Nucleosomal DNA regulates the core-histone-binding subunit of the human Hat1 acetyltransferase

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Background: In eukaryotic cells, newly synthesized histone H4 is acetylated at lysines 5 and 12, a transient modification erased by deacetylases shortly after deposition of histones into chromosomes. Genetic studies in *Saccharomyces cerevisiae* revealed that acetylation of newly synthesized histones H3 and H4 is likely to be important for maintaining cell viability; the precise biochemical function of this acetylation is not known, however. The identification of enzymes mediating site-specific acetylation of H4 at Lys5 and Lys12 may help explain the function of the acetylation of newly synthesized histones.

Results: A cDNA encoding the catalytic subunit of the human Hat1 acetyltransferase was cloned and, using specific antibodies, the Hat1 holoenzyme was purified from human 293 cells. The human enzyme acetylates soluble but not nucleosomal H4 at Lys5 and Lys12 and acetylates histone H2A at Lys5. Unexpectedly, we found Hat1 in the nucleus of S-phase cells. Like its yeast counterpart, the human holoenzyme consists of two subunits: a catalytic subunit, Hat1, and a subunit that binds core histones, p46, which greatly stimulates the acetyltransferase activity of Hat1. Both p46 and the highly related p48 polypeptide (the small subunit of human chromatin assembly factor 1; CAF-1) bind directly to helix 1 of histone H4, a region that is not accessible when H4 is in chromatin.

Conclusions: We suggest that p46 and p48 are core-histone-binding subunits that target chromatin assembly factors, chromatin remodeling factors, histone acetyltransferases and histone deacetylases to their histone substrates in a manner that is regulated by nucleosomal DNA.

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Received: 27 October 1997
Revised: 20 November 1997
Accepted: 20 November 1997

Published: 17 December 1997

Current Biology 1997, 8:96–108
http://biomednet.com/elecref/0960982200800096

Background

In proliferating cells, the bulk of histone synthesis occurs during S phase of the cell cycle, and histone assembly into chromosomes is tightly coupled to the passage of the DNA replication fork [1]. Core histone tetramers are assembled onto DNA via two distinct reactions that occur concomitantly behind the DNA replication fork. The redistribution of parental core histone tetramers ($H3_2{\cdot}H4_2$) onto the two nascent chromatids, known as parental nucleosome segregation, occurs without extensive histone acetylation [2]. In contrast, the assembly of newly synthesized histones H3 and H4 into nucleosomes, known as *de novo* nucleosome assembly, involves the participation of cellular protein(s) such as chromatin assembly factor 1 (CAF-1), which forms a stable complex with newly synthesized and acetylated histones H3 and H4 and in some way targets these histones specifically to sites of DNA synthesis [3–6].

In all species that have been examined thus far, newly synthesized H4 is acetylated transiently at two, and possibly three [5], specific residues within the amino-terminal domain of the protein. The two best-documented acetylation sites in newly synthesized H4 (lysine residues at positions 5 and 12) are distinct from the sites modified by the histone acetyltransferases involved in transcriptional regulation [7,8]. In addition to H4, newly synthesized H3 is also acetylated at specific sites [8,9], although perhaps not in all cell types [9].

In *Saccharomyces cerevisiae*, the amino-terminal domain of either H3 or H4 alone is sufficient for cell viability and nucleosome assembly; the amino-terminal domains of H3 and H4 can in fact be exchanged without significantly impairing viability [10]. In contrast, deletion of the amino-terminal domains of both newly synthesized H3 and H4 results in cells that lose viability during S phase of the cell cycle and elicits a nucleosome-assembly defect [10]. Taken together, these data argue that the amino-terminal domains of H3 and H4, possibly as acetylated proteins, perform an essential, but redundant, function in maintaining cellular viability, presumably because of their role in nucleosome assembly. In addition, acetylation of the newly synthesized histones and linker histone deficiency have been found to stimulate transcription synergistically in a *Xenopus* cell-free system for

DNA-synthesis-dependent nucleosome assembly [11]. Recently, an enzyme that generates the specific pattern of acetylation at Lys5 and Lys12 characteristic of newly synthesized H4 has been identified in *S. cerevisiae* [12,13]. The yeast Hat1 acetyltransferase consists of two polypeptide subunits: a catalytic subunit encoded by the *HAT1* gene and an accessory subunit encoded by *HAT2*. The Hat2p protein enhances the activity of the Hat1p catalytic subunit by increasing its affinity for histone H4.

