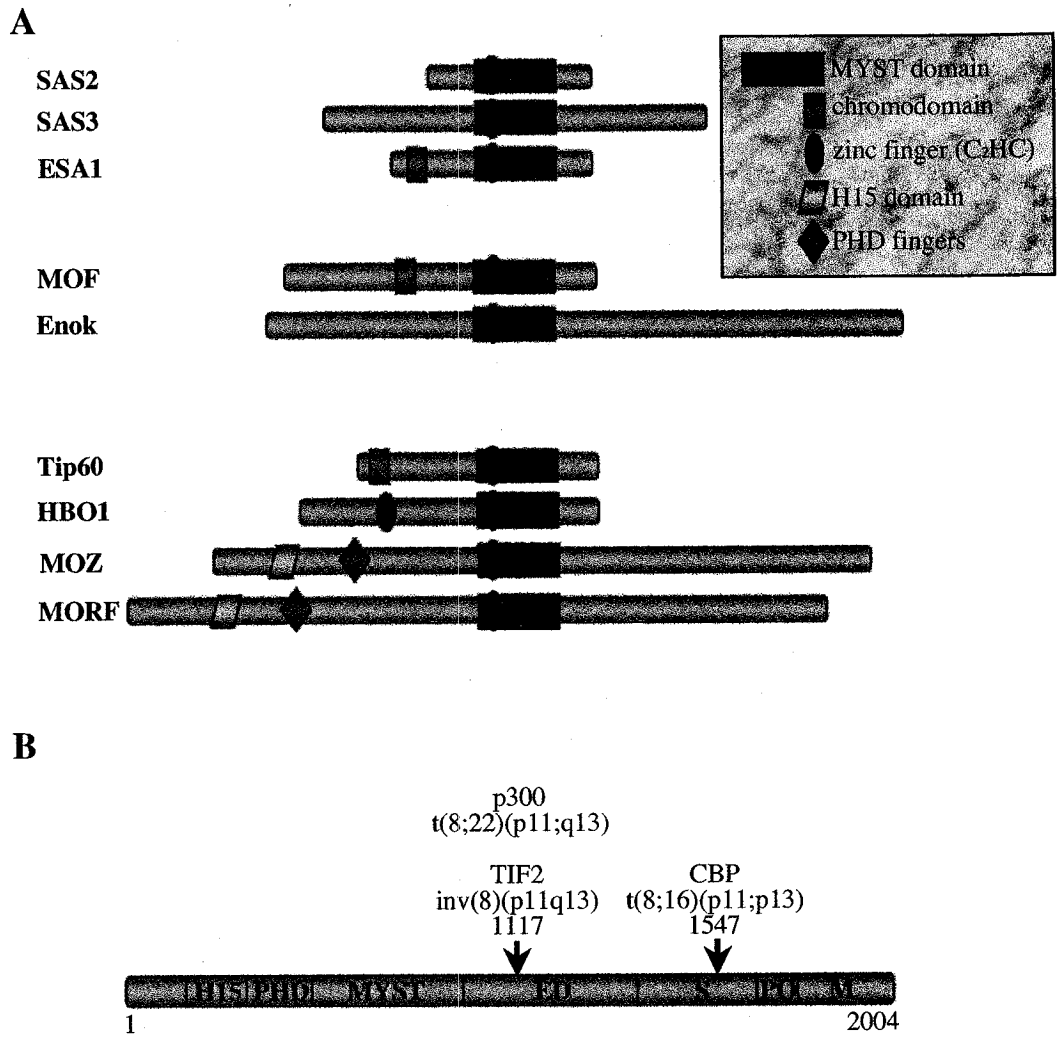

Figure 7. The MYST proteins.

(A) **Alignment of conserved MYST domain proteins.** Structural domains are illustrated and defined in the legend.

(B) **Schematic representations of chromosomal abnormalities associated with MOZ.** The breakpoints are indicated by arrows, and numbers at their ends represent the amino acid positions. Structural domains of MOZ are labeled as follows: H15, linker histones H1- and H5-like module; PHD, *p*lant *h*omeo*d*omain zinc fingers; MYST, MYST acetyltransferase domain; ED, Glu/Asp-rich acidic regions; S, Ser-rich domain; PQ, Pro/Gln-stretch; and M, Meth-rich domain.

HAT family	*Organisms*	*Complex(es)*	*Function(s)*
GNAT			
Hat1	Yeast to human	HAT1B	Histone deposition
Gcn5	Yeast to human	SAGA, ADA, A2	Coactivator
PCAF	Human, mouse	PCAF	Coactivator
Elp3	Yeast	elongator	Transcription elongation
Hpa2	Yeast		
MYST			
Sas2	Yeast	SASI	Silencing
Sas3	Yeast	NuA3	Silencing
Esa1	Yeast	NuA4	Cell cycle progression, DNA repair
MOF	*Drosophila*	MSL	Dosage compensation
Enok	*Drosophila*	n.d.	Brain development
Chameau	*Drosophila*	n.d.	Silencing
Tip60	Human	Tip60	DNA repair, apoptosis, coactivator, corepressor
HBO1	Human	ORC	Replication
MOZ	Human	n.d.	Coactivator
MORF	Human	n.d.	Coactivator
p300/CBP			
p300	Yeast to human		Coactivator
CBP	Yeast to human		Coactivator
Basal transcription factors			
TAFII250	Yeast to human	TFIID	TBP-associated factor
TFIIIC	human	TFIIIC	RNA polymerase III transcription initiation
Nut1	Yeast	mediator	Transcription initiation
Nuclear receptors coactivators			
SRC-1	Human, mouse	n.d.	Nuclear receptor coactivators
ACTR	Human, mouse	n.d.	Nuclear receptor coactivators
TIF2	Human, mouse	n.d.	Nuclear receptor coactivators

Table 2. Summary of known and putative HATs. n.d., not determined.

Yeast MYST proteins

SAS2

Sas2 (something about silencing) was identified in *Saccharomyces cerevisiae* in two genetic screens for genes involved in transcriptional silencing (Ehrenhofer-Murray et al., 1997; Reifsnyder et al., 1996). These genetic experiments showed that Sas2 has opposite regulatory effects, depending on the silenced locus. Sas2 mutants are defective in silencing at telomeres and HML locus in sir1 mutant cells, but display improved silencing at a mutated HMR locus and at rDNA (Ehrenhofer-Murray et al., 1997; Meijsing and Ehrenhofer-Murray, 2001; Reifsnyder et al., 1996). Biochemical studies have indicated that Sas2 is part of the SAS-I complex (Meijsing and Ehrenhofer-Murray, 2001; Osada et al., 2001). This complex contains the proteins Sas4, Sas5, and Cac1, the largest subunit of the chromatin assembly factor CAF-I, and the nucleosome assembly factor Asf1. Mutations in the acetyl-CoA binding motif of Sas2 were shown to disrupt its ability to mediate HML and telomere silencing, suggesting that the HAT activity of Sas2 is important in mediating silencing (Osada et al., 2001). Recently, it was shown that recombinant Sas2 has HAT activity that absolutely requires Sas4 and is stimulated by Sas5 (Sutton et al., 2003). The recombinant SAS complex (Sas2, Sas4 and Sas5) from *Escherichia coli*, acetylates H4K16 and H3K14, but interestingly, the yeast native SAS-I complex was unable to acetylate nucleosomal histones (Sutton et al., 2003). This finding suggests the possibility that the SAS-I complex may acetylate free histones prior to their deposition onto DNA by Asf1 or CAF-I. Sas2 was shown to block the spreading of silencing from telomere regions mediated by the deacetylase Sir2 (Kimura et al., 2002; Suka et al., 2002). Heterochromatin formation at telomeres is mediated by Sir2, a H4K16

deacetylase that promotes Sir protein interaction with hypoacetylated histones H3 and H4 (Grunstein, 1997b). Sas2 opposed deacetylation by Sir2 at telomeric regions by acetylating H4K16 to prevent telomeric heterochromatin from spreading into adjacent telomeric regions (Kimura et al., 2002; Suka et al., 2002).

Sas3

Sas3 was isolated as a gene related to Sas2 and involved in HML silencing (Ehrenhofer-Murray et al., 1997; Reifsnyder et al., 1996). However, Sas3 does not contribute to telomere silencing (Reifsnyder et al., 1996). Sas3 was shown to be the catalytic HAT subunit of the NuA3 (Nucleosomal Acetyltransferase of histone H3) complex (John et al., 2000). This complex primarily acetylates histone H3 on nucleosomal template, and this activity requires the zinc finger region found in the MYST domain of Sas3 (Howe et al., 2001; John et al., 2000; Takechi and Nakayama, 1999). Sas3 mediates interaction of the NuA3 complex with Spt16, a component of the yeast CP (Cdc68/Pob3) and mammalian FACT (facilitates chromatin transcription) involved in transcription elongation and DNA replication (Brewster et al., 1998; Orphanides et al., 1998). This interaction suggests that the NuA3 complex might function in concert with FACT-CP to stimulate transcription or replication by coupling its acetyltransferase activity.

Esa1

Esa1 (essential sas-related acetyltransferase 1) was first identified as a HAT that is essential for growth in yeast (Clarke et al., 1999; Smith et al., 1998). Esa1 is part of

the NuA4 (Nucleosomal Acetyltransferase of histone H4) complex, which acetylates primarily nucleosomal histone H4 (Allard et al., 1999). The NuA4 complex contains eleven subunits, including Esa1, which are essential for yeast growth (Allard et al., 1999; Choy et al., 2001; Eisen et al., 2001; Galarneau et al., 2000; Loewith et al., 2000). The Esa1 chromodomain is also essential for yeast viability (Yan et al., 2000). Esa1 is targeted to a small subset of promoters in an activator-specific manner. For examples, Esa1 is recruited to ribosomal protein and to PHO5 promoters (Reid et al., 2000; Vogelauer et al., 2000). Recently, it was demonstrated in budding yeast that the NuA4 complex is recruited specifically to DNA double-strand breaks via one of its subunits, Arp4, which binds histone H4 tails (Bird et al., 2002). This piece of data shows the importance of histone tail acetylation in DNA repair.

Drosophila MYST proteins

MOF

Dosage compensation ensures that males with a single X chromosome have the same amount of X-linked gene products as females with two X chromosomes (Muller, 1932). Acetylation of lysine 16 on histone H4 is a hallmark of dosage compensation (Smith et al., 2000a). MOF (males absent on the first) has been demonstrated to acetylate H4K16 and to be required for dosage compensation in male flies (Akhtar and Becker, 2000). MOF is part of a complex containing six gene products: MSL1 (male-specific lethal 1), MSL2, MSL3, MLE (maleless), roX1 (RNA on the X1) and roX2 non-coding RNAs (Gu et al., 1998; Gu et al., 2000; Smith et al., 2000a). This complex binds the X chromosome at numerous sites and results in a significant increase of acetylated H4K16

(Smith et al., 2000a). Mutation in any protein member of the MSL complex causes male-specific lethality and lack of enrichment of H4K16 acetylation or of the MSL complex itself on the male X chromosome (Hilfiker et al., 1997). MOF was shown to activate transcription when fused to a Gal4 DNA binding domain and this activation requires an intact acetyl-CoA binding region (Akhtar and Becker, 2000).

Enok

Enok (enoki mushroom) encodes a putative histone acetyltransferase of the MYST family and is essential for normal development of the mushroom bodies, centers for olfactory learning and memory in *Drosophila* (Scott et al., 2001). A single amino acid change in the zinc finger motif of the putative HAT domain creates the same phenotype as the full gene deletion (Scott et al., 2001). This study has demonstrated an important function of acetylation in *Drosophila* brain development.

Chameau

Recombinant chameau was shown to acetylate histone *in vitro* (Grienenberger et al., 2002). Chameau suppresses PEV (position effect variagation) and is required for mediating HOX gene silencing by the Polycomb group proteins (Grienenberger et al., 2002). The acetyltransferase activity of chameau is required for these processes, since a mutation in the catalytic domain no longer rescues PEV modification (Grienenberger et al., 2002). Interestingly, like SAS2 and SAS3, chameau links histone acetylation to gene silencing.

Human MYST proteins

TIP60

Yeast two-hybrid analysis has identified Tip60 (Tat interactive protein 60kDa) as an interaction partner for HIV1-Tat protein. It was later identified as a histone acetyltransferase (Kamine et al., 1996; Yamamoto and Horikoshi, 1997). Recently, it was shown that Tip60 histone acetyltransferase activity is controlled by phosphorylation by the cyclin B/Cdc2 complex (Lemercier et al., 2003). Interestingly, the HIV1-Tat protein inhibits Tip60 histone acetyltransferase activity and also abolishes the transactivation of the Mn-dependent superoxide dismutase (Mn-SOD) gene, which is activated by Tip60 (Creaven et al., 1999). These data suggest that by interacting with Tip60, Tat hinders the expression of cellular genes, such as Mn-SOD, that normally interferes with the efficient replication of HIV1 virus. Tip60 was also shown to interact with class I nuclear hormone receptors and potentiates their transactivation activity in a ligand-dependent manner (Brady et al., 1999; Gaughan et al., 2001). In addition to its histone acetyltransferase activity, Tip60 is part of a complex consisting of fourteen subunits of which some display ATPase, DNA helicase and DNA binding activities (Ikura et al., 2000). Ectopic expression of Tip60 lacking histone acetyltransferase activity resulted in cells defective in double-strand DNA break repair and loss of apoptotic competence (Ikura et al., 2000). These results suggest that histone acetylation is linked to important biological processes such as DNA repair and apoptosis. Recently, Tip60 has been demonstrated to be implicated in Alzheimer's disease by forming a complex with the cytoplasmic tail of APP (amyloid-β precursor protein) protein and the nuclear adaptor protein Fe65 to stimulate transcription (Cao and Sudhof, 2001). The interaction with APP and Fe65 and

consequently transcription activation requires intact Tip60 MYST domain (Cao and Sudhof, 2001). Numerous proteins (HIV1 Tat, interleukin-9 receptor, androgen receptor, Fe65, endothelin receptor A, cytosolic phospholipase A2, E26 transforming-specific leukaemia gene TEL) have been shown to interact with Tip60 in two-hybrid screens (Brady et al., 1999; Cao and Sudhof, 2001; Kamine et al., 1996; Lee et al., 2001; Nordentoft and Jorgensen, 2003; Sheridan et al., 2001; Sliva et al., 1999). Tip60 has also been implicated in negative regulation of gene expression by binding to CREB and STAT3 transcription factors and TEL protein (Gavaravarapu and Kamine, 2000; Nordentoft and Jorgensen, 2003; Xiao et al., 2003). So, depending on the signaling pathway, Tip60 can either be a coactivator through its interaction with nuclear hormone receptor or a corepressor by associating with CREB.

HBO1

The origin recognition complex (ORC), the minichromosome maintenance (MCM) proteins and the Cdc6 protein play important roles in eukaryotic DNA replication by binding at replication origins (Bell and Stillman, 1992; Cocker et al., 1996; Diffley, 1996). HBO1 (histone acetyltransferase bound to ORC 1) has been shown to possess intrinsic histone acetyltransferase activity and interact with the largest subunit of ORC, ORC1, and to MCM2 protein (Burke et al., 2001; Iizuka and Stillman, 1999). These results suggest a role of histone acetylation by HBO1 in the process of DNA replication (Iizuka and Stillman, 1999). Like Tip60, HBO1 was shown to interact with the androgen receptor (AR) (Sharma et al., 2000). The interaction between HBO1 and AR was

enhanced in the presence of dihydrotestosterone both *in vitro* and *in vivo*. Unlike Tip60, HBO1 represses AR-mediated transcription (Sharma et al., 2000).

MOZ

MOZ was identified as a gene associated with acute myeloid leukemia (AML) (Borrow et al., 1996). The chromosomal translocation t(8:16)(p11;p13) results in in frame fusion of the CBP gene at 16p13 to the MOZ gene at 8p11. The breakpoint occurs at MOZ codon 1547 and CBP codon 266 (Borrow et al., 1996; Panagopoulos et al., 2000) (Figure 7B). The p300 gene, a homologue of the CBP gene, was also observed to be fused to MOZ gene in AML (Chaffanet et al., 2000; Kitabayashi et al., 2001b). Because the product of t(8:16)(p11;p13) contains two acetyltransferase domains, it raises the possibility that the MOZ-CBP fusion protein could mediate leukemogenesis via aberrant chromatin acetylation and thus alterations in gene expression. Another abnormality associated with AML is inv(8)(p11q13), which fuses MOZ and the nuclear receptor transcriptional coactivator TIF2 (Aguiar et al., 1997; Carapeti et al., 1998; Carapeti et al., 1999; Liang et al., 1998) (Figure 6B). MOZ-TIF2 retains the histone acetyltransferase domains of both proteins and also the CBP binding domain of TIF2. Again, recruitment of CBP by MOZ-TIF2 to promoters could result in abnormal histone acetylation and promote cancer.

3.3 Regulation of histone acetyltransferases

The regulation of HATs is a field that has not been extensively explored. Recently, several papers have shed light on the mechanisms by which mammalian cells control HAT activity.

One mechanism involved in regulating HAT activity is to regulate their stability. Tip60 is required for DNA repair and apoptosis following irradiation of human cells (Ikura et al., 2000). Recently, it was shown that DNA damage induces a signaling pathway leading to Tip60 accumulation. Interestingly, the ubiquitin ligase, Mdm2, interacts with Tip60 and induces its ubiquitination and proteasome-dependent degradation (Legube et al., 2002). These results suggest that Tip60 intracellular levels are highly controlled in cells in part by modulating the protein stability.

Post-translational modification is another regulating mechanism for HATs. Phosphorylation of CBP by cyclin E/cyclin-dependent kinase 2 stimulates its HAT activity and is important for the progression to S phase (Ait-Si-Ali et al., 1998). Signaling via Ca^{2+}/calmodulin dependent kinase IV (CaMKIV) has been shown to activate CBP-dependent transcription (Hu et al., 1999). Recently, it was demonstrated that phosphorylation of CBP by CaMKIV is responsible for CREB/CBP-dependent activation of neural cells genes (Impey et al., 2002). Steroid hormones regulate expression of target genes by binding their corresponding nuclear receptors. Binding of the ligand to the nuclear receptor favors the recruitment of the coactivators CBP, CARM1 and GRIP-1. Methylation of CBP by the histone methyltransferase CARM1 outside its KIX domain plays a critical role in GRIP-1-dependent transcriptional activation and hormone-induced gene activation (Chevillard-Briet et al., 2002). Interestingly,

methylation of the CBP KIX domain by CARM1 blocks CREB activation by disabling its interaction with CBP (Xu et al., 2001). Thus, CARM1 is a direct positive activator for nuclear receptors, but an indirect modulator by inhibition of the CREB-dependent pathway.

The activity of HAT can also be modulated through the recruitment of HDACs. For example, the recruitment of the histone deacetylase HDAC6 by sumoylated p300 mediates transcriptional repression (Girdwood et al., 2003). Some complexes containing both HAT and HDAC activities have been characterized (Yamagoe et al., 2003).

3.4 HAT and cancer

Alteration in chromatin remodeling by improper targeting of HATs or HDACs to certain loci, functional inactivation of HATs, and overexpression of HDACs can induce tumorigenesis (Jacobson and Pillus, 1999).

Inactivation of HAT activity due to gene mutations or the inhibitory action of viral proteins is associated with cancer. For example, functional mutations in one CBP allele are associated with Rubinstein-Taybi syndrome, a condition that predisposes to cancer (Kalkhoven et al., 2003). Missense mutations and loss of heterozygosity at the p300 locus are associated with colorectal and breast cancers (Giles et al., 1998). The interaction between oncogenic viral proteins, such as the adenovirus E1A and the SV40 T-antigen, with p300, CBP and PCAF antagonizes the normal expression of cellular genes. This can be explained by the fact that oncogenic viral proteins can either inhibit the HAT activity or the interaction between coactivators and transcription factors like p53 (Goodman and Smolik, 2000). CBP, p300 and PCAF are associated with p53-mediated

transcription. Inhibition of coactivator function through mutation or binding of oncogenic viral proteins can inhibit p53-mediated apoptosis or cell growth arrest (Groosman, 2001).

Several HAT-associated chromosomal translocations have been discovered in leukemia patients. For example, t(8;16)(p11;13), which results in the fusion between the MOZ and CBP genes, was reported in patients with acute myeloid leukemia (AML) (Borrow et al., 1996). This fusion protein retains the HAT domains of the two coactivators and has been shown to inhibit Runx1-dependent transcription and differentiation of M1 cells into monocytes/macrophages (Kitabayashi et al., 2001a). Translocations that fuse the MOZ and TIF2 or MLL and CBP genes, have also been associated with AML (Liang et al., 1998).

4. The RUNX family of transcription factors

The Runx transcription factors are composed of a DNA binding subunit, α, and a non-DNA binding subunit, β (Ito, 1999). There are three mammalian genes encoding the α subunit, termed Runx1, 2, and, 3. They share a high degree of sequence similarity within most of their coding regions (Levanon et al., 1994). Runx proteins consist of several functional modules: the Runt domain and transcriptional activation and repression domains (Kanno et al., 1998). Runx proteins are able to either increase or inhibit the transcriptional activity of target genes, most likely depending on the specific cell type as well as the particular target gene. The Runt domain, named after the *Drosophila* runt gene, is an evolutionarily conserved 128-amino acid region that is responsible for both

DNA binding and heterodimerization with CBFβ (Ito, 1999; Kamachi et al., 1990). CBFβ enhances DNA binding of the Runt domain, but does not contact DNA itself (Kamachi et al., 1990; Ogawa et al., 1993). Another important role of CBFβ is that its dimerization with Runx proteins protects them against proteolytic degradation by the ubiquitin-proteasome system (Huang et al., 2001; Zhao et al., 2003). Within their respective cell lineages, the maximal expression of Runx transcription factors occurs during the developmental transition from proliferation to differentiation. So, Runx proteins are very important in promoting differentiation of specific cell types. There is a functional relationship between Runx proteins and TGF-β/BMP signaling. Smad transcription factors, the downstream targets of TGF-β/BMP signaling pathways, were shown to interact with Runx1, 2, and 3 to activate or repress target genes (Alliston et al., 2001; Hanai et al., 1999a).

4.1 Runx1, a key regulator for hematopoietic cell differentiation

Runx1 was initially identified as a gene in the breakpoint of t(8;21) (Miyoshi et al., 1991). Translocations involving Runx1 produce chimeric proteins such as Runx1-ETO and TEL-Runx1 in several types of acute leukemia (Miyoshi et al., 1993; Miyoshi et al., 1991). Mutations in the Runx1 DNA binding domain were found in different patients with leukemia, so alterations of Runx1 itself may contribute to leukemogenesis (Osato et al., 1999).

The development of the hematopoietic system is regulated by a series of transcription factors that control both the generation of hematopoietic stem cells and the lineage commitment and differentiation of the progenitor cells. Runx1 is a master

regulator of hematopoiesis because mice lacking Runx1 died *in utero* due to lack of fetal liver hematopoiesis and massive hemorrhages mainly observed in the central nervous system (Wang et al., 1996). Expression of Runx1 can restore definitive erythropoiesis and myelopoiesis in an *in vitro* assay using Runx1-deficient embryonic stem cells (Okuda et al., 2000). Runx1 regulates the transcription of a large number of hematopoietic-specific genes, including cell surface receptors such as the T-cell antigen receptor (TCR) and macrophage colony-stimulating factor (M-CSF) receptor; neutrophil elastase, and cytokines such as interleukin (IL)-3 and granulocyte macrophage colony-stimulating factor (GM-CSF) (Cameron et al., 1994; Nuchprayoon et al., 1994; Prosser et al., 1992; Rhoades et al., 1996; Takahashi et al., 1995). The expression of these genes also depends on the presence of adjacent binding sites for lineage-restricted transcription factors, such as c-Myb, C/EBPα, and Ets family members (Hernandez-Munain and Krangel, 1995; Wotton et al., 1994; Zhang et al., 1996). These results demonstrate that Runx1 functions as a key regulatory switch that controls the formation of the hematopoietic stem cell.

4.2 Runx2, an osteoblast-specific regulator

Like other Runx proteins, Runx2 binds DNA through its runt domain. The presence of a glutamine/alanine (Q/A) region adjacent to the runt domain was shown to inhibit the heterodimerization between Runx2 and CBFβ (Thirunavukkarasu et al., 1998) (Figure 8A). The first amino-terminal 19 amino acids as well as the Q/A region, in the context of the native protein, were shown to activate transcription (Thirunavukkarasu et al., 1998). The carboxy-terminal region of Runx2, termed the PST domain (because it is rich in proline, serine and threonine) contains consensus phosphorylation sites for ERK,

PKC and PKA (Selvamurugan et al., 2000). Within the PST domain are also found a nuclear localization signal (NLS) and both an activation and a repression domain separated by a nuclear matrix targeting signal (Figure 8A). Nuclear targeting of Runx2 to subcellular foci was shown to be important for the activation of the osteocalcin gene (Zaidi et al., 2001). The last five amino acids of Runx2 (VWRPY) were shown to recruit the TLE family of corepressors and thus function as a repression domain (Thirunavukkarasu et al., 1998).

Runx2 is a bone-related transcription factor (Ducy et al., 2000). It is essential for mesenchymal cell differentiation into osteoblasts (Ducy, 2000). Homozygous $Runx2^{-/-}$ mice showed a complete lack of functional osteoblasts and are devoid of mineralized bone or hypertrophic cartilage (Otto et al., 1997). Mutations in the human Runx2 gene, mainly in the runt domain, caused cleidocranial dysplasia, an autosomal disease characterized by the absence of clavicles, open fontanelles, supernumerary teeth and short stature (Mundlos et al., 1997). Mutational analyses suggest that cleidocranial dysplasia is caused by the inability of the Runx2 mutants to transmit the bone morphogenic protein (BMP) signal and to regulate the target genes required for the induction of osteogenesis (Zhang et al., 2000).

