Repression domain of the yeast global repressor Tup1 interacts directly with histones H3 and H4

Diane G. Edmondson,[1] M. Mitchell Smith,[2] and Sharon Y. Roth[1,3]

[1]Department of Biochemistry and Molecular Biology, University of Texas M.D. Anderson Cancer Center (UTMDACC), Houston, Texas 77030 USA; [2]Department of Microbiology, University of Virginia School of Medicine, Charlottesville, Virginia 22908 USA

Repression of yeast *a* cell-specific genes by the global repressor Ssn6/Tup1 has been linked to a specific organization of chromatin. We report here that Tup1 directly interacts with the amino-terminal tails of histones H3 and H4, providing a molecular basis for this connection. This interaction appears to be required for Tup1 function because amino-terminal mutations in H3 and H4 that weaken interactions with Tup1 cause derepression of both *a* cell-specific and DNA damage-inducible genes. Moreover, the Tup1 histone-binding domain coincides with the previously defined Tup1 repression domain. Tup1/histone interactions are negatively influenced by high levels of histone acetylation, suggesting a mechanism whereby the organization of chromatin may be modulated in response to changing environmental signals.

[*Key Words:* Ssn6/Tup1; chromatin; repression; histones; transcription]

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The yeast Ssn6/Tup1 repressor complex provides a unique paradigm for repression of transcription. These complexes repress diverse genes that maintain cell type or respond to changing physiological conditions. For example, Ssn6/Tup1 is required to mediate repression of cell type-specific genes by the α2 repressor (Keleher et al. 1992) and is also required for repression of glucose-repressible genes such as *SUC2* (Carlson et al. 1984; Trumbly 1986; Williams et al. 1991), DNA damage-inducible genes such as *RNR3* (Zhou and Elledge 1992), and genes regulated by oxygen such as *ANB1* (Zitomer and Lowry 1992). Neither Ssn6 nor Tup1 binds directly to DNA, and these factors appear to be targeted to particular promoters through interactions with specific DNA-binding proteins such as α2 (Komachi et al 1994; Smith et al. 1995; Treitel and Carlson 1995; Tzamarias and Struhl 1995). If directed to specific promoters via fusion to a heterologous DNA-binding domain, either Ssn6 or Tup1 can mediate repression (Keleher et al. 1992; Tzamarias and Struhl 1994). Although repression by Ssn6 requires Tup1 (Keleher et al. 1992), Tup1 can repress transcription in the absence of Ssn6 (Tzamarias and Struhl 1994). Tup1, then, may serve as the active repressor subunit of the complex, whereas Ssn6 may mediate or stabilize interactions between Tup1 and various targeting proteins.

Ssn6 (Cyc8) is a large (107-kD) phosphoprotein that contains 10 tandem copies of a tetratricopeptide repeat (TPR) (Schultz and Carlson 1987; Schultz et al. 1990). This domain appears to mediate protein–protein interactions. The first three TPR motifs are required for interaction with Tup1, whereas most or all of the TPRs are capable of interacting with α2 (Smith et al. 1995; Tzamarias and Struhl 1995). Different repeats within the TPR domain are required for repression of different sets of genes. Only the first 3 repeats are required for repression of **a** cell-specific genes, for example, but all 10 repeats are required for repression of DNA damage-inducible genes (Tzamarias and Struhl 1995). The ability to interact with multiple proteins is consistent with the proposal that Ssn6 acts as an adapter between Tup1 and various DNA-binding proteins.

Several functional domains have been identified in Tup1. The carboxy-terminal half of Tup1 contains eight repeats of a 43-amino-acid sequence rich in aspartate and tryptophan (WD-40 repeats) (Fong et al. 1986; Williams and Trumbly 1990; Mukai et al. 1991). This domain also serves as a protein interaction domain, and WD repeats 1 and 2 directly interact with α2 (Komachi et al. 1994). The amino-terminal 72 amino acids of Tup1 are required for interaction with Ssn6 and for multimerization of Tup1 (Tzamarias and Struhl 1995). In addition, two alanine-rich repression domains have been identified in Tup1 using Tup1–lexA fusion proteins (Tzamarias and Struhl 1994).

Despite the detailed characterization of these two proteins, the mechanisms by which Ssn6/Tup1 confer repression are unknown. Given the involvement of Ssn6/Tup1 in the repression of several independently regu-

[3]**Corresponding author.**

lated genes, this complex is likely to interact with some moiety common to multiple promoters. Possible targets include basal transcription factors or components of chromatin. Evidence exists to support both of these possibilities. Ssn6/Tup1 repression of an **a** cell-specific reporter can be reconstituted in vitro, in the absence of chromatin, on addition of recombinant α2 protein and nuclear extracts from cells overexpressing Ssn6 and Tup1 (Herschbach et al. 1994), indicating that Tup1 may act on a component of the basal transcription machinery. However, the modest repression (2- to 4-fold) observed in these experiments is much lower than that observed in vivo (>200-fold), indicating that some factors may be limiting or missing in these nuclear extracts.

Other experiments suggest that Ssn6/Tup1 repression is mediated in vivo through organization of chromatin. Repression of **a** cell-specific genes in haploid α and diploid **a**/α cells is linked to a precise and stable positioning of nucleosomes adjacent to the α2 operator (Roth et al. 1990; Shimizu et al. 1991). In **a** cells, where α2 is not expressed, Ssn6/Tup1 is not targeted to **a** cell-specific promoters and nucleosomes are randomly positioned at these loci. Mutation of either *SSN6* or *TUP1* results in derepression of the **a** cell-specific genes and perturbation of the placement and stability of nucleosomes adjacent to the α2 operator (Cooper et al. 1994). Stable nucleosome positioning and complete repression require not only α2, Ssn6, and Tup1, but also the amino-terminal "tail" of histone H4 (Roth et al. 1992). These factors appear to cooperate to create a repressive region of chromatin. Organized chromatin structures are also observed for other genes regulated by Ssn6/Tup1 (Hirschorn et al. 1992; Matallana et al. 1992).

If Ssn6/Tup1 functions by modulating chromatin structure, these factors might directly interact with nucleosomal proteins. The interaction of the silent information regulators, Sir3 and Sir4, with histones provides a precedent for such interactions (Hecht et al. 1995). We report here that a previously defined repression domain in Tup1 interacts specifically with the amino termini of histones H3 and H4 and that these interactions are influenced by the acetylation state of the histones. Moreover, mutations in the amino-terminal region of H3 or H4 that abolish or weaken interactions with Tup1 compromise Tup1-mediated repression. Our data strongly suggest that Ssn6/Tup1 complexes may directly modulate chromatin structure to repress gene expression.

Results

Tup1 interacts with histones H3 and H4

Current models propose that Ssn6/Tup1 complexes are targeted to specific promoters through interactions with DNA-binding proteins, such as α2, and that once recruited to a promoter, Tup1 initiates repression (Keleher et al. 1992; Tzamarias and Struhl 1994, 1995). Given the link we had observed previously between chromatin structure and Tup1-mediated repression of the **a** cell-specific genes (Shimizu et al. 1991; Roth et al. 1992; Cooper et al. 1994), we investigated whether Tup1 might directly interact with histones.

We first assayed these interactions by a FAR Western blot analysis. Acid-soluble yeast nuclear proteins enriched in histones were separated by SDS-PAGE, electroblotted, and probed with in vitro-translated, ^{35}S-labeled Tup1 (Tup1; Fig. 1A), or an unprogrammed translation reaction ("No RNA;" Fig. 1A). The labeled Tup1 bound to three proteins, but no signal was obtained with the unprogrammed translate. The bands interacting with

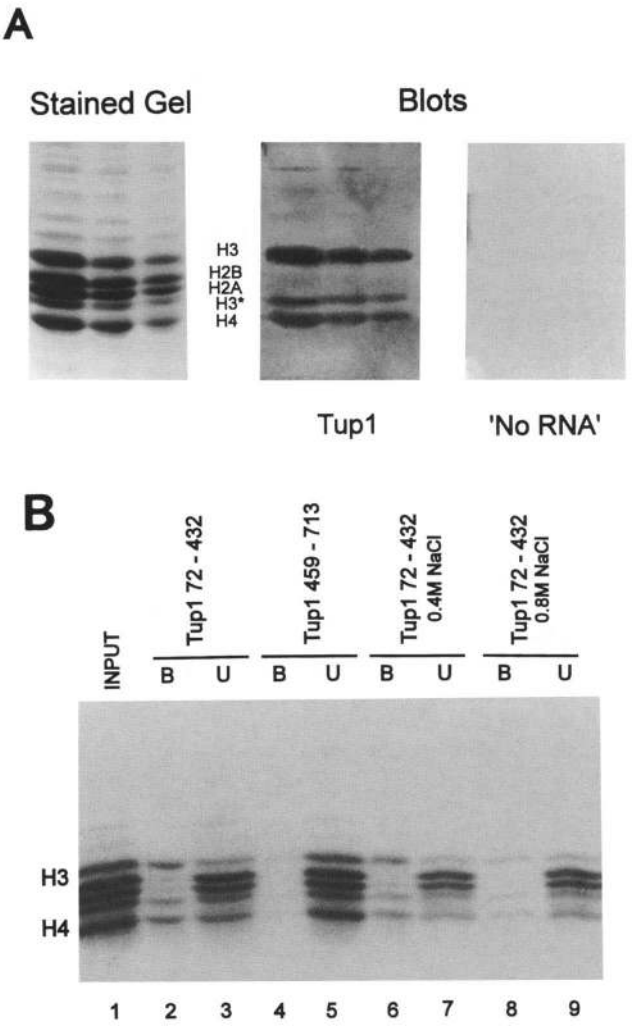

Figure 1. Tup1 interacts with histones H3 and H4. (*A*) FAR Western blot analysis. Samples of yeast histones were separated by SDS-PAGE, electroblotted to PVDF membrane, and probed with an ^{35}S-labeled Tup1 probe (Tup1) or with an unprogrammed reticulocyte lysate "probe" ("No RNA"). Parallel lanes were stained with Coomassie Brilliant blue R (stained gel). The three lanes contain decreasing loads of histone. (*B*) Binding of H3 and H4 to Tup1–Sepharose. Cyanogen bromide-activated Sepharose beads were coupled to purified recombinant $Tup1_{(72-432)}$ or $Tup1_{(459-713)}$ proteins. Yeast histone samples were added to the beads and allowed to bind. Bound (B, lanes *2,4,6,8*) and unbound (U, lanes *3,5,7,9*) fractions were then analyzed by SDS-PAGE and Coomassie blue staining. An aliquot of the input histone protein sample is shown in lane *1*.

Tup1 were identified by mobility and confirmed by Western blot analysis (data not shown) as histones H3, H4, and a spontaneous breakdown product of histone H3 (H3*; Fig. 1A; sometimes called P1*; Bohm et al. 1981; Bortvin and Winston 1996) that is commonly seen in histone preparations from a number of organisms. Varying amounts of this autolysed H3 were observed in individual yeast histone preparations (data not shown, but see panels in Fig. 4A, below, for examples). Importantly, Tup1 did not bind to histones H2A or H2B, indicating that binding to H3 and H4 is unlikely to be mediated by nonspecific charge interactions, because H2A and H2B are also highly positively charged. Also, no interactions were observed between Tup1 and other small, highly basic molecules such as lysozyme (data not shown).

To confirm interaction of Tup1 with histones H3 and H4, we investigated the ability of free histones to bind to Tup1 immobilized on Sepharose beads. We were unable to express full-length Tup1 in bacteria but were able to express two partial Tup1 products: Tup1/amino acids 72–432 and Tup1/amino acids 459–713. Purified proteins were coupled to Sepharose beads and incubated with yeast nuclear proteins (as above), and then the bound and unbound fractions were analyzed by SDS-PAGE. Histones H3 and H4 bound to the $Tup1_{(72-432)}$-coupled beads (Fig. 1B, lane 2), whereas histones H2A and H2B remained in the unbound fraction (Fig. 1B, lane 3). None of the core histones bound to the $Tup1_{(459-713)}$-coupled beads (Fig. 1B, lane 4). Interestingly, the $Tup1_{(72-432)}$ protein (which binds histones H3 and H4) contains the putative repression domain of Tup1, whereas $Tup1_{(459-713)}$ contains the WD repeats of Tup1 (see Fig. 4, below).

We then examined the stability of the Tup1–histone complex under conditions of high ionic strength. Histones bound to $Tup1_{(72-432)}$-coupled beads were washed first with binding buffer and then with buffer containing 0.4 M or 0.8 M NaCl. Even in the presence of 0.8 M NaCl, significant amounts of histones H3 and H4 remained bound to the $Tup1_{(72-432)}$-coupled beads (Fig. 1B, lane 8). Histones were not precipitated nonspecifically by high salt because H3 and H4 are not found in the bound fraction when $Tup1_{(459-713)}$-coupled beads are mixed with histones in high salt (data not shown). The persistence of Tup1/histone interaction under conditions of high salt suggests that, once formed, the interaction is fairly insensitive to ionic interactions.

Tup1 interacts with underacetylated forms of histones H3 and H4

Interestingly, only a fraction of the total H3 and H4 bound to the $Tup1_{(72-432)}$-beads, suggesting that Tup1 may only bind to specifically modified isoforms of these histones. Acetylation is a common modification of histones and has been correlated with transcriptional regulation. In yeast, genes that are silenced are associated with hypoacetylated forms of H4 (Braunstein et al. 1993), and a similar association has been observed in both *Drosophila* and mammals (Turner et al. 1990, 1992; Jeppesen and Turner 1993; Bone et al. 1994; for review, see Turner 1991). To investigate whether interactions between Tup1 and H3 or H4 might be influenced by levels of histone acetylation, we performed a FAR Western blot analysis on yeast histone isoforms resolved by acid–urea electrophoresis, again using in vitro-translated Tup1 as probe.

Five isoforms of H4 are distinguished by acid–urea electrophoresis (Fig. 2A), and these bands represent different levels of H4 acetylation (Allis et al. 1980). The identity of these bands was confirmed using an antibody that recognizes multiply acetylated forms of H4 (Lin et al. 1989), as well as acetylated H2A in yeast (Fig. 2C). Multiple isoforms of H3 are also apparent in the stained acid–urea gel, and at least three acetylated isoforms of

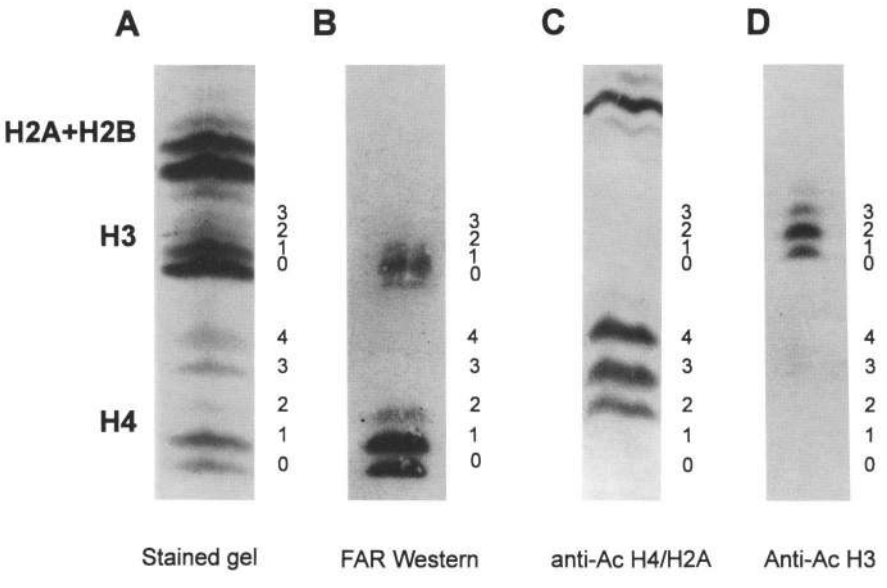

Figure 2. Analysis of Tup1 binding to acetylated isoforms of H3 and H4. Yeast histones were resolved by acid–urea gel electrophoresis. The positions of differentially acetylated isoforms are indicated by numbers to the *right* of each lane (0 = unacetylated, 1 = monoacetylated, 2 = diacetylated, 3 = triacetylated, 4 = tetra-acetylated). (*A*) Coomassie blue stained gel. (*B*) FAR Western blot using radiolabeled Tup1 probe. (*C*) Western blot using anti-acetylated H4/H2A antibodies. (*D*) Western blot using anti-acetylated H3 antibodies. Note the absence of Tup1 binding to tri- and tetra-acetylated forms of H3 and H4.

H3 are detected in this histone preparation (Fig. 2D) by an antibody directed against acetylated H3.

Tup1 interacts solely with the unacetylated, monoacetylated, and diacetylated forms of histone H4 and preferentially binds monoacetylated H3 (Fig. 2B). No Tup1 binding was observed to the more highly (tri- and tetra-) acetylated forms of either histone. The binding of Tup1 to underacetylated histones is consistent with the long-standing correlation between histone deacetylation and transcriptional repression (Turner 1991) and suggests a mechanism whereby interactions between Tup1 and the histones might be modulated easily.

The amino termini of histones H3 and H4 are required for interaction with Tup1

Acetylation of H3 and H4 occurs at highly conserved lysine residues in the amino-terminal regions of these histones (Turner 1991). Because binding of Tup1 to these histones is influenced by acetylation, these interactions are likely to involve these amino-terminal regions. Moreover, we have shown previously that deletions or point mutations in the amino-terminal region of H4 affect both nucleosome positioning and repression of the **a** cell-specific genes (Roth et al. 1992), further indicating that this histone domain is important to Tup1-mediated repression.

To determine whether the amino-terminal region of H4 is required for a direct interaction with Tup1, we prepared histones from the strains of yeast used in the aforementioned regulatory studies. These isogenic strains contain either wild-type H4 (Fig. 3A, lanes 2,3), a deletion of amino acids 4–19 [del(4–19); lane 1], or a deletion of amino acids 4–14 [del(4–14); lane 4] (Kayne et al. 1988). The FAR Western blot analysis revealed that whereas Tup1 bound quite well to H3 in all three histone preparations, it bound well only to H4 isolated from wild-type cells (Fig. 3A, lanes 2′, 3′). Deletion of amino acids 4–14 weakened Tup1 binding (lane 4′) by 50%–60% (relative to wild-type H4) as indicated by densitometric scanning of the H4 signal in the stained gels and corresponding autoradiograms shown in Figure 3 (see Materials and methods section). Importantly, deletion of amino acids 4–19 in H4 abolished interaction of this his-

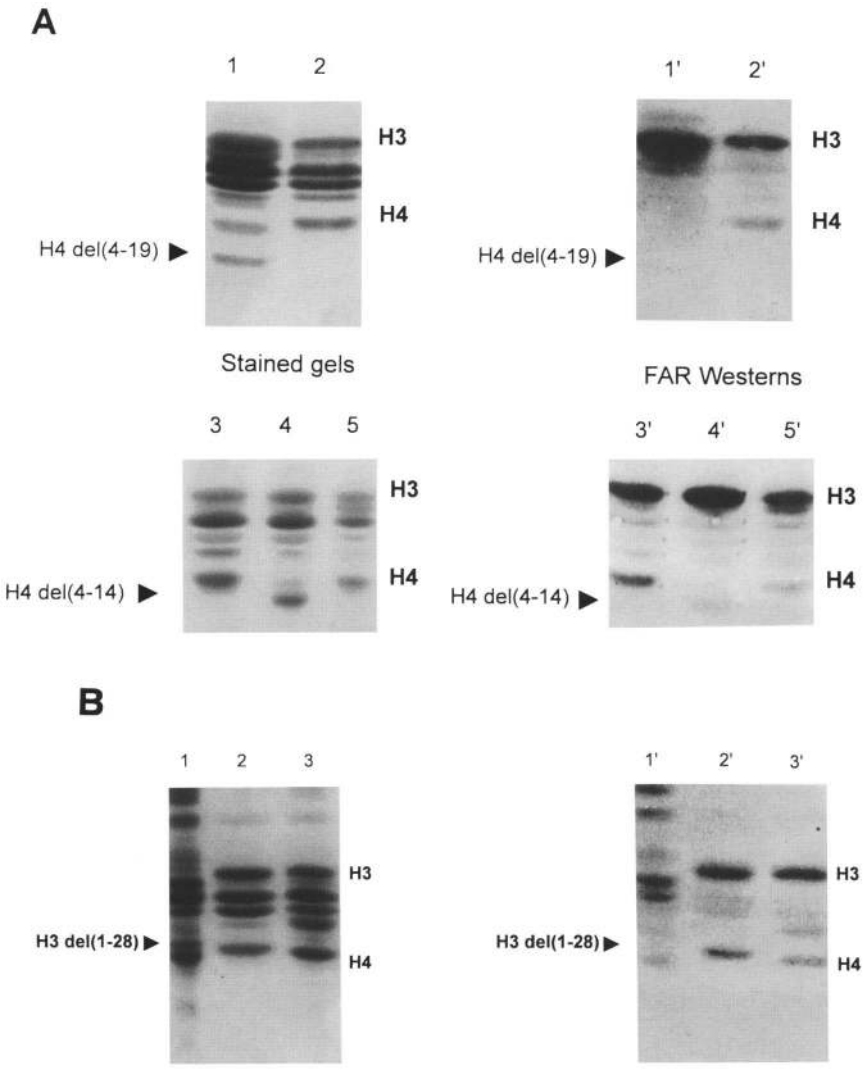

Figure 3. Tup1 interacts with the amino termini of histones H3 and H4. (*A*) Histone samples, prepared from isogenic strains carrying wild-type histones (lanes *2,2′*, *3,3′*), deletion of amino acids 4–19 in H4 (lanes *1,1′*), deletion of amino acids 4–14 in H4 (lanes *4,4′*), or a point mutation in H4 converting lysine16 to glycine (lanes *5,5′*) were analyzed by Coomassie staining (*left*) or by FAR Western blot (*right*) using a Tup1 probe. Arrowheads denote positions of the H4 del(4–19) and H4 del(4–14) gene products. (*B*) Histone samples were prepared from isogenic strains carrying either wild-type H3 and H4 (lanes *2,2′*), wild-type H3 and mutated H4 carrying substitutions of glutamine for the lysines normally present at residues 12 and 16 [H4mut-(K12Q,K16Q)] (lanes *3,3′*), or H3 deleted for amino acids 1–28 together with the H4 (K12Q,K16Q) mutation (lanes *1,1′*). Parallel lanes were analyzed by Coomassie blue staining (*left*, lanes *1–3*) or by FAR Western blot (*right*, lanes *1′–3′*) using a Tup1 probe. Position of H3 del(1–28) is indicated by the arrowhead.

tone with Tup1 (Fig. 3A, lane 1′). H4 del(4–19) also failed to bind Tup1–Sepharose columns, whereas wild-type H3 in the same histone preparation bound well (data not shown). Together, these results suggest that amino acids 15–19 in H4 are critical for Tup1/H4 interactions, consistent with our previous finding that repression of **a** cell-specific genes is perturbed in the presence of H4 del(4–19) (Roth et al. 1992).

We also examined the effects of specific point mutations in H4 on Tup1/H4 interactions. Histones were isolated from isogenic yeast strains bearing either wild-type H4 (Fig. 3A, lane 3) or a substitution of glycine for the lysine residue normally present at position 16 (K16G) (Fig. 3A, lane 5). In a separate experiment, we examined a double point mutation in H4 that substitutes glutamine for lysines 12 and 16 (K12Q,K16Q) (Megee et al. 1995) (Fig. 3B, lane 3). This double point mutation alters the mobility of H4 in SDS gels such that it migrates slightly faster than wild-type H4 (Fig. 3B, cf. lanes 2 and 3). Again, in a Far Western blot analysis, Tup1 bound to wild-type H3 and wild-type H4 (Fig. 3A, lane 3′; Fig. 3B, lane 2′), but both H4 mutations weakened Tup1 binding by 50%–60% (Fig. 3A, lane 5′; Fig. 3B, lane 3′).

We then investigated whether Tup1 interacts with the amino-terminal region of histone H3. Histones were isolated from a strain containing a deletion of the first 28 amino acids in H3 del(1–28) (Morgan et al. 1991) together with the H4 double point mutation (K12Q,K16Q) described above. This form of H3 mimics an H3 peptide produced upon digestion of nucleosome core particles with trypsin, and hence this fragment migrates just above H4 in SDS gels (Fig. 3B, arrowhead, lane 1) (Bohm et al. 1981). No binding of Tup1 to this truncated form of H3 was observed (Fig. 3B, arrowhead, lane 1′), even though higher levels of nonspecific binding to other proteins occurred in this experiment. H3 del(1–28) also failed to bind Tup1–Sepharose columns (data not shown). Taken together, these data indicate that the amino termini of both H3 and H4 are critical for mediating interaction with Tup1.

Histone mutations compromise Tup1 function

If interaction with H3 and H4 is important to Tup1 function, loss of repression should accompany the loss of Tup1/histone interactions. As described above, H4 del(4–19), which abolished interaction with Tup1, was shown previously to cause a partial derepression of **a** cell-specific genes (Roth et al. 1992). Interestingly, whereas both H4 del(4–14) and H4 (K16G) exhibited weakened interactions with Tup1, derepression was only observed in the presence of the H4 (K16G) mutation in previous studies. Perhaps the strong interaction of Tup1 with H3 in these strains can suppress the effects of decreased interaction with H4 del(4–14) but cannot completely compensate for the weakened interaction with H4 (K16G), consistent with the idea that amino acids 15–19 in H4 are critically important for Tup1 binding and function.

To further test the role of histones in Tup1-mediated repression, we examined repression in strains containing the H4 double point mutation (K12Q,K16Q), this H4 mutation in combination with the H3 del(1–28) mutation, and the H3 del(1–28) mutation alone. The expression of reporter genes responsive to two separate pathways of Tup1-mediated repression, an **a** cell-specific reporter (α2op–CYC1–LacZ) and a DNA damage-inducible reporter (RNR2–LacZ), was monitored in the presence of the histone mutations or in isogenic wild-type cells.

As expected, both reporter genes are repressed in the strain containing wild-type H4 and wild-type H3 (MSY590 and MSY890; Table 1). A modest (three- to four-fold) derepression of the α2op–CYC1–LacZ construct is observed in the strain containing the H4 double point mutation alone (MSY612). We also observed derepression (sixfold) of the α2op–CYC1–LacZ reporter in a strain containing H3 del(1–28) and wild-type H4 (MSY892; Table 1, cf. to MSY 890). Interestingly, elevated expression of an uninduced GAL1 (glucose-repressible) promoter has also been observed in the presence of H3 amino-terminal deletions (Mann and Grunstein 1992). An even greater derepression of the α2op–CYC1–LacZ reporter is observed when the H4 mutation is combined with the H3 del(1–28) truncation (MSY577; Table 1). β-Galactosidase expression is increased 13-fold relative to expression in the wild-type strain. This increase is not observed with a reporter plasmid that is not subject to Tup1 repression (CYC1–LacZ). The expression of the CYC1–LacZ reporter is decreased in the presence of the histone mutations, indicating H3 and H4 may be important to the activation of this promoter, as has been reported for other genes (Mann and Grunstein 1992; Fisher-Adams and Grunstein 1995). This decrease may also reflect a previously reported requirement of Tup1 function for the activation of the CYC1 promoter (Zhang and Guarente 1994). Significantly, β-galactosidase expression from the α2op–CYC1–LacZ reporter in the presence of the combined H3 and H4 mutations reaches almost 20% of the fully nonrepressed level of the CYC1–LacZ construct in these cells.

Repression of the DNA damage-inducible reporter RNR2–LacZ is also compromised by histone mutation (Table 1B). Again, modest derepression (threefold) is observed in the presence of H3 del(1–28) alone. A greater derepression (ninefold) is observed when this H3 mutation is combined with the H4 (K12Q,K16Q) mutation, even though this H4 mutation alone has no effect on the repression of RNR2–LacZ. The greater effect of the coupled mutations on both the **a** cell-specific and DNA damage-inducible reporter genes is consistent with the combined loss of interaction between Tup1 and H3 del(1–28) and weakened interaction between Tup1 and H4 (K12Q,K16Q) observed above. These results may also indicate a redundancy of H3 and H4 functions in Tup1-mediated repression. We are currently analyzing several combinations of H3 and H4 mutations to further test this idea (W. Zhang and S. Roth, unpubl.). Importantly, the derepression of at least two separate classes of genes regulated by Tup1 in the presence of the histone muta-

Table 1. *Histone mutations compromise Tup1 repression*

A. **a** *cell-specific reporter*

Strain	H3	H4	α2op–CYC1–LacZ	Fold derepression	CYC1–LacZ
MSY590	wild type	wild type	7 ± 3	1	3533 ± 1126
MSY612	wild type	K12Q,K16Q	24 ± 1	3	2216 ± 742
MSY577	del(1–28)	K12Q,K16Q	94 ± 57	13	544 ± 158
MSY890	wild type	wild type	16 ± 5	1	N.D.
MSY892	del(1–28)	wild type	90 ± 22	6	N.D.

B. DNA damage-inducible reporter

Strain	H3	H4	RNR2–LacZ	Fold derepression
MSY590	wild type	wild type	16 ± 14	1
MSY612	wild type	K12Q,K16Q	9 ± 10	<1
MSY577	del(1–28)	K12Q,K12Q	143 ± 28	9
MSY890	wild type	wild type	57 ± 15	1
MSY892	del(1–28)	wild type	187 ± 75	3

β-Galactosidase activities (expressed as nmoles/min per mg protein) were determined in cell extracts using ONPG as substrate as described in Materials and methods. Values represent the average of at least two independent transformants and at least three independent assays (in duplicate) of each transformant. Standard deviations (±) are as shown. All strains are isogenic. MSY890 and MSY892 carry H3 and H4 genes on a plasmid, whereas these genes are integrated in all other strains.

tions reported here indicates that H3 and H4 play a central role in Tup1-mediated repression.

The Tup1 repression domain is required for interaction with histones H3 and H4

Deletion constructs of *TUP1* (Fig. 4B) were generated to identify domains that interact with H3 and H4. These constructs were translated in vitro and used to probe strips of electroblotted histones (Fig. 4A; summarized in Fig. 4C).

We first examined amino-terminal fragments of Tup1 for histone-binding activity. A fragment containing amino acids 1–72 exhibited minimal histone binding. Increased binding was observed with a fragment containing amino acids 1–120 (27%), and 82%–94% of full-length binding was observed with longer fragments containing amino acids 1–253 or 1–508. Strong binding was also observed with longer Tup1 fragments containing amino acids 1–594 and 1–674 (data not shown). These results indicate the histone-binding domain is located between amino acids 72 and 508.

We then examined carboxy-terminal fragments of Tup1 to determine the carboxy-terminal border of the histone-binding domain. A fragment containing amino acids 316–713 exhibited significant histone binding (32% of full length). In agreement with the data presented in Figure 1B, fragments containing amino acids 386–713 or 432–713 exhibited minimal histone-binding activity. These results suggest that the carboxy-terminal boundary of the histone-binding domain extends to amino acid 385, yet the above studies indicated little difference in the binding of Tup1 peptides containing amino acids 1–253 and 1–508. Therefore, we confirmed the histone-binding properties of amino acids carboxy-terminal to residue 253 using a bacterially expressed Tup1 peptide containing amino acids 252–673. Sepharose beads were coupled to the purified protein and histone binding was assayed as described in Figure 1B. As a control, Sepharose beads were subjected to the same coupling reaction conditions in the absence of added Tup1 protein (mock; Fig. 4D). Histones H3 and H4 bound to the $TUP1_{(252–673)}$-coupled beads (Fig. 4D, lane 2), whereas histones H2A and H2B were found completely in the unbound fraction (Fig. 4D, lane 3; also, cf. to Fig. 1B, lanes 4,5). None of the core histones bound to the mock beads (Fig. 4D, lane 4). As with histones bound to $Tup1_{(72–432)}$-coupled beads (Fig. 1B), H3 and H4 bound to $TUP1_{(252–673)}$-coupled beads were resistant to elution in up to 0.8 M NaCl (data not shown), indicating that these interactions are relatively insensitive to ionic conditions. Thus, we conclude that amino acids carboxy-terminal to residue 253 have significant histone-binding activity.

Taken together, our data (Fig. 1B, Fig. 4) indicate that a region of Tup1 spanning amino acids 73–385 interacts with histones H3 and H4 and that the strongest histone-binding domain is located between amino acids 121 and 316. Previously, repression domains were defined in Tup1 between amino acids 72 and 389 (Tzamarias and Struhl 1994). This region mediates all repressive functions of Ssn6/Tup1 (Tzamarias and Struhl 1994, 1995) and substantially overlaps the histone-binding domain defined here (Fig. 5), strongly suggesting that Tup1/histone interactions are directly required for Tup1-mediated repression.

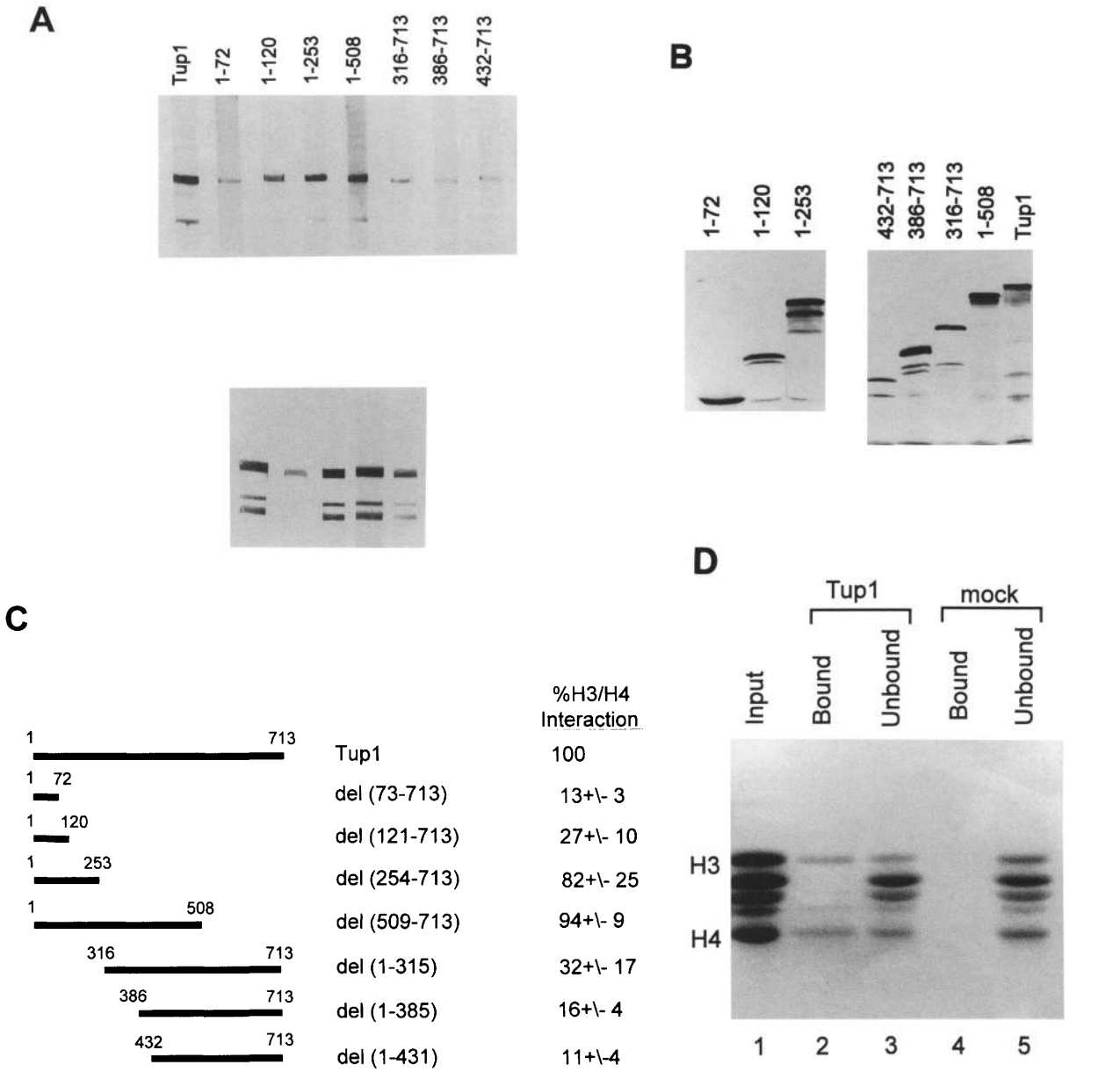

Figure 4. The repression domain of Tup1 interacts with histones H3 and H4. (*A*) Representative Far Western blot analysis of the ability of various Tup1 deletion mutants to bind to H3 and H4. Strips of preparative histone gels were probed with individual Tup1 derivatives as indicated at *top*. Two independent experiments are shown, using different histone preparations and independently translated probes. (*B*) In vitro-translated probes (as indicated at *top*) displayed by SDS-PAGE. (*Right*) An autoradiograph of a 10% gel; (*left*) the autoradiograph of a 22% gel. (*C*) Summary of the Tup1 deletion mutants analyzed, and their ability to interact with H3 and H4. The structures of Tup1 and Tup1 deletion derivatives are indicated schematically. Numbers correspond to the amino acids retained in each, and the center column denotes amino acids that are deleted. The *right* hand column indicates each derivative's ability to interact with H3 and H4 relative to intact Tup1. Histone binding was quantitated as described in the Materials and methods. (*D*) Binding of H3 and H4 to $Tup1_{(252–673)}$–Sepharose. Cyanogen bromide-activated Sepharose beads were coupled to purified recombinant $Tup1_{(252–673)}$ protein or were "mock" coupled in the absence of added protein. Yeast histone samples were added to the beads and allowed to bind. Bound and unbound fractions were then analyzed by SDS-PAGE and Coomassie blue staining. (Lane *1*) Input histone protein sample; (lane *2*), fraction bound to $Tup1_{(252–673)}$–Sepharose; (lane *3*) fraction that did not bind to $Tup1_{(252–673)}$–Sepharose; (lane *4*) fraction bound to mock-coupled Sepharose; (lane *5*) fraction that did not bind to mock-coupled Sepharose.

Discussion

Chromatin represses transcription in vivo and in vitro, but often this repression is nonspecific, affecting the basal transcription of most genes, or is associated with specialized heterochromatic structures important for silencing (for review, see Lewin 1994). Our data indicate that chromatin may also be actively recruited by repres-

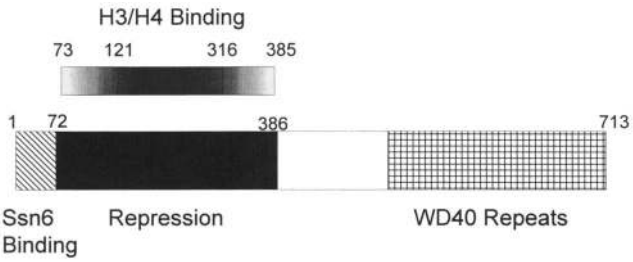

Figure 5. Functional domains of Tup1. The locations of the WD40 repeats, the Ssn6 interaction domain, and the previously defined Tup1 repression domain (Komachi et al. 1994; Tzamarias and Struhl 1994, 1995) are shown schematically. The H3/H4 binding domain defined by this work is depicted above Tup1, showing the correspondence between the histone-binding domain and the repression domain.

sor proteins such as Tup1 to establish or maintain repression of specific, euchromatic genes.

Mechanism of repression by Ssn6/Tup1

Several independent lines of evidence suggest that repression by Ssn6/Tup1 is mediated through the organization of chromatin. First, we have demonstrated previously that positioned nucleosomes are linked to the repression of the **a** cell-specific genes (for review, see Roth 1995). Tup1 and Ssn6 are targeted to these promoters via interactions with the cell type-specific repressor α2 (Komachi et al. 1994; Smith et al. 1995), and disruption of either *SSN6* or *TUP1* leads to loss of positioning as well as loss of repression (Cooper et al. 1994). Organized nucleosomes have also been observed in the promoter region of the glucose-repressible gene *SUC2*, and this structure is again perturbed by mutations in *SSN6* or *TUP1* (Matallana 1992). Second, Tup1 directly interacts with the amino termini of histones H3 and H4, and mutations in these histones that disrupt Tup1/histone interactions cause a partial derepression of both **a** cell-specific genes and DNA damage-inducible genes. Finally, the histone-binding domain of Tup1 overlaps previously defined repression domains (Tzamarias and Struhl 1994, 1995), further suggesting these two Tup1 functions are linked physically.

Although these data strongly indicate that chromatin is important to repression by Tup1, other factors may also contribute. Recently, a *CDC28* related kinase [*ARE1* (*SRB10*)] that is part of the yeast RNA polymerase II holoenzyme was identified genetically as a factor required for repression of the **a** cell-specific genes (Kuchin et al. 1995; Wahi and Johnson 1995), suggesting that Tup1 might interact directly with general transcription factors. However, many kinases have multiple targets in the cell, and phosphorylation of a factor, perhaps even Ssn6 or Tup1, may be required to stabilize interactions between Tup1 and neighboring nucleosomes. Alternately, Tup1 might interact with general transcription factors to initiate repression and subsequently organize chromatin to maintain the repressed state.

The Tup1 histone-binding domain

The domain in Tup1 that exhibits strongest binding to histones H3 and H4 (amino acids 121–385) contains few charged residues. The absence of an acidic region in the histone-binding domain and the stability of Tup1–H3/H4 interactions in high salt argues against a nonspecific electrostatic association between these proteins, as does the lack of Tup1 interaction with other highly charged proteins such as H2A, H2B, and lysozyme. The Tup1 histone-binding domain is rich in proline, serine, and alanine (Williams and Trumbly 1990). Remarkably, 27 of the 35 proline residues in Tup1 are located in this interval. A stretch rich in glutamine is also found in the strong histone-binding domain (amino acids 181–198), but a longer glutamine repeat is found upstream (amino acids 97–118) in a region that binds less well to histones. At present, it is unclear what features of the histone-binding domain are important for interaction with H3 and H4. No obvious homologies between this region and histone-binding domains defined in other proteins, such as N1 (Kleinschmidt and Seiter 1988) or Sir3 (Hecht et al. 1995), have been detected.

The ability of Tup1 to interact with the spontaneously generated fragment of histone H3 (H3*; Fig. 1A) present in some of our histone preparations but not the genetically engineered H3 amino-terminal deletion suggests that a very specific region within the H3 amino terminus is required for interaction with Tup1. The "autolytic" H3 fragment has been identified as a peptide containing amino acids 21–135 or 23–135 (Bohm et al. 1981; Bortvin and Winston 1996), whereas the genetically engineered truncation only contains amino acids 29–135 (Morgan et al. 1991). Amino acids 21–28 of histone H3, therefore, may be critical for the Tup1/H3 interaction. Interestingly, this domain contains two potential sites of acetylation, as well as a potential phosphorylation site.

Mechanism of nucleosome positioning

Our data suggest a molecular model for positioning of nucleosomes adjacent to the α2 operator (Fig. 6). Recruitment of Ssn6/Tup1 by α2 most likely establishes the position of the first nucleosome through Tup1/histone interactions. Multimerization of Tup1 or of Ssn6/Tup1 complexes might then provide a template for the propagation of positioned nucleosomes farther downstream. Ssn6 has been proposed to interact with Tup1 multimers (Tzamarias and Struhl 1995) and could conceivably serve as a framework for the stabilization of Tup1 polymers that in turn could propagate nucleosome positioning. Such a framework would help to explain the extent of the positioned arrays observed adjacent to the α2 operator (>3 kb at *STE6*; Simpson et al. 1993). Loss of Ssn6, then, would be predicted to destabilize positioning, whereas loss of Tup1 would be predicted to cause more drastic alterations in nucleosome locations. These predictions are consistent with changes observed in the chromatin structure of *STE6* in the absence of Ssn6 or Tup1 (Cooper et al. 1994).

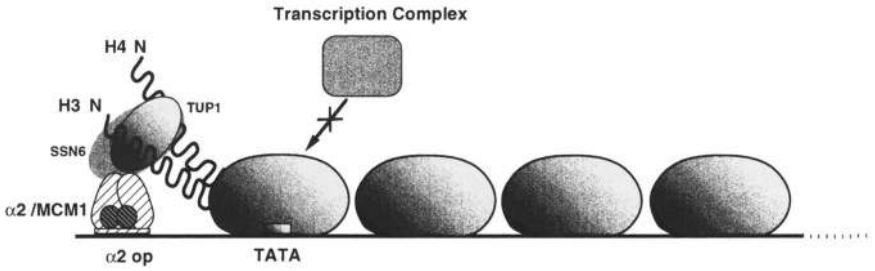

Figure 6. Model for Tup1-mediated nucleosome positioning. The Ssn6/Tup1 repressor complex is recruited to promoters of **a** cell-specific genes via direct interaction with α2. Tup1 then serves as a "bridge" between α2/MCM1 and the neighboring nucleosome through interactions with the amino termini of H3 and H4, nucleating an array of positioned nucleosomes adjacent to the α2/MCM1 binding site. Ssn6/Tup1 multimers may also play a role in the propagation of the positioned nucleosomes downstream (see text). The positioned nucleosome array contributes to repression by blocking access to transcription factors.

Different targets, different structures?

Ssn6/Tup1 complexes are targeted to different promoters by specific DNA-binding proteins, and it is clear that different regions of Ssn6 and/or Tup1 are required for repression of different sets of genes (Tzarmarias and Struhl 1995). The combinatorial nature of this repression may be somewhat analogous to the combinatorial action of some transcriptional activators. In both cases, different partners are utilized to direct the regulation of particular promoters. It remains to be seen whether the modularity of Ssn6/Tup1 repression is reflected in a range of different chromatin structures organized by these factors. For example, the positioning of nucleosomes at the **a** cell-specific genes might be established through the recruitment of two Ssn6/Tup1 complexes by the homodimer of α2 bound to the α2 operator. Alternatively, one molecule of α2 may interact with Ssn6, whereas the other interacts with Tup1, increasing the affinity of Ssn6/Tup1 binding at **a** cell-specific promoters and leading to the highly organized chromatin structures associated with these genes. Interactions between Ssn6/Tup1 and other DNA-binding factors may occur with different affinities and/or stoichiometries, leading to less stable or more accessible structures associated with inducible genes. Moreover, the binding of Tup1 to less highly acetylated isoforms of H3 and H4 suggests a pathway by which chromatin-induced repression might be reversed in response to changing cellular needs. Further characterization of the interactions between Ssn6/Tup1 and various DNA-binding factors and further examination of the chromatin structures of genes regulated by these factors will determine the validity of these speculations.

Relevance to other repressors

H3 and H4 participate in silencing at telomeres and the silent mating loci (Kayne et al. 1988; Thompson et al. 1994) through interactions with two regulatory proteins, Sir3 and Sir4 (Johnson et al. 1990; Hecht et al. 1995). Like Ssn6/Tup1, these proteins are targeted to specific sites via interactions with DNA-binding proteins (for review, see Roth 1995). Sir3/Sir4 complexes then cooperate with other silencing factors to nucleate a silenced chromatin domain. Recruitment of specialized chromatin "organizers" to specific promoters may be a common mechanism of transcriptional repression.

The histones and, in particular, the amino termini of histones H3 and H4 are highly conserved between species, raising the possibility that interactions between transcriptional repressors and histones might also occur in other systems. The *Polycomb* group genes (PcG) in *Drosophila* provide intriguing candidates for this type of regulation. (for review, see Orlando and Paro 1995). These genes repress homeotic genes and other developmental regulatory factors to aid in the establishment of body pattern and segmentation. A mammalian homolog, *Bmi-1*, has been identified, and *bmi-1*-deficient mice suffer homeotic transformations similar to those caused by PcG mutations in flies (Van der Lugt et al. 1994). Like Ssn6/Tup1, the PcG genes form a complex that may be targeted to genes through interactions with specific DNA-binding proteins such as *hunchback*. Repression of *Ultrabithorax* by *hunchback* has been proposed to involve a specialized chromatin structure established by PcG complexes (Bienz 1992). Moreover, the PcG protein *extra sex combs* contains multiple WD repeats (Sathe and Harte 1995). It will be interesting to determine whether these complexes also interact with nucleosomes via specific interactions with histones H3 and H4.

Interactions between Ssn6/Tup1 and the histones furnish a physical and functional link between transcriptional regulators and chromatin. Post-translational modifications such as acetylation and phosphorylation may be used to fine tune this regulation and make it responsive to environmental or developmental cues. These global regulators provide important clues as to how chromatin may be remodeled to reprogram gene expression.

Materials and methods

Yeast strains

Saccharomyes cerevisiae strains were propagated according to standard procedures (Rose et al. 1990) in either rich media (YEPD) or selective media (SC lacking the appropriate amino

Table 2. *Strain list*

Strain	Genotype	Reference
PKY999	*MATα, ade2-101, arg4-1, his3–Δ200, leu2-3, leu2,112, lys2-801, trpΔ901, ura3-52, thr, tyr, Δhhf1:HIS3, Δhhf2::LEU2,* pUK499 (*HHF2:URA3*)	Kayne et al. (1988)
PK917	isogenic to PKY999 except pPK917[*hhf2* del(4–14)] instead of pUK499	Kayne et al. (1988)
PKY918	isogenic to PKY999 except pPK918[*hhf2* del(4–19):*URA3*] instead of pUK499	Kayne et al. (1988)
PKY505	isogenic to PKY999 except *MAT***a** and pPK305[*hhf2* Gly-16 *URA3*] instead of pUK499	Johnson et al. (1990)
MSY590	MATα, ura3-52, lys2-Δ201, leu2-3, HHT1, HHF1Δ(*hht2–hhf2*)	Megee et al. (1995)
MSY577[a]	isogenic to MSY590 except *Mat***a,** *hht1-2* del(1–28), *hhf1-32*(H4 K12Q,K16Q) instead of *HHT1, HHF1*	P. Megere and M. Smith
MSY612[a]	isogenic to MSY590 except *MAT***a,** *hhf1-32* instead of *HHF1*	Megee et al. (1995)
MSY890	*MATα, ura3-52, leu2-3,-112 lys2Δ201* Δ(*hht1 hhf1*) Δ(*hht2 hhf2*) pMS337[*CEN ARS LEU2 hht1-1 HHF1*]	Morgan et al. (1991)
MSY892	isogenic to MSY890 except pMS358[*CEN ARS LEU2 hht1-2 HHF1*] instead of pMS337	Morgan et al. (1991)
YPH49	MAT**a**/α *ura3-52/ura3-52, ade2-101/ade2-101, lys2-801/lys2-801, trp1Δ1/trp1Δ1*	Rose et al. (1990)

[a]The silent mating loci are derepressed in these strains by the H4 mutation, providing α2 expression.

acids). Strains used are presented in Table 2. Yeast were transformed with various plasmids according to Hill et al. (1991).

Plasmids

Tup1 constructs

A *Pst*I–*Hin*dIII fragment of the Tup1 clone pFW28 (Williams and Trumbly 1990) was excised and cloned into the *Pst*I–*Hin*dIII sites of pBluescript II (Stratagene). An oligomer corresponding to nucleotides 478–492 of Tup1 with *Nco*I and *Xho*I sites on the 5′ end and the KS primer (Stratagene) were used to PCR a full-length copy of Tup1, which was then cloned into the *Nco*I and *Sal*I site of pCITE2 (Novagen). Tup1 truncations were made as follows: Tup1–72, Tup1–119, Tup1–253, Tup1–506, Tup1–594, and Tup1–674 were made by cloning the *Nco*I–*Mlu*I, the *Nco*I–*Nhe*I, the *Nco*I–*Bam*H1, the *Nco*I–*Bst*EII, the *Nco*I–*Bgl*II, and the *Nco*I–*Eco*RI fragments from pCite/Tup1 into the *Nco*I–*Bgl*II sites of pCITE2 (the 3′ junction between the Tup1 deletion and pCITE was blunted when the fragments did not have complementary ends). The remaining Tup1 deletion clones were generated by PCR with oligomers corresponding to the appropriate Tup1 sequences and engineered to contain restriction sites for cloning and were cloned into either the *Nco*I–*Sal*I sites or the *Nco*I–*Bam*H1 sites of pCITE2.

For bacterial expression of Tup1, three clones were constructed by cloning Tup1 fragments into the pRset vector (Invitrogen). A *Bam*HI–*Eco*RI fragment of Tup1 was used to construct $Tup1_{(252–673)}$, whereas DNA fragments corresponding to $Tup1_{(72–432)}$ and $Tup1_{(459–713)}$ were generated by PCR with oligomers containing the appropriate Tup1 sequences. The fusion proteins produced by these vectors have a "histidine" tag fused to the amino terminus.

β-Galactosidase reporter constructs

The RNR2–LacZ (pNN405) construct was generously provided by S. Elledge (Elledge and Davis 1989). The CYC1–LacZ construct was a generous gift of D. Stillman (University of Utah, Salt Lake City) (plasmid pM629). A double-stranded oligonucleotide corresponding to the α2 operator present in the STE6 promoter was cloned into a unique *Sal*I site in M629, such that the operator was inserted between the UAS and TATA sequences in the CYC1 promoter (plasmid $pM629_{\alpha 2op}$).

Histone purification

Histones were isolated from YPH49 or other strains in Table 2 by a combination of procedures described by Braunstein et al. (1993) and Davie et al. (1981). One or two liters of yeast were grown to a density of $\sim 2\times10^8$ cells/ml and centrifuged in a Beckman JA-10 rotor for 5 min at 5000 rpm at 4°C. The cell pellet was washed once in sterile water, then resuspended in 50 ml of 0.1 mM Tris (pH 9.4), 10 mM DTT and incubated for 15 min at 30°C with gentle shaking. The cells were centrifuged as above, washed in 100 ml of 1.2 M Sorbitol, 20 mM HEPES (pH 7.4), respun, then resuspended in the same buffer with 2 ml of 10 mg/ml Zymolyase. Cells were incubated at 30°C for 45–60 min with gentle shaking, until they could be easily "squashed" between a glass slide and coverslip (Roth and Simpson 1992). Following the addition of 100 ml of ice-cold 1.2 M Sorbitol, 20 mM PIPES, 1 mM $MgCl_2$ (pH6.8), the cells were centrifuged again in a JA10 rotor (5 min, 4°C, 3.5K). Cell pellets were resuspended in 50 ml of ice-cold NIB (nuclei isolation buffer; 0.25 M sucrose, 60 mM KCl, 14 mM NaCl, 5 mM $MgCl_2$, 1 mM $CaCl_2$, 15 mM MES at pH 6.6, 1 mM PMSF, 0.8% Triton X-100), held on ice water for 20 min, and spun at 4K in a JA10 rotor for 5 min. The NIB wash was repeated two times. The cells were then washed three times in "A" wash (10 mM Tris at pH 8.0, 0.5% NP-40, 75 mM NaCl, 30 mM sodium butyrate, 1 mM PMSF; Davie et al. 1981), holding on ice water for 15 min for the first two washes and 5 min for the final wash. The cell pellet was then washed in "B" wash (10 mM Tris at pH 8.0, 0.4 M NaCl, 30 mM sodium butyrate, 1 mM PMSF; Davie et al. 1981) one time for 10 min holding on ice water. After centrifugation, the cells were resuspended again in B buffer and centrifuged immediately. The histones were extracted by resuspending the pellet in 10 ml of cold 0.4 N H_2SO_4 and holding in ice water for 30 min, vortexing occasionally. Debris was removed from the solution by spinning for 10 min at 10K. The supernatant, which contains the extracted histones, was precipitated by adding 100% TCA to a final concentration of 20%. After 30 min incubation in ice water, the protein pellet was collected by centrifugation for 30 min, at 12K in a Beckman JS 13.1 rotor. Pellets were washed in acidified acetone (acetone + 1% HCl), then acetone, and then air dried. The histones were resuspended in ~2 ml of 10 mM Tris (pH 8.0) and stored at −20°C.

Far Western blot analysis

Proteins were separated by SDS-PAGE as described (Allis et al. 1980; Allis and Wiggins 1984 (22% gel, 60:0.4 acrylamide/bisacrylamide)). After equilibration in transfer buffer (running buffer plus 10% methanol), gels were semidry-blotted (Fisher Scientific; 2 hr at 500 mA) to polyvinylidene difluoride (PVDF) membrane (MSI) that was prewet in methanol and then equilibrated in transfer buffer. After transfer, the blots were stained for 10 min with Ponceau Red (Sambrook et al. 1989) and the positions of the histone bands were marked. The blots were then blocked for 2 hr in 0.05% Tween 20 in PBS (137 mM NaCl, 3 mM KCl, 7 mM Na_2HPO_4, 15 mM K_2HPO_4 at pH 7.9–8.0), for 2 hr in 1% BSA in PBS, then rinsed for 10 min in phosphate-buffered saline (PBS) (Kleinschmidt and Seiter 1988). Blots were then wrapped in plastic wrap and stored overnight to 2 weeks at 4°C.

Full-length Tup1 or deletion derivatives were translated in vitro in the presence of ^{35}S-methionine using the Promega TnT-coupled transcription/translation kit. Probes were diluted with 400 μl of 1× Promega translation buffer or with 40 mM HEPES (pH 7.4), 40 mM DTT, placed in a microcon10 (Amicon) column, and spun for 20 min at 10,000*g* in a microcentrifuge at room temperature. An aliquot of the purified probe (typically 2 μl) was analyzed by SDS-PAGE, and a separate aliquot was taken for scintillation counting. The remaining probe was diluted in 3 ml of PBS containing 1% goat serum, 0.3% BSA. Blots were prehybridized for 10 min in this buffer without probe, then incubated for 2 hr at room temperature in a 50-ml conical tube containing the probe. The tubes were mechanically rotated throughout the binding reaction. After 2 hr, blots were washed in PBS for 4 × 5 min, allowed to air dry, then exposed to film or to PhosphorImager screens (Molecular Dynamics).

Quantitation of full-length Tup1 binding to mutant histone H4 proteins (as in Fig. 3) was performed by densitometric scanning of Coomassie-stained histone gels and autoradiographs of corresponding FAR Western blots. The signal in the H4 region of the FAR Western blot was normalized according to the H4 signal in the corresponding stained gel to determine the level of Tup1 binding/load of H4. Binding of Tup1 to each mutant form of H4 was then normalized to that of Tup1 binding to wild-type H4 (set at 100%).

Quantitation of Tup1 fragment binding to wild-type histones (as in Fig. 4) was performed with Molecular Dynamics ImageQuant software. In this case, strips of preparative histone blots containing equal loads of histone (within each experiment) were probed with different Tup1 fragments and the level of H3/H4 binding by full-length Tup1 was set to 100%. The data summarized in Figure 4C represent the average of at least three (more typically five) independent FAR Western blots for each Tup1 probe. All probes were translated to similar specific activities, and differential binding of the Tup1 derivatives to H3 and H4 was not a function of differential labeling owing to methionine content. For example, the 1–72, 1–120, and 1–253 Tup1 constructs all contain the same three methionine residues (positions 45, 52, and 69), yet the 1–253 fragment exhibited significantly more histone binding than did the other two fragments.

Tup1–Sepharose affinity chromatography

The Tup1 fusion proteins were expressed in bacterial cells [BL21(DE3)] and purified using Novagen His-Bind resin and buffers according to the manufacturer-supplied procedures. The $Tup1_{(72–432)}$ protein was isolated under nondenaturing conditions. The $Tup1_{(252–673)}$ and $Tup1_{(459–713)}$ fusion proteins were isolated in 6 M urea using the denaturing protocol. The proteins were renatured by sequential dialysis through 4 M, 2 M, and 1 M urea in PBS and then through several changes of PBS.

Cyanogen bromide-activated Sepharose beads were purchased from Pharmacia, and Tup1 proteins were coupled to the beads as described in Harlow and Lane (1988). Histone proteins in binding buffer (20 mM Tris at pH 8.0, 150 mM NaCl, 1 mM EDTA, 0.1% Triton X-100, 1 mM glutamate, 1 mM DTT) were added to ~25 μl of Tup1–beads or mock-coupled beads that had been washed extensively in binding buffer. After incubation at room temperature for 5–15 min, beads were collected by centrifugation (microcentrifuge at 700*g* for 2 min, room temperature). The supernatant was saved (unbound fraction), and the beads were washed for 3 × 5 min with 1 ml of binding buffer. For salt wash experiments, beads were washed two times in binding buffer followed by a wash in binding buffer containing 0.4 or 0.8 M NaCl. The washed beads were resuspended directly in 2× SDS-PAGE buffer, and all samples were analyzed by SDS-PAGE and visualized by staining with Coomassie Brilliant blue R (Sambrook et al. 1989).

β-Galactosidase assays

β-Galactosidase activities of reporter genes in wild-type cells or in strains carrying histone mutations were quantitated by standard ONPG (*O*-nitrophenyl-β-D-galactoside) assays following preparation of cell extracts by a glass bead procedure (Rose et al. 1990).

Preparation of antibodies to acetylated H3

A peptide corresponding to the first 20 amino acids of yeast histone H3 with acetyl groups arbitrarily placed at lysines 9 and 18 was purchased (Baylor College of Medicine Protein Chemistry Core Facility) and used to generate polyclonal rabbit antibodies (Bethyl Laboratories).

Acid–urea gels

Acid–urea gels were prepared and run according to Allis et al. (1980). After an overnight "preelectrophoresis," the gels were warmed to room temperature and "teeth" were cast. The gels were then "scavenged" for 1 hr with 20 μl of 8 M urea, 5% acetic acid, 0.6 M β-mercaptoethanol. Approximately 50 μg of histone preparation [TCA precipitated and resuspended in loading buffer (4 M urea, 0.02% pyronin Y, 4% β-mercaptoethanol, 5% acetic acid)] was loaded per lane, and 31-cm gels were run for 17,000 Vh at 4°C. After electrophoresis, gels were equilibrated in 0.7% acetic acid, then semidry-blotted in the same buffer, or were stained with Coomassie Brilliant blue R. Blotted membranes were processed for either FAR Western or traditional Western blot analysis.

Western blots

Blotted PVDF membranes were blocked for 2 hr to overnight in 5% Carnation nonfat dry milk in TBS (10 mM Tris at pH 7.5, 150 mM NaCl). Membranes were then incubated at 37°C overnight with either anti-acetylated H3 antibodies (1:1000 dilution in 1× TBS with 1% goat serum and 0.3% BSA) or anti-acetylated H4 antibodies ("penta," Lin et al. 1989) (1:500 dilution). The next day, membranes were washed 6 × 5 min with 1× TBS prior to incubation with 1:1000 dilution of goat anti-rabbit IgG, conjugated to alkaline phosphatase (Boehringer Mannheim) in TBS with 10% goat serum. Membranes were then washed again (6 × 5 min in 1× TBS) and were developed with 66 μl of NBT (5% stock in 70% dimethylformamide) and 33 μl of BCIP (5% in

dimethylformamide) per 10 ml of alkaline phosphatase buffer (100 mM Tris at pH 9.5, 10 mM NaCl, 5 mM $MgCl_2$).

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