Essential and redundant functions of histone acetylation revealed by mutation of target lysines and loss of the Gcn5p acetyltransferase

Wenzheng Zhang, James R.Bone, Diane G.Edmondson, Bryan M.Turner and Sharon Y.Roth

Department of Biochemistry and Molecular Biology, U.T.M.D. Anderson Cancer Center, Houston, TX 77030, USA and Chromatin and Gene Expression Group, Anatomy Department, University of Birmingham Medical School, Birmingham B15 2TT, UK

Introduction

The packaging of eukaryotic DNA into chromatin influences every process that utilizes DNA as a template, including transcription, recombination, replication and repair. The nucleosome core particle serves as the basic repeat unit of chromatin and comprises an octamer of the four core histones with ~146 bp DNA wound around the outside (van Holde, 1989). Nucleosomes are folded together into higher order structures, and both individual nucleosomes and more highly folded forms of chromatin limit the access of trans-acting factors to DNA, thereby inhibiting transcription and other processes (Edmondson and Roth, 1996).

Histones contain a central, globular domain which is important for histone–histone interactions and nucleosome formation (van Holde, 1989). In addition, relatively unstructured and highly charged N-terminal tail domains extrude from the histone octamer, and these tails are important for DNA–histone interactions within and between nucleosomes, as well as for nucleosome–nucleosome interactions (Hansen, 1997). In addition, these N-terminal domains function in histone deposition and nucleosome assembly during S phase, in transcriptional regulation, and in the maintenance of genome integrity (Megee et al., 1995; Turner, 1995; Roth and Allis, 1996; Ura et al., 1997). Post-translational acetylation of lysine residues within these N-terminal tails facilitates binding of transcriptional activators to nucleosomal DNA (Lee et al., 1993), at least in part through neutralization of the positive charge associated with the lysine ε-amino group (Turner, 1991; Wolfe and Pruss, 1996). Acetylation also affects higher order packing of chromatin (Fletcher and Hansen, 1996) and interactions of non-histone proteins with the histones (Edmondson et al., 1996).

Recent discoveries that indicate several transcriptional activators or co-activators possess histone acetyltransferase activity provide a molecular basis for a long-standing correlation between histone acetylation and transcriptional activation (Wade et al., 1997). Yeast Gcn5p and its Tetrahymena homolog were the first of this class of proteins to be identified as catalytic subunits of histone acetyltransferase type A (HAT A) activities (Brownell et al., 1996). Gcn5p is part of larger complexes that contain Ada2p, Ada3p, Ada5p and/or other proteins (Marcus et al., 1994, 1996; Horiuichi et al., 1995; Candau and Berger, 1996; Roth and Allis, 1996; Grant et al., 1997; Roberts and Winston, 1997). The Gcn5p–Ada5 complex was initially described as a transcriptional adaptor, based on the finding that it is needed by several acidic transactivators for the activation of transcription (Berger et al., 1992), and the ability of components of the complex to interact with both these transactivators and basal transcription proteins such as TBP (Silverman et al., 1994; Barlev et al., 1995). Interactions between the acetyltransferase complex and DNA-binding transactivators suggest a mechanism by which these enzymes might be directed to specific regions of the genome for transcriptional activation. Moreover, mutations in GCN5 that eliminate Gcn5p acetyltransferase activity in vitro are defective in transcriptional activation in vivo (Candau et al., 1997; Wang et al., 1998).
mutations in gcn5 cells

1993; Roth and Allis, 1996; Johnson
tionally active and potentially active chromatin (Turner,
acetylation of K16 is commonly enriched in transcrip-
and K8 and K16 of H4 (Kuo
in vitro
Recombinant Gcn5p preferentially acetylates K14 of H3
defects (Grunstein, 1990; Smith, 1991; Roth, 1995).
Accordingly, mutation of multiple sites in H3 and/or H4
in yeast confers specific transcriptional and cell growth
questions and to examine the importance of specific
those acetylated at Gcn5p target sites. To address these
acetylation of lysines other than those recognized by Gcn5p
and that
H4 likely serves both redundant and unique functions,
suggest that acetylation of particular lysines in H3 and
Gcn5p is required for acetylation of sites
H3 and H4 that are not recognized by the recombinant
enzyme in vitro. Moreover, our findings indicate that
GCN5 is required for normal progression of the cell cycle
and that a crucial balance of histone acetylation at specific
lysines is essential for cell viability.