Multiple signaling pathways activate Runx2 activity (Figure 8B). One is the mitogen-activated protein kinase (MAPK) pathway (Xiao et al., 2000). This pathway can be stimulated by several signals including those initiated by extracellular matrix (ECM), osteogenic growth hormone like fibroblast growth factor-2 (FGF-2), and mechanical loading (Nugent and Iozzo, 2000; Wang et al., 2002; Xiao et al., 2002; Xiao et al., 1998; Ziros et al., 2002) (Figure 8B). These signals induce Runx2 phosphorylation and

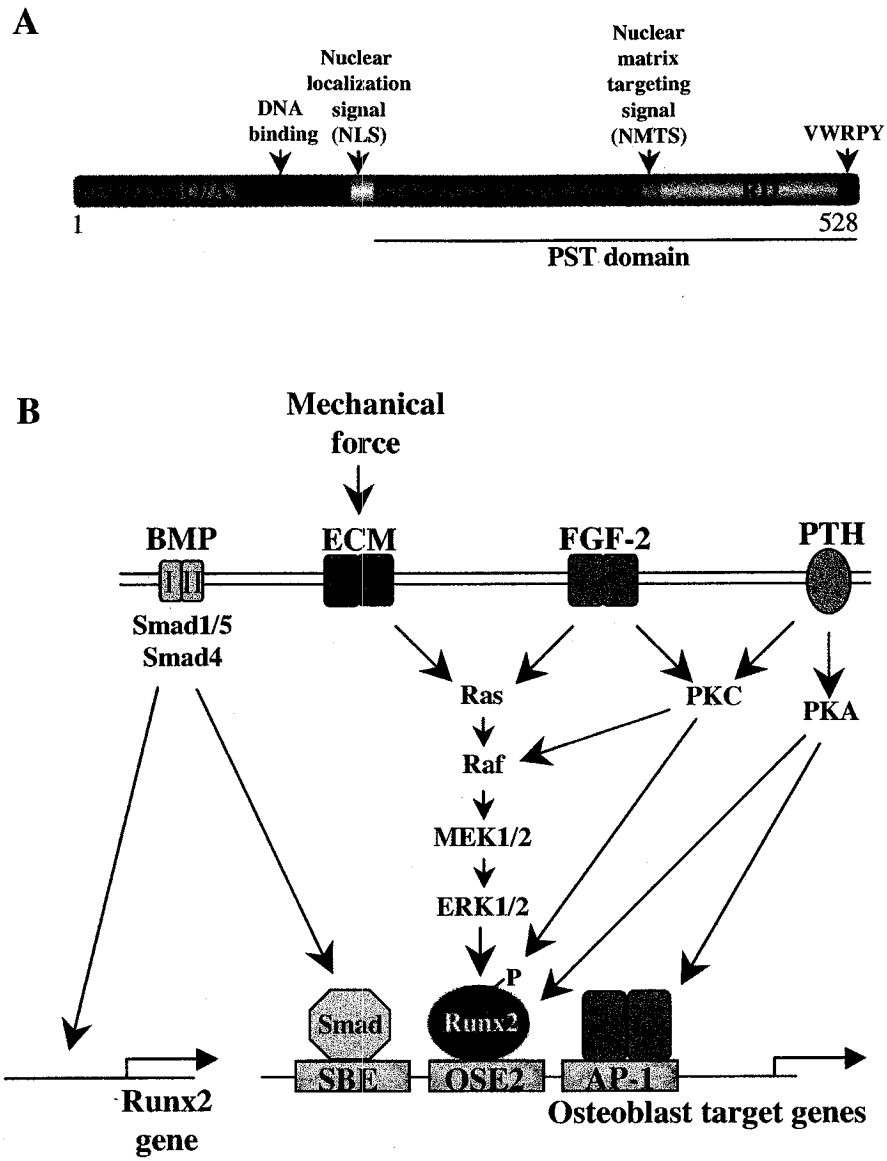

Figure 8. Signaling pathways involved in Runx2 regulation.

(A) **Schematic representation of the Runx2 protein.** Activation domains (AD) are in orange, repression domain (RD) in blue, the Q/A region in yellow, the runt DNA binding domain is in red, the NLS in white and the NMTS is in green.

(B) **Overview of signal transduction pathways affecting Runx2 activity.**

consequently its DNA binding and transactivation activity on OSE2 (osteocalcin sequence element 2) element (Xiao et al., 1997; Xiao et al., 2002; Ziros et al., 2002). Recently, it was shown that FGF2 stimulates Runx2 expression and its transactivation activity by stimulating the PKC signaling pathway (Kim et al., 2003) (Figure 8B). Another signaling pathway involved in Runx2 activation is initiated by the parathyroid hormone (PTH), an important regulator of calcium homeostasis and bone formation. Binding of PTH to its receptor activates two signaling pathways: the protein kinase A and the protein kinase C pathways. These can phosphorylate or upregulate transcription factors like CREB, AP-1 and Runx2 (Karaplis and Goltzman, 2000). It was shown that PTH stimulates the collagenase-3 promoter by a PKA-dependent pathway that phosphorylates Runx2 and up-regulates c-Fos/c-Jun via the phosphorylation of CREB (Hess et al., 2001; Selvamurugan et al., 2000). Mutations of Runx2 or c-Fos/c-Jun binding site, abolished PTH stimulation of the collagenase-3 promoter (Selvamurugan et al., 1998). The close proximity of c-Fos/c-Jun and Runx2 binding sites suggests that Runx2 physically interacts with c-Fos and/or c-Jun to form a complex that stimulates collagenase-3 promoter (D'Alonzo et al., 2002). PTH can stimulate proliferation of osteoblasts through PKC culminating in enhanced ERK signaling (Swarthout et al., 2001) (Figure 8B). Little is known about the mechanisms by which PKC and ERK are activated by PTH with regards to gene expression. Genes related to cell proliferation might be potential targets. BMPs are well-known inducers of osteoblast differentiation. Signals initiated by the binding of BMPs to their receptors are transduced by the transcription factors Smad1, 5, and 8 (Baker and Harland, 1997). Runx2 expression and activity are regulated by BMPs (Chen et al., 1998a). For example, BMPs stimulate

osteoblast differentiation by activating Runx2 through its interaction with Smad1 (Hanai et al., 1999b). BMP also transcriptionally activates Runx2 and mediates its interaction with Smad5 to induce osteoblast-specific gene expression in C2C12 mesenchymal progenitor cells (Lee et al., 2000).

Runx 2 can either be a transcriptional activator or repressor depending on the cell type as well as the particular target genes. Runx2 can stimulate expression of several genes in skeletal tissues, such as osteocalcin, osteopontin, bone sialoprotein, collagenase-3, ameloblastin and RANKL (Dhamija and Krebsbach, 2001; Ducy et al., 1997; Geoffroy et al., 1995; Otto et al., 2003). Several coactivators, such as Rb, p300, YAP and TAZ, were shown to interact with Runx2 and stimulate osteocalcin gene expression (Cui et al., 2003; Sierra et al., 2003; Thomas et al., 2001; Yagi et al., 1999). The repression of the p21 promoter by Runx2 and the histone deacetylase HDAC6 suggests that it may also interact with Runx2 in osteoblasts to regulate tissue-specific gene expression (Westendorf et al., 2002).

4.3 Runx3 is a tumor suppressor for gastric cancer

Two different laboratories have demonstrated that $Runx3^{-/-}$ knockout mice manifested severe limb ataxia due to defective development of proprioceptive neurons in the dorsal root ganglia (Inoue et al., 2002; Levanon et al., 2002). In another study, $Runx3^{-/-}$ mice exhibited gastric mucosa hyperplasia due to stimulated proliferation and suppressed apoptosis in epithelial cells. These mice died shortly after birth, apparently due to starvation (Li et al., 2002). This hyperplasia is caused by a reduction in sensitivity to both the growth-suppressive effect and the apoptosis-inducing activity of TGF-β (Li et

al., 2002). These interesting results suggest that Runx3 is a major growth regulator of gastric epithelial cells. Indeed, at advanced stages of cancer, it was demonstrated that human gastric epithelial cells did not significantly express Runx3 gene because it was inactivated by hemizygous deletion and methylated at its promoter. A rare missense loss-of-function mutation in the runt domain of Runx3 was also observed (Li et al., 2002). Exogenous expression of Runx3 in MKN28 cells, which do not express Runx3 and form rapidly growing tumors, greatly reduced tumor growth, suggesting that Runx3 is a tumor suppressor (Li et al., 2002).

TGF-β signaling pathway activates Smad2/3 transcription factors. These were shown to interact with Runx3 and mediate transactivation of specific promoters (Park et al., 2003). This observation fits nicely with the results obtained in $Runx3^{-/-}$ mice in which the TGF-β signaling pathway is interrupted in gastric cancer because of the lack of Runx3 function. Thus, Runx3 is an integral part of the TGF-β induced signaling pathway and contributes to its tumor-suppressive activities.

5. Rationale for the thesis project

The chromatin structure is a highly organized DNA-protein complex and serves as a barrier to chromatin template nuclear processes such as transcription, replication, recombination and repair. How the repressive chromatin structure is regulated is a question of great importance. In 1996, the identification of the first nuclear histone acetyltransferase, GCN5, in *Tetrahymena thermophila,* opened a new chapter in chromatin biology (Brownell et al., 1996). Since this exciting discovery, the identification and the functional characterization of new genes encoding histone

acetyltransferases provided important knowledge for understanding the unsolved mysteries of the chromatin structure. The identification of a fusion between MOZ and CBP genes in patients with acute myeloid leukemia provided the first link between histone acetylation and cancer (Borrow et al., 1996). In addition, MOZ is a very interesting protein because it contains a putative acetyl-CoA binding motif and is part of the MYST family of proteins associated with multiple cellular functions. No biochemical and functional studies were documented for MOZ in 1998 when I started my PhD project.

The first part of my thesis project (Chapter II) focuses on the identification and functional characterization of a new histone acetyltransferase called MORF (MOZ-related factor) (Champagne et al., 1999). Interestingly, MORF displays high sequence homology to MOZ and is composed of four parts: an amino-terminal domain containing two C4HC3 PHD-zinc fingers, a putative HAT domain, an acidic region, and a carboxy-terminal domain rich in serine and methionine residues. Biochemical and functional studies demonstrated that MORF is a histone acetyltransferase and contains several modules characteristic of a transcriptional regulator. This study led us to characterize MOZ, which indicates that MOZ is functionally similar to MORF (Champagne et al., 2001)

The second part of my project (Chapter III) focuses on the biological function of MOZ and MORF. We have demonstrated that both MOZ and MORF activation domains interact with Runx2, an osteoblast specific transcription factor, and potentiate Runx2-dependent transcriptional activation of the osteocalcin gene, a marker for osteoblast differentiation (Pelletier et al., 2002).

In the last part of my project (Chapter IV), we have demonstrated that Runx2, MOZ and TAZ synergistically stimulate the osteocalcin promoter. Stimulation of cells with phorbol 12-myristate 13-acetate (PMA) increases the synergistic effect of Runx2, MOZ and TAZ on the osteocalcin promoter. We have also shown that the interaction between full-length MOZ and Runx2 is very weak. Interestingly, the interaction of Runx2 with MOZ and TAZ is regulated by PMA (Pelletier et al., 2004). These results demonstrate how MOZ and MORF regulate Runx-dependent transcription.

Understanding the molecular mechanisms by which HATs are in control of gene regulation is of great importance for the diagnosis of treatment of diseases such as cancer. This study thus provides new insights into the function of MOZ and MORF and how they are implicated in leukemogenesis and osteoblast differentiation.

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CHAPTER II

IDENTIFICATION OF A HUMAN HISTONE ACETYLTRANSFERASE RELATED TO MONOCYTIC LEUKEMIA ZINC FINGER PROTEIN

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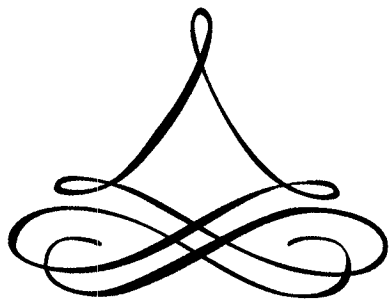

1. ABSTRACT

We describe here the identification and functional characterization of a novel human histone acetyltransferase, termed monocytic leukemia zinc finger protein (MOZ)-related factor (MORF). MORF is a 1781-residue protein displaying significant sequence similarity to MOZ. MORF is ubiquitously expressed in adult human tissues, and its gene is located at human chromosome band 10q22. MORF has intrinsic histone acetyltransferase activity. In addition to its histone acetyltransferase domain, MORF possesses a strong transcriptional repression domain at its N terminus and a highly potent activation domain at its C terminus. Therefore, MORF is a novel histone acetyltransferase that contains multiple functional domains and may be involved in both positive and negative regulation of transcription.

2. INTRODUCTION

In eukaryotes, DNA is packaged into chromatin, a highly organized DNA-protein complex that fulfills important functions not only as a structural element in preserving genetic information but also as an active player in controlling gene activity (1,2). How chromatin structure is regulated by DNA-binding transcription factors remains a central issue in studies of eukaryotic gene regulation. One regulatory mechanism involves acetylation of ε-amino groups of specific lysine residues located at the flexible N termini of core histones (1-5). While transcriptionally silent heterochromatin is usually hypoacetylated, transcriptionally active euchromatin is hyperacetylated (6-8). Mechanistically, histone acetylation affects nucleosome stability and/or internucleosomal interaction, or interferes with the interaction of histone tails with other proteins (4,5,9-11).

Histone acetyltransferases and deacetylases are the enzymes responsible for governing dynamic levels of histone acetylation at various chromatin domains *in vivo* (12,13). Histone deacetylases have been found to be associated with transcriptional repression (14,15). On the other hand, histone acetyltransferase (HAT) [1/] activity is intrinsic to several known transcriptional coactivators, including GCN5 (16,17), PCAF (18), p300 (19), CBP (19,20) and others (reviewed in 5,13,15,21-23).

Aberrant histone acetylation may lead to tumorigenesis (24,25). One piece of supporting evidence is that the CBP gene is frequently rearranged in cancers (26-29). Interestingly, one of the translocation partners involved is the MOZ gene (26,30). MOZ itself contains a putative acetyl CoA-binding motif and shares its putative HAT domain with the yeast proteins SAS2, SAS3 and ESA1 (31,32), *Drosophila* MOF (33) and human TIP60 (34). Among these proteins, ESA1 and TIP60 have been shown to possess

intrinsic HAT activity (32,34), whereas recombinant MOF does not exhibit any detectable HAT activity (33). Intriguingly, the putative yeast HATs, SAS2 and SAS3, have been implicated in both positive and negative regulation of gene expression (31,35), and ESA1 has recently been found to be required for cell cycle progression (36). Furthermore, MOF, which is required for dosage compensation in male flies, has been shown to be targeted to the X chromosome of *Drosophila* (33,37). For human MOZ, no biochemical or functional studies have been documented.

In this paper, we report the identification of a new human HAT, termed MORF (for MOZ-related factor), and further show that MORF possesses functional domains characteristic of a transcriptional regulator. Our results suggest the direct involvement of MORF and its related protein MOZ in transcriptional regulation and thus provide new insights into how abnormal forms of MOZ lead to tumorigenesis.

3. MATERIALS AND METHODS

3.1 Molecular cloning

cDNA library screening, plasmid construction and DNA sequencing were performed following standard procedures. Northern analyses on polyA-RNA blots (Clontech) were carried out according to the manufacturer's instructions. The reporter tk-Luc was derived from pGL2 (Promega) by insertion of the thymidine kinase (tk) core promoter (-105/+52). Gal4-tk-Luc was constructed from tk-Luc by insertion of 5 copies of the Gal4-binding site upstream from the tk promoter. Gal4-E4-Luc was derived from Gal4-tk-Luc by replacement of the tk region with the adenoviral E4 core promoter from 3TP-Lux (38).

3.2 Fluorescence in situ hybridization

FISH was performed on human lymphocytes as described (39), using as the probe a 5.8 kb MORF cDNA fragment biotinylated with dATP using the BioNick labeling kit (Gibco BRL).

3.3 Protein expression and purification

Full-length MORF was expressed in Sf9 cells as a fusion protein with the Flag epitope tag (IBI-Kodak) using a recombinant baculovirus generated with the Bac-To-Bac baculovirus system (Gibco BRL). The expressed fusion protein, f-MORF, was affinity-purified on M2 agarose and eluted with Flag peptide (0.1 mg/ml; IBI-Kodak). The Flag-tagged PCAF protein, f-PCAF, was similarly produced in and purified from Sf9 cells using the PCAF recombinant baculovirus previously described (18). Maltose-binding protein (MBP)-MORF mutants were expressed in E. coli, purified on amylose resin (New

England Biolabs), eluted with maltose (10 mM) and used directly for further analyses. For all affinity purification, buffer B (20 mM Tris-HCl pH8.0, 10% glycerol, 5 mM MgCl2, 0.1% NP-40 and protease inhibitors) containing 0.5 M KCl was used as lysis and washing buffers. In all elution buffers, the concentration of KCl was reduced to 0.15 M.

3.4 Western blot analysis

Affinity-purified f-MORF and f-PCAF proteins were electro-transferred to BioTrace nitrocellulose membranes (Gelman Sciences) and probed with M2 anti-Flag antibody (IBI-Kodak). Blots were developed with Supersignal chemiluminescent substrate (Pierce).

3.5 HAT assay

HAT activity was determined by analyzing incorporation of [3H]- or [14C]-labeled acetyl groups into histones. To measure HAT activity, Whatman P81 filter-binding assays were used (40,41). A typical reaction (20 ml) contained 75 nCi of [3H]acetyl CoA (4.7 Ci/mmol; Amersham Life Science) and 2 mg of calf thymus histones (type IIa; Sigma). The reaction mixture was incubated at 30°C for 10 min and then processed as described (40,41). For acetyllysine peptides (19), 30 mg were used per reaction. To distinguish which histones were acetylated, each reaction (20 ml) contained 2.5 nCi of [14C]acetyl CoA (51 mCi/mmol; Amersham Life Science) and 0.5 mg of HeLa octamers, nucleosomes or oligonucleosomes. The reaction was carried out at 30°C for 30 min and stopped by the addition of 10 ml of 3xSDS sample buffer, followed by separation on 15% SDS-PAGE gels and subsequent fluorography or phosphoimaging analysis using a

FUJIX BAS 100 phosphoimager (18). While the latter assay distinguishes which histones are acetylated, the P81-filter binding assay is more reliable and convenient for quantitative determination of HAT activity.

3.6 Reporter gene assays

SuperFect transfection reagent (Qiagen) was used to transiently transfect a luciferase reporter (200 ng) and/or mammalian expression plasmids (200 ng) into NIH3T3 or 293T cells. pBluescript KSII(+) was used to normalize the total amount of plasmids used in each transfection and the plasmid CMV-b-Gal (50 ng) was cotransfected for normalization of transfection efficiency. After 48 hrs, luciferase activity of transfected cells was determined using D-(-)-Luciferin (Boehringer Mannheim) as the substrate. Galactosidase activity was measured using Galacto-Light Plus™ (Tropix, Perkin-Elmer Co.) as the substrate. The chemiluminescence from activated Luciferin or Galacto-Light Plus™ was measured on a Luminometer Plate Reader (Dynex). Each transfection was performed at least 3 times to ensure that consistent results were obtained.

To verify the expression of Gal4 fusion proteins, expression plasmids were transfected to 293T cells; total or nuclear extracts were prepared for Western blotting analysis using a monoclonal anti-Gal4 antibody (Santa Cruz Biotech., RK5C1). Total extracts were prepared as described above using buffer B containing 0.15 M KCl. For nuclear extracts, transfected cells were washed twice with PBS and lysed in situ using 5 ml (for a 10 cm dish) of ice-cold hypotonic lysis buffer (20 mM HEPES pH 7.6, 20% glycerol, 10 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 0.1% Triton X-100, 25 mM NaF, 25 mM b-glycerophosphate, 1 mM DTT and protease inhibitors). After 5 min, cell

lysates were harvested by scraping and centrifuged for 5 min at 500 rpm on a Beckman swinging bucket tabletop centrifuge to pellet the nuclei. 0.1 ml of hypotonic lysis buffer containing 0.5 M NaCl was used to extract the nuclei. After being rotated for 20 min at 4°C and brief centrifugation, the supernatants were collected and used as nuclear extracts for Western blotting analysis.

4. RESULTS AND DISCUSSION

4.1 Cloning of MORF

With known and putative HATs as baits, we performed BLAST and PSI-BLAST searches (42) against various sequence databases. During these searches, we found a partial human cDNA clone (GenBank accession no. AB002381). This partial clone encodes a polypeptide displaying significant sequence similarity to MOZ. To obtain the complete coding sequence, we screened human cDNA libraries. PCR and sequence analyses of a majority of positive cDNA clones indicated that the full-length MORF clone encodes a polypeptide consisting of 1781 residues (Fig. 1A). The remaining positive clones were found to encode the polypeptides MORFa and MORFb, with 109 and 292 residues inserted between P372 and D373 of MORF, respectively (Fig. 1B). Database searches and amino acid sequence comparison indicated that MORF is homologous to MOZ (identity, 60%; similarity, 66%). As shown in Fig. 1A, MORF is composed of four parts: an N terminal region containing two C4HC3 PHD-zinc fingers, a putative HAT domain, an acidic region and a C terminal Ser/Met-rich domain. PHD-zinc fingers are putative protein-protein interaction motifs found in numerous proteins implicated in gene regulation, including the transcriptional regulator ATRX (43,44), the corepressor KAP-1/TIF1-b (45,46) and the helicase protein Mi2 (47-51). Interestingly, the latter two are known transcriptional repressors. The putative HAT domain of MORF is homologous to that shared by MOZ (26), HBO1(GenBank accession no. AF074606), TIP60 (34), MOF (52), SAS2 (31), SAS3 (31) and ESA1 (32). The acidic and Ser/Met-rich domains of MORF do not display obvious sequence similarity to known proteins

other than MOZ. These structural features of MORF suggest that it may be a HAT with novel properties.

As shown in Fig. 2A, Northern blot analyses of polyA-RNA from various human tissues indicated that MORF is ubiquitously expressed, most abundantly in heart, pancreas, testis and ovary. The expression of MORF is low in lung but detectable. FISH analyses revealed that the MORF gene is located at human chromosome band 10q22.2 (Figs. 2B & C). A juvenile polyposis tumor suppressor locus has been mapped to 10q22 (53). Furthermore, this band is abnormal in a patient with biphenotypic acute leukemia (54), and amplification of a candidate gene located at 10q22.1-q23.1 has been correlated with the metastasis of bladder cancers (55). Therefore, the MORF gene is located at a chromosomal region that is rearranged in several neoplasms.

4.2 HAT activity of MORF

Next we asked if MORF is really a HAT. To test this, we tried to express full-length MORF as a Flag-tagged fusion protein in Sf9 insect cells. Due to unknown reasons, the recombinant baculovirus was not stable and the expression level of f-MORF was very low. Because of the limited amount of MORF available, Western analysis with an anti-Flag antibody was used to determine the concentration of Flag-tagged MORF (f-MORF) in affinity-purified preparations (Fig. 3A, lane 2). For such Western analyses, Flag-tagged PCAF (f-PCAF; lane 1), which could be highly expressed with a similar system and affinity-purified to near homogeneity (18), was utilized for comparison. To determine HAT activity of full-length MORF, both f-MORF and f-PCAF were affinity-

purified and subjected to HAT assays. As shown in Fig. 3B, affinity-purified f-MORF was much more active than f-PCAF.

To determine the subtrate specificity, HeLa histones and oligonucleosomes were labeled with f-MORF in the presence of [14C]acetyl CoA, resolved by SDS-PAGE and subjected to fluorography or phophoimaging analysis. As shown in Fig. 3C, f-MORF preferentially acetylated histones H3 and H4, whereas f-PCAF preferentially acetylated H3. Like f-PCAF, f-MORF was autoacetylated. When oligonucleosomes were used substrates, f-MORF preferentially acetylated H4, whereas f-PCAF preferentially acetylated H3. Taken together, these results indicate that MORF is a potent HAT.

4.3 Characterization of the HAT domain of MORF

To map the HAT domain, we expressed several MORF fragments in E. coli as a protein fused to the following affinity tags: 6xHis, glutathione S-transferase and MBP. Among these, only the MBP fusion proteins could be expressed in soluble forms (Fig. 4A). These fusion proteins were affinity-purified on amylose resin (Fig. 4B). HAT assays were performed with these purified MBP-fusion proteins. As shown in Fig. 4C, MBP-A efficiently acetylated histones. Deletion of residues 361-425 increased HAT activity by 4-fold (Fig. 4C, compare MBP-A and -B), suggesting that residues 361-425 negatively regulate the activity of the HAT domain. On a molar basis, MBP-B was found to be about 40-fold less active than f-MORF. Further deletion of residues 426-460 abolished the activity (Fig. 4C, MBP-C). Deletion of residues 554-587, which contains the putative acetyl CoA-binding motif, inactivated the enzyme (Fig. 4C, MBP-D).

Altogether, these results indicate that residues 426-716 of MORF constitute its HAT domain. These results also surpport that MORF has intrinsic HAT activity.

As shown in Fig. 1, MORFα contains an insertion of 109 residues between P372 and D373 of MORF. Since residues 361-425 of MORF serve as a negative regulator for the HAT domain, we tested if the MORFα fragment corresponding to residues 426-716 of MORF has distinct HAT activity. For this, residues 361-825 of MORFa was expressed and purified as an MBP-fusion protein. This fusion protein (MBP-E; Fig. 4C) was as active as MBP-A, suggesting that the insertion of 109 residues between P372 and D373 of MORF does not relieve the inhibitory effect of residues 361-425 on the HAT domain.

To determine which histones are acetylated, substrates labeled with MBP-A or MBP-E were separated by SDS-PAGE and subjected to fluorography or phosphoimaging analysis. As shown in Fig. 4D, MBP-A and MBP-E preferentially acetylated free histones H3 and H4. Unlike MBP-A, MBP-E was autoacetylated. When oligonucleosomes were used, no detectable acetylation was observed with either MBP-A or MBP-E (data not shown).

Next we sought to assess which lysine of histone H4 is acetylated. For this, histone H4 acetyllysine peptides (Fig. 5A; ref. 19) were used as substrates. As shown in Figs. 5B-D, MBP-A, -B and -E acetylated Lys-5, -8, -12, and -16 of histone H4.

Consistent with the fact that MORF shares a conserved HAT domain with TIP60 and ESA1 (Fig. 1A; refs. 32,34), these results indicate that the substrate specificity of MORF is similar to that reported for TIP60 and ESA1. With free core histones as a substrate, it has been reported that TIP60 and ESA1 acetylate H2A, H3 and H4 (32,34). Furthermore, TIP60 acetylates Lys-5, -8, -12 and -16 of histone H4 (56). However, unlike MORF,

ESA1 and TIP60 were found to be unable to acetylate nucleosomal histones H2A, H3 and H4 (32,34). Interestingly, unlike full-length MORF, MBP-A is unable to efficiently acetylate nucleosomal histones. In the case of TIP60, only the HAT domain was analyzed (34), so it is still unclear whether full-length TIP60 is able to acetylate nucleosomal histones. It is unclear whether this difference is due to different assay conditions employed in different studies.

The substrate specificity of MORF is clearly different from that of other HATs such as GCN5, PCAF, p300 and CBP (16,18-20,57,58). One complicating factor is that most HATs exist as multisubunit complexes in vivo and recombinant catalytic subunits display properties (e.g. substrate specificity and specific activity) different from the corresponding complexes (59,60). This may partially explain why f-MORF is more active than MBP-B since some Sf9 cellular proteins may tightly associate with f-MORF and affect its function. Relevant to this, recombinant Drosophila MOF was found to be inactive although it is expected to be an active HAT in vivo (33). Another complicating factor is that properties of HATs are affected by the assay conditions employed (57). Therefore, further biochemical studies are needed to fully elucidate the properties of MORF in vivo and to compare them with those of other HATs.

4.4 Transcriptional ability of MORF

Since MORF has intrinsic HAT activity, we next examined if MORF is able to regulate transcription when tethered to a promoter. For this, a series of constructs was engineered to express MORF or its deletion mutants fused to the Gal4 DNA-binding domain (Fig. 6A). As shown in Fig. 6B, full-length MORF and its mutant DCoA weakly

repressed transcription (by 2- and 4- fold, respectively). In contrast, the deletion mutant N426 activated transcription by 5.9-fold. The weak effects of these fusion proteins on transcription may be due to their low protein expression levels, which were undetecable by Western blotting analysis with a monoclonal anti-Gal4 antibody (data not shown). These results suggest that there is an activation domain located at the C terminal part of MORF and that the N terminal region (residues 1-426) counteracts the function of this activation domain.

To map the activation domain, we tested several deletion mutants and found that one mutant (N1268) activated transcription by 333-fold (Fig. 6B). This mutant had minimal effects on tk-Luc, a reporter lacking Gal4-binding sites, suggesting that the observed effect on Gal4-tk-Luc is dependent on specific recruitment to the Gal4-binding sites. To further define the activation domains, two deletion mutants (N1564 and N1493) were constructed. While N1564 stimulated transcription by 46-fold, N1493 was inactive. Western analysis indicated that N1493 was well expressed as N1564 and N1493 (Fig. 7A). Therefore, a transcriptional activation domain is located at the Ser-rich region of MORF and the Met-rich region is required for the optimal function of this activation domain.

As shown above, the N terminal region of MORF counteracts the function of its C terminal activation domain. This could be due either to the N terminal region binding to the C terminal activation domain and then inhibiting its activating function, or to the N terminal region itself being a transcriptional repression domain. To test the latter possibility, a series of constructs containing truncations from the C terminus of MORF was engineered and tested in reporter assays (Fig. 6A). As shown in Fig. 6C, with the

reporter Gal4-E4-Luc, C426 and C352 repressed transcription by 43- and 33-fold, respectively. On the other hand, the deletion mutants C215 and C207 down-regulated transcription to a lesser extent (by 5.9- and 3.7-fold, respectively). The expression level of C207 was lower than the others (Fig. 7B). These mutants had minimal effects on 3TP-Lux, which lacks Gal4-binding sites (38). Therefore, the observed repression on Gal4-E4-Luc is dependent on specific promoter tethering, suggesting that the N terminal region of MORF constitutes an active repression domain.

We then decided to investigate possible repression mechanisms. PHD-zinc fingers of Mi2b have been found to be required (but not sufficient) for direct interaction with the histone deacetylase HDAC1 (48), raising the question of whether the PHD-zinc finger-containing repression domain of MORF directly interacts with HDAC1. To test this, HDAC1 was synthesized in vitro in reticulocyte lysates and subjected to pull-down assays with MBP-MORF(1-426) immobilized on amylose resin. These assays revealed, however, that there was no detectable interaction between MORF(1-426) and HDAC1 (data not shown). To further substantiate this, equivalent amounts of MBP and MBP-MORF(1-426) were immobilized on amylose resin and incubated with 293T cellular extracts. Subsequently, the amylose resin was extensively washed, and bound proteins were eluted and subjected to histone deacetylase assays. These assays revealed that MBP-MORF(1-426) did not retain more histone deacetylase activity than MBP (data not shown), suggesting that MORF(1-426) does not interact with a histone deacetylase. Consistent with this, transcriptional repression mediated by MORF(1-426) could not be relieved by treatment with the histone deacetylase inhibitor trichostatin A (data not

shown). Taken together, these results indicate that the repression mediated by MORF (1-426) operates through a mechanism other than recruitment of a histone deacetylase.

5. CONCLUSION

The data presented here demonstrate that MORF is a new HAT containing multiple functional domains. In additional to its HAT domain, MORF possesses a potent transcriptional activation domain located at its C terminus. This reflects a theme already described for p300 and CBP, both of which contain two activation domains independent of their HAT domains (reviewed in 5,13, 22-23). On the other hand, PCAF does not appear to have additional activation domains besides its HAT domain (18). Different from known HATs, MORF contains a strong transcriptional repression domain located at its N terminus. This repression domain contains two PHD-zinc fingers. Similar zinc fingers have been found in known transcriptional repressors, e.g. TIF1-b/KAP-1 (45,46,61) and Mi2 (47-51). Unlike MORF, Mi2 interacts with HDAC1 though its PHD-zinc fingers (48,50,51). MORF shares its HAT domain with a family of proteins, including human MOZ (26) and TIP60 (34), Drosophila MOF (52), and yeast SAS2, SAS3 and ESA1 (32,52). Interestingly, while MOF is considered to be involved in gene activation, SAS2 and SAS3 are involved in both positive and negative regulation of gene expression (31,34,52). Our findings on MORF add another level of complexity to the function of this new family of proteins.

In summary, we have identified a new human protein termed MORF. MORF is ubiquitously expressed and its gene is located at chromosome band 10q22, a region rearranged in several neoplasms. MORF has intrinsic HAT activity. Unlike known HATs, MORF possesses a transcriptional repression domain at its N terminus and an activation domain at its C terminus. Based on these findings, we speculate that through their multiple functional domains, MORF and its homolog MOZ participate in both

positive and negative regulation of gene expression in vivo. Therefore, this study also illuminates how abnormalities of the MOZ gene lead to leukemogenesis.

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Figure 1 Primary structure of MORF and its isoforms.

A. Comparison of amino acid sequences of MORF and MOZ (GenBank accession no. U47742). The sequences were aligned using the Bestfit program (Genetics Computer Group, Inc.). Putative domains are marked by dark lines at right. Cys and His residues coordinating zinc binding in the PHD- and C2HC-zinc fingers are shown in bold. Two putative nuclear localization signals (NLS1 and NLS2) and a putative acetyl CoA-binding site are also indicated. A vertical arrow denotes the insertion site of extra residues in alternatively-spliced variants.

B. Sequences of extra amino acids of MORFα (*top*) and MORFβ (*bottom*) inserted between P372 and D373 of MORF.

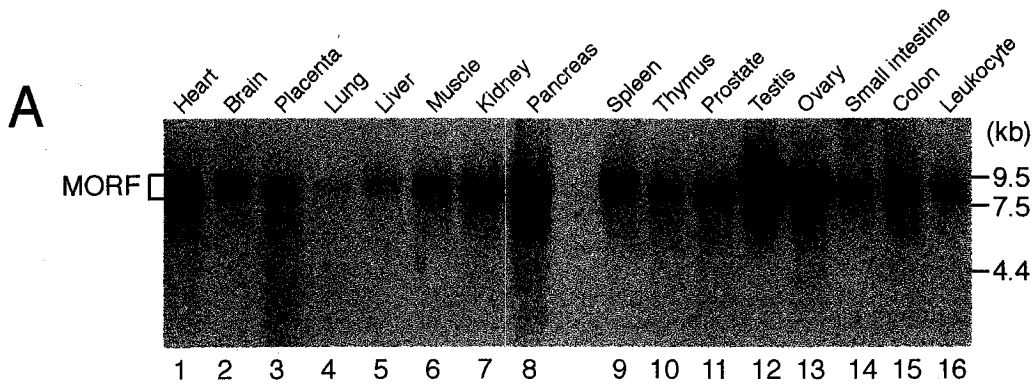

A
MORF 1 MVKLANPLYTEWILEAIQKIKKQKQRPSERRICHAVSTSHGLDKKTVSEQ 50
MOZ 1 MVKLANPLYTEWILEAIKKVKKQKQRPSERRICNAVSSSHGLDRKTVLEQ 50
51 LRLSVQDGSVLKVTNKGLASYKDPDNPGRPSSVKPGTFPKSAKGSRGSCN 100
51 LELSVKDGTILKVSNKGLNSYKDPDNPGRIALPKP.....RNHGKLD 92
101 DLRNVDWNKLLRRAIEGLEEPNGSSLKNIEKYLRSQSDLTSTTNNPA... 147
93 NKQNVDWNKLIKRAVEGLAESGGSTLKSIERFLKGQKDVSALPGGSAASG 142
148 PQQRLRLGAKRAVNNGRLLKDGPQYRVNYGSLDGKGAPQYPSAFPSSLPP 197
143 FHQQLRLAIKRAIGHGRLLKDGPLYRLNTKATNVDGKESCESL..SCLPP 190
198 VSLLPHEKDQPRADPIPICSFCLGTKESNREKKPEELLSCADCGSSGHPS 247
191 VSLLPHEKDKPVAEPIPICSFCLGTKEQNREKKPEELISCADCGNSGHPS 240
248 CLKFCPELTTNVKALRWQCIECKTCSACRVQGRNADNMLFCDSCDRGFHM 297
241 CLKFSPELTVRVKALRWQCIECKTCSSCRDQGKNADNMLFCDSCDRGFHM 290
298 ECCDPPLSRMPKGMWICQVCRPKKKGRKLLHEKAAQIKRRYAKPIGRPKN 347
291 ECCDPPLTRMPKGMWICQICRPKKKGRKLLQKKAAQIKRRYTNPIGRPKN 340
348 KLKQ.....RLLSVTSDRGSMNAFTGR 369
341 RLKKQNTVSKGPFSKVRTGPGKGRKRKITLSSQSASSSSEEGYLERIDGL 390
370 G...SPDTEIKINIK..... 381
391 DFCRDSNVSLRPNKKTKGLIDGLTKFFTPSPDGRKARGEVVDYSEQYRIR 440
382 .....QESADVNVIGNKDVVTEEDLDVFKQAQE 409
441 KRGNRKSSTSDWPTDNQDGWDGKQENRERLPGSQEIMTEKDMELPRDIQE 490
410 LSWRKIECESGVEDCGRYPSVIEFGKYEIQTWYSSPYPQEYARLPKLYLC 459
491 QALQKVGVTGPPDPQVRCPSVIEFGKYEIHTWYSSPYPQEYSRLPKLYLC 540
460 EFCLKYMKSKNILLRESKKCGWFHPPANEIYRRKDLSVPEVDGNMSKIYC 509
541 EFCLKYMKSRTILQQHMKKCGWFHPPANEIYRKNNISVPEVDGNVSTIYC 590
510 QNLCLLAKLPLDHKTLYYDVEPPLFYVLTKNDEKGCHLVGYLSKEKLCQQ 559
591 QNLCLLAKLPLDHKTLYYDVEPPLFYVLTQNDVKGCHLVGYFSKEKHCQQ 640
560 KYNVSCIMIMPQHQRQGFGRPLIDFSYLLSRREGQAGSPEKPLSDLGRLS 609
641 KYNVSCIMILPQYQRKGYGRPLIDFSYLLSKRRGQAGSPEKPLSDLGRLS 690
610 YLAYWKSVILEYLYHHHERHISIKAISRATGMCPHDIATTLQHLHMIDKR 659
691 YMAYWKSVILECLYHQNDKQISIKKLSKLTGICPQDITSTLHHLRMLDFR 740
660 DGRFVIIRREKLILSHMEKLKTCSRANELDPDSLRWTPILTSNAAVSEER 709
741 SDQFVIIRREKLIQDHMAKLQLNLRPVDVDPECLRWTPVIVSNSVVSEEE 790
710 RE.....AEKEAERLMEQASCWEKEEQEILSTRANSR...QSP 744
791 EEEAEEGENEEPQCQERELEISVGKSVSHENKEQDSYSVESEKKPEVMAP 840
745 AKVQSKNKYLHSPESRPVTGE...RGQ.....LLEL 772
841 VSSTRLSKQVLPHDSLPANSQPSRRGRWGRKNRKTQERPGDKDSKLLLER 890
773 SKESSEEESEEEDEEEEEEEEEEDEEE..... 801
891 TSSAPQEQYGECGEKSEATQEQYTESEEQLVASEEQPSQDGKPDLPKRRL 940
802 .....EEEEE 806
941 SEGVEPWRGQLKKSPEALKCRLTEGSERLPRRYSEGDRAVLRGPSESSEE 990
807 EEEEEENIQSSPPRLTKPQSVAIKRKRPPVLKKKRGRKRRINSGVTTET 856
991 EEEPESPRSSSSPPILTKP...TLKRKKPFLHRRRRVRKRKHHNSSVVTET 1037
857 ISETTEVLNEPPDNSDEERPMPQLEPTCEI...EVEEDGRKPVLRKAFQH 903
1038 ISETTEVLDEPPEDSDSERPMPRLEPTFEIDEEEEEDENELFPREYFRR 1087
904 .....QPGKKRQTERERGGKDNHCFKNADPCRNNMNDDSSNLKEGSK 944
1088 LSSQDVLRCQSSSKRKSKDEERDEESDDADDTPILKPVSLLRKRDVKNSP 1137
945 DNPE...PLKCKQVWPKGTKRGLSKWRQNKERKTGPKLNLYTPPETPMEP 991
1138 LEPDTSTPLKKKKGWPKGKSRKPIHWKKRPGRKPGFKLSREIMPVS.... 1183
992 DEQVTVEEQKETSEGKTSPSPIRIEEEVKETGEALLPQEENRREETCAPV 1041
1184 TQACVIEPIVSIPKAGRKPKIQESEETVEPKEDMPLPEERKEEEEMQAEA 1233
1042 SPNTSPGEKPEDDLIKPEEEEEEEEEEEEEEEEEGEEEEGGGNVEKDPD 1091
1234 EEAEGEEEDAASSEVPAASPADSSNSPETETKEPEVEEE..... 1273
1092 GAKSQEKEEPEISTEKEDSARLDDHEEEEEEDEEPSHNEDHDADDEDDSH 1141
1274 EEKPRVSEEQRQSEEEQQRLEEPEPEEEEDAAAETAQNDDHDADDEDDGH 1323
1142 MESA.EVEKEELP.RESPKEVLENQETPLDLNVQPGHSNPEVLMDCGVDL 1189
1324 LESTKKKELEEQPTREDVKEEPGVQESPLDANMQKSREKIKDKEETELD. 1372
1190 TASCNSEPKELAGDPEAVPESDEEPPPGEQAQKQDQKNSKEVDTEPKEGN 1239
1373 .....SEEEQPSHDTSVV...SEQMAGSEDDHEEDGHTKEELIELKEEEE 1414
1240 PATMEIDSETVQAVQSLTQ.ESSEQDDTPQDCAETQEACRSLQNYTRADQ 1288
1415 IPHSELDLETVQAVQSLTQEESSEHEGAYQDCEETLAACQTLQSYTQADE 1464
1289 SPQIATTLDDCQQSDHSSPVSSVHSHPQQSVRSVNSPSVPALENSYAQIS 1338
1465 DPQM.SMVEDCHASEHNSPISSVQSHPSQSVRSVSSPNVPALESSGYTQIS 1513
1339 PDQSAISVPSLQNMETSPMMDVPSVSDHSQQVVDSGFSDLGSIESTTENY 1388
1514 PEQGSLSAPSMQNMETSPMMDVPSVSDHSQQVVDSGFSDLGSIESTTENY 1563
1389 ENPSSYDSTMGGSICGNGSSQNSCSYSNL.TSSSLTQSSCAVTQQMSNIS 1437
1564 ENPSSYDSTMGGSICGNSSSQSSCSYGGLSSSSSLTQSSCVVTQQMASMG 1613
1438 GSCSMLQQTSISSPPTCSVKSPQGCVVERPPSSSQQ..... 1473
1614 SSCSMMQQSSVQPAANCSIKSPQSCVVERPPSNQQQQPPPPPQQPQPPP 1663
1474 .....LAQCSMAANFTP 1485
1664 PQPQPAPQPPPPQQQPQQQPQPQPQQPPPPPPQQQPPLSQCSMNNSFTP 1713
1486 PMQLAEIPET.SNANIGLYERMGQSDFGAGHYPQPSATFSLAKLQQLTNT 1534
1714 APMIMEIPESGSTGNISIYERI.PGDFGAGSYSQPSATFSLAKLQQLTNT 1762
1535 LID.HSLPYSHSAAVTSYANSASLSTPLSNTGLVQLSQSPHSVPGGPQAQ 1583
1763 IMDPHAMPYSHSPAVTSYATSVS....LSNTGLAQLAPS.HPLAGTPQAQ 1807
1584 ATMTPPPNLTPPPMNLPPPLLQRNMAASNIGISHSQRLQTQIASKGHISM 1633
1808 ATMTPPPNLASTTMNLTSPLLQCNMSATNIGIPHTQRLQGQMPVKGHISI 1857
1634 RTKSASLSPAAATHQSQIVGRS.QTVAMQGPARTLTMQRGMNMSVNLMPA 1682
1858 RSKSAPL.PSAAAHQQQLYGRSPSAVAMQAGPRALAVQRGMNMGVNLMPT 1906
1683 PAYNVNSVNMNMNYLNAMNGYSMSQPMMNSGYHSNHGYMNQTPQYPMQMQ 1732
1907 PAYNVNS..MNMNYLNAMNSYRMTQPMMNSSYHSNPAYMNQTAQYPMQMQ 1954
1733 MGMMGTQPYAQQPMQTPPHGNMMYTAPGHHGYMN.TGMSKQSLNGSYMRR 1781
1955 MGMMGSQAYTQQPMQPNPHGNMMYTGPSHHSYMNAAGVPKQSLNGPYMRR 2004
PhD zinc fingers
NLS1
C2HC
Acetyl-CoA
HAT
Acidic
NLS2
Acidic
Ser-rich
Met-rich
B
MORFα
373 GRGQKTKVCT TPSSGHAASG KDSSSRLAVT DPTRPGATTK ITTTSTYISA
423 STLKVNKKTK GLIDGLTKFP TPSPDGRRSR GEIIDFSKHY RPKKKVSQKQ
473 SCTSHVLAT
MORFβ
373 GRGQKTKVCT TPSSGHAASG KDSSSRLAVT DPTRPGATTK ITTTSTYISA
423 STLKVNKKTK GLIDGLTKFP TPSPDGRRSR GEIIDPSKHY RPKKKVSQKQ
473 SCTSHVLATG TTQKLKPPPS SLPPPTPISG QSPSSQKSST ATSSPSPQSS
523 SSQCSVPSLS SLTTNSQLKA LPDGLSHTIP LRDSLAKRDT PSYAPPKRMR
573 RKTPELSSTAK SKAHFPC
623 TTPLKKHRML GRLKYKV

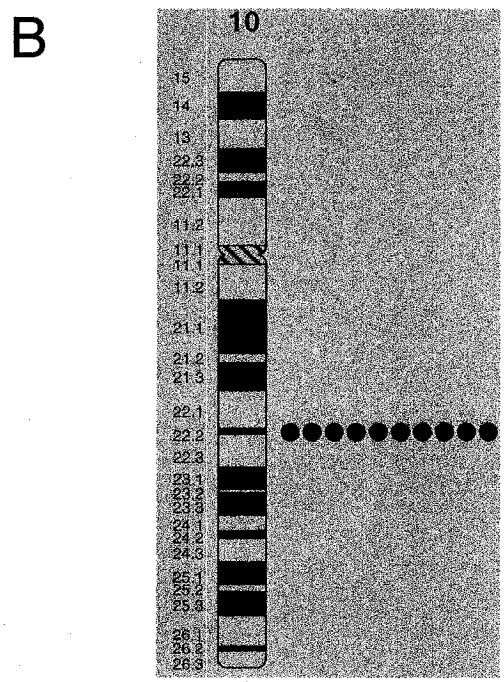
Figure 2 Analysis of the MORF gene.

A. Expression of MORF among different adult human tissues. PolyA-RNA blots were probed with an MORF cDNA fragment corresponding to its 3' untranslated region. Relative positions of RNA markers are indicated at right. Each lane contains 2 μg of polyA-RNA; hybridization with an actin cDNA probe confirmed that similar amounts of RNA are present in all lanes (data not shown).

B. Ideogram illustrating the chromosomal localization of MORF. Human blood lymphocytes were used for FISH. The hybridization efficiency was 86%, i.e. 86 of 100 mitotic figures checked showed this localization. Each dot represents double FISH signals (*C*) detected.

C. Example of FISH mapping. Left panel shows FISH signals on chromosome 2 (indicated by an arrow) while right panel shows the same mitotic figure stained with DAPI to identify chromosomes.


A
Heart
Brain
Placenta
Lung
Liver
Muscle
Kidney
Pancreas
Spleen
Thymus
Prostate
Testis
Ovary
Small intestine
Colon
Leukocyte
(kb)
MORF
9.5
7.5
4.4
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16


B
10

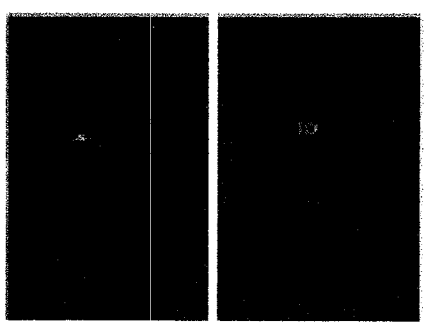
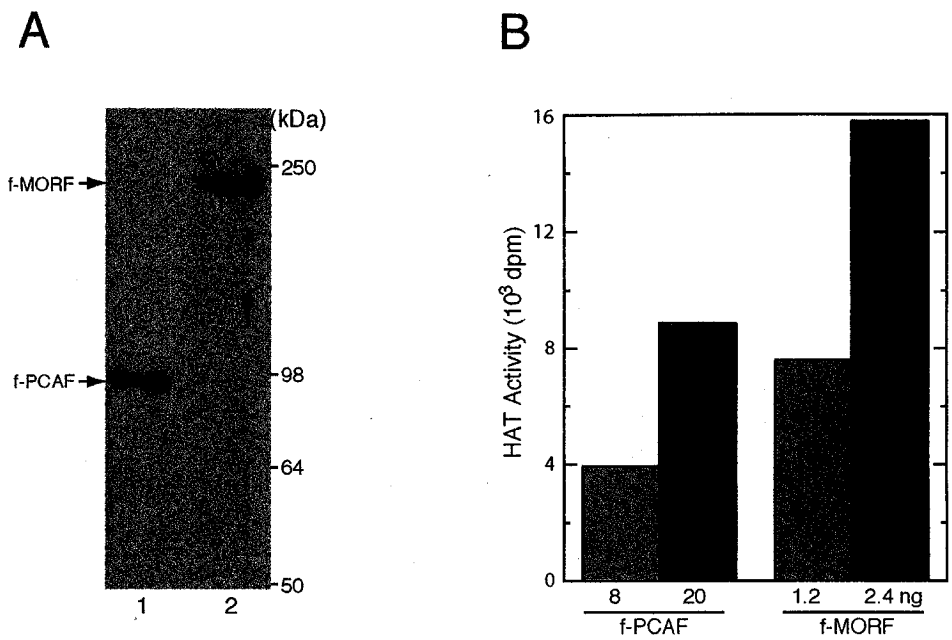

C

Figure 3 HAT activity of full-length MORF.

A. Western analysis of f-MORF. The Flag-tagged MORF protein, f-MORF, was expressed in Sf9 cells and affinity-purified on M2 agarose. Western analysis of f-MORF with an anti-Flag antibody is shown here (lane 2). For comparison, f-PCAF was similarly prepared and analyzed (lane 1).

B. HAT activity of f-PCAF and f-MORF determined by P81-filter binding assays. Both f-MORF and f-PCAF were affinity-purified on M2 agarose from infected Sf9 insect cell extracts. During affinity purification, a buffer containing 0.5 M KCl was used for extensive washing; under such conditions, with uninfected Sf9 cell extracts, equivalent amounts of M2 agarose retained minimal HAT activity (data not shown). The amount (in ng) of f-PCAF and f-MORF used in the assays are indicated at the bottom. The amount of f-MORF was estimated based on Western analyses similar to that shown in (*A*) except that various amounts of f-PCAF were used to ensure that signals from such analyses were proportional to the molar amounts of Flag-tagged proteins tested.

C. Substrate specificity of f-MORF. 0.5 μg of HeLa core histones (lanes 1-3) or oligonucleosomes (lanes 4-6) was incubated with no enzyme (lanes 1 and 4), f-PCAF (2.4 pmol; lanes 2 and 5) or f-MORF (0.06 pmol; lanes 3 and 6) in the presence of [^{14}C]acetyl CoA. After separation on SDS-PAGE gels, [^{14}C]-labeled histones were detected by fluorography or phosphoimaging.


A
(kDa)
250
f-MORF
f-PCAF
98
64
50
1
2
B
HAT Activity (10^3 dpm)
16
12
8
4
0
8
20
1.2
2.4 ng
f-PCAF
f-MORF

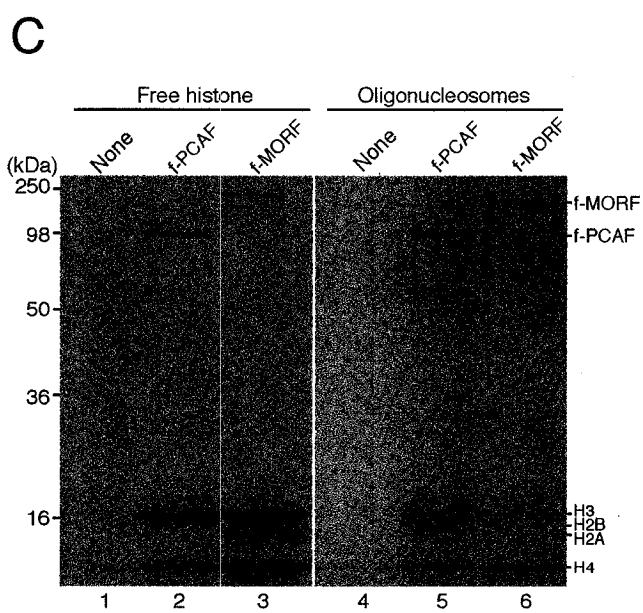

C
Free histone
Oligonucleosomes
None
f-PCAF
f-MORF
None
f-PCAF
f-MORF
(kDa)
250
98
50
36
16
f-MORF
f-PCAF
H3
H2B
H2A
H4
1
2
3
4
5
6

Figure 4 Characterization of the HAT domain of MORF.

A. Schematic representation of MBP-MORF fragments used to determine intrinsic HAT activity of MORF. The putative acetyl-CoA binding site is indicated by the tripeptide motif GYG.

B. Analysis of affinity-purified MBP-MORF fragments. MBP-A, -B, -C, -D and -E (0.4 μg per lane) were resolved on 10% SDS-PAGE and stained with Coomassie brilliant blue R-250.

C. HAT activity of MBP-MORF fragments determined by P81-filter binding assays. HAT activity is expressed in acetyl groups transferred (dpm) per pmol of MBP-fragments.

D. Substrate specificity of MBP-A and MBP-E. 0.5 μg of HeLa core histones was incubated with 2.5 pmol of MBP-A (lane 1) or MBP-E (lane 3) in the presence of [^{14}C]acetyl CoA. After separation on SDS-PAGE gels, [^{14}C]-labeled histones were detected by fluorography or phosphoimaging.

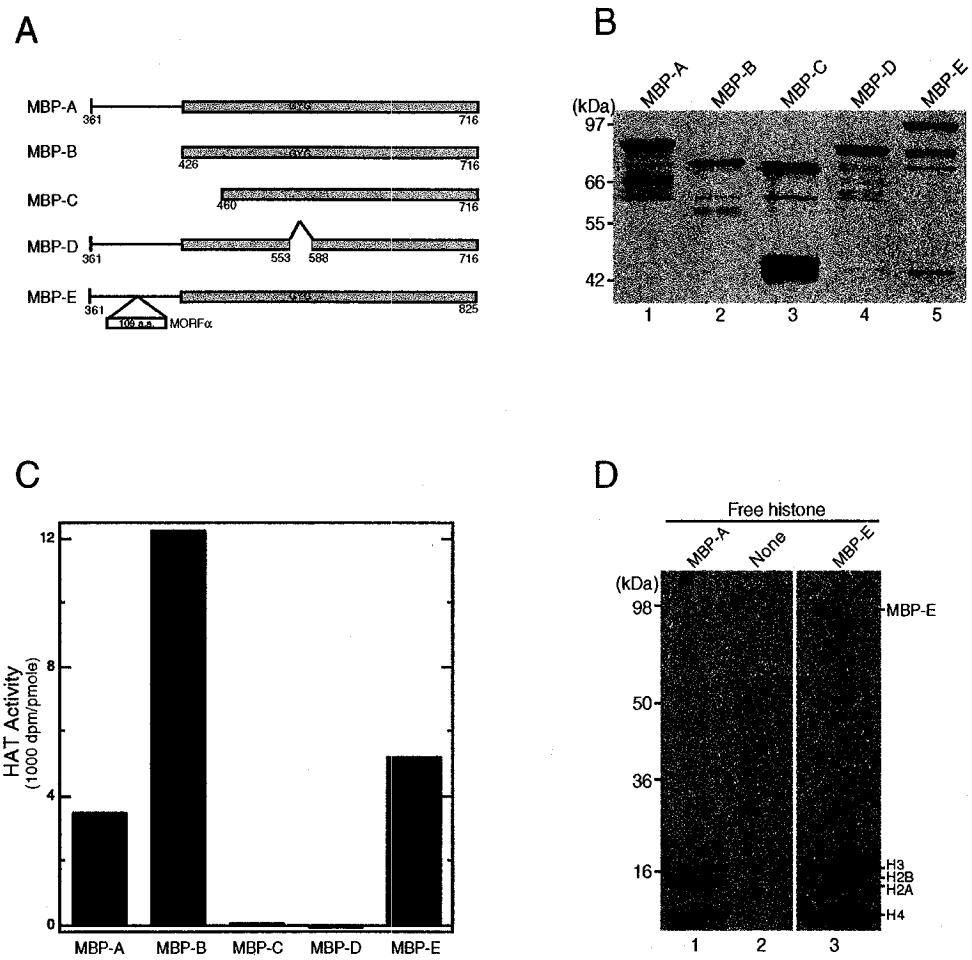

A
MBP-A
361
716
MBP-B
426
716
MBP-C
460
716
MBP-D
361
553
588
716
MBP-E
361
109 a.a.
MORFα
825
B
(kDa)
97
66
55
42
MBP-A
MBP-B
MBP-C
MBP-D
MBP-E
1
2
3
4
5
C
HAT Activity
(1000 dpm/pmole)
12
8
4
0
MBP-A
MBP-B
MBP-C
MBP-D
MBP-E
D
Free histone
MBP-A
None
MBP-E
(kDa)
98
50
36
16
MBP-E
H3
H2B
H2A
H4
1
2
3

Figure 5 Identification of lysine residues of histone H4 acetylated by MORF fragments. Chemically synthesized acetyllysine peptides (***A***) were incubated with MBP-A (***B***), -B (***C***) and -E (***D***), and incorporated [^{3}H]-acetyl groups were quantified by P81 filter-binding assays. For each enzyme, the activity observed with the wild-type peptide was arbitrarily set to 100% to calculate the relative activity when acetyllysine peptides were used.

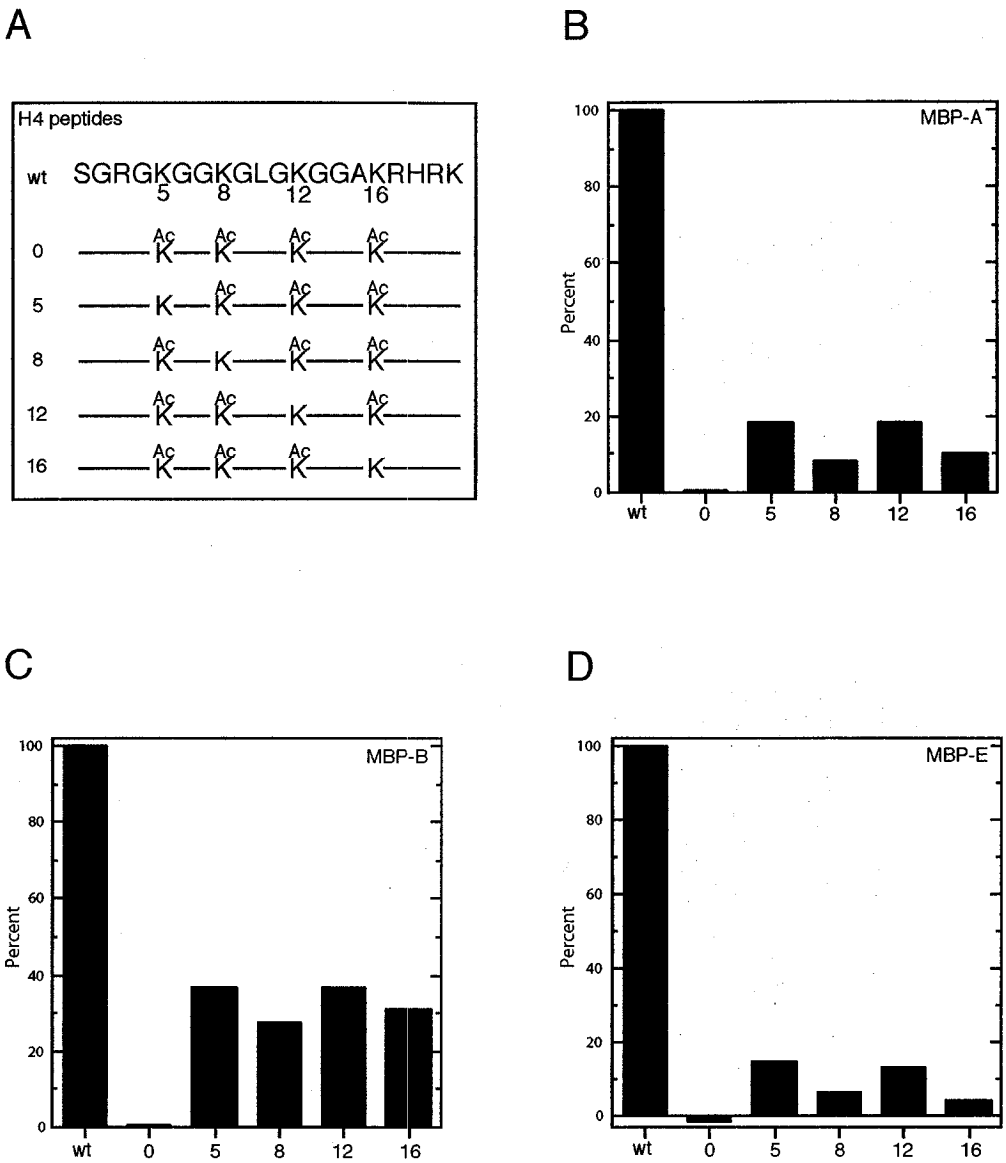

A
H4 peptides
wt SGRGKGGKGLGKGGAKRHRK
5 8 12 16
0
5
8
12
16
B
MBP-A
Percent
100
80
60
40
20
0
wt 0 5 8 12 16
C
MBP-B
Percent
100
80
60
40
20
0
wt 0 5 8 12 16
D
MBP-E
Percent
100
80
60
40
20
0
wt 0 5 8 12 16

Figure 6 Mapping of activation and repression domains of MORF.

A. Schematic representation of MORF and its deletion mutants. The name for each mutant is given at left, and residues that each mutant contains are also indicated. The mutant ΔCoA lacks residues around the putative acetyl CoA-binding site (from 554 to 587).

B. Transcriptional activation by the C terminal mutants of MORF. Mammalian vectors were constructed to express MORF or its deletion mutants fused to the C terminus of the Gal4 DNA-binding domain (residues 1-147) and cotransfected into NIH3T3 cells with the Gal4-tk-Luc reporter. Luciferase activities were normalized to internal β-galactosidase controls and used to calculate relative activation potential (the activity without an effector was arbitrarily set to 1.0).

C. Transcriptional repression by the N terminal mutants of MORF. Mammalian constructs expressing the indicated Gal4 fusion proteins were engineered as above and cotransfected into NIH3T3 cells with the reporter Gal4-E4-Luc or 3TP-Lux.

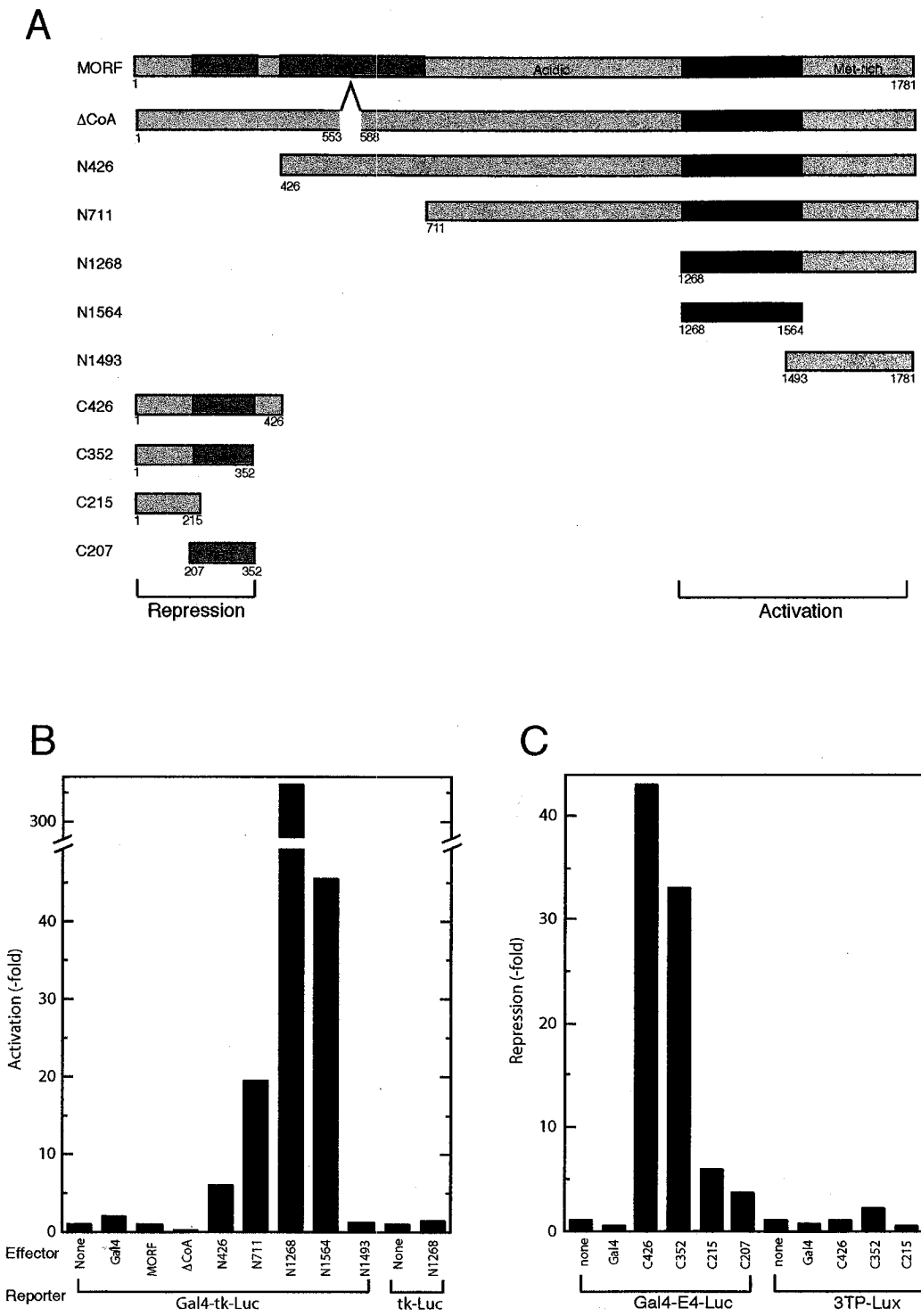
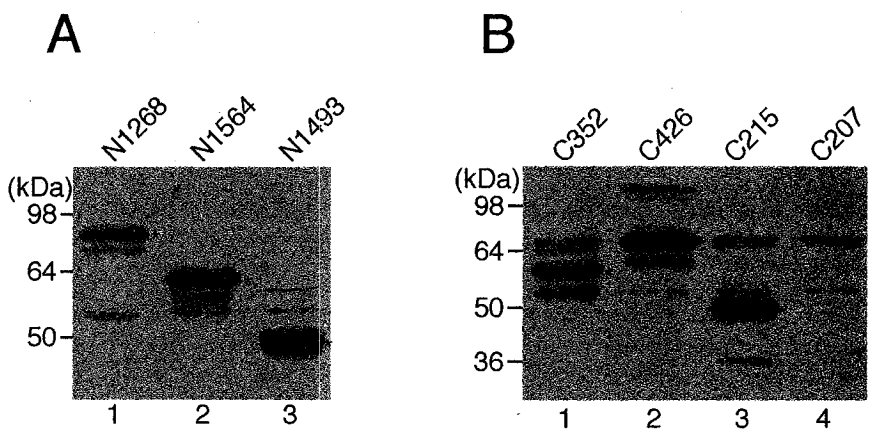

A
MORF
Acidic
Met-rich
1
1781
ΔCoA
553
568
N426
426
N711
711
N1268
1268
N1564
1564
N1493
1493
C426
C352
352
C215
215
C207
207
Repression
Activation
B
Activation (-fold)
300
40
30
20
10
0
Effector
None
Gal4
MORF
ΔCoA
N426
N711
N1268
N1564
N1493
None
N1268
Reporter
Gal4-tk-Luc
tk-Luc
C
Repression (-fold)
40
30
20
10
0
none
Gal4
C426
C352
C215
C207
Gal4-E4-Luc
3TP-Lux

Figure 7 Expression of MORF Gal4-fusion proteins.

Mammalian vectors expressing indicated Gal4 fusion proteins were transfected into 293T cells. Total extracts (***A***) or nuclear extracts (***B***) were prepared for Western analysis with an anti-Gal4 antibody. Western analysis of total extracts for the Gal4-fusion proteins C426, C352, C215 and C207 yielded various non-specific bands, so nuclear extracts were used.

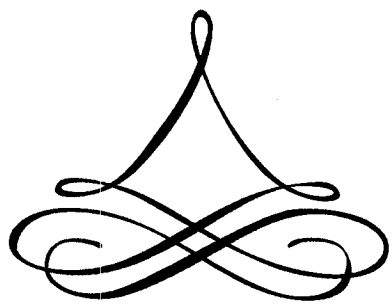

A
N1268
N1564
N1493
(kDa)
98
64
50
1
2
3
B
C352
C426
C215
C207
(kDa)
98
64
50
36
1
2
3
4

CHAPTER III

MOZ AND MORF HISTONE ACETYLTRANSFERASES INTERACT WITH THE RUNT-DOMAIN TRANSCRIPTION FACTOR RUNX2

Nadine Pelletier, Nathalie Champagne, Stefano Stifani, Xiang-Jiao Yang

Oncogene, (2002), Vol. 21, 2729-2740



PREFACE

Chromatin-modifying enzymes are recruited to specific promoters via DNA-binding transcription factors. In Chapter II, we have characterized and demonstrated that MORF is a histone acetyltransferases with transcriptional repression and activation domains, suggesting that it might be transcriptional coregulator. Indeed, we have found that both MOZ and MORF interact with Runx2 transcription factor, a key player for osteoblast differentiation and thus bone formation. This chapter focuses on this novel biological function of MOZ and MORF.

1. ABSTRACT

The monocytic leukemia zinc finger protein MOZ and its homologue MORF have been implicated in leukemogenesis. Both MOZ and MORF are histone acetyltransferases with weak transcriptional repression domains and strong transcriptional activation domains, suggesting that they may function as transcriptional coregulators. Here we describe that MOZ and MORF both interact with Runx2 (or Cbfa1), a Runt-domain transcription factor that is known to play important roles in T cell lymphomagenesis and bone development. Through its C-terminal SM (serine- and methionine-rich) domain, MORF binds to Runx2 in vitro and in vivo. Consistent with this, the SM domain of MORF also binds to Runx1 (or AML1), a Runx2 homologue that is frequently altered by leukemia-associated chromosomal translocations. While MORF does not acetylate Runx2, its SM domain is able to potentiate Runx2-dependent transcriptional activation. Moreover, endogenous MORF is required for transcriptional activation by Runx2. Unexpectedly, Runx2 negatively regulates the transcriptional activation potential of the MORF SM domain. Like that of MORF, the SM domain of MOZ also physically and functionally interacts with Runx2. These results identify Runx2 as an interaction partner of MOZ and MORF and suggest that both acetyltransferases are involved in regulating transcriptional activation mediated by Runx2 and its homologues.

2. INTRODUCTION

Deregulation of gene expression at the transcriptional level is a hallmark of oncogenic transformation. Indeed, genes of many DNA-binding transcription factors are frequently rearranged in cancer patients (Look, 1997; Semenza, 1998). In the past decade, numerous studies have established that DNA-binding transcription factors recruit coactivators or corepressors to specific promoters to regulate transcription. Transcriptional coactivators have been found to possess histone acetyltransferase (HAT) activity (reviewed in Brown *et al.*, 2000; Kouzarides, 2000; Sterner & Berger, 2000; Jenuwein & Allis, 2001; Nakatani, 2001). Analogous to DNA-binding transcription factors, these coactivators are also frequent targets of cancer-associated chromosomal rearrangements. Consistent with this notion, leukemia-associated chromosomal translocations have been found to alter the well-known transcriptional coactivators CBP and p300 (Borrow *et al.*, 1996; Ida *et al.*, 1997; Rowley *et al.*, 1997; Satake *et al.*, 1997; Sobulo *et al.*, 1997; Taki *et al.*, 1997; Chaffanet *et al.*, 1999; Jacobson & Pillus, 1999; Chaffanet *et al.*, 2000; Lavau *et al.*, 2000; Panagopoulos *et al.*, 2000; Kitabayashi *et al.*, 2001a). For example, the CBP and p300 genes were discovered to be rearranged in the reciprocal translocations t(8;16)(p11;q13) and t(8;22)(p11;q13), respectively (Borrow *et al.*, 1996; Chaffanet *et al.*, 1999; Chaffanet *et al.*, 2000; Panagopoulos *et al.*, 2000). In both cases, the fusion partner was identified as the monocytic leukemia zinc finger protein (MOZ) (Fig. 1). In several leukemia patients with inv(8)(p11q13), the MOZ gene was shown to be linked to the TIF2 gene (Carapeti *et al.*, 1998; Liang *et al.*, 1998; Carapeti *et al.*, 1999). The human MORF (MOZ-related factor) gene was mapped to 10q22 (Champagne *et al.*, 1999), and was recently discovered to be fused to the CBP gene in a leukemia patient with t(10; 16)(q22;p13) (Panagopoulos *et al.*, 2001). As indicated by the breakpoints shown in Fig.

1, one common feature about these chromosomal abnormalities is that they produce aberrant proteins with the C-terminal SM (serine- and methionine-rich) domain of MOZ or MORF replaced by the C-terminal part of CBP, p300 or TIF2, suggesting that removal of the SM domains may contribute to leukemogenesis. These domains have been shown to possess transcriptional activation potential (Champagne *et al.*, 1999; Champagne *et al.*, 2001). One interesting but unaddressed question is whether MOZ and MORF interact with transcription factors and directly regulate transcription.

Both MOZ and MORF are members of the MYST protein family. The acronym MYST is from its four founding members: MOZ (Borrow *et al.*, 1996), YBF2/SAS3 (Reifsnyder *et al.*, 1996; Takechi & Nakayama, 1999), SAS2 (Reifsnyder *et al.*, 1996), and TIP60 (Kamine *et al.*, 1996; Yamamoto & Horikoshi, 1997; Ran & Pereira-Smith, 2000). Additional MYST proteins include yeast ESA1 (Smith *et al.*, 1998; Clarke *et al.*, 1999), *Drosophila* MOF (Hilfiker *et al.*, 1997; Akhtar & Becker, 2000) and Enok (Scott *et al.*, 2001), and human HBO1 (Iizuka & Stillman, 1999). Except for SAS2 and Enok, MYST family members have been shown to possess HAT activity. Due to their distinct domains, members of this family have diverse functions, including roles in epigenetic control (Reifsnyder *et al.*, 1996; Hilfiker *et al.*, 1997; Akhtar *et al.*, 2000; Galarneau *et al.*, 2000; Ikura *et al.*, 2000), transcriptional regulation (Allard *et al.*, 1999; Brady *et al.*, 1999; John *et al.*, 2000; Sharma *et al.*, 2000; Brown *et al.*, 2001; Hassan *et al.*, 2001; Reid *et al.*, 2001), DNA replication (Iizuka & Stillman, 1999; Burke *et al.*, 2001), DNA repair (Ikura *et al.*, 2000), chromatin assembly (Meijsing & Ehrenhofer-Murray, 2001; Osada *et al.*, 2001), cell cycle progression (Smith *et al.*, 1998; Clarke *et al.*, 1999; Yan *et al.*, 2000; Howe *et al.*, 2001) and cellular signaling (Cao & Sudhof, 2001; Lee *et al.*,

2001; Sheridan *et al.*, 2001). Since they possess several unique domains (Fig. 1), MOZ and MORF may function differently from other MYST members. A recent report on *Querkopf* mice has yielded good insight into the function of MORF *in vivo* (Thomas *et al.*, 2000). Because of integration of a gene-trapping reporter at a 5'-noncoding exon of the MORF gene, these mice produce MORF transcripts only at a residual level. Homozygotes display defects in calvarial bone and cerebral cortex development. This important finding suggests that if it is a transcriptional coregulator, MORF may interact with transcription factors important for bone or cerebral cortex development. However, this intriguing possibility remains to be investigated.

The Runt-domain transcription factor Runx1 (also known as AML1 or Cbfa2) is an essential regulator of fetal liver hematopoiesis (Westendorf & Hiebert, 1999; Wheeler *et al.*, 2000), and its gene is frequently rearranged in leukemia patients (Speck *et al.*, 1999). Runx2 (also known as Cbfa1 or AML3) displays extensive sequence similarity to Runx1, and functions as a novel oncogenic effector for T-cell lymphoma (Vaillant *et al.*, 1999; Blyth *et al.*, 2001). It also plays an essential role in controlling osteoblast differentiation and bone formation (Ducy *et al.*, 2000; Wheeler *et al.*, 2000). Here we present data to show that MOZ and MORF physically and functionally interact with Runx2. The results strongly suggest that MOZ and MORF are involved in regulating gene expression controlled by Runx2 and its homologues.

3. RESULTS

3.1 Identification of Runx2 as a MORF interaction partner

Compared to other MYST family members, MOZ and MORF possess unique structural domains (Figs. 1 & 2A). The N-terminal part of MORF constitutes a weak transcriptional repression domain, whereas the C-terminal SM domain acts as a strong transcriptional activation domain (Fig. 1), suggesting that MORF may be a transcriptional coregulator. To corroborate this contention, we sought to identify functional partners of MORF. Since it is widely expressed in adult human tissues (Champagne *et al.*, 1999), MORF may interact with different target transcription factors. To identify these targets, we took several approaches, one of which was to use an *in vitro* protein-protein interaction assay to test transcription factors available to us. In this assay, Flag-MORF was immobilized on anti-Flag M2 agarose and used to retain transcription factors synthesized *in vitro*. MEF2C, MEF2D, Oct1, Oct2, Smad2, Smad3, FKHR and Sox9 were first analyzed, and none of these transcription factors were retained by M2 agarose immobilized with Flag-MORF (data not shown), indicating that these transcription factors are not interaction partners of MORF.

During the course of these experiments, the *Querkopf* mutation was reported to decrease MORF transcripts to a residual level and cause developmental defects in cerebral cortex and calvarial bones (Thomas *et al.*, 2000), suggesting that MORF may regulate gene expression during neurogenesis and bone development. It is well known that Runx2 plays a major role in bone morphogenesis (reviewed in Ducy *et al.*, 2000; Wheeler *et al.*, 2000), so we asked whether it interacts with MORF. To test this, Runx2 was synthesized *in vitro* and subjected to the Flag-MORF binding assay just described.

As shown in Fig. 2B, Runx2 was retained by M2 agarose pre-incubated with Sf9 extracts containing Flag-MORF (lanes 1 & 3), but not by M2 agarose pre-incubated with plain Sf9 extracts (lane 2), indicating that Runx2 specifically interacts with MORF.

3.2 Interaction of the SM domain of MOZ and MORF with Runx2

We next mapped the domain(s) of MORF that mediates its binding to Runx2. For this, we expressed MORF fragments (Fig. 2A) as Flag-tagged fusion proteins in 293 cells with or without HA-tagged Runx2. Expressed proteins were immunoprecipitated on anti-Flag M2 agarose, and precipitated proteins were eluted with Flag peptide and subjected to Western blotting analyses. HA-Runx2 co-immunoprecipitated with fragment SM, but not with fragment N, H or NH (Fig. 2A, C & data not shown), indicating that the SM domain of MORF interacts with Runx2 in vivo.