Interestingly, Hat2p is structurally related to another yeast protein, known as Cac3p or Msi1p, which was recently identified as the small subunit of *S. cerevisiae* CAF-1 [14]. Disruption of the *CAC3* gene results in the same phenotypes as disruption of *CAC1* or *CAC2* (which encode the other two subunits of the CAF-1 protein), namely sensitivity to ultraviolet light and derepression of transcriptional silencing by adjacent telomeres [14]. In contrast, disruption of *HAT2* has essentially no phenotype [13]. This provides genetic evidence that Cac3p is required for CAF-1 activity and that Hat2p and Cac3p are functionally distinct, despite their structural similarity [5]. An analogous situation may also exist in higher eukaryotes, given that both mouse and human cells contain homologues of yeast Hat2p and Cac3p. The two mammalian proteins, known as p46 and p48 (or RbAp46 and RbAp48, because they were originally purified by virtue of their binding to a retinoblastoma (Rb) protein affinity column [15]) are encoded by different genes and are ubiquitously expressed in mouse tissues [16]. The two proteins are highly related to each other (90% identical and 93% similar), but only p48 is part of human CAF-1 [5]. More recently, both p46 and p48 were also found to co-purify with the HDAC1 and HDAC2 histone deacetylases [17,18], suggesting that p46 and p48 may also play a role in transcriptional repression. In addition, disruption of the genes encoding the yeast histone deacetylase Rpd3p and, to a lesser extent, Hda1p, both of which have amino-acid sequence similarity to HDAC1 and HDAC2, results in the accumulation of chromosomal histone H4 acetylated at Lys5 and Lys12, arguing that Rpd3p and Hda1p play a role in deacetylation of newly synthesized H4 [19].

By analogy with the yeast system, we surmised that p46 or p48 might be involved in histone acetylation in human cells. This paper provides biochemical evidence that the Hat1 enzyme is conserved in both subunit composition and substrate specificity between yeast and human cells, and that p46, but not p48, is the core-histone-binding subunit of the Hat1 holoenzyme. The p46 subunit and the related p48 polypeptide bind directly to helix 1 within the histone-fold domain of histone H4, thereby increasing the affinity of the Hat1 catalytic subunit for its substrate and, as a result, increasing H4 acetylation.

Results

Human *Hat1* cDNA

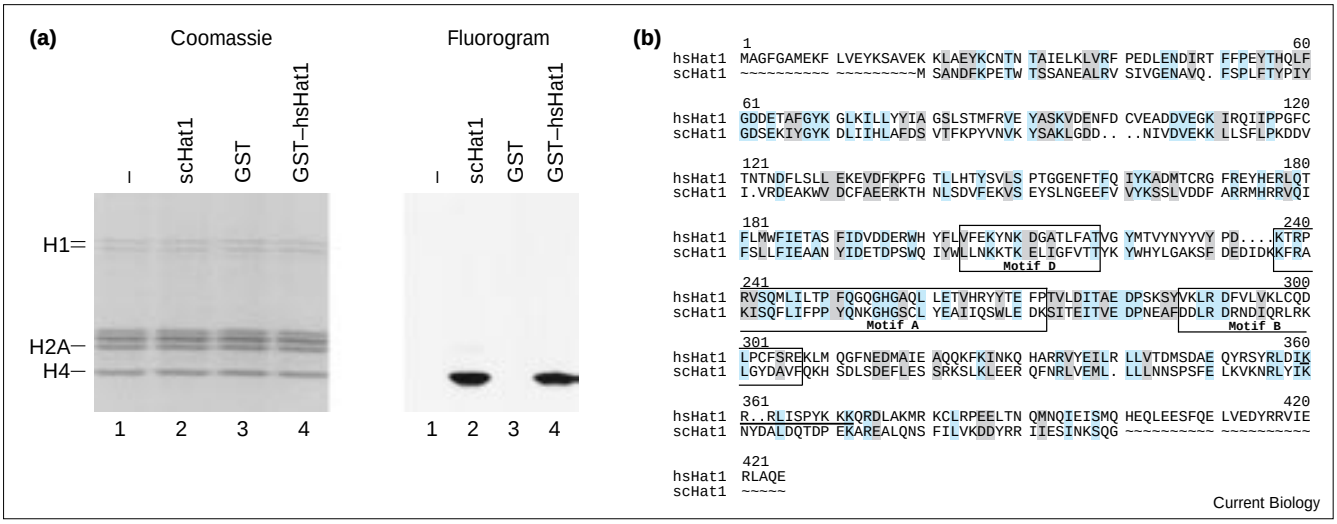
A partial cDNA clone (ID 24027, GenBank accession number T78280) encoding a potential human orthologue of *S. cerevisiae* Hat1p was identified by screening the Merck–Washington University database of expressed sequence tags (ESTs) [20], and a full-length cDNA was subsequently isolated. In order to determine whether this cDNA encodes a histone acetyltransferase (HAT), the protein was expressed as a glutathione-S-transferase (GST) fusion in *Escherichia coli*, purified and used in HAT assays. As shown in Figure 1a (lanes 2,4), both *S. cerevisiae* Hat1p and the protein expressed from the human EST cDNA clone 24027 had intrinsic histone H4 acetyltransferase activity when expressed in *E. coli*. The specific activity of the human Hat1 subunit purified from *E. coli* was much lower than that of the Hat1 enzyme purified from human cells, however (data not shown; see below). Consequently, a large amount of Hat1 and a long exposure to film were required to obtain the fluorogram in Figure 1a.