Results

Synthetic growth defects induced by H3 K14
mutations in gcn5 cells

To test the importance of acetylation at specific sites
in H3 and H4 in vivo, we engineered a set of isogenic GCN5
or gcn5 yeast strains in which the endogenous H3 and H4
loci (HHT1-HHF1 and HHT2-HHF2) are disrupted and
in which these histones are provided by a plasmid-borne
copy of HHT2-HHF2. Specific mutated versions of these
histone genes were then introduced by a plasmid shuffle
protocol (Materials and methods). We initially focused on
the mutation of H3 since this histone is the primary in vitro
target of both Gcn5p (Kuo et al., 1996) and native Gcn5p-
containing complexes isolated from yeast (Grant et al.,
1997). In particular, we mutated the major substrate site
for rGcn5p in H3, lysine 14 (K14) to arginine or to
arginine (R) or glycine (G) had only modest effects on the doubling
G1. Interestingly, this lengthened doubling time is associated with a moderately increased proportion
of cells in G2, and a decreased proportion of cells in G1 (Figure 3).
Conversion of K14 in H3 to glutamine (Q), arginine
(R) or glycine (G) had only modest effects on the doubling
time of these cells was extended from 172 min
(3-AT) due to inefficient activation of genes involved in
histidine biosynthesis. Therefore, we first compared
growth of GCN5 or gcn5 cells containing specific histone
mutations on this media (Figure 1). As expected, in the
presence of wild-type histones gcn5 cells grew noticeably
more slowly than GCN5 cells on 3-AT-containing media.
Even in rich media, gcn5 cells exhibited a lengthened
doubling time (172 min) relative to wild-type cells (104
min; Figure 2). Interestingly, this lengthened doubling
time is associated with a moderately increased proportion
of cells in G2, and a decreased proportion of cells in G1 (Figure 3).
Conversion of K14 in H3 to glutamine (Q), arginine
(R) or glycine (G) had only modest effects on the doubling
time of GCN5 cells (Figures 1 and 2), indicating that
permanent fixation of a positive or neutral charge at this
site does not abrogate essential H3 functions. In contrast,
gcn5 cells bearing these mutations exhibited markedly
increased doubling times (Figure 2). In rich media, the
doubling time of these cells was extended from 172 min
(wild-type H3) to 264 (H3 K14→Q) or 275 min (H3
K14→R). A depletion of cells in G1 is observed in both
the GCN5 and gcn5 cell populations, with an increased
number of cells in G2 (Figure 3). These results are similar
to previously observed delays in progression through
G2/M for cells bearing several different mutations in the
N-termini of histones H3 or H4 (Megee et al., 1990, 1995;
Durrin et al., 1991; Morgan et al., 1991).
Functions of histone acetylation

Fig. 2. Doubling times of cells bearing H3 mutations. Exponential doubling times of GCN5 or gcn5 cells containing wild-type H3 or mutations of K14 or K9 in rich media (YPD).

Importantly, the common phenotype for all three of the K14 H3 (Q, R and G) mutations indicates that the loss of the lysine residue is the most significant change and that the change in charge may be less important than the change in structure of this residue upon acetylation. Moreover, the synergistic growth defects we observe when GCN5 null mutations are combined with these H3 mutations suggest redundancies both in functions of specific acetylation events and in the enzymes that mediate these events.

**GCN5 is required for acetylation of H3 K9 and H3 K18 in vivo**

In contrast to the K14 mutations above, mutation of K9 in H3 did not exhibit a synergistic effect on doubling time of gcn5 cells. Cells lacking GCN5 and bearing K9→Q or K9→R mutations divide in rich media every 184 or 188 min, respectively (Figure 2), similar to the doubling time of the gcn5 cells carrying wild-type H3. Both mutations show an increased number of cells with a 2N content of DNA (data not shown).

To further investigate possible connections between acetylation at specific sites and Gcn5p function, we examined the acetylation states of wild-type or various mutant forms of H3 established in vivo, in the presence or absence of GCN5. Histones were isolated from cells bearing these mutations and probed with H3-specific antibodies (Figure 4). One antiserum, αH3, recognizes both acetylated and unacetylated forms of H3 (data not shown). Two other antisera, αH3 Ac K9,14 (generously provided by D.Allis, University of Rochester) and αH3Ac K9,18 (Edmondson et al., 1996) recognize specific acetylated forms of H3 (data not shown).

The wild-type, K14→R and K9→R forms of H3 all reacted equally well with the αH3 antiserum, regardless of whether they were isolated from GCN5 or gcn5 cells (Figure 4B and D). As expected, deletion of the H3 tail (Δ3–29; Figure 4B, lane 6) abolishes the reaction with this antibody. Interestingly, the K14→R form of H3 isolated from GCN5 cells reacted strongly with the αH3 Ac K9,14 antiserum (Figure 4C, lane 3). Since the H3 K14→R mutation inevitably eliminates acetylation at K14, the high degree of reactivity with the αH3 Ac K9,14 antibody indicates that this histone is well acetylated at K9. Consistent with these observations, the K9→R version of H3 (isolated from GCN5 cells) reacted very poorly with the αH3 Ac K9,14 antiserum (Figure 4C, lane 5). Since K14 is still available for acetylation in this mutant, the reduced reactivity with this antibody could reflect a bias in the specificity of the antibody towards K9 or a predominance of K9 acetylation (over K14) in bulk populations of wild-type histones. It is also possible that mutation at K9 might decrease acetylation at K14, resulting in decreased antibody recognition. In any case, this antibody clearly reacts well with H3 acetylated at K9.

To determine whether GCN5 is also required for acetylation of K18 in H3 in vivo, wild-type or K9→R mutant forms of H3 were probed with the αH3 Ac K9,18 antiserum. When isolated from GCN5 cells, both wild-type and K9→R H3 reacted well with this antiserum (Figure 4E, compare lanes 2 and 4 with lanes 1 and 3). These data indicate that GCN5 is required for full acetylation of K9 in H3 in vivo.
Fig. 4. Acetylation of multiple sites in the H3 N-terminus is dependent on Gcn5p in vivo. Histones were isolated from GCN5 or gcn5 cells containing wild-type or a mutated version of H3, as indicated. (+) indicates wild-type GCN5 or H3. (A) Histones are displayed on an SDS gel, stained with Coomassie Blue. Only the region of the gel containing the histones is shown. (B) A duplicate of the gel displayed in (A) was blotted to PVDF and probed with an antiserum specific for H3. (C) Another duplicate of the gel in (A) (except for the omission of lane 6) was transferred to PVDF and probed with the αH3 Ac K9,14 antiserum. (D) Histones from the indicated cells [~50% of the mass used in gel displayed in (A)] were separated by SDS–PAGE, blotted to PVDF and probed with the αH3 specific antiserum. (E) A duplicate of the gel in (D) was probed with the αH3 AcK9,18 antiserum.

In vivo, native Gcn5p–Adap or SAGA complexes (Grant et al., 1997) might exhibit an expanded substrate specificity, including K9 and K18 in H3. Alternatively, Gcn5p might influence acetylation of these sites indirectly.

Redundant functions of H4 acetylation for cell growth

Several studies have examined the function of the four highly conserved lysines (K5, K8, K12 and K16) in the H4 N-terminus in yeast, and mutations in one or more of these sites directly affect cell growth (Megee et al., 1990, 1995; Durrin et al., 1991; Morgan et al., 1991). Interestingly, simultaneous mutation of all four sites to glutamine leads to a decreased proportion of cells in G1 with an increased number of cells in G2 (Megee et al., 1995). This cell-cycle defect is rescued by the addition of a single lysine, independent of the site of insertion in the H4 N-terminus. These experiments suggest that lysines
Functions of histone acetylation

located at multiple positions in H4 can provide overlapping or redundant functions needed for normal cell growth. To further link the functions of these residues to their acetylation state, we examined the effects of mutations in K8 and K16 or in K5 and K12 in cells containing or lacking GCN5.

Conversion of the previously identified Gcn5p target sites in H4, K8 and K16, to Q or to R slowed growth in the presence of GCN5, as predicted by previous findings (Megee et al., 1990, 1995; Durrin et al., 1991; Morgan et al., 1991; Figure 6). In both GCN5 and gcn5 cells containing the K8/16 H4 mutations, an increased proportion of cells with a G2 content of DNA was observed (Figure 3; data not shown). Interestingly, combination of the K8/K16→Q H4 mutations with loss of GCN5 did not relieve or exacerbate the gcn5 slow growth phenotype on 3-AT-containing media (Figure 1) or on rich media (Figure 6), but conversion of these residues to R significantly enhanced the gcn5 slow growth phenotype (Figure 6), resulting in a doubling time of 239 min.

Strikingly, mutation of the two other acetylation sites in H4, K5 and K12, also induced an extreme slow growth phenotype in the absence of GCN5 (257 min for K5/12→Q and 273 min for K5/12→R; Figure 6) and accumulation of cells in G2/M (data not shown). In this case, both R and Q mutations exhibited very similar effects on cell growth, indicating that the loss of acetylatable lysines at these positions has ramifications beyond the alteration in charge.

Since previous studies indicated that rGcn5p acetylates only K8 and K16 in H4, we were surprised to observe a significant amount of acetylation of the K8/16→R or Q peptide.
forms of H4 by rGcn5p in vitro (Figure 5B, compare labelling of H4 in lanes 3 and 6 with that in 1, 9 and 12). These data suggest that, under some conditions, K5 and/or K12 might be acetylated by rGcn5p. To investigate the possibility that the acetylation states of K5 and K12 in H4 might also be influenced in vivo by GCN5, we probed wild-type and mutant forms of this histone with antibodies specific for particular acetylation states (Turner et al., 1992). We first probed H4 isolated from GCN5 or gcn5 cells with an antibody raised against a non-acetylated H4 peptide that recognizes non-, mono- and diacetylated forms of H4 (B. Turner, unpublished observations) but does not recognize more highly acetylated forms of this histone (Figure 7B). H4 isolated from GCN5 cells exhibited slightly lower reactivity with this antibody compared with H4 isolated from gcn5 cells, suggesting that H4 is more highly acetylated when GCN5 is present. Consistent with this, we found that loss of GCN5 caused a diminution of acetylation at each of the four acetylation sites in H4 (Figure 7C–F), as determined by a lowered reactivity with antibodies specific for acetylation at each individual site. Changes in acetylation levels at multiple sites in H4 provide an explanation for the synergistic effects in cell growth that we observe upon concomitant GCN5 loss and mutation of either the K5/12 or the K8/16 lysine pairs. Furthermore, our data also support a redundancy in at least some functions of acetylation at these four sites.

**Essential functions of acetylation revealed by concomitant mutation of H3 and H4 in gcn5 cells**

The possibility of redundant functions for acetylation of particular sites in H3 versus H4 was tested by examining the effects of combined mutations in these histones in GCN5 or gcn5 cells.

Combination of H3 K14→Q and H4 K8/K16→Q mutations had relatively minimal effects on cell growth in the presence of GCN5 (136 min doubling time; Figure 8), indicating that this combination of mutations does not overly interfere with H3 and H4 functions. In gcn5 cells, however, this combination of mutations results in a severe growth defect (351 min doubling time; Figure 8), significantly worse than the individual H3 or H4 mutations described above. Severe defects in cell growth were also observed in gcn5 cells bearing mutations at other sites (K9 in H3 and K5/12 in H4), but in this case the defect is more similar to that seen with cells bearing the H4 K5/12 mutation alone (Figures 6 and 8). An increased proportion of cells in G2 was observed in the presence of all of these combined mutations, in both GCN5 and gcn5 cells (Figure 3; data not shown).

Strikingly, combination of the H3 K14→R and H4 K8/K16→R mutations in gcn5 cells is lethal. We were repeatedly unable to complete a plasmid shuffle protocol to introduce these histone alleles into gcn5 cells. In these experiments, both a URA3-marked plasmid bearing wild-type histones H3 and H4 and a TRP1-marked plasmid bearing the mutated H3 and H4 genes were introduced into cells disrupted for the endogenous H3 and H4 genes. After growth in Ura+ media, cells that had lost the URA3-marked, wild-type histone plasmid were selected on 5-FOA-containing media. Although we typically obtained thousands of 5-FOA-resistant colonies from GCN5 cells, very few were recovered from gcn5 cells (Table I), and these were subsequently determined (by Southern analysis; data not shown) to have undergone rearrangement events such that they no longer contained the mutant histone alleles. We were also unable to recover this combination of mutated H3, H4 and gcn5 alleles from sporulation of diploid cells heterozygous for both the mutant histones and GCN5, although all other expected combinations of alleles were observed at the expected frequency (>30 tetrads dissected; data not shown). This synthetic lethality is limited to particular target sites of rGcn5p, since we successfully recovered gcn5 colonies bearing combined H3 K9→R and H4 K5/12→R mutations after plasmid shuffle (Table I; Figure 8). Interestingly, we find that deletion of the N-terminal tail of either H3 (residues 3–29) or H4 (residues 4–19) in gcn5 cells is also lethal (Table I; unpublished sporulation analysis), indicating that a critical level of histone acetylation must be maintained for cell viability.

**Nucleosome assembly in GCN5 or gcn5 cells is not grossly changed by mutation of either Gcn5p-target or deposition-related acetylation sites**

The effects of specific lysine mutations on cell growth in rich media in the absence of gcn5 could reflect either defective organization of chromatin or defective regulation of transcription, or both. To determine whether these mutations affect chromatin organization at the level of nucleosome assembly, we examined the superhelical density of the endogenous 2μ plasmid in these various strains. Each nucleosome imparts one negative supercoil to a circular plasmid, so comparison of plasmid topoisomer distributions provides an estimate of the relative number of nucleosomes assembled onto the plasmid in vivo.

We focused on strains containing combined H3 and H4 mutations, since these exhibited the most severe growth defects (Figure 9). No gross changes in superhelical density of the 2μ plasmid were observed in any of these strains (Figure 9). Other strains bearing single H3 or double H4 mutations in isolation were also examined, but again, no gross changes in topoisomer distribution were
Fig. 8. Combined H3 and H4 mutations severely compromise growth of \textit{gcn5} cells. Exponential doubling times of \textit{GCN5} or \textit{gcn5} strains containing the indicated forms of H3 and H4 in rich media are presented in minutes.

Table I. Plasmid shuffle of histone alleles

<table>
<thead>
<tr>
<th>H3</th>
<th>H4</th>
<th>GCN5 colonies\textsuperscript{a}</th>
<th>5-FOA\textsuperscript{b}</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>WT</td>
<td>+</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>WT</td>
<td>Δ</td>
<td>+</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>K14R</td>
<td>WT</td>
<td>Δ</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>WT</td>
<td>K8,16R</td>
<td>+</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>WT</td>
<td>K8,16R</td>
<td>Δ</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>K9R</td>
<td>K5,12R</td>
<td>+</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>K9R</td>
<td>K5,12R</td>
<td>Δ</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>K14R</td>
<td>K8,16R</td>
<td>+</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>WT</td>
<td>K8,16R</td>
<td>Δ</td>
<td>6\textsuperscript{b}</td>
</tr>
<tr>
<td>WT</td>
<td>del 3–29</td>
<td>+</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>WT</td>
<td>del 4–19</td>
<td>Δ</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>WT</td>
<td>del 4–19</td>
<td>Δ</td>
<td>4\textsuperscript{b}</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Multiple independent colonies for each strain were chosen and confirmed to contain the appropriate wild-type or mutated histone allele by Southern blotting.

\textsuperscript{b}Upon Southern analysis, all of these colonies were found to contain rearrangements in the mutated H3 or H4 alleles.

observed (data not shown). At most, a change in linking number corresponding to a change of one to two nucleosomes per plasmid was observed in \textit{gcn5} cells carrying the combined K14→Q H3, K8/16→Q H4 alleles relative to \textit{GCN5} cells bearing these same mutations. In this case, however, the increased topoisomer density would indicate that the plasmid actually carries an increased number of nucleosomes in these \textit{gcn5} cells.

Interestingly, even simultaneous mutation of all deposition-related lysines in H3 (K9) and H4 (K5/12) to R (Figure 9) did not cause a gross loss of nucleosomes from the plasmid, comparable, for example, with that observed by others upon simultaneous loss of both the H3 and H4 N-terminal tails (Ling et al., 1996). Our results indicate that acetylation of these lysines is not required absolutely for histone deposition and nucleosome assembly in vivo, although certainly acetylation may facilitate these processes.

\textbf{Mutation of target lysines affects transcriptional activation by GCN5}

\textit{GCN5} is required for transcriptional activation by several acidic transactivator proteins in yeast, including heterologous VP16 derivatives (Berger et al., 1992; Marcus et al., 1994). A truncated form of VP16 (residues 413–470) fused to the Gal4p DNA binding domain (\textit{GAL4–VP16}\textsubscript{413–470}) strongly activates a reporter construct bearing a Gal4p binding site in wild-type cells (Barlev et al., 1995). Only 2 units of activity are observed in the absence of \textit{GAL4–VP16}\textsubscript{413–470} (data not shown) whereas >600 units are observed in wild-type cells in the presence of the activator (Figure 10A). As expected, ~9-fold less activation of the reporter by \textit{GAL4–VP16}\textsubscript{413–470} is observed in the absence of \textit{GCN5} (Figure 10A). Previous studies have demonstrated that Gal4p–VP16 expression is not changed in \textit{gcn5} cells (Berger et al., 1992), and we find that the copy number of the reporter construct is also unchanged in these cells or in the cells harboring the various histone mutations described below (data not shown). Decreases in reporter expression, therefore, reflect ineffective activation by Gal4p–VP16.

The K14→Q mutation in H3 does not suppress the
Fig. 9. Nucleosome density on the endogenous 2μ plasmid in GCN5 or gcn5 cells is not grossly affected by acetylation site mutations in H3 and/or H4. The distribution of 2μ plasmid topoisomers in extracts from the indicated strains was examined after chloroquin gel electrophoresis, as described in Materials and methods. The mean topoisomer distribution is not grossly changed in the various strains, indicating a similar density of nucleosomes present on the plasmid in these cells. All samples were resolved in the same gel, but a darker exposure of the fourth lane is presented to facilitate comparisons.

gcn5 transcriptional phenotype but does cause a 2-fold hyperactivation of the reporter in GCN5 cells (Figure 10A). Conversion of K14 to R decreases reporter expression slightly in GCN5 cells but does not exacerbate the transcriptional defect in the absence of GCN5. Conversion of K14 to G (data not shown) or mutation of K9 to either Q or R increases reporter expression 2- to 3-fold in both GCN5 and gcn5 cells. Interestingly, increased activation of other genes, including the natural GAL1 promoter, in the presence of other H3 N-terminal mutations has been observed previously (Mann and Grunstein, 1992).

Fig. 10. Transcriptional activation by Gal4–VP16 in GCN5 and gcn5 cells containing H3 and/or H4 acetylation site mutations. β-Galactosidase activity resulting from transcriptional activation of a Gal4–VP16- and Gcn5p-dependent reporter plasmid was measured in extracts from GCN5 or gcn5 strains (as indicated) containing (A) mutations in K14 or K9 of histone H3, (B) mutations in K5,12 or K8,16 of histone H4, or (C) combined mutations in H3 and H4 (as indicated). Averages of activities from multiple colonies of each strain, assayed at least twice are shown. Standard errors were typically 10–20% of the values shown.

gene (Figure 10C). Indeed, the level of activation in the gcn5 cells reached 68% of that achieved in the GCN5 cells. These data support an important role for acetylation of specific lysines in H3 and H4 for Gcn5p-mediated activation of transcription.

Discussion

The recent identification of a bevy of regulatory proteins as histone acetyltransferases and deacetylases implies clear connections between this histone modification, gene expression and cell growth. However, since some acetyltransferases recognize both non-histone and histone substrates (Gu and Roeder, 1997; Imhof et al., 1997), the importance of histone acetylation relative to acetylation of other targets has come into question. We directly tested the importance of histone acetylation to cell growth and transcription by combining mutations in substrate lysines in histone H3 and H4 with null alleles of the GCN5 acetyltransferase in yeast. Our data indicate that GCN5 is required for full levels of acetylation at multiple sites in
H3 and H4 in vivo. These acetylation events are important for normal progression of the cell cycle and for transcriptional activation. Moreover, our data demonstrate that a critical overall level of acetylation is essential for cell viability.

Acetylation neutralizes the positive charge associated with the ε-amino group of lysine moieties and therefore weakens interactions with the negatively charged DNA. If charge neutralization is a primary purpose of acetylation by Gcn5p, mutation of substrate lysines in H3 and H4 to the neutral amino acid glutamine or to the charged amino acid arginine might suppress or enhance, respectively, the requirement for Gcn5p for transcriptional activation or for normal cell growth. However, in all but one case (involving concomitant conversion of K14 in H3 and K8/16 in H4), the Q and R mutations we examined elicited very similar effects upon cell growth and transcription. Our results indicate that loss of the acetylatable lysines has consequences beyond changes in charge, likely reflecting alterations in structure of the tail domains (Hansen, 1997) or in the association of non-histone, regulatory proteins with these domains (Edmondson et al., 1996). Acetylation also likely increases the mobility of the tail domains (Cary et al., 1982; Hong et al., 1993; Ura et al., 1997), and our mutations may restrict such mobility.

**Redundant and essential functions of GCN5**

Multiple acetyltransferase activities have been identified previously in yeast extracts (Travis et al., 1984; Grant et al., 1997). In addition, other specific acetyltransferases have been identified, including yeast TAF130 (Mizzen et al., 1996). The synthetic growth defects we observed upon mutation of target sites and loss of GCN5 predict that at least some of these other activities will have substrate specificities overlapping with, but not identical to, those of Gcn5p. Indeed, acetyltransferase complexes with a predominant specificity for H4, rather than the preference of H3 exhibited by Gcn5p, have been isolated from yeast extracts (Grant et al., 1997). Such additional acetyltransferase activities that acetylate wild-type H3 and/or H4 in gen5 cells could minimize the effects of GCN5 loss, but when these histones are mutated such that they are no longer substrates for any of these enzymes, more serious defects in cell growth should ensue, as we observed.

The synthetic growth defects and the synthetic lethality that occurs in gen5 cells carrying certain histone mutations also indicate that Gcn5p affects acetylation of additional targets in H3, H4 or other proteins in vivo that are not recognized by the recombinant protein in vitro. Consistent with this, we find that full acetylation of multiple lysines in H3 and H4 is dependent on GCN5 in vivo. In principle, this might reflect an interdependence of acetylation events, i.e. acetylation of one lysine might be required prior to acetylation of another. However, examination of the acetylation states of single site substitution mutants in H4 indicates that all four sites in this histone are acetylated independently (H. Johnson, Ph.D. thesis, University of Birmingham). Our data suggesting additional targets for Gcn5p in vivo complement recent findings by Grant et al. (1997) that indicate complexes isolated from yeast containing Gcn5p/Ada2p also acetylate histone H2B. Thus, Gcn5p can acetylate at least three of the four core histones in vitro and this enzyme influences acetylation of multiple sites within at least two of these histones in vivo.

Our finding that concomitant mutation of the three lysines recognized by recombinant Gcn5p in vitro, K14 in H3 and K8, K16 in H4, to glutamine can largely bypass the need for Gcn5p in activation of transcription by Gal4–Vp16 in vivo clearly supports an important role for the acetylation of these residues in Gcn5p-mediated activation events. Since GCN5 is required for full acetylation of K9 in H3 and of all four sites in H4, it is possible that other combinations of mutations might have similar effects. However, conversion of K9 in H3 and K5, K12 in H4 to glutamine did not bypass the need for GCN5 for Gal4–Vp16 activation, and conversion of these sites to arginine was not synthetically lethal with the GCN5 null allele. Therefore, acetylation events at these various sites in H3 and H4 have at least some unique functions in transcriptional regulation and cell growth. In future experiments, it will be interesting to examine other combinations of mutations, for example K9→Q in H3 with K8, K16→Q in H4 or K14→Q in H3 with K5, K12→Q in H4, for their effects on these processes.

**Roles for Gcn5p-mediated acetylation in cell growth**

The defective progression of gen5 cells (containing wild-type histones) through G2/M may be due to defective transcription of genes needed for cell-cycle advancement, or it may indicate that Gcn5p-mediated acetylation is important for changes in chromatin structure that occur during G2 in preparation for mitosis, or both. Our results are consistent with previous studies (Morgan et al., 1991; Megee et al., 1995) that indicate the N-termini of H3 and H4 are required for normal cell-cycle progression and that the H4 N-terminus plays a role in maintenance of genome integrity. Our data now link these processes more directly to acetylation of these histones. Future studies will examine possible connections between H3 and H4 acetylation and particular cell cycle or DNA damage checkpoints.

Cells bearing GCN5 disruptions are temperature sensitive for growth at 37°C, and this lethality is not rescued by any of the H3 or H4 mutations we examined (W.Zhang, unpublished observations). Our finding that deletion of GCN5 is lethal even at the permissive temperature of 30°C when combined with a truncation of either the H3 or the H4 N-terminus is reminiscent of findings by Ma et al. (1996). These researchers uncovered a set of hsl (histone synthetic lethal) mutations, all of which also exhibited lethality in combination with N-terminal deletions in H3 or H4. Interestingly, two of the HSL genes, HSL1 and HSL7, and a dosage-dependent suppressor of hsl1 and hsl7 mutations, OSS1, are all regulators of the Swel1p kinase, an important cell-cycle regulator. Like GCN5, HSL1 and HSL7 are not essential genes, but cells lacking these genes exhibit a defect in progression through G2/M. These similarities may simply reflect the involvement of the H3 and H4 tail domains in multiple processes affecting cell-cycle progression, but they also suggest the intriguing possibility that GCN5 functions, particularly histone acetylation, might be directly linked to cell-cycle controls via Swel1p or the HSL genes.
Materials and methods

Yeast culture
S. cerevisiae strains were propagated according to standard procedures in rich media (YPD) or appropriate selective media (SC or SD) as needed. In some experiments, cells were also cultured in the presence of 10–100 mM 3-AT (100 mM in plate depicted in Figure 3). Strains used in this study are listed in Table II. All are isogenic to DY881 and DY882 (generously provided by P.Heitler, Johns Hopkins University), and are derived from S288C. Yeast were transformed according to Hill et al. (1991). To measure doubling times, cells were diluted into YPD and counted at defined intervals during exponential growth.

Disruption of HHT1-HHF1 and HHT2-HHF2
Homologous recombination was used to replace the coding regions of the two endogenous H3 and H4 gene cassettes with vector and marker sequences. To disrupt HHT1-HHF1, DNA fragments flanking the H3–H4 coding region (corresponding to base pairs 1–212 and 1543–1800, where 615 denotes the A residue of the H3 initiator ATG codon) were created by PCR using genomic yeast DNA as the template. These fragments were cloned in tandem into the polylinker of a disruption vector, pRS405 (a gift from P.Heitler, Johns Hopkins University), carrying the selectable marker. The resulting construct was linearized with restriction endonucleases and used to transform yeast. The disruption vectors were selected by their growth on medium lacking uracil. The resulting transformants were confirmed to have lost the selectable marker. The resulting diploid was then sporulated and identified (WZY42 and WZY43) and confirmed to contain both of the replacement alleles, so that the plasmid borne copy of HHT2-HHF2 is only source of histones H3 and H4 in these cells. Sequences of PCR primers used in these studies will be gladly provided upon request.

In vitro mutagenesis of HHT2-HHF2 cells
A 1.8 kb SpeI fragment was isolated from Ycp50-copy II (HHT2-HHF2) (generously provide by Dr M.A.Osley, Sloan Kettering), a plasmid bearing wild-type H3–H4 copy II alleles with their natural promoter and a URA3 selectable marker. This diploid was then sporulated and Ura-. His+ haploid strains of both mating types were identified (WZY42 and WZY43) and confirmed to contain both of the above replacement alleles, so that the plasmid borne copy of HHT2-HHF2 is only source of histones H3 and H4 in these cells. Sequences of PCR primers used in these studies will be gladly provided upon request.
Plasmid name   H3   H4   Restriction sites introduced   Sites used in cloning   Starting plasmid   Cloning method or fragment inserted
---   ---   ---   ---   ---   ---   ---
pWZ414-F8   Δ3–29   none   EcoRI–XhoI   prS414   OLE PCR fragment
-F12   WT   WT   none   SmaI–AflII   prS414   Blunted SpeI fragment of Ycp50-copyII
-F13   WT   WT   AflIIb   EcoRI–AflII   prWZ414-F12   MORPH, Oligo WZ33c
-F14   Δ3–29   WT   none   EcoRI–AflII   prWZ414-F13   EcoRI–AflII fragment of pWZ414-F8
-F15   WT   Δ4–19   AflIIb   prWZ414-F13   OLE PCR fragment
-F30   K9Q   WT   SacI   prWZ414-F13   MORPH, Oligo WZ50c
-F53   K9R   WT   SacI, SacII   SacII–BanHI   prWZ414-F43   Oligos WZ85, 86d
-F31   K14Q   WT   SacII   AflII–BanHI   prWZ414-F13   OLE PCR fragment
-F36   K14G   WT   SacI   AflII–BanHI   prWZ414-F13   OLE PCR fragment
-F43   K14R   WT   SacII   AflII–BanHI   prWZ414-F13   OLE PCR fragment
-F23   WT   K9Q   SacII   prWZ414-F13   MORPH, Oligo WZ37d
-F33   WT   K9Q   SacII, BglII   prWZ414-F23   MORPH, Oligo WZ53d
-F51   WT   K5.12Q   SacII, AvrII, BglII   BglII–SacII   prWZ414-F33   Oligos WZ83, 84d
-F52   WT   K5.12R   SacII, AvrII, BglII   BglII–SacII   prWZ414-F33   Oligos WZ81, 82d
-F47   WT   K8.16Q   SacII, SacI, BglII   BglII–SacII   prWZ414-F33   Oligos WZ74.75d
-F49   WT   K8.16R   SacII, SacI, BglII   BglII–SacII   prWZ414-F33   Oligos WZ79.80d
-F48   K14Q   K8.16Q   SacII (2'), SacII, BglII   BamHI–EcoRI   prWZ414-F47   BamHI–EcoRI fragment of pWZ414-F31
-F50   K14R   K8.16R   SacII (2'), SacI, BglII   BamHI–EcoRI   prWZ414-F49   BamHI–EcoRI fragment of pWZ414-F43
-F54   K9Q   K5.12Q   SacI, SacII, AvrII, BglII   BamHI–EcoRI   prWZ414-F51   BamHI–EcoRI fragment of pWZ414-F30
-F55   K9R   K5.12R   SacII (2'), SacI, AvrII, BglII   BamHI–EcoRI   prWZ414-F52   BamHI–EcoRI fragment of pWZ414-F53

---

Other plasmid constructions
To create a LYS2-marked GAL4–VP16 construct, a 2.8 kb Apal–PvuII fragment from pPC97–VP16,413–470 was ligated to the Apal–SmaI sites of pRS317. This plasmid was named pWZGP. To create LYS2-marked integrating versions of the H3 Δ3–29, the H4 Δ4–19, and the combined H3 K14R, H4 K8.16R alleles (to confirm the synthetic lethality of these alleles upon segregation with gen5 null alleles during sporulation), PvuII fragments containing these alleles were isolated from pWZ414-F14, pWZ414-F15 and pWZ414-F50, respectively, and ligated into PvuII-cut pRS404, yielding pWZ404-F14, pWZ404-F15 and pWZ404-F50. A PvuII fragment containing the LYS2 promoter and coding region was isolated from pMPK1 (a gift from M.Kladde, Pennsylvania State University) and ligated into the unique PvuII site of pWZ404-F14, pWZ404-F15 and pWZ404-F50.

Deletion of GCN5
GCN5 was deleted in WZY63 using a UR43 marked disruption vector as described previously (Marcus et al., 1994) to create WZY73 (URA+) (Table II). After confirmation of GCN5 loss by Southern blot analysis, cells that had lost the UR43 marker were selected on 5-FOA-containing media (WZY76). The plasmid Ycp50-copyII was introduced into these cells, and the TRP1-marked pWZ414-F13 was lost after growth in Trp– media. Trp–cells were identified by replica plating onto Trp– media. Mutant histone alleles were then introduced into this strain (WZY81) by plasmid shuffle.

Plasmid shuffle of histone alleles
Mutant histone alleles on TRP1-marked plasmids (pWZ414 series) were introduced into GCN5 (WZY42) or gen5 (WZY81) cells carrying wild-type histones on the UR43-marked Ycp50-copy II plasmid. After growth in Ura– media, cells that had lost the UR43 plasmid were selected by growth on 5-FOA-containing media. Loss of the wild-type histone alleles and the presence of the mutant histone alleles were confirmed by Southern blot analysis.

β-Galactosidase assays
β-Galactosidase assays were performed as described following transformation of strains listed in Table II with plasmid pWZGP (GALA–VP16,413–470 construct from above) and plasmid pLGSD5 (PNAS 79-7410–7414), which carries the β-galactosidase gene under the control of a single Gal4p binding site. Multiple colonies of each strain were assayed at least twice (more commonly three to five times), and the data presented are the averages of these multiple measurements.

Southern blotting
Genomic yeast DNA was isolated by a glass bead procedure as described previously (Rose et al., 1990), digested with appropriate restriction enzymes and resolved by agarose electrophoresis. The DNA was then transferred from the gel to GENESCREEN membranes (NEN) by Posiblot (Stratagene). DNA blots were probed with randomly primed, 32P-labeled probes, washed and exposed to X-ray film as described.

Topoisomerase analysis
Total genomic yeast DNA prepared as described above (Rose et al., 1990) was resolved on 0.8% agarose gels containing 10 μg/ml chloroquine by electrophoresis at 75 V for 24 h. After transfer to a membrane as above, DNA was probed with sequences specific for the endogenous 2μ plasmid.

FACS analysis
Cells (15 ml) were grown to a density of 0.5–1×107 cells/ml and harvested by centrifugation at 1000 g on a tabletop centrifuge. The cells were washed with one volume of phosphate-buffered saline (PBS), then resuspended in one volume (15 ml) of 70% ethanol and fixed at room temperature for 1 h with agitation. Cells were then rehydrated in PBS for 2 h at room temperature. Approximately 5×106 cells were RNase treated in a volume of 0.5 ml of 200 mM Tris pH 8.0, 20 mM EDTA and 1 mg/ml RNase A overnight at 37°C. Cells were pelleted in a microfuge and resuspended in 0.1 ml of PBS and counted. Cells (1.5×107) were then stained with 50 mM propidium iodide in a final volume of 0.1 ml of PBS for 2 h at room temperature in the dark. The cells were diluted with 400 μl of PBS, sonicated briefly and then counted on a Coulter EPICS XL-100 MCL flow-cytometer.

Histone preparation, acetyltransferase assays and Western blot analysis
Histones were isolated, resolved by SDS–PAGE and blotted to PVDF as described previously (Edmondson et al., 1996). Blots were blocked...
in 5% dry milk dissolved in 1× TBS (0.15 M NaCl, 0.1 M Tris-HCl, pH 7.5) for 2 h at room temperature with agitation. Antisera were diluted in 3% BSA, 10% goat serum in 1× TBS and blots were incubated with this mixture at room temperature overnight. Dilutions of total antisera were: αH3 1:500; αH3 AcK9,14 1:8000; αH3 AcK9,18 1:4000; R41/5 (αH4 AcK5 in Figure 7) 1:500; R12/8 (αH4 AcK8 in Figure 7) 1:8000; R20/12 (αH4 AcK12 in Figure 7) 1:5000 and R14/16 (αH4 AcK16 in Figure 7) 1:1000. The H3 AcK9,18 antibody is described in Edmondson et al. (1996). The site-specific H4 antibodies are described in Turner et al. (1989). Blots were then washed six times for at least 5 min in 1× TBS and incubated with the secondary antibody (goat anti-rabbit conjugated to alkaline phosphatase; Pierce Biochemicals) diluted 1:1000 in 10% goat serum in 1× TBS for 2 h at room temperature. Blots were then washed six times in 1× TBS and developed for alkaline phosphatase according to the manufacturer’s protocol.

Histone acetyltransferase assays were performed as described by Brownell et al. (1996).

Acknowledgements

DNA sequencing was carried out in part by the UTMDCG core DNA sequencing facility. We thank Olga Yarygina for help in cloning and preparation of several plasmids, and Anjie Watson for help in cloning the H3–H4 disruption vectors. We are also grateful to C. David Allis for helpful discussions and for the H3 K9,14 antisera, M. Mitchell Smith for advice in performance and interpretation of FACS, David Stillman for the gift of DY881 and DY882 yeast and for comments on the manuscript, Shelley Berger for the GAL4–VP16 plasmid, the reporter plasmid, a GCN5 disruption vector and many helpful discussions and comments on the manuscript, Jerry Workman for sharing results prior to publication and Karen Hensley for help with many of the graphics. This work was supported by grants to S.Y. from the NIH (GM51189), the Robert A. Welch Foundation (G1371) and developmental funds from the M.D. Anderson Cancer Center Core grant, and by a grant to B.M.T. from the Wellcome Trust programme (No. 045030/Z/95). J.R.B. is supported by grants to S.Y. R. from the NIH (GM51189), the Robert A. Welch Foundation (G1371) and developmental funds from the M.D. Anderson Cancer Center Core grant, and by a grant to B.M.T. from the Wellcome Trust programme (No. 045030/Z/95). J.R.B. is supported by the American Cancer Society. D.G. E. is supported by the Theodore L. Scientific Achievement Fellowship.

References

Functions of histone acetylation


Received February 23, 1998; revised and accepted March 30, 1998