Except for the PQ insertion (Fig. 1), the SM domain of MOZ is highly similar to that of MORF, so MOZ may also interact with Runx2. To test this, the SM domain of MOZ was expressed as a Flag-tagged fusion protein and subjected to co-immunoprecipitation and Western blotting analysis. As shown in Fig. 2D (upper panel), this fusion protein was expressed and purified as expected. Importantly, Runx2 specifically co-immunoprecipitated with Flag-MOZ-SM (Fig. 2D, lower panel). Therefore, the SM domain of MOZ is able to interact with Runx2.

MBP (maltose-binding protein) pull-down assays were used to test whether the interaction between Runx2 and the SM domain of MORF is direct. As shown in Fig. 2E (lanes 1-3), MBP-SM, but not MBP, retained Runx2, supporting that Runx2 directly interacts with the SM domain of MORF. Fragments S and M were also expressed and

analyzed (Fig. 2A). Like MBP-SM, MBP-S and MBP-M specifically associated with Runx2 (Fig. 2E, lanes 4-9), indicating that both fragments S and M bind to Runx2. This finding suggests that the overlapping region of fragments S and M may contribute to the binding.

3.3 Mapping the MORF-binding sites in Runx2

To locate the MORF-binding site(s), Runx2 deletion mutants (Fig. 3A) were produced in reticulocyte lysates and used for analysis of interaction with MBP-SM. Deletion mutants 1-373, 1-235 and 235-528 (Fig. 3A) were first tested. As shown in Fig. 3B, full-length Runx2 (lanes 1-3), deletion mutants 1-373 (lanes 4-6) and 235-528 (lanes 10-12) specifically associated with MBP-SM, whereas deletion mutant 1-235 (lanes 7-9) did not display specific binding to MBP-SM. These results suggest either that residues 235-373 of Runx2 contain a MORF-binding site or that Runx2 has two binding sites with one residing within residues 1-373 and the other within residues 374-528. To distinguish between these two possibilities, we analyzed mutants 235-468, 374-528 and 235-373 (Fig. 3A). As shown in Fig. 3C, mutants 235-468 (lanes 1-3) and 374-528 (lanes 4-6), but not 235-373 (lanes 7-9), specifically interacted with MBP-SM. This finding is consistent with the possibility that Runx2 possesses two MORF-binding sites. For the one within residues 1-373, the whole region may be required for binding since neither 1-235 (Fig. 2B, lanes 7-9) nor 235-373 (Fig. 2C, lanes 7-9) interacted with Runx2. To determine whether residues 374-468 are sufficient for MORF binding, deletion mutant 374-468 (Fig. 3A) was expressed and analyzed for binding. As shown in Fig. 3C (lanes 10-12), this mutant specifically interacted with MBP-SM, indicating that residues 374-468

constitute a MORF-binding site. To further map this binding site, we tested the following deletion mutants: 235-427, 235-440, 235-458 and 410-528 (Fig. 3A). As shown in Fig. 3D, MBP-SM interacted with 235-440 and 235-458, but only minimally with 235-427 and 410-528. Together, these results indicate that residues 1-373 and 374-440 of Runx2 constitute two MORF-binding sites in vitro.

To verify this conclusion, we examined how deletion mutants 1-468 and 1-373 interact with the SM domain of MORF in vivo. For this, Flag-SM, HA-Runx2 and HA-tagged Runx2 mutants were expressed for co-immunoprecipitation. As shown in Fig. 3E, full-length Runx2 and mutant 1-468 co-immunoprecipitated similarly with Flag-SM, whereas mutant 1-373 weakly co-immunoprecipitated with Flag-SM, indicating that the MORF-binding site within residues 374-468 of Runx2 is much stronger than that within residues 1-373 in vivo. These results further support the conclusion that Runx2 has two MORF-binding sites.

Residues 374-468 of Runx2 display high sequence similarity to the corresponding region of Runx1, raising the interesting possibility that the SM domain of MORF may interact with Runx1. To test this, the SM domain of MORF was expressed as a Flag-tagged fusion protein with or without HA-tagged Runx1 in 293 cells. Expressed proteins were subjected to immunoprecipitation and Western blotting analyses. Consistent with the sequence similarity between Runx1 and Runx2, HA-Runx1 specifically co-immunoprecipitated with Flag-SM (Fig. 3F), indicating that the SM domain of MORF also interacts with Runx1.

3.4 Acetylation of Runx2 by MORF

Physical association of MORF with Runx2 suggests that they may functionally interact with each other. CBP, p300 and PCAF have been found to acetylate non-histone proteins (reviewed in Kouzarides, 2000; Sterner & Berger, 2000) and MORF contains HAT activity comparable to that of PCAF (Yang et al., 1996; Champagne et al., 1999), so MORF may acetylate Runx2 and regulate its function. To test this, Runx2 was expressed in Sf9 cells and affinity-purified as a Flag-tagged fusion protein. Flag-Runx2 was analyzed for acetylation. As previously reported (Champagne et al., 1999), Flag-MORF efficiently acetylated itself and histones (Fig. 4, lane 1). By contrast, acetylation of Flag-Runx2 by Flag-MORF was undetectable (lane 2), indicating that MORF is unable to acetylate Runx2.

3.5 Effect of MOZ and MORF on Runx2-dependent transcriptional activation

The SM domain of MORF physically interacts with Runx2 (Fig. 2), and is known to possess transcriptional activation potential (Champagne et al., 1999), so it may potentiate Runx2-dependent transcription. To test this, we employed the 6OSE2-Luc reporter, which contains six tandem Runx2-response elements upstream from the luciferase gene (Ducy & Karsenty, 1995; McLarren et al., 2000). As previously reported (McLarren et al., 2000), exogenous expression of Runx2 resulted in 12-fold activation of reporter gene expression in ROS17/2.8 osteoblastic cells (Fig. 5A). Importantly, this transactivation was stimulated in a dose-dependent manner when increasing amounts of Flag-SM were co-expressed with a fixed amount of Runx2 (Fig. 5A). In the absence of co-transfected Runx2, expression of Flag-SM had a minimal effect on luciferase reporter activity (Fig.

5A), indicating that the observed transactivation by Flag-SM is Runx2-dependent. To test whether the functional cooperation between Runx2 and Flag-SM is cell line-dependent, similar assays were carried out in two other cell lines. In 293 cells, exogenous expression of Runx2 resulted in 107-fold activation of reporter gene expression, and this transactivation was dramatically increased (13.3-fold) when Flag-SM was co-expressed (Fig. 5B). In NIH3T3 cells, exogenous expression of Runx2 activated reporter gene expression 96-fold, and this transactivation was further increased (2.2-fold) when Flag-SM was co-expressed (Fig. 5C). To assure that these results are related to native Runx2-dependent promoters, we analyzed the luciferase reporter OG2-Luc, which contains a mouse osteocalcin promoter fragment (-147/+13) upstream from the luciferase gene (Ducy & Karsenty, 1995). As shown in Fig. 5D, exogenous expression of Runx2 activated the reporter gene expression 1.5-fold, and this transactivation was further stimulated (2.0-fold) when Flag-SM was co-expressed. Taken together, these findings indicate that the SM domain of MORF potentiates Runx2-dependent transcription in different cell lines.

Like that of MORF, the SM domain of MOZ binds to Runx2 (Fig. 2E), so we tested whether it also regulates Runx2-dependent transcription. As shown in Fig. 5E, co-expression of this SM domain with Runx2 led to potentiation of Runx2-dependent transcription.

To determine how endogenous MORF affects Runx2-dependent transcriptional activation, we utilized fragment M of MORF as a dominant-negative mutant (Fig. 2A). This fragment is able to interact with Runx2 (Fig. 2E), but possesses no transcriptional activation potential (Champagne et al., 1999). Therefore, if overexpressed, fragment M

may compete with endogenous MORF for Runx2 binding and interfere with the ability of endogenous MORF to regulate Runx2-dependent transcription (Fig. 6A). As shown above, exogenous expression of Runx2 stimulated luciferase reporter activity (Fig. 6B). Importantly, in a dose-dependent manner, expression of fragment M inhibited transcriptional activation by Runx2 (Fig. 6B). Similar results were obtained with ROS17/2.8 cells (Fig. 6C). Fragment M was found to be unstable, so it could not be highly expressed to exert more dramatic effects (data not shown). Together, these findings suggest that endogenous MORF is required for Runx2-dependent transcriptional activation.

To substantiate this conclusion with different assays, we utilized antisense inhibition. Co-transfection of an antisense contruct that contains a 1.6-kb MORF cDNA fragment inhibited Runx2-dependent transcriptional activation in a dose-dependent manner, whereas the corresponding sense construct had minimal effects (Fig. 6D). RNA interference has been recently reported to be functional in cultured mammalian cells (Elbashir et al., 2001), so we asked how co-transfection of the sense and antisense contructs may affect Runx2-dependent transcriptional activation. As shown in Fig. 6D, co-transfection of both constructs produced stronger effects. These results provide additional support for the above conclusion that endogenous MORF is required for Runx2-dependent transcriptional activation.

3.6 Effects of Runx2 on the transcriptional potential of the MORF SM domain

It has been established that when tethered to promoters, the SM domain of MORF activates transcription (Champagne et al., 1999). The physical interaction of Runx2 with

the SM domain of MORF (Fig. 2) also raises the intriguing possibility that Runx2 might regulate the function of this domain. To test this, the reporter Gal4-tk-Luc (Fig. 7A) was transfected along with expression plasmids for Gal4-SM and Runx2. Importantly, Runx2 inhibited transcription activated by Gal4-SM (Fig. 7B). To determine the specificity of this inhibition, we assessed effects of Runx2 on transcription dependent on the VP16 transcriptional activation domain. Runx2 did not inhibit transcriptional activation by Gal4-VP16 (data not shown), suggesting that the observed inhibition is specific. To determine whether the inhibition is due to secondary effects of Runx2-dependent expression of cellular genes, a Runx2 point mutant (L175D) was engineered. A similar Runx1 mutant is known to be defective in transcriptional activation (Strom et al., 2000). Like wild-type Runx2, the L175D point mutant inhibited transcriptional activation by Gal4-SM (data not shown). Therefore, Runx2 is able to negatively regulate the transcriptional activation potential of the SM domain of MORF (Fig. 7C, upper part).

4. DISCUSSION

4.1 Interaction of MOZ and MORF with Runx2

The results presented herein demonstrate that Runx2 binds to MOZ and MORF. First, in vitro binding assays indicate that Runx2 associates with full-length MORF (Fig. 2A-B). Second, immunoprecipitation experiments reveal that Runx2 binds to the SM domain but not the PHD fingers or the HAT domain of MORF (Fig. 2). Third, in agreement with its high sequence homology with the corresponding region of MORF, the SM domain of MOZ interacts with Runx2 in vivo (Fig. 2D). Fourth, Runx2 possesses two MORF-binding sites, with one consisting of the N-terminal 373 residues and the other located within residues 374-440 (Fig. 3). The latter appeared to be the stronger binding site in vivo (Fig. 3E). Finally, consistent with its significant sequence similarity to Runx2, Runx1 interacts with the SM domain of MORF (Fig. 3F). Therefore, the SM domains of MOZ and MORF mediate their specific binding to Runx2 and its homologue Runx1.

4.2 Regulation of Runx2 by MOZ and MORF

Physical association of MORF with Runx2 suggests that MORF may regulate the function of Runx2. Since numerous transcription factors have been shown to be acetylated and regulated by HATs (Kouzarides, 2000; Sterner & Berger, 2000), MORF may acetylate and regulate Runx2. Under the conditions employed, no acetylation of Runx2 by MORF was detected (Fig. 4). On the other hand, overexpression of the SM domain of MOZ or MORF potentiated Runx2-dependent transcriptional activation in different cell lines (Fig. 5), and residues 374-440 of Runx2 were found to be essential for this potentiation (data not shown). Moreover, endogenous MORF appeared to be required

for transcriptional activation by Runx2 (Fig. 6). These results indicate that Runx2 recruits MORF (or MOZ) to specific promoters to activate transcription (Fig. 6A). Independently, another group recently discovered that MOZ stimulates transcription mediated by Runx1 (Kitabayashi et al., 2001b).

Residues 374-440 of Runx2 constitute the major MORF-binding site in vivo (Fig. 3E). This region is highly conserved in Runx1 and contains a PPxY motif that may mediate binding of the transcriptional coactivators YAP65 and TAZ (Yagi et al., 1999; Kanai et al., 2000). Moreover, Runx1 interacts with the transcriptional coactivator p300, and the p300-binding site is located to a region corresponding to residues 374-440 of Runx2 (Kitabayashi et al., 1998), so Runx2 may also bind p300. Through its C-terminal part, Runx2 interacts with the transcriptional activator HES-1 (McLarren et al., 2000). Therefore, Runx2 may recruit multiple transcriptional activators. This may be one reason that dominant-negative inhibition, antisense inhibition or RNA interference did not have more dramatic effects (Fig. 6).

MORF possesses HAT activity (Champagne et al., 1999), so it may regulate transcription by acetylating chromatin. How its HAT and SM domains coordinate with each other to regulate Runx2-dependent transcription in vivo is an interesting issue that awaits further investigation. The conclusion that Runx2 recruits MORF to activate transcription does not exclude the possibility that MORF may function with other transcription factors. Besides its HAT and SM domains, MORF possesses other domains (e.g. PHD fingers, Fig. 1) that may mediate its binding to other transcription factors. Furthermore, MORF is expressed in many tissues (Borrow et al., 1996; Champagne et al., 1999), and Querkopf mice possess multiple defects (Thomas et al., 2000). It would be

interesting to identify other transcription factors that also recruit MORF (or MOZ) as a transcriptional coactivator.

4.3 Regulation of MORF by Runx2

Physical association of MORF with Runx2 also suggests that Runx2 may regulate the function of MORF. Consistent with this, exogenous expression of Runx2 inhibited transcriptional activation by the SM domain of MORF in 293 cells (Fig. 7). The L175D point mutant of Runx2 was found to have a similar inhibitory effect (data not shown), suggesting that the inhibition by Runx2 is independent of its ability to activate transcription. Runx1 was found to have similar effects (data not shown). Besides roles as transcriptional activators, Runx1 and Runx2 also function as repressors. When artificially recruited to promoters by the Gal4 DNA-binding domain, Runx2 was found to function as a repressor (Aronson et al., 1997). Through its C-terminal end, Runx2 recruits the Groucho/TLE family of transcriptional corepressors to repress transcription (Thirunavukkarasu et al., 1998; McLarren et al., 2000). Runx1 has been shown to repress transcription from the p21Waf1/Cip1 promoter by recruiting the Sin3A deacetylase complex (Lutterbach et al., 2000). Related to this, MORF possesses a weak repression domain at its N-terminal end (Champagne et al., 1999). Therefore, it is tempting to propose that Runx2 binds to MORF and inhibits transcription mediated by other transcription factors that interact with MORF (Fig. 7C, lower part).

4.4 Roles of MOZ and MORF proteins in oncogenesis

The results presented herein also shed light on how MOZ and MORF may contribute to oncogenesis. First, Runx2 functions as a novel oncogenic effector for T-cell lymphoma (Vaillant et al., 1999; Blyth et al., 2001). Since Runx2 interacts with MOZ and MORF, their expression levels may affect the role of Runx2 in the development of T-cell lymphoma. Second, Runx1 is an important regulator of fetal liver hematopoiesis, and its gene is frequently rearranged in leukemia patients (Speck et al., 1999; Westendorf & Hiebert, 1999). The SM domain of MORF interacts with Runx1 (Fig. 3F) and positively regulates its transcription ability (data not shown), so expression levels of MORF and its homologue MOZ may affect roles of aberrant Runx1 proteins in leukemogenesis. Third, MOZ and MORF genes have been found to be rearranged in leukemia patients (Fig. 1) (Borrow et al., 1996; Carapeti et al., 1998; Liang et al., 1998; Carapeti et al., 1999; Chaffanet et al., 1999; Jacobson & Pillus, 1999; Chaffanet et al., 2000; Panagopoulos et al., 2000; Kitabayashi et al., 2001a; Panagopoulos et al., 2001). A common feature of the chromosomal rearrangements involved is that they generate aberrant proteins with the SM domain of MOZ or MORF replaced by the C-terminal part of CBP, p300 or TIF2. The SM domains of MOZ and MORF associate with Runx2, so these chromosomal abnormalities may deregulate MOZ-, MORF- and Runx2-dependent gene expression.

In summary, through their SM domains, MOZ and MORF interact with Runx2. MORF stimulates Runx2-dependent transcriptional activation, whereas Runx2 negatively regulates the transcriptional activation potential of MORF. Further investigation of how MOZ and MORF modulate expression of genes controlled by Runx2 and its homologues

should shed light on the molecular mechanisms whereby MOZ, MORF and Runx proteins regulate cell proliferation and differentation in vivo.

5. MATERIALS AND METHODS

5.1 Materials

Expression plasmids for MOZ, MORF and some of their deletion mutants have been described previously (Champagne et al., 1999; Champagne et al., 2001). Additional mutants were generated by PCR with Expand thermostable DNA polymerase (Roche). The luciferase reporter Gal4-tk-Luc has been described (Champagne et al., 1999). In the reporter 6OSE2-Luc, the luciferase gene is under the control of six tandem copies of osteocalcin-specific element 2 (OSE2) (Ducy & Karsenty, 1995). The reporter OG2-Luc contains a mouse osteocalcin promoter fragment (-147/+13) upstream from the luciferase gene (Ducy & Karsenty, 1995). Mammalian expression plasmids for mouse Runx2 and human Runx1b (a Runx1 isoform) have been previously described (Ducy & Karsenty, 1995; Thirunavukkarasu et al., 1998; McLarren et al., 2000; McLarren et al., 2001). A 1.6-kb BamHI fragment consisting of 5'-UTR and the coding sequence for residues 1-362 of human MORF was cloned into the BamHI site of pLXSN to generate sense and antisense constructs.

5.2 Expression of MORF and Runx2 in insect cells

Flag-tagged MORF was expressed in and purified from Sf9 cells as previously described (Champagne et al., 1999). Flag-tagged Runx2 was expressed in Sf9 cells via a bacmid generated with the Bac-to-Bac baculovirus expression system (Gibco BRL), and the expressed Runx2 protein was affinity-purified on M2 agarose (Sigma). For purity assessment, purified Flag-MORF and Flag-Runx2 were resolved by reducing SDS-PAGE and stained with Coomassie Blue R-250 (Bio-Rad).

5.3 Protein-protein interaction assays

For analysis of in vitro interaction between MORF and Runx2, Sf9 extracts containing Flag-MORF were incubated with M2 agarose and unbound proteins were removed by extensive washing with buffer B (20 mM Tris-HCl [pH 8.0], 0.15 M KCl, 10% glycerol, 5 mM MgCl2, 0.1% NP-40 and protease inhibitors). Agarose beads were then incubated with Runx2 synthesized in vitro in the presence of L-[35S]methionine (Amersham Pharmacia Biotech) by use of the TNT-T7 coupled reticulocyte lysate system (Promega). After agitation at 4^0C for 30 min, the beads were washed four times with buffer B, and bound proteins were subsequently analyzed by reducing SDS-PAGE and autoradiography. In vitro MBP binding and co-immunoprecipitation assays were carried out as described (Wang et al., 1999; Wang et al., 2000).

5.4 HAT assays

Purified Flag-Runx2 (0.2 mg) was mixed with Flag-MORF (0.05 mg) in a 20 ml reaction containing 50 mM Tris-HCl pH 8.0, 10% glycerol, 1 mM DTT, 0.1 mM EDTA, 1 mM PMSF, 10 mM sodium butyrate (Sigma) and 2.5 nCi [14C]acetyl-CoA (51 mCi/mmol; Amersham Pharmacia Biotech) and processed as described (Mizzen et al., 1996; Champagne et al., 1999).

5.5 Reporter gene assays

For these assays, plasmids were prepared using double CsCl ultra-centrifugation, butanol extraction and ethanol precipitation. Transfection and reporter assays were performed as described (Champagne et al., 1999; Wang et al., 1999).

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Figure 1 Schematic illustration of chromosomal abnormalities associated with MOZ and MORF.

The breakpoints are indicated with arrows, and numbers at their ends represent the amino acid positions. Structural domains of MOZ and MORF are labeled as follows: H15, linker histones H1- and H5-like module; PHD, plant homeodomain zinc fingers; MYST, MYST acetyltransferase domain; ED, Glu/Asp-rich acidic region; S, Ser-rich domain; PQ, Pro/Gln-stretch; and M, Met-rich domain. Also indicated are the transcriptional repression and activation domains of MORF.

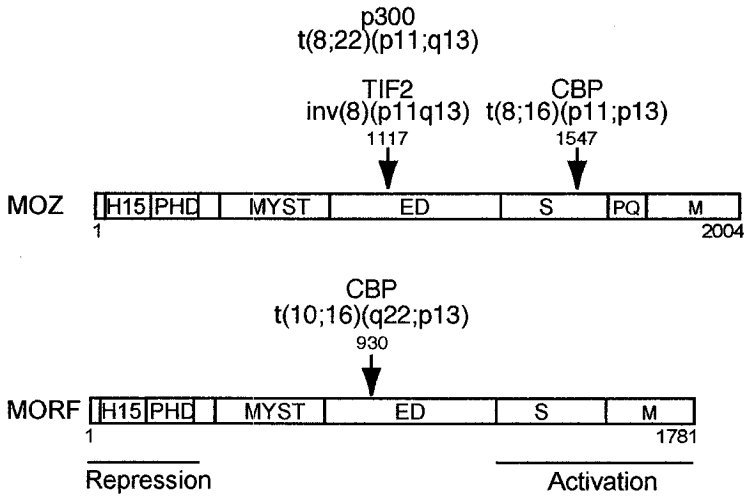

p300
t(8;22)(p11;q13)
TIF2
inv(8)(p11q13)
1117
CBP
t(8;16)(p11;p13)
1547
MOZ
H15
PHD
MYST
ED
S
PQ
M
1
2004
CBP
t(10;16)(q22;p13)
930
MORF
H15
PHD
MYST
ED
S
M
1
1781
Repression
Activation

Figure 2 Interaction of MORF with Runx2.

(A) Schematic representation of MORF and its fragments. Structural domains of MORF are labeled as in Fig. 1. MORF fragments are depicted by bold lines, and numbers indicate the positions of amino acid residues. The acidic region of MORF could not be expressed in E. coli or mammalian cells. Runx2-binding ability of each fragment is summarized at right

(B) Binding of full-length MORF to Runx2. Extracts from uninfected SF9 cells (lane 2) or those infected with Flag-MORF-expressing baculovirus (lane 3) were incubated with anti-Flag M2 agarose and [35S]-labeled Runx2. Specifically bound proteins were analyzed by reducing SDS-PAGE and autoradiography. Lane 1 (input) represents 10% [35S]-labeled Runx2 used for each binding reaction.

(C, D) Interaction of Runx2 with the SM domains of MORF and MOZ. Flag-SM (C) or Flag-MOZ-SM (D) expression plasmid was transfected into 293 cells with or without HA-Runx2 expression plasmid, and extracts were prepared for immunoprecipitation (IP). Eluted proteins were analyzed by immunoblotting with a-Flag (top) or a-HA (bottom) antibody.

(E) Association of MORF fragments with Runx2. Bacterial extracts expressing MBP, MBP-SM, MBP-S, or MBP-M were incubated with amylose agarose and [35S]-labeled Runx2, and specifically bound proteins were analyzed as in (B).

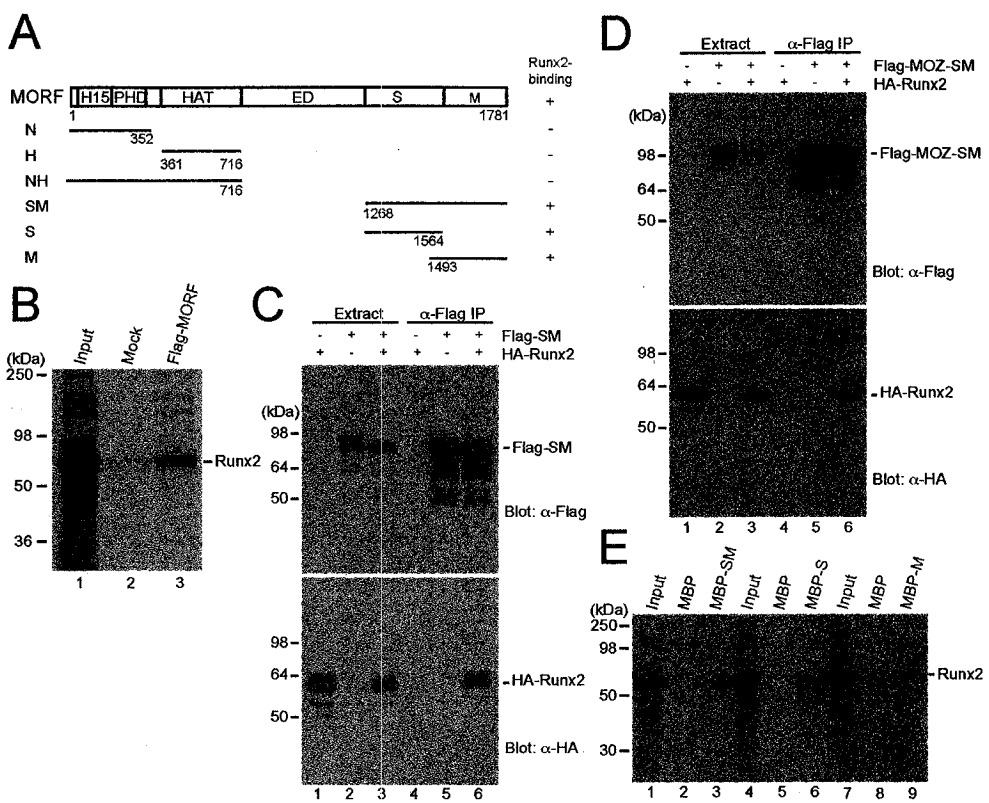

A
MORF
H15
PHD
HAT
ED
S
M
1
1781
N
352
H
361
716
NH
716
SM
1268
S
1564
M
1493
Runx2-binding
+
-
-
-
+
+
+
B
Input
Mock
Flag-MORF
(kDa)
250
98
50
36
Runx2
1 2 3
C
Extract
α-Flag IP
Flag-SM
HA-Runx2
(kDa)
98
64
50
Flag-SM
Blot: α-Flag
HA-Runx2
Blot: α-HA
1 2 3 4 5 6
D
Extract
α-Flag IP
Flag-MOZ-SM
HA-Runx2
(kDa)
98
64
50
Flag-MOZ-SM
Blot: α-Flag
HA-Runx2
Blot: α-HA
1 2 3 4 5 6
E
Input
MBP
MBP-SM
Input
MBP
MBP-S
Input
MBP
MBP-M
(kDa)
250
98
50
30
Runx2
1 2 3 4 5 6 7 8 9

Figure 3 Mapping MORF-binding sites of Runx2.

(A) Schematic representation of Runx2 and its deletion mutants. Runx2 domains are labeled as follows: Runt, Runt-related DNA-binding domain; NLS, nuclear localization signal; and WRPY, WRPY-motif required for binding to the Groucho/TLE family of transcriptional corepressors. MORF-binding ability of each mutant is summarized at right.

(B-D) Interaction of Runx2 and mutants with the SM domain of MORF. Bacterial extracts expressing MBP or MBP-SM were incubated with amylose agarose and [35S]-labeled Runx2. Bound proteins were analyzed by reducing SDS-PAGE and autoradiography. Input represents 10% [35S]-labeled Runx2 or mutants used for each binding assay.

(E) Binding of Runx2 and deletion mutants to the SM domain of MORF. Flag-SM expression plasmid was transfected into 293 cells with or without expression plasmids for HA-tagged Runx2 or mutants, and extracts were prepared for immunoprecipitation and immunoblotting as in Fig. 2C.

(F) Binding of Runx1 to the SM domain of MORF. Flag-SM expression plasmid was transfected into 293 cells with or without HA-Runx1, and extracts were prepared for immunoprecipitation and immunoblotting as in Fig. 2C.

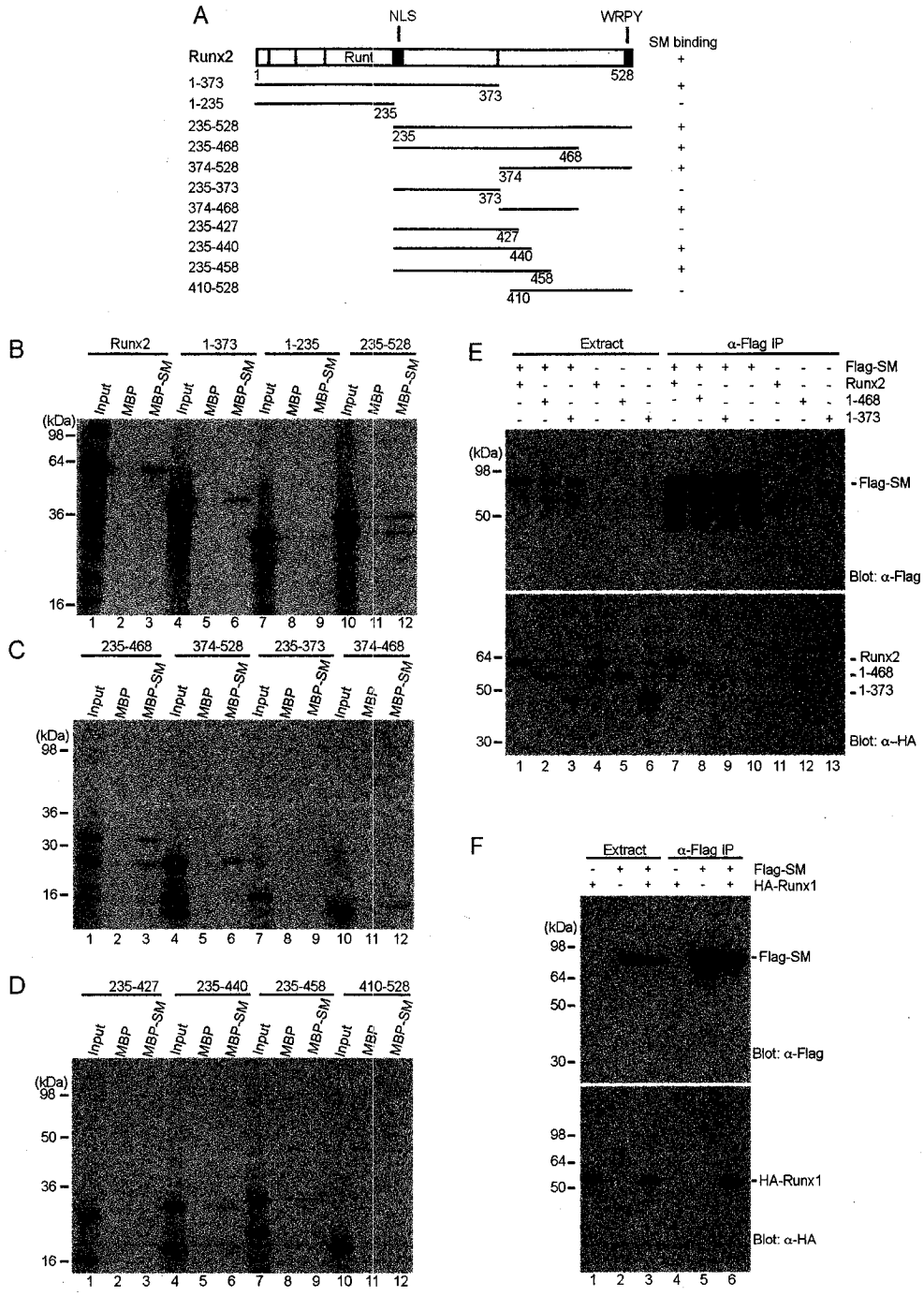

A
NLS
WRPY
SM binding
Runx2
Runt
1
528
1-373
373
+
1-235
235
-
235-528
235
+
235-468
468
+
374-528
374
+
235-373
373
-
374-468
+
235-427
427
-
235-440
440
+
235-458
458
+
410-528
410
-
B
Runx2
1-373
1-235
235-528
Input
MBP
MBP-SM
(kDa)
98
64
36
16
1 2 3 4 5 6 7 8 9 10 11 12
C
235-468
374-528
235-373
374-468
(kDa)
98
36
30
16
1 2 3 4 5 6 7 8 9 10 11 12
D
235-427
235-440
235-458
410-528
(kDa)
98
50
36
16
1 2 3 4 5 6 7 8 9 10 11 12
E
Extract
α-Flag IP
Flag-SM
Runx2
1-468
1-373
(kDa)
98
50
Flag-SM
Blot: α-Flag
64
50
30
Runx2
1-468
1-373
Blot: α-HA
1 2 3 4 5 6 7 8 9 10 11 12 13
F
Extract
α-Flag IP
Flag-SM
HA-Runx1
(kDa)
98
64
50
30
Flag-SM
Blot: α-Flag
98
64
50
HA-Runx1
Blot: α-HA
1 2 3 4 5 6

Figure 4 Acetylation of Runx2 by MORF.

Histones (lane 1, 1 mg) or Flag-Runx2 (lane 2, 0.2 mg) was subject to acetylation by Flag-MORF (0.05 mg) in the presence of [14C]acetyl-CoA. Acetylated proteins were detected by reducing SDS-PAGE and subsequent phosphoimaging. Flag-Runx2 and Flag-MORF proteins were expressed in and affinity-purified from Sf9 cells.

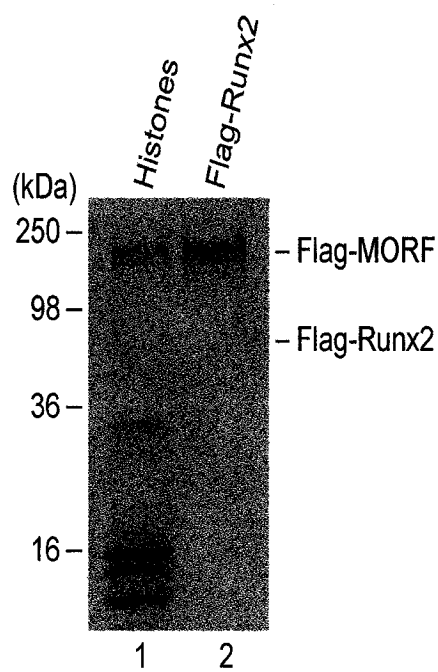

Histones
Flag-Runx2
(kDa)
250 –
98 –
36 –
16 –
– Flag-MORF
– Flag-Runx2
1
2

Figure 5 Activation of Runx2-dependent transcription by the SM domains of MORF and MOZ.

The 6OSE2-Luc or OG2-Luc luciferase reporter (0.2 mg) was transfected into ROS17/2.8, 293 or NIH3T3 cells along with an internal control plasmid (CMV-b-Gal, 0.05 mg) and expression plasmids for Runx2, Flag-SM (**A-E**) and/or Flag-MOZ-SM (**D**) at indicated amounts. The normalized luciferase activity from the transfection with the reporter alone was arbitrarily set to 1.0. Average values of at least three independent experiments are shown with standard deviation indicated by error bars. Expression of Runx2, Flag-SM or Flag-MOZ-SM had minimal effects on the CMV-b-Gal reporter activity.

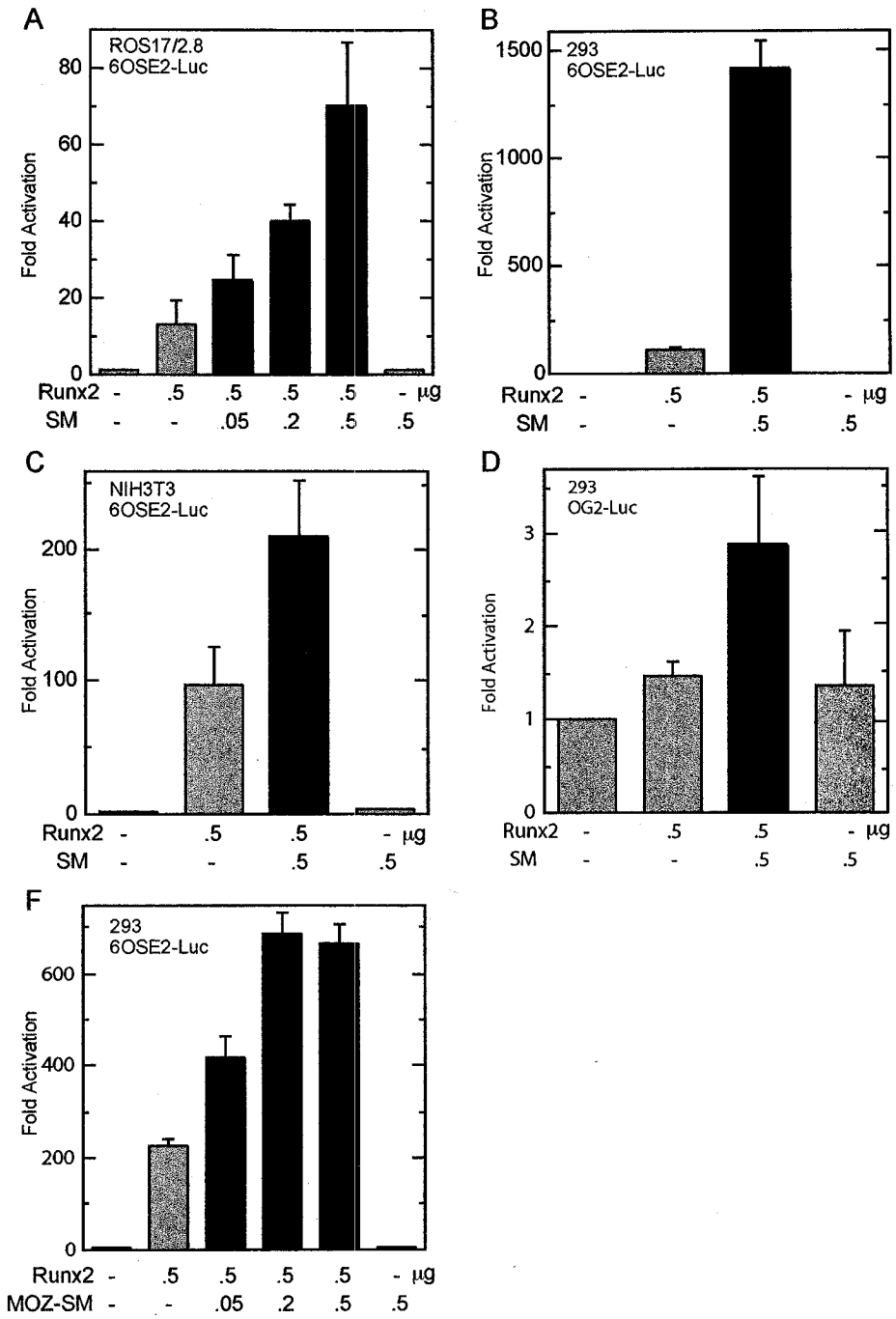

A
ROS17/2.8
6OSE2-Luc
Fold Activation
0
20
40
60
80
Runx2 - .5 .5 .5 .5 - µg
SM - - .05 .2 .5 .5
B
293
6OSE2-Luc
Fold Activation
0
500
1000
1500
Runx2 - .5 .5 - µg
SM - - .5 .5
C
NIH3T3
6OSE2-Luc
Fold Activation
0
100
200
Runx2 - .5 .5 - µg
SM - - .5 .5
D
293
OG2-Luc
Fold Activation
0
1
2
3
Runx2 - .5 .5 - µg
SM - - .5 .5
F
293
6OSE2-Luc
Fold Activation
0
200
400
600
Runx2 - .5 .5 .5 .5 - µg
MOZ-SM - - .05 .2 .5 .5

Figure 6 Role of endogenous MORF in Runx2-dependent transcription.

(A) Model illustrating how fragment M may interfere with role of MORF in Runx2-dependent transcription.

(B-C) The luciferase reporter, 6OSE2-Luc (0.2 mg), was transfected into 293 (B) or ROS17/2.8 (C) cells with an internal control plasmid (CMV-b-Gal, 0.05 mg) and expression plasmids for Runx2 and the M domain of MORF (Flag-tagged) at indicated amounts. The normalized luciferase activity from the transfection with Runx2 expression plasmid as the only effector was arbitrarily set to 100%. Average values of seven independent experiments are shown with standard deviation indicated by error bars.

(D) The luciferase reporter, 6OSE2-Luc (0.2 mg), was transfected into ROS17/2.8 cells with an internal control plasmid (CMV-b-Gal, 0.05 mg), Runx2 expression plasmid, sense construct (S) and/or antisense contruct (AS) at the indicated amount. Reporter activities were measured and analyzed as in (A-B).

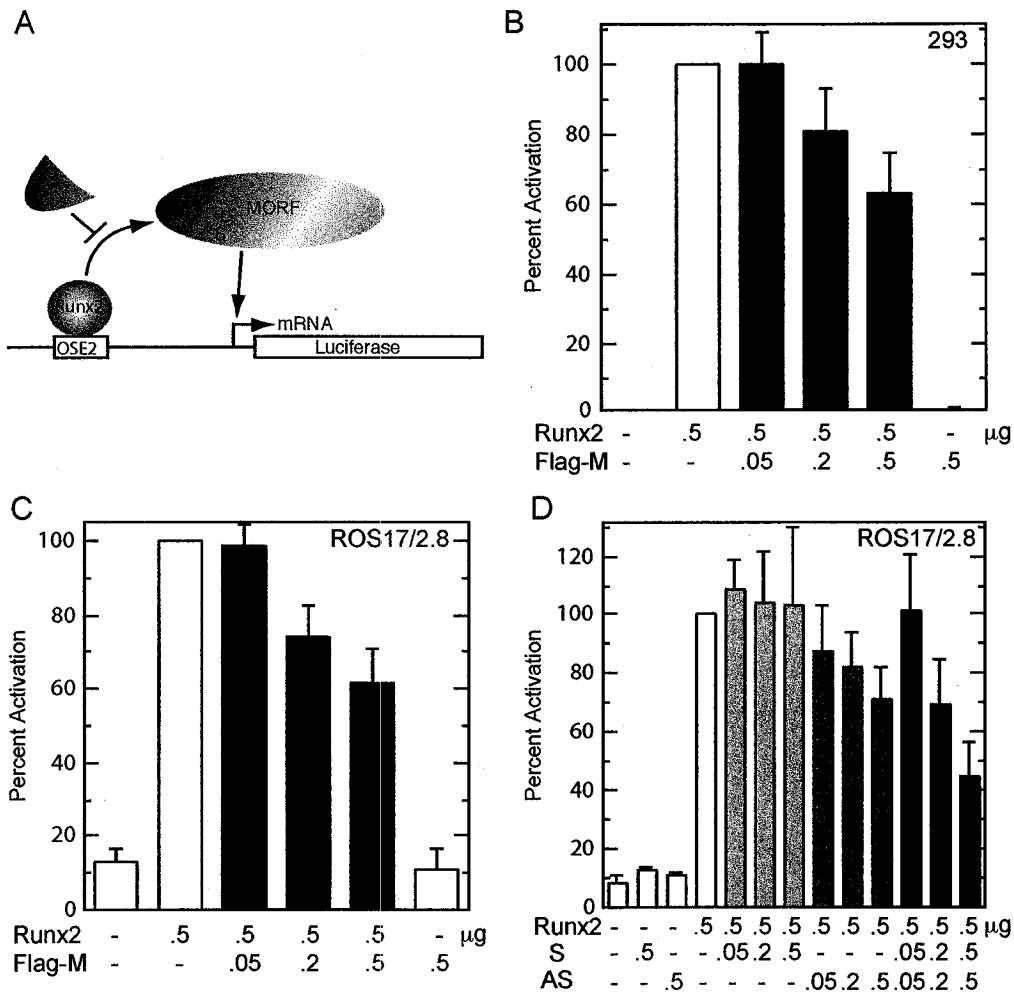
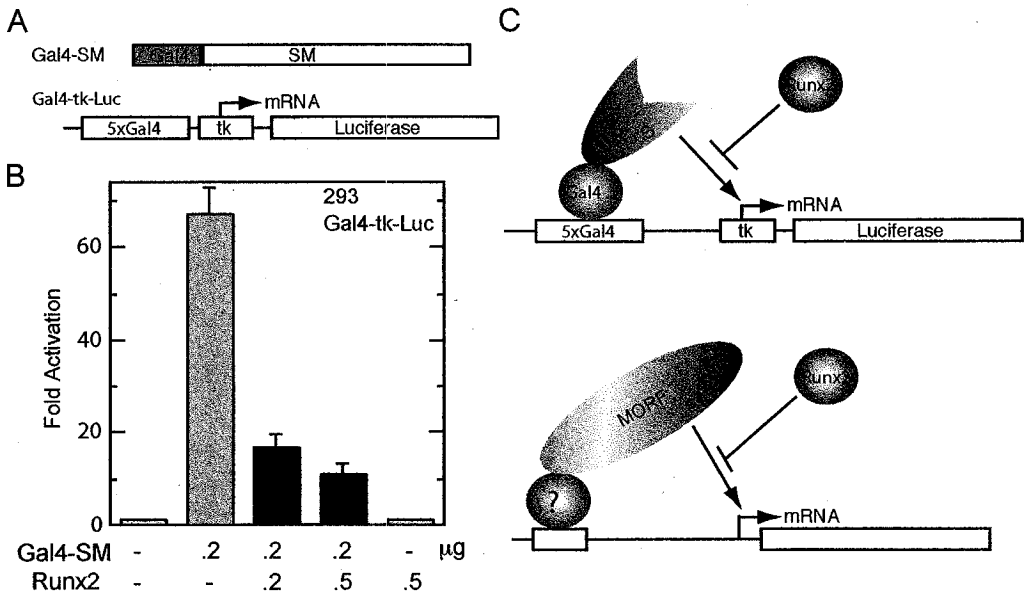

A
MORF
Runx2
mRNA
OSE2
Luciferase
B
293
Percent Activation
100
80
60
40
20
0
Runx2 - .5 .5 .5 .5 - µg
Flag-M - - .05 .2 .5 .5
C
ROS17/2.8
Percent Activation
100
80
60
40
20
0
Runx2 - .5 .5 .5 .5 - µg
Flag-M - - .05 .2 .5 .5
D
ROS17/2.8
Percent Activation
120
100
80
60
40
20
0
Runx2 - - - .5 .5 .5 .5 .5 .5 .5 .5 .5 .5 µg
S - .5 - - .05 .2 .5 - - - .05 .2 .5
AS - - .5 - - - - .05 .2 .5 .05 .2 .5

Figure 7 Effect of Runx2 on the transcriptional activation potential of MORF.

(A) Schematic illustration of the Gal4-SM fusion protein and the Gal4-tk-Luc reporter.

(B) Effect of Runx2 on transcriptional activation by Gal4-SM. The Gal4-tk-Luc luciferase reporter (0.2 mg) was transfected into 293 cells along with an internal control plasmid (CMV-b-Gal, 0.05 mg) and expression plasmids for Runx2 and Gal4-SM at indicated amounts. The normalized luciferase activity from the transfection without any effectors was arbitrarily set to 1.0. Average values of at least three independent experiments are shown with standard deviation indicated by error bars.

(C) Models illustrating how Runx2 inhibits transcription activated by Gal4-SM (top) and MORF (bottom). An unidentified DNA-binding transcription factor(s) may recruit MORF to activate transcription, and Runx2 inhibits the transcription activation.

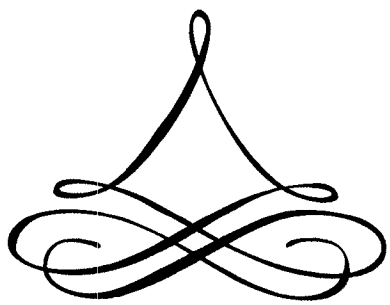

A
Gal4-SM
SM
Gal4-tk-Luc
mRNA
5xGal4
tk
Luciferase
B
293
Gal4-tk-Luc
Fold Activation
60
40
20
0
Gal4-SM - .2 .2 .2 - µg
Runx2 - - .2 .5 .5
C
Runx2
Gal4
mRNA
5xGal4
tk
Luciferase
MORF
?
Runx2
mRNA

CHAPTER IV

SIGNAL-DEPENDENT REGULATION OF RUNX2 TRANSCRIPTIONAL ACTIVITY BY MOZ AND THE WW-DOMAIN COACTIVATOR TAZ

Nadine Pelletier, Siew-Lee Goh, Dengshun Miao, Nathalie Champagne, David Goltzman and Xiang-Jiao Yang

To be modified for submission



PREFACE

Regulation of histone acetyltransferases is an area that has not been extensively explored. In the previous chapter, we demonstrated that MOZ and MORF are coregulators of Runx2. Here we report that the WW-domain protein TAZ, another coregulator of Runx2, synergizes with MOZ to potentiate Runx2-dependent activation of the osteocalcin promoter. Moreover, this synergistic effect is regulated by cell signaling.

1. ABSTRACT

The transcription factor Runx2 is essential for osteoblastic differentiation and thus bone formation. Several coactivators have been shown to interact with and activate Runx2-dependent transcription. Here, we demonstrate that Runx2 collaborates with the coactivators MOZ and TAZ to regulate the osteocalcin promoter. Coimmunoprecipitation studies indicate that the interaction of Runx2 with the coregulators TAZ and MOZ is relatively weak, suggesting that the interaction might be regulated. Interestingly, we found that the interaction was stimulated when 293 cells were treated by PMA. Furthermore, the synergistic activation by MOZ, TAZ and Runx2 was stimulated when cells were treated by PMA. These results suggest that PMA activates signaling pathways important for regulation of the osteocalcin promoter by MOZ, TAZ and Runx2.

2. INTRODUCTION

Chromatin plays an important role in controlling different nuclear processes and its structure is regulated by different mechanisms, including histone acetylation (Horn and Peterson, 2002; Kornberg and Lorch, 1999). Since identification of the first histone acetyltransferases (HATs) in the mid-1990s (Bannister and Kouzarides, 1996; Brownell et al., 1996; Kleff et al., 1995; Mizzen et al., 1996; Ogryzko et al., 1996; Yang et al., 1996), various proteins have been shown to possess intrinsic HAT activity. Importantly, these enzymes also acetylate transcription factors, leading to either transcriptional activation or inhibition. Therefore, HATs regulate transcription by modifying chromatin as well as transcription factors.

Known HATs are grouped into different families, three of which are Gcn5/PCAF, p300/CBP and the MYST family of proteins. The acronym MYST is from its four founding members: human MOZ (monocytic leukemia zinc finger protein) (Borrow et al., 1996), yeast Ybf2 (renamed Sas3, for something about silencing 3) (Reifsnyder et al., 1996; Takechi and Nakayama, 1999), yeast Sas2 (Reifsnyder et al., 1996), and mammalian TIP60 (HIV Tat-interacting 60 kDa protein) (Kamine et al., 1996; Ran and Pereira-Smith, 2000; Yamamoto and Horikoshi, 1997). A third MYST protein in *S. cerevisiae* is Esa1 (essential Sas2-related acetyltransferase 1) (Clarke et al., 1999; Smith et al., 1998). In *Drosophila*, there are five members, including Mof (male-absent on the first) (Akhtar and Becker, 2000; Hilfiker et al., 1997), Enok (Enoki mushroom) (Scott et al., 2001), Chameau (Camel in French) (Grienenberger et al., 2002), and two uncharacterized MYST proteins (CG6121 and CG1894). In humans, besides MOZ and TIP60, there are hMOF (ortholog of *Drosophila* Mof), HBO1 (HAT bound to ORC1; Chameau ortholog) (Iizuka and Stillman, 1999) and MORF (MOZ-related factor)

(Champagne et al., 1999). These proteins play roles in diverse cellular programs. Compared to members of the Gcn5/PCAF and p300/CBP families, MYST proteins are much more diverse in domain organization, multiprotein complex formation and biological function (Utley and Cote, 2003).

MOZ and MORF possess transcriptional repression and activation domains (Champagne et al., 1999; Champagne et al., 2001; Kitabayashi et al., 2001), suggesting that these two HATs are potential transcriptional coregulators. Indeed, MORF is present in a transcriptional coactivator complex associated with the nuclear receptor PPARα (Surapureddi et al., 2002). Both MOZ and MORF physically and functionally interact with Runx1 and Runx2 (Bristow and Shore, 2003b; Kitabayashi et al., 2001; Pelletier et al., 2002), two Runt-domain transcription factors important for cell growth and differentiation in different tissues (Ducy, 2000; Speck, 2001; Westendorf and Hiebert, 1999; Wheeler et al., 2000). In agreement with this, downregulated expression of mouse MORF, known as Querkopf (squarehead in German), leads to defects in osteogenesis and neurogenesis (Thomas et al., 2000). Although it is quite clear that MOZ and MORF function as transcriptional coactivators for Runx1 and Runx2, little is known about how the coactivator function of MOZ and MORF is regulated.

In addition to MOZ and MORF, other proteins, such as p300, Rb and YAP (Yes-associated protein) and its homolog TAZ, have been shown to function as transcriptional coactivators for Runx1 or Runx2 (Cui et al., 2003; Kitabayashi et al., 1998; Sierra et al., 2003; Thomas et al., 2001; Yagi et al., 1999). p300 possesses intrinsic HAT activity, whereas Rb functions as a transcriptional corepressor. YAP and TAZ are particularly intriguing since they possess WW-domains that interact with a PPxY motif located at the

C-terminal part of Runx1 or Runx2. Moreover, YAP and TAZ are considered to be signaling adaptors, and their subcellular localization is regulated by nucleocytoplasmic trafficking (Kanai et al., 2000). Upon specific phosphorylation, 14-3-3 proteins bind to TAZ and YAP and sequester them in the cytoplasm. In light of these findings, we investigated how MOZ may interact with TAZ in regulating Runx2-dependent transcription. Here we describe that MOZ synergizes with TAZ and upregulates Runx2 transcriptional activity in a signal-dependent fashion.

3. MATERIALS AND METHODS

3.1 Materials

Expression plasmids for MOZ and TAZ have been described previously (Kanai et al., 2000; Kitabayashi et al., 2001). In the reporter 6OSE2-Luc, the luciferase gene is under the control of six tandem copies of osteocalcin-specific element 2 (OSE2) (Ducy & Karsenty, 1995). The reporter OG2-Luc contains a mouse osteocalcin promoter fragment (-147/+13) upstream from the luciferase gene (Ducy & Karsenty, 1995). Mammalian expression plasmids for mouse Runx2 and human Runx1c (a Runx1 isoform) have been previously described (Ducy & Karsenty, 1995; Thirunavukkarasu et al., 1998; McLarren et al., 2000; McLarren et al., 2001).

3.2 Reporter gene assays

For these assays, plasmids were prepared using double CsCl ultra-centrifugation, butanol extraction and ethanol precipitation. Transfection and reporter assays were performed as described (Champagne et al., 1999; Wang et al., 1999). 293 (human embryonic kidney), ROS17/2.8 (rat osteoblast sarcoma) and NIH 3T3 cells were used for reporter gene assays. For stimulation assays 293 cells were treated or untreated with PMA (50 ng/ml) in the presence or absence of U0126 (10 μM).

3.3 Protein-protein interaction

To examine the interaction between Runx proteins and the coactivators MOZ and TAZ, the Flag-MOZ or the Flag-TAZ expression plasmids were cotransfected into 293 cells with or without expression plasmid for Runx1 or hemagglutinin (HA)-tagged Runx2. A 5-μg portion of each plasmid was used to transfect 5 X 10^5 cells (in a 10-cm

dish) with 20 μl of SuperFect transfection reagent (Qiagen). Thirty-six hours after transfection, the cells were treated with phorbol 12-myristate 13-acetate (PMA) and/or phytohemaglutinin (PHA). Twelve hours later, the cells were washed twice with cold PBS and collected in 0.5 ml of cold buffer K (20 mM sodium phosphate pH 7.0, 150 mM KCl, 30 mM sodium pyrophosphate, 0.1% NP-40, 5 mM EDTA, 10 mM NaF, 0.1 mM Na_3VO_4 and 1 mM phenylmethylsulfonyl fluoride (PMSF), and protease inhibitors). Cell extracts were prepared for affinity purification on M2 agarose beads (Sigma). Beads were washed four times with buffer K and bound proteins were eluted with Flag peptide (Sigma). Eluted proteins were subsequently resolved by SDS-polyacrylamide gel electrophoresis (10% polyacrylamide) and transferred to nitrocellulose membranes for Western analysis with the anti-Flag, anti-HA or anti-Runx1 antibody. Blots were developed with Supersignal chemiluminescent substrates (Pierce).

4. RESULTS

4.1 MOZ and TAZ are coactivators of Runx2

In previous study, we demonstrated that the MOZ and MORF activation domains interact with and potentiate Runx2-dependent transactivation of the osteocalcin promoter (Pelletier et al., 2002). In addition to MOZ and MORF, the coactivator TAZ has been shown to interact with Runx2 and potentiate its transactivation of the osteocalcin promoter (Cui et al., 2003). These results prompted us to investigate whether MOZ and TAZ synergistically activate Runx2-dependent transcription. Because full-length MORF is difficult to express, we used full-length MOZ expression plasmid. To test our hypothesis, the luciferase reporters 6OSE2-Luc and OG2-Luc were analysed. The 6OSE2-Luc contains six tandem Runx2-response elements upstream of the luciferase gene, and the OG2-Luc contains –147/+13 promoter fragment of the mouse osteocalcin gene (Ducy and Karsenty, 1995; McLarren et al., 2000) (Figure 1a). Exogenous expression of Runx2 stimulated the 6OSE2-Luc but not the OG2-Luc reporter in 293 (Figure 1b, c). This is different from what we previously reported when a higher amount (0.2 μg) of Runx2 was used (Pelletier et al., 2002). Like the MOZ activation domain, full-length MOZ potentiates Runx2-dependent transcription, supporting the previous observation that TAZ stimulates Runx2 activity (Cui et al., 2003). Interestingly, when MOZ and TAZ were co-expressed, Runx2 activity was stimulated further in 293 cells. In the absence of co-transfected Runx2, expression of MOZ and TAZ has a minimal effect on the luciferase reporter activity (Figure 1b, c), indicating that the observed transactivation by MOZ and TAZ is Runx2-dependent. To test whether the functional cooperation between Runx2, MOZ and TAZ is cell line-dependent, similar assays were carried out in two other cell lines. Different from NIH3T3 cells, exogenous expression of

Runx2 did not activate either reporter gene expression in ROS17/2.8 cells (Figure 1d-f). However, MOZ potentiated Runx2 activity in both cell lines (Figure 1d, e). TAZ stimulates Runx2-dependent activation in ROS17/2.8 and 293 cells. Exogenous expression of MOZ, TAZ and Runx2 led to 165-fold and 10-fold activation of the 6OSE2-Luc and OG2-Luc reporter gene expression, respectively, in ROS17/2.8 cells, and to 90-fold activation of OG2-Luc in NIH3T3 (Figure 1d-e). Together, these results suggest that MOZ and TAZ synergize to upregulate the transcriptional activity of Runx2.

4.2 The interaction between MOZ and Runx2 is regulated by PMA treatment

We have demonstrated that MOZ activation domain potentiates Runx2-dependent transactivation of the osteocalcin promoter (Pelletier et al., 2002). It was recently reported that the human MIP-1α promoter is regulated by MOZ and Runx1 was up-regulated when Jurkat T cells were stimulated by PHA/PMA (Bristow and Shore, 2003a). The synergistic activity between MOZ and Runx1 was greatly enhanced when Jurkat T cells were treated with PMA/PHA. Because the interaction between full-length MOZ and Runx2 is very weak (Figure 2, lane 1), we asked if the interaction between MOZ and Runx2 is regulated. Although PHA treatment did not have any effect (data not shown), the interaction between MOZ and Runx2 was enhanced dramatically when 293 cells were stimulated with PMA/PHA (Figure 2, lanes 3-4). The interaction between MOZ and Runx2 was at the highest when cells were treated for 12 hours with PMA/PHA (Figure 2, lane 4). After 12 hours the binding between MOZ and Runx2 decreased. These results implied that PMA/PHA activates signaling pathways regulating the interaction between MOZ and Runx2.

4.3 The interaction between TAZ and Runx2 is regulated by PMA treatment

The coactivator TAZ has been shown to interact with Runx2 and increase its transcriptional activity (Cui et al., 2003). Because the interaction between TAZ and Runx2 is relatively weak (Figure 3, lane 1) and the interaction between MOZ and Runx2 is regulated by PMA/PHA, we investigated if the interaction between TAZ and Runx2 is regulated by PMA. Indeed, like MOZ and Runx2, the interaction between TAZ and Runx2 is stimulated and peaks after 12 hours of PMA/PHA treatment (Figure 3, lane 2-4). These findings demonstrated that the interaction between TAZ and Runx2 is regulated by PMA/PHA treatment.

4.4 The phosphorylation state of Runx1 is important for its interaction with MOZ

Because the activation domain of Runx1 is similar to that of Runx2 and PMA stimulates the interaction between MOZ and Runx2, we sought to investigate whether PMA could regulate the interaction between MOZ and Runx1. The interaction between MOZ and Runx1 is stronger than that of MOZ and Runx2 (Figure 4, lane 4 and Figure 2, lane 1). PMA stimulation did not enhance MOZ and Runx1 interaction dramatically (Figure 4, lane 6), which is consistent with the observation that the interaction between MOZ and Runx1 was initially strong.

PMA induces the PKC and the MAPK signaling pathways. The downstream kinases of these pathways eventually lead to phosphorylation of target proteins, so we decided to investigate if the Runx1 mutant M11, in which 11 potential ERK phosphorylation sites in its carboxy-terminal domain are mutated, could still mediate its

interaction with MOZ. M11 was shown to interact weakly with MOZ, suggesting the importance of serine and/or threonine residues for the interaction between MOZ and Runx1 (Figure 4, lane 5). Interestingly, the interaction between MOZ and M11 was increased when the cells were stimulated with PMA (Figure 4, lane 7). Because the interaction between MOZ and M11 is very weak, PMA stimulation could induce phosphorylation at the interaction interface of MOZ and M11, leading to their increase in the binding. So, serine and/or threonine residues appear to be important for MOZ and Runx1 interaction, but are not essential for PMA-stimulated interaction between MOZ and Runx1.

4.5 The synergistic activity of MOZ, TAZ and Runx2 is stimulated by PMA treatment

Because PMA stimulated the interaction of Runx2 with MOZ and TAZ, we wanted to investigate if the synergistic activity observed between MOZ, TAZ and Runx2 is modulated by PMA. The transcriptional activation of the 6OSE2-Luc mediated by Runx2 was stimulated when 293 cells were treated with PMA. Interestingly, the MEK kinase inhibitor, U0126, inhibited the induction of Runx2 activity by PMA (Figure 5, lane 2). In the presence of PMA, MOZ and Runx2 strongly activate the reporter gene expression, but not when cells were treated with both PMA and U0126 (Figure 5, lane 3). These results are in agreement with previous data showing that the interaction between MOZ and Runx2 is stronger after the cells were treated with PMA. Furthermore, PMA induces the synergistic effect of TAZ and Runx2, but U0126 has little effect on the transcriptional activity (Figure 5, lane 4). MOZ, TAZ and Runx2 activated the reporter

gene expression. This synergistic effect is stimulated by PMA but not when 293 cells were treated with U0126 (Figure 5, lane 5). These results suggest that MOZ activates Runx2 transcriptional activity through an MEK-1-dependent pathway.

5. DISCUSSION

5.1 Regulation of Runx2 by MOZ and TAZ

A physical association of the coactivators MOZ and TAZ with the transcription factor Runx2 was previously reported (Cui et al., 2003; Pelletier et al., 2002). Independently, MOZ and TAZ have been shown to upregulate Runx2 transcriptional activity (Cui et al., 2003; Pelletier et al., 2002), but no reports have described about how MOZ and TAZ cooperate to regulate Runx2 activity. The results presented herein demonstrate that MOZ and TAZ synergistically activate Runx2-dependent transcription of the osteocalcin promoter (Figure 1).

The recruitment of coactivators with histone acetyltransferase activity by transcription factors is very important for opening chromatin structure and gene activation. Several coactivators, like Rb and p300, have been shown to interact with and participate in Runx2-dependent activation of the osteocalcin promoter (Sierra et al., 2003; Thomas et al., 2001). It was demonstrated that the acetylation of histones H3 and H4 of the osteocalcin locus is increased when ROS17/2.8 cells are treated with vitamin D (Shen et al., 2002). This increase in histones H3 and H4 acetylation of the osteocalcin locus is not mediated by p300 (Sierra et al., 2003). Because MOZ has intrinsic histone acetyltransferase activity for histones H3 and H4, it is possible that MOZ could mediate acetylation of the osteocalcin promoter by interacting with Runx2 (Champagne et al., 2001).

5.2 PMA treatment stimulates the interaction of Runx2 with MOZ and TAZ

We have previously reported that MOZ activation domain interacts with Runx2 (Pelletier et al., 2002). Immunoprecipitation studies have demonstrated that full-length MOZ and TAZ interact weakly with Runx2 (Figures 2 and 3). It was recently reported that the human MIP-1α promoter is regulated by MOZ and Runx1 (Bristow and Shore, 2003a). The synergistic activity between MOZ and Runx1 was greatly enhanced when Jurkat T cells were treated with PMA/PHA. This result prompted us to investigate whether the interaction of Runx2 with MOZ and TAZ is regulated by PMA/PHA. Although PHA treatment didn't have any effect (data not shown), Figures 2 and 3 clearly demonstrated that the interactions of Runx2 with MOZ and TAZ are regulated and their maximal interactions occur after twelve hours of PMA treatment. Immunoprecipitation data revealed that MOZ interacts weakly with TAZ and that this interaction is not modulated by PMA (data not shown). These results suggest that signaling pathway activated by PMA regulate the interactions.

The MAPK kinase, the PKC and the PKA signaling pathways were shown to regulate Runx2 transcriptional activity, but neither were shown to modulate the interaction of Runx2 with coactivators (Franceschi and Xiao, 2003; Kim et al., 2003; Xiao et al., 2000). PMA activates the PKC pathway which can diverge to and stimulate the MAPK kinase one's. Thus, the interactions could either be regulated by the PKC and/or the MAPK kinase pathway. Further experiments are required to investigate this possibility.

Because MOZ binds Runx1, we investigated whether PMA could also stimulate the MOZ/Runx1 interaction. PMA treatment did not have dramatic effect on this

interaction, which is consistent with our and others' results indicating that MOZ interacts strongly with Runx1 (Kitabayashi et al., 2001) (Figure 4). Interestingly, PMA stimulates the association of MOZ with Runx1 mutant (M11) in which 11 potential ERK phosphorylation sites were mutated. Since the interaction between MOZ and M11 is very weak, the binding interfaces of MOZ and M11 might be permissive to phosphorylation events mediated by the PKC and/or the MAPK kinase signaling pathways that are independent of Runx1 mutations.

5.3 PMA treatment stimulates the coactivator activity of MOZ and TAZ

Since the interaction of Runx2 with MOZ and TAZ is regulated by PMA treatment, we investigated whether the synergistic activation by these members is also modulated by PMA. As previously reported, the transcriptional activity of Runx2 is stimulated by the PKC and the MAPK signaling pathways (Kim et al., 2003; Xiao et al., 2002; Xiao et al., 2000) (Figure 5). Interestingly, the stimulation of Runx2 activity by PMA is inhibited when cells were treated with the MEK kinase inhibitor U0126. The stimulation of MOZ/Runx2 transcriptional activity by PMA is consistent with our results showing that the MOZ/Runx2 interaction is increased by PMA. Because U0126 inhibits PMA-dependent stimulation of MOZ/Runx2, it is tempting to speculate that the MOZ and Runx2 interaction is MAPK pathway-dependent. U0126 has less effect on PMA-stimulated TAZ/Runx2 activity, suggesting that the PKC pathway may also be involved in the TAZ/Runx2 interaction. The synergistic activity of MOZ, TAZ and Runx2 on luciferase gene expression is modulated by PMA, and U0126 diminishes PMA-dependent stimulation of MOZ, TAZ and Runx2 (Figure 5). Thus depending on the signaling

pathway, the phosphorylation state of Runx2 might determine Runx2-specific coactivators partners.

In summary, the coactivators MOZ and TAZ synergize to enhance Runx2 transcriptional activity. The interaction of Runx2 with MOZ and TAZ increased when cells were treated with PMA. Consistent with this, PMA treatment enhanced the synergy between MOZ and TAZ in activating the osteocalcin promoter. These results provide new insights into the molecular mechanisms by which MOZ, TAZ and Runx2 regulate cell proliferation and differentiation.

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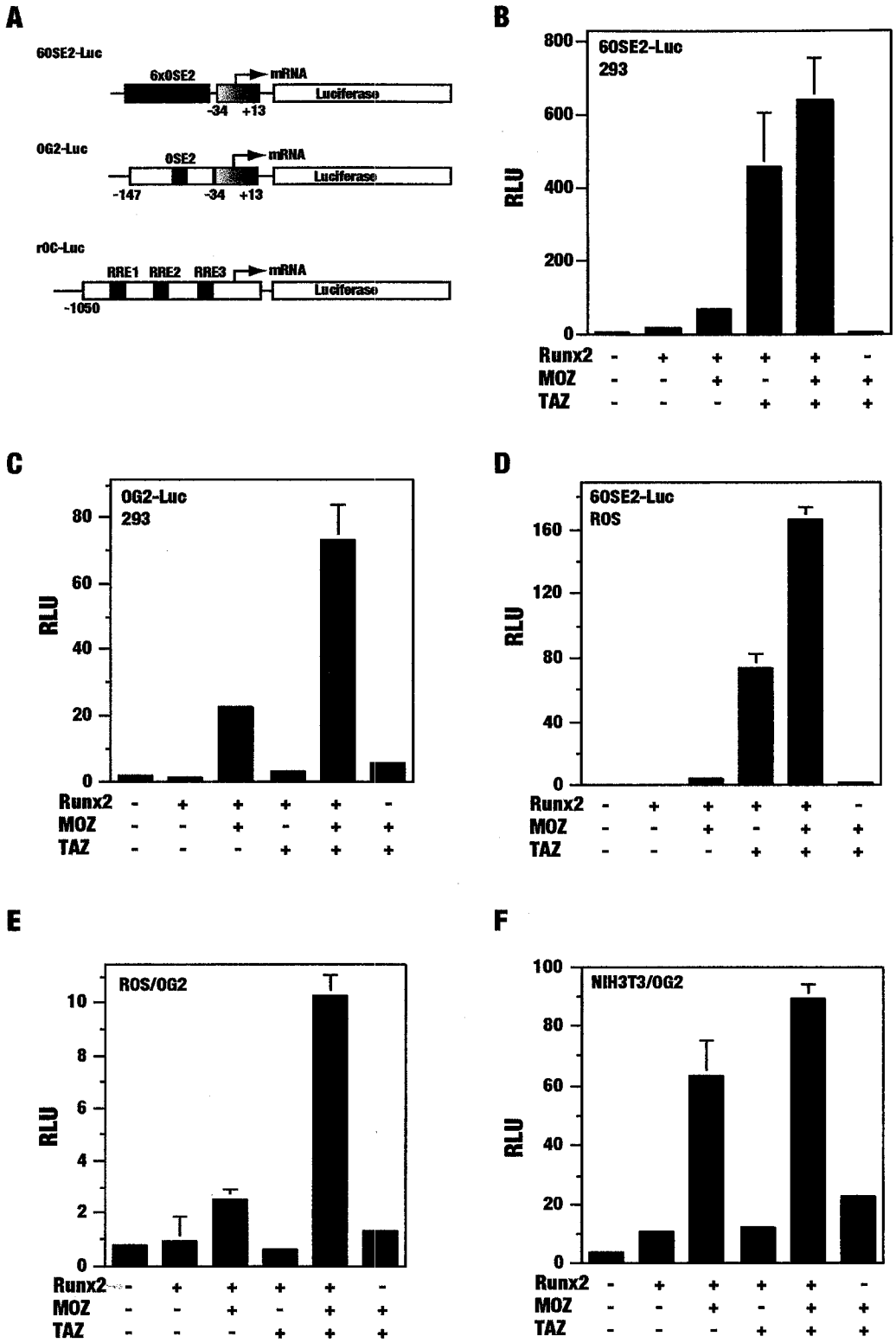
Figure 1 MOZ and TAZ are coactivators for Runx2.

(A) Schematic representation of the luciferase reporters 6OSE2-Luc and OG2-Luc.

(B-F) Activation of Runx2-dependent transcription by MOZ and TAZ. Luciferase reporters (0.4 μg) were transfected into 293, ROS17/2.8 or NIH3T3 cells along with an internal control plasmid (CMV-β-Gal, 0.05 μg) and expression plasmids for Runx2 (0.05 μg), Flag-MOZ and/or Flag-TAZ (0.1 μg) and CBFβ2 (0.2 μg). Luciferase activities were measured and normalized with internal β-galactosidase controls. Mean values of at least three independent experiments are shown with standard deviations indicated by error bars.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6OSE2-Luc
6xOSE2
mRNA
Luciferase
-34 +13
OG2-Luc
OSE2
mRNA
Luciferase
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rOC-Luc
RRE1 RRE2 RRE3
mRNA
Luciferase
-1050
B
6OSE2-Luc
293
RLU
800
600
400
200
0
Runx2 - + + + + -
MOZ - - + - + +
TAZ - - - + + +
C
OG2-Luc
293
RLU
80
60
40
20
0
Runx2 - + + + + -
MOZ - - + - + +
TAZ - - - + + +
D
6OSE2-Luc
ROS
RLU
160
120
80
40
0
Runx2 - + + + + -
MOZ - - + - + +
TAZ - - - + + +
E
ROS/OG2
RLU
10
8
6
4
2
0
Runx2 - + + + + -
MOZ - - + - + +
TAZ - - - + + +
F
NIH3T3/OG2
RLU
100
80
60
40
20
0
Runx2 - + + + + -
MOZ - - + - + +
TAZ - - - + + +

Figure 2 PMA stimulates the interaction between MOZ and Runx2.

Flag-MOZ expression plasmid was transfected into 293 cells with HA-Runx2 expression plasmid. Thirty-six hours post transfection, the cells were treated for 3, 6, 12, 16 and 24 hours with PMA (50 ng/ml). After the indicated time of PMA treatment, cell extracts were prepared for immunoprecipitation (IP). Eluted proteins were analysed by immunoblotting with α-Flag (top) or α-HA (middle) antibody. Bottom panel corresponds to cellular extracts immunoblotted with α-HA antibody.

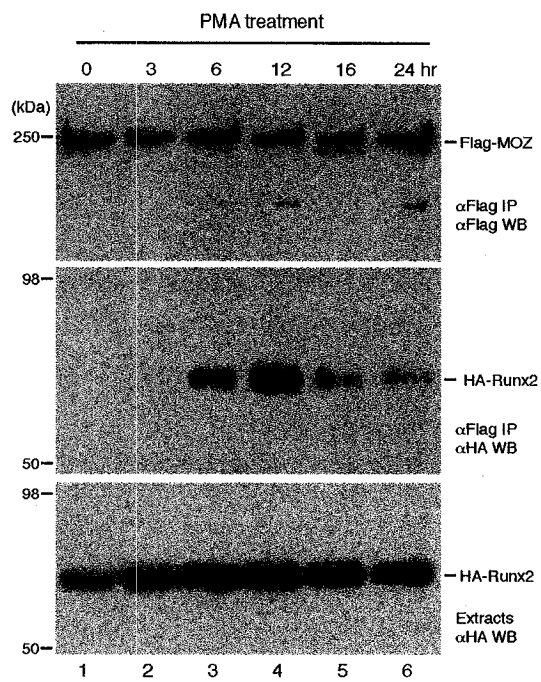

PMA treatment
0
3
6
12
16
24 hr
(kDa)
250
Flag-MOZ
αFlag IP
αFlag WB
98
HA-Runx2
αFlag IP
αHA WB
50
98
HA-Runx2
Extracts
αHA WB
50
1
2
3
4
5
6

Figure 3 PMA stimulates the interaction between TAZ with Runx2.

Flag-TAZ expression plasmid was transfected into 293 cells with HA-Runx2 expression plasmid. Thirty-six hours post transfection, the cells were treated for 3, 6, 12, 16 and 24 hours with PMA (50 ng/ml). After the indicated time of PMA treatment, cell extracts were prepared for immunoprecipitation (IP). Eluted proteins were analysed by immunoblotting with α-Flag (top) or α-HA (bottom) antibody.

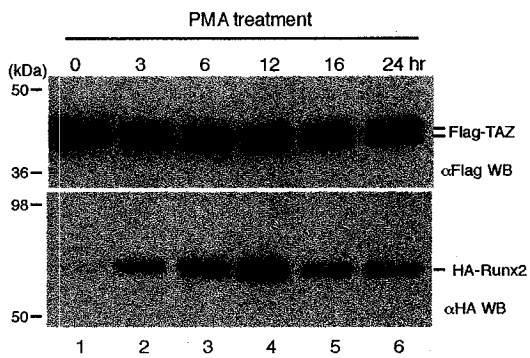

PMA treatment
(kDa)
0
3
6
12
16
24 hr
50
36
98
50
Flag-TAZ
αFlag WB
HA-Runx2
αHA WB
1
2
3
4
5
6

Figure 4 The phosphorylation state of Runx1 is important for its interaction with MOZ.

Flag-MOZ expression plasmid was transfected into 293 cells with or without Runx1 or M11 expression plasmid. Thirty-six hours post transfection, cells co-transfected with Flag-MOZ and Runx1 or M11 were treated for 12 hours with PMA (50 ng/ml). Once stimulation was completed, cell extracts were prepared for immunoprecipitation (IP). Eluted proteins were analysed by immunoblotting with α-Flag (top) or α-Runx1 (middle) antibody. Bottom panel corresponds to cellular extracts immunoblotted with α-Runx1 antibody.

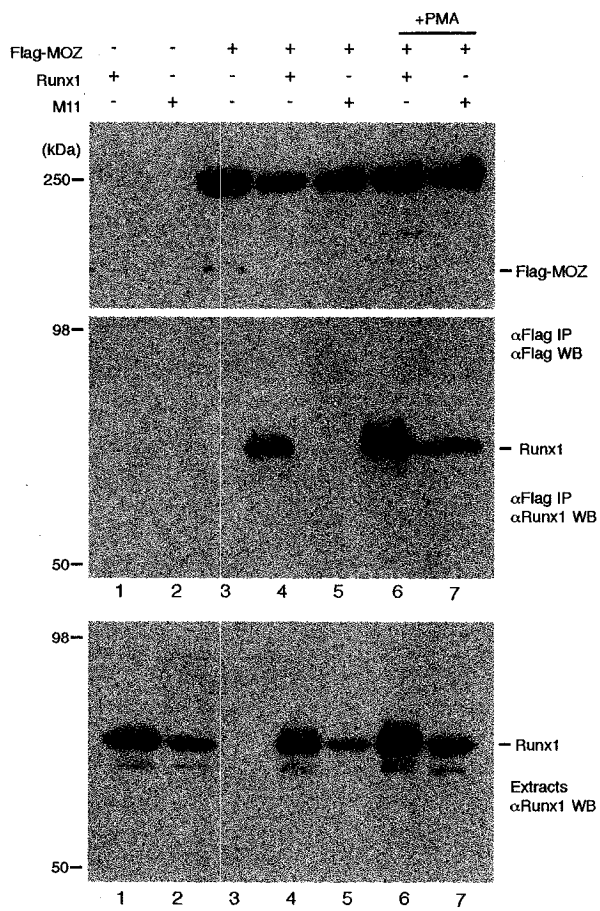
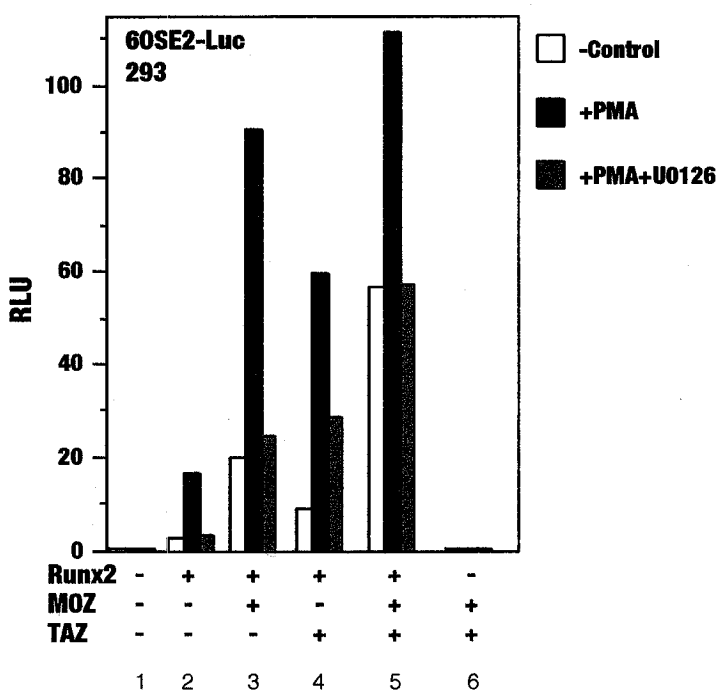

+PMA
Flag-MOZ - - + + + + +
Runx1 + - - + - + -
M11 - + - - + - +
(kDa)
250
Flag-MOZ
98
αFlag IP
αFlag WB
Runx1
αFlag IP
αRunx1 WB
50
1 2 3 4 5 6 7
98
Runx1
Extracts
αRunx1 WB
50
1 2 3 4 5 6 7

Figure 5. PMA enhances the synergistic effect of MOZ, TAZ and Runx2.

The luciferase reporter 6OSE2-Luc (0.4 μg) was transfected into 293 along with an internal control plasmid (CMV-β-Gal, 0.05 μg) and expression plasmids for Runx2 (0.05 μg), Flag-MOZ and/or Flag-TAZ (0.1 μg) and CBFβ2 (0.2 μg). Thirty-six hours post transfection, the cells were treated or untreated for 12 hours with PMA (50 ng/ml) in the presence or absence of U0126 (10 μM). Luciferase activities were measured and normalized to internal β-galactosidase controls. Average values of at least three independent experiments are shown with standard deviations indicated by error bars.


6OSE2-Luc
293
-Control
+PMA
+PMA+U0126
RLU
100
80
60
40
20
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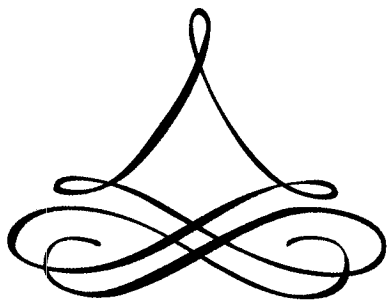
Runx2 - + + + + -
MOZ - - + - + +
TAZ - - - + + +
1 2 3 4 5 6

CHAPTER V

GENERAL DISCUSSION



In Chapters II-IV, I have described the functional characterizations of MOZ and MORF. Basically, we have identified a new member of the MYST family, MORF, which shows high sequence similarity to the MOZ protein. Biochemical studies have revealed that MORF contains intrinsic histone acetyltransferase activity. Furthermore, we have demonstrated that MORF possesses different functional domains. A weak repression domain and a strong activation domain were mapped at the amino- and carboxy-terminal regions of MORF, respectively. Interestingly, we have identified Runx1 and Runx2 as two transcription factors interacting with both MOZ and MORF. These HATs potentiate Runx2-dependent transcriptional activity of the osteocalcin promoter, thus suggesting, for the first time, that MOZ and MORF may play a role in bone differentiation. Most importantly, we have demonstrated that MOZ synergizes with TAZ to activate the osteocalcin promoter and that PMA treatment enhances this synergy. In this chapter, I discuss these findings and their relationship with osteoblast differentiation and diseases such as acute myeloid leukemia.

1. MOZ and MORF are coactivators with histone acetyltransferase activity

The identification and functional characterization of new genes encoding histone acetyltransferases are of great importance for understanding the regulation of the chromatin structure. Histone acetylation is now well known to modulate the chromatin structure and thus transcription (Wolffe, 1998). Importantly, a link between histone acetylation and cancer was suggested by the discovery of a fusion between the MOZ and CBP (or TIF2) genes in patients with acute myeloid leukemia (AML) (Borrow et al., 1996; Carapeti et al., 1998; Chaffanet et al., 2000; Kitabayashi et al., 2001b).

Sequence database searches using BLAST and PSI-BLAST identified a partial human cDNA, encoding MORF (MOZ related factor). MORF displays sequence similarity to MOZ (identity, 60%; similarity, 66%) (Figure 1). Biochemical studies have revealed that both MOZ and MORF contain intrinsic histone acetyltransferase activity (Champagne et al., 1999; Champagne et al., 2001; Pelletier et al., 2003). The MYST domains of MORF and MOZ display subtle differences in substrate specificity. For example, the MORF MYST domain was able to acetylate free histones H4 and H3 and, to a lesser extent, histone H2A. The MOZ MYST domain acetylates histone H2A more efficiently then that of MORF (Champagne et al., 2001; Kitabayashi et al., 2001a). Alignment of the MYST domains of several MYST family members, such as Esa1, Sas2, Sas3, MOZ and Tip60, indicates a central core region, with high conserved residues, that mediates interaction with acetyl-CoA (Yan et al., 2000). The flanking regions of this core region are less conserved and were shown to determine histone specificity (Yan et al., 2000). Full-length MORF was capable of acetylating histones H4, H3 and H2A, much more efficiently then its MYST domain. So amino acids sequences outside the MYST domain are also important in determining substrate specificity (Champagne et al., 1999). Interestingly, we have shown that unlike its MYST domain, MORF acetylates nucleosomal histones (Champagne et al., 1999; Champagne et al., 2001). One possible explanation is that other domains of MORF might be required for nucleosomal histones acetylation. Prior to biochemical studies, full-length MORF was purified from insect cells (Pelletier et al., 2003). During the purification process, Sf9 cellular proteins might have been co-purified with MORF and played an important role in mediating nucleosomal histone acetylation. Since the MYST domain was expressed and purified

from *Escherichia coli*, essential proteins for MORF-dependent nucleosomal histones acetylation might have been absent. Relevant to this, histone acetyltransferases do not work alone *in vivo*. Instead, they are part of multisubunit complexes (Naar et al., 2001; Roth et al., 2001). For example, recombinant forms of Gcn5 fail to acetylate nucleosomal histones suggesting that other subunits of SAGA or ADA complexes are important for regulating histone tail accessibility in nucleosomes (Brownell et al., 1996).

Because histone acetylation is linked to gene activation and several histone acetyltransferases such as p300/CBP contain repression and activation domains, we have investigated whether MOZ and MORF are able to regulate transcription when tethered to a promoter (Grunstein, 1997; Janknecht and Hunter, 1996; Kwok et al., 1994; Workman and Kingston, 1998). Interestingly, a weak repression domain was mapped at the amino-terminal region of MOZ and MORF (Champagne et al., 1999; Champagne et al., 2001) (Figure 1). Coactivators like p300, CBP and HBO1 also contain repression domains (Gregory et al., 2002; Sharma et al., 2000; Snowden et al., 2000). We have demonstrated that the amino-terminal region of MORF does not associate with NAD-independent histone deacetylase activity and that treatment with trichostatin A did not relieve the transcriptional repression (Champagne et al., 1999). Because the histone deacetylase activity of class III HDACs is not sensitive to trichostatin A, it is still possible that MOZ and MORF mediate repression activity through association with these HDACs. Interestingly, some complexes containing both histone acetyltransferase and histone deacetylase activities have been characterized (Yamagoe et al., 2003). Another possibility is that MOZ and MORF repress transcription through post-translational modification of their amino-terminal domains. Post-translational modifications could be

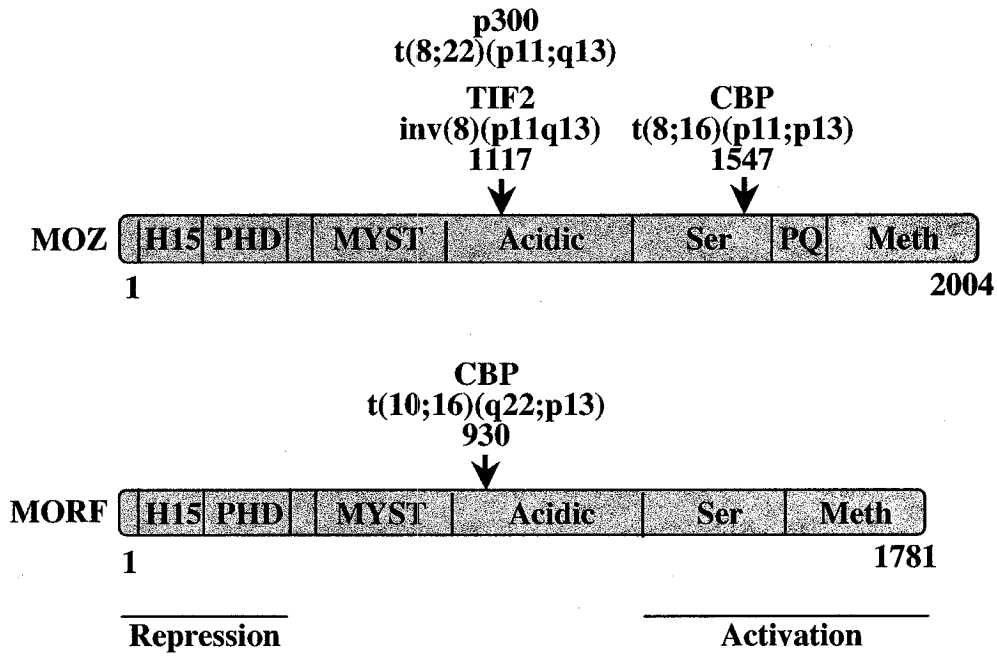


Figure 1. Shematic representation of MOZ and MORF histone acetyltransferases. Structural domains of MOZ and MORF are labeled as follows: H15, linker histones H1- and H5-like module; PHD, *p*lant *h*omeo*d*omain zinc fingers; MYST, MYST acetyltransferase domain; ED, Glu/Asp-rich acidic regions; S, Ser-rich domain; PQ, Pro/Gln-stretch; and M, Meth-rich domain.

required for the recruitment of repressor and/or histone deacetylase to mediate transcriptional repression. Recently, it was demonstrated that sumoylation of p300 within the repression domain is required for the recruitment of HDAC6 and p300-mediated transcriptional repression (Girdwood et al., 2003). The PHD module within the amino-terminal domain of MOZ and MORF might be involved in mediating repression activity. It was demonstrated that the corepressor KAP-1, via its PHD module, recruits the Mi-2α subunit of NuRD to repress transcription (Schultz et al., 2001). Furthermore, some reports provide evidence that PHD modules are capable of mediating protein-protein interaction (Fair et al., 2001; O'Connell et al., 2001).

An activation domain was mapped at the carboxy-terminal region of MOZ or MORF (Champagne et al., 1999; Champagne et al., 2001; Kitabayashi et al., 2001a) (Figure 1). The carboxy-terminal regions of MOZ and MORF are composed of serine- and methionine-rich domains. Previous results have indicated that the transcriptional activation domain is located at the serine-rich region, and the methionine-rich domain is required for the optimal function of the activation domain (Champagne et al., 1999; Champagne et al., 2001; Kitabayashi et al., 2001a). Interestingly, the activation domain of MOZ was weaker than that of MORF, raising the question whether the PQ-stretch in the activation domain of MOZ reduces its activity (Champagne et al., 2001). Our and other's results have indicated that the MYST domain of MOZ or MORF is not required for transcriptional activation (Champagne et al., 1999; Champagne et al., 2001; Kitabayashi et al., 2001a). No transcriptional activity was observed for tethered full-length MOZ and MORF (Champagne et al., 1999; Kitabayashi et al., 2001a). These results suggest that their repression domains might counteract the function of their

activation domains. Because MOZ and MORF contain transcriptional regulatory domains, they might be transcriptional coregulators.

2. The Runx proteins are targets of MOZ and MORF

It is well known that chromatin-modifying enzymes are recruited to specific promoters via transcription factors (Natarajan et al., 1999; Neely et al., 1999; Yudkovsky et al., 1999). For example, the recruitment of histone acetyltransferase by transcription factor to promoter favors the opening of the chromatin structure after histone acetylation (Narlikar et al., 2002). A report on *Querkopf* mice has yielded good insight into the function of MORF (Thomas et al., 2000). Mice homozygous for the *Querkopf* mutation display defects in brain and skeletal development and gastrointestinal function (Thomas et al., 2000). A key player for osteoblast differentiation and bone formation is the Runx2 transcription factor (Ducy, 2000). Our results indicate that full-length MORF interacts with Runx2 (Pelletier et al., 2002). Furthermore, mapping analysis has indicated that MORF and Runx2 interact with each other through their activation domains (Pelletier et al., 2002). Because the MOZ activation domain is highly similar to that of MORF (identity, 72%; similarity, 75%), it is not surprising that MOZ also binds Runx2. In addition, we have demonstrated that MORF activation domain interacts with Runx1, an important regulator for hematopoietic cell-specific genes (Pelletier et al., 2002; Wang et al., 1996). Independently, another report revealed that MOZ interacts with Runx1 and functions as a potent coactivator (Kitabayashi et al., 2001a). Therefore, the activation domains of MOZ and MORF mediate the interaction with Runx1 and Runx2 both *in vitro* and *in vivo*.

Although capable of acetylating histones, histone acetyltransferases are also able to acetylate other proteins (Gu and Roeder, 1997). Several nonhistone substrates have been identified *in vitro*, particularly for p300/CBP and PCAF (Boyes et al., 1998; Gu and Roeder, 1997; Zhang and Bieker, 1998). Interestingly, MOZ could acetylate Runx1, suggesting that MOZ may regulate Runx1 function through acetylation (Kitabayashi et al., 2001a). MORF was not able to acetylate Runx2 (Pelletier et al., 2002). The variability in the amino acid sequence of Runx1 and Runx2 and the fact that the interaction between MOZ and Runx1 is stronger than between MORF and Runx2 could be the explanation.

Because MOZ and MORF are ubiquitously expressed in human tissues and *Querkopf* mice display multiple biological defects, other transcription factors might recruit MOZ and MORF to specific promoters. Accordingly, MORF was shown to be part of the PRIC (PPARα-interacting cofactor) complex that interacts with PPARα (Surapureddi et al., 2002). PPARα is a member of the nuclear receptor superfamily and plays a central role in fatty acids metabolism. Also, sustained activation of PPARα by peroxisome proliferators has been shown to induce hepatocellular carcinomas in rats and mice (Yu et al., 2003). Interestingly, it was previously reported that the inhibition of osteoblast differentiation by PPARγ suppresses the expression of Runx2 and interferes with its transactivation activity (Jeon et al., 2003). Thus, MORF could interact with either Runx2 or PPAR to induce differentiation of mesenchymal cells to specific cell lineages. Runx2 is a master regulator for osteoblast differentiation, but it was recently shown to be also important for endothelial cell differentiation and angiogenesis (Sun et

al., 2001). So, in addition to its role in osteoblast differentiation, MORF might be very important for adipocyte and endothelial cell differentiation.

Runx1 and Runx2 function as oncogenic effectors for T-cell lymphomas (Blyth et al., 2001; Wotton et al., 2002). Furthermore, Runx1 gene is frequently rearranged in leukemia patients (Westendorf and Hiebert, 1999). MOZ and MORF might regulate genes affecting the development of T-cell lymphomas and acute myeloid leukemia (AML). The MOZ and MORF genes have been found to be rearranged in AML patients (Borrow et al., 1996; Carapeti et al., 1998; Carapeti et al., 1999; Chaffanet et al., 2000; Chaffanet et al., 1999; Deguchi et al., 2003; Liang et al., 1998; Panagopoulos et al., 2001; Panagopoulos et al., 2000; Panagopoulos et al., 2003) (Figure 1). These chromosome translocations have yielded aberrant proteins where the activation domains of MOZ and MORF are replaced by the carboxy-terminal part of CBP, p300 or TIF2. The MOZ-CBP fusion protein inhibits Runx1-dependent transcription (Kitabayashi et al., 2001a). By competing with endogenous MOZ/MORF and/or p300/CBP, the MOZ-CBP fusion protein may interact with Runx1 and inhibit its activity through the repression domain of MOZ. The MOZ-CBP fusion protein retains two histone acetyltransferase domains, so aberrant acetylation of Runx1 and/or chromatin might affect Runx1 function and gene regulation.

3. Signal-dependent regulation of Runx2 transcriptional activity by MOZ and TAZ

Transcription factors can recruit multiple coactivators to specific promoters. For example, p300/CBP and PCAF were shown to regulate positively the activity of p53 transcription factor (Scolnick et al., 1997). In addition to MOZ, MORF and p300, Runx2

interacts with the coactivators Rb, YAP (Yes-associated protein) and its homolog TAZ (Cui et al., 2003; Kanai et al., 2000; Pelletier et al., 2002; Sierra et al., 2003; Thomas et al., 2001; Yagi et al., 1999). Because TAZ is a coactivator of Runx2, we investigated whether MOZ and TAZ could stimulate Runx2-dependent activation of the osteocalcin promoter. Indeed, our results clearly indicate that MOZ and TAZ are coactivators for Runx2-dependent activation of the osteocalcin promoter. Previous studies have demonstrated that the active osteocalcin promoter is associated with acetylated histones H3 and H4 and that the regulation of osteocalcin by p300 requires Runx2 and the vitamin D_3 receptor but not the histone acetyltransferase activity of p300 (Shen et al., 2002; Sierra et al., 2003). To date, no histone acetyltransferase has been shown to acetylate the osteocalcin promoter. Thus, it is tempting to speculate that MOZ is important for acetylation-dependent chromatin remodeling and activation of the osteocalcin promoter.

Runx1, a master regulator of haematopoiesis, regulates cytokine and cell cycle genes (Linggi et al., 2002; Lutterbach et al., 2000; Takahashi et al., 1995; Taylor et al., 1996). Previously reported is the regulation of the MIP-1α promoter by Runx1 and MOZ. Stimulation of Jurkat T-cells with PMA/PHA greatly enhances Runx1 and MOZ synergistic activity of the MIP-1α promoter (Bristow and Shore, 2003). Because the interactions of Runx2 with MOZ and TAZ are relatively weak, we speculated that their interaction might by regulate by PMA/PHA. Although PHA did not have any effect, treatment of 293 cells with PMA increases dramatically the interaction of Runx2 with MOZ and TAZ. The strongest interaction of Runx2 with MOZ and TAZ occurs after twelve hours of PMA treatment, indicating that this response is indirect and probably requires novel protein synthesis. The MAPK signaling pathway was shown to mediate

the interaction between Elk-1 and p300 (Li et al., 2003). The phosphorylation of Elk-1 by MAPK enhances its interaction with p300 but, most importantly, Elk-1 exhibits new interactions with p300. These interaction changes render more potent the histone acetyltransferase activity of p300 that is critical for chromatin remodeling and gene activation. It will be very interesting to investigate whether the histone acetyltransferase activity of MOZ is regulated by PMA.

The signaling pathways regulating Runx2 activity are just beginning to be understood. It is known that Runx2 transcriptional activity is modulated by PKA, PKC and MAPK pathways (Franceschi and Xiao, 2003). The PKC and the MAPK signaling pathways are stimulated when cells are treated with PMA. We have demonstrated that Runx2 transcriptional activity is stimulated by PMA and this stimulation is inhibited when the cells are also treated with the MEK inhibitor U0126. The synergistic effect of Runx2 and MOZ is also sensitive to U0126, suggesting that the MAPK pathway regulates directly and/or indirectly their interaction and activity. Interestingly, U0126 has less effect on Runx2 and TAZ transactivation activity, suggesting that the PKC pathway might be involved. The synergistic activity of Runx2 with MOZ and TAZ is increased when cells are treated with PMA and this is inhibited when the cells are treated with U0126.

The MAPK pathway can be stimulated by a variety of signals including those initiated by extracellular matrix, fibroblast growth factor-2 (FGF-2), mechanical loading and parathyroid hormone (PTH) (Franceschi and Xiao, 2003). All these pathways are important for bone formation, although PTH is indirectly involved in osteoclast differentiation by changing the phenotype of osteoblasts (Partridge et al., 1994).

Generally, PTH treatment of osteoblasts results in decreased expression of many of the genes involved in bone formation while increasing expression of some genes involved in bone resorption (Swarthout et al., 2002). It is important to note that osteocalcin mRNA is stabilized in rat osteoblast-like cells treated with PTH (Noda et al., 1988). It would be very interesting to investigate which physiological signal is important for Runx2-mediated increase interaction and transactivation with the coactivators MOZ and TAZ.

4. Do MOZ and MORF have redundant functions?

We have identified MORF as a new member of the MYST family that shows high sequence similarity to MOZ (Champagne et al., 1999). MOZ and MORF are coactivators with histone acetyltransferase activities and were shown to regulate Runx-dependent transactivation (Kitabayashi et al., 2001a; Pelletier et al., 2002). Since MOZ and MORF are functionally similar proteins, do they have redundant biological functions?

MOZ and MORF are ubiquitously expressed in human tissues and both are most abundantly expressed in testis and ovary (Borrow et al., 1996; Champagne et al., 1999). Although there is no report on the temporal expression of MOZ during the mouse development, the mouse homologue of MORF, querkopf, is expressed strongly in the mesoderm surrounding the cartilage at ~E15.5 (Thomas et al., 2000). These cells have the potential to differentiate into osteoblasts. The expression of querkopf is downregulated as cells differentiate. Although in homozygous mice, the querkopf mRNA is expressed at 10%, the majority (65%) of these mice died around the time of weaning. This suggests that the temporal expression of MOZ might occur late during mouse development, because MOZ fails to rescue mice with the querkopf mutation.

MOZ interacts more strongly with Runx1 than Runx2 (Kitabayashi et al., 2001a; Pelletier et al., 2002). Because the expression of MOZ is ubiquitous, MOZ can regulate haematopoietic and osteoblast specific genes in collaboration with Runx1 and Runx2 transcription factors, respectively. We and others have demonstrated that PHA and/or PMA stimulate Runx1 and Runx2 transcriptional activity by MOZ (Bristow and Shore, 2003; Pelletier et al., 2004). Most importantly, we have demonstrated that the interaction of Runx2 with MOZ and TAZ increases dramatically when 293 cells were treated with PMA, but the interaction between MOZ and Runx1 was not affected by PMA (Pelletier et al., 2004) (Figure 2). At the molecular level, in haematopoietic cells, since the interaction of Runx1 with MOZ is already strong, PMA/PHA treatment could affect only the synergistic activity of Runx1 and MOZ. In osteoblast cells, PMA might not only modulate the synergistic activity of Runx2 with MOZ and TAZ but also their physical interaction.

Overall, MOZ and MORF functions can be attributed to haematopoietic and osteoblast cells. We have shown that signaling pathways can affect how MOZ and MORF function in different cells. Regulation of the Runx2 interaction with MOZ and TAZ by PMA treatment yields new insight into the molecular mechanism by which Runx2 regulate osteoblast differentiation.

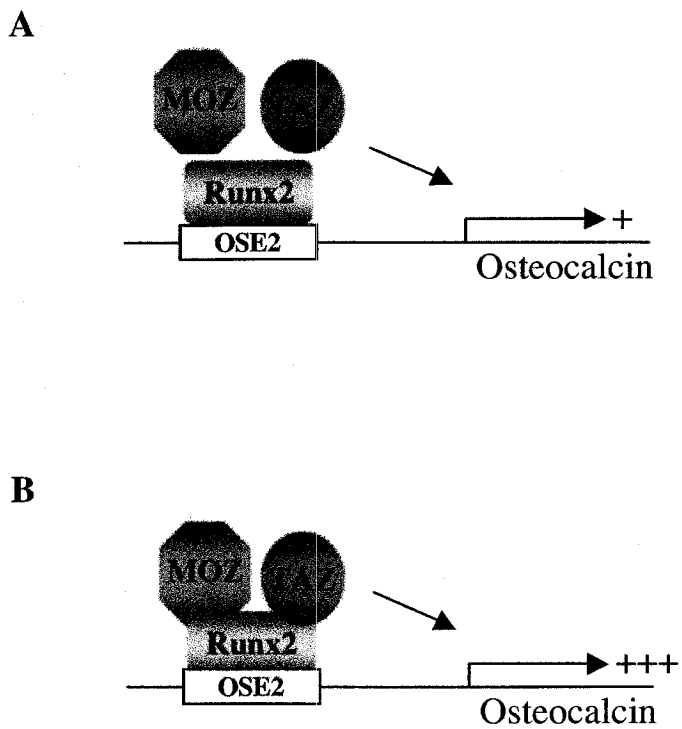


Figure 2. Model for Osteocalcin gene activation.

(A) MOZ, TAZ and Runx2 activate the osteocalcin promoter. The interactions of Runx2 with MOZ and TAZ are weak.

(B) PMA induces the interaction of Runx2 with MOZ and TAZ and stimulates their synergistic activity on the osteocalcin promoter.

5 References

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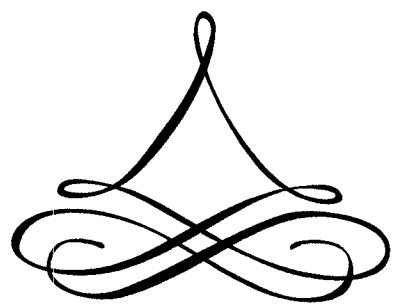
CHAPTER VI

CONTRIBUTION TO ORIGINAL RESEARCH



1. Identification and characterization of MORF, a new member of the MYST family of histone acetyltransferases. Demonstrate that MORF contains histone acetyltransferase activity and possesses several modules characteristic of a transcriptional coregulator. These findings might explain the implication of MORF and MOZ in leukemogenesis.

2. Demonstrated that MOZ and MORF activation domains bind and stimulate Runx2-dependent activation of the osteocalcin gene. This finding suggests a novel role of MOZ and MORF in cell proliferation and differentiation.

3. Demonstrated that MOZ, TAZ and Runx2 synergistically modulate the osteocalcin promoter. The importance of signaling pathways activated by PMA in the regulation of the osteocalcin promoter by MOZ, TAZ and Runx2 is described. This finding leads to the proposal of how transcription factors in conjunction with coactivators regulate osteoblast differentiation via signaling pathways.

From: "Hooker, Sophie (AJ's)" <S.Hooker@nature.com>
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To: s.hooker@nature.com
Subject: signed waivers

Dear Madam Hooker,

Attached is a request for signed waivers.

Sincerely,
Nadine Pelletier