The sequence of human Hat1 is 29% identical and 55% similar to that of *S. cerevisiae* Hat1p and the similarity is distributed throughout the entire open reading frame (Figure 1b). Particularly noteworthy is the presence of the D, A and B HAT signature motifs (boxed in Figure 1b) previously identified by the PROBE multiple sequence alignment algorithm [21]. On the basis of their presence in many enzymes that acetylate substrates other than histones [21], these amino-acid sequence motifs are likely to participate in either the binding of acetyl-coenzyme A or enzyme catalysis, rather than in histone recognition. In addition to human Hat1, database searches identified a *Caenorhabditis elegans* gene that encodes a putative nematode homologue of Hat1. Therefore, as might be expected from the high degree of conservation of newly synthesized H4 acetylation [9], the Hat1 polypeptide is conserved in at least three widely divergent organisms.

Purification of the human Hat1 holoenzyme

In order to find out whether the Hat1 catalytic subunit was associated with other proteins, antibodies raised against a synthetic peptide derived from the amino terminus of Hat1 were used for immunoaffinity purification of the Hat1 enzyme from human 293 cells, as summarized in Figure 2a. This purification took advantage of the isolation from crude antisera of antibodies that dissociate from Hat1 under acidic conditions. As a final step in the purification, the Hat1 enzyme was sedimented through a glycerol gradient and the fractions analyzed for HAT activity and by SDS–polyacrylamide gel electrophoresis. As shown in Figure 2b, a HAT activity co-sedimented with two prominent polypeptides of apparent molecular masses 46 kDa and 50 kDa in SDS–polyacrylamide gels. Western blotting analysis identified the lower, 46 kDa, polypeptide as Hat1 (Figure 2c, left panel). The 50 kDa polypeptide in the

Figure 1



The human Hat1 cDNA encodes the catalytic subunit of a histone H4 acetyltransferase. (a) A crude extract from *E. coli* cells expressing *S. cerevisiae* Hat1 (lane 2), or human GST–Hat1 expressed in *E. coli* and affinity purified using glutathione–Sepharose beads (lane 4) was assayed for HAT activity with [^{3}H]acetyl-coenzyme A and total histones as substrates. The histones were resolved by electrophoresis through an SDS–18% polyacrylamide gel, which was stained with Coomassie blue and subjected to fluorography. Negative control reactions were performed without enzyme (lane 1) or with GST alone (lane 3). (b) Sequence alignment of *Homo sapiens* Hat1 (hsHat1) and *S. cerevisiae* Hat1 (scHat1). Residues that are identical among the two proteins are indicated by blue shading, while similar residues are shaded gray. Motifs D, A and B, which are present in a large number of N-acetyltransferases including enzymes that acetylate substrates other than histones, are boxed [21]. A region in hsHat1 with similarity to the bipartite nuclear localization signal of nucleoplasmin [27] is underlined.

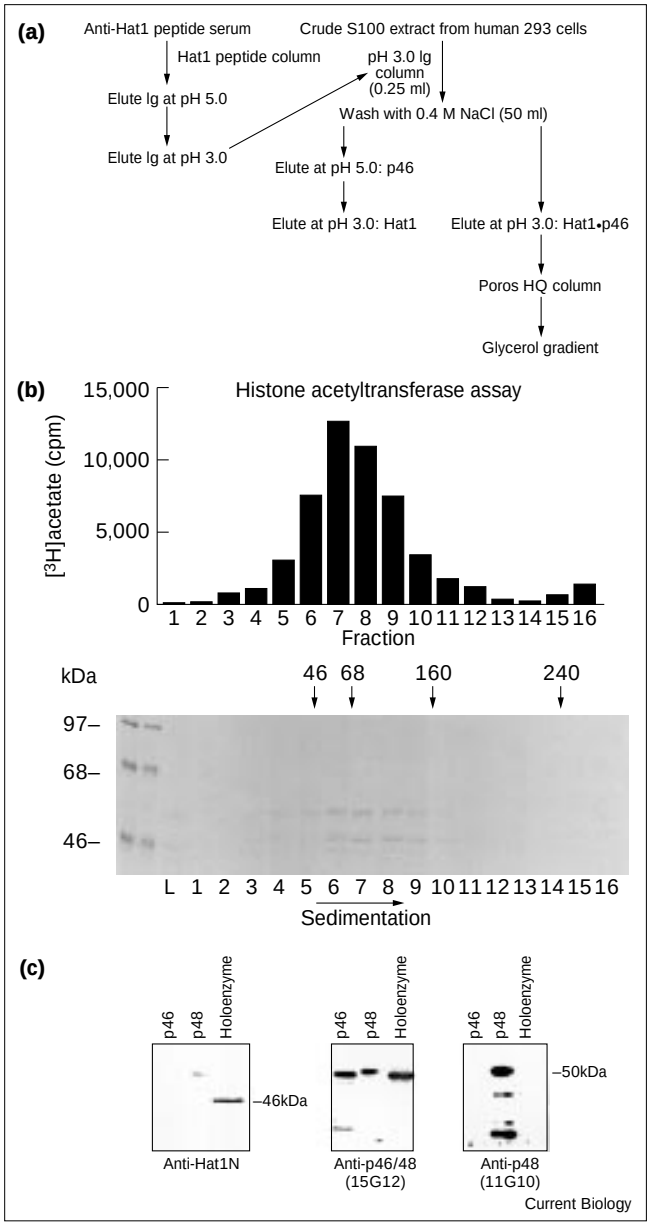
purified Hat1 holoenzyme sample was recognized by monoclonal antibodies that react equally well against both p46 and p48, such as antibody 15G12 (Figure 2c, middle panel) or antibodies 12B1 and 19H9 (data not shown). In contrast, p48-specific monoclonal antibodies such as antibody 11G10 (Figure 2c, right panel) or antibody 13D10 (data not shown) did not detect any polypeptide in the purified Hat1 holoenzyme preparation, indicating that the polypeptide co-purifying with Hat1 was p46 and not p48. In addition, western blots of purified Hat1 holoenzyme failed to detect the presence of the Rb p110 polypeptide (data not shown).

The p46 subunit stimulates the acetyltransferase activity of Hat1

Hat1 and p46 reversibly dissociated under mildly acidic conditions (Figure 2a). This provided a simple way to isolate the two subunits of the Hat1 holoenzyme in a purified form, to address the importance of p46 for HAT activity (Figure 3a). Although the Hat1 polypeptide was clearly the catalytic subunit of the enzyme (Figure 1), its activity in the absence of p46 was very weak (Figure 3b). Titration of increasing amounts of p46 in the presence of a constant amount of Hat1 catalytic subunit stimulated the activity of the enzyme (approximately 15-fold with a 3–4-fold molar excess of p46 over Hat1; Figure 3b).

As p46 had no intrinsic HAT activity (Figure 3b) and its yeast orthologue Hat2p enhances binding of the holoenzyme to the amino-terminal domain of H4 [13], it seemed plausible that p46 might stimulate Hat1 by binding directly to core histones and thereby promoting their association with Hat1. To test this hypothesis with purified proteins, we performed binding assays with GST–histone fusion proteins (purified from *E. coli)* and either the Hat1 holoenzyme or its individual subunits. The holoenzyme (Hat1·p46) bound efficiently to H4 and, to a lesser extent, to histone H2A, but did not bind to either histone H2B or H3 (Figure 4). In keeping with its low specific activity, the Hat1 catalytic subunit alone did not bind tightly to any of the four core histones (Figure 4). In contrast, recombinant p46 or the related p48 protein purified from *E. coli* bound to both H2A and H4 equally well. Surprisingly, recombinant p46 and p48 expressed and purified from Sf9 cells (Figure 4) and p46 purified from human cells (data not shown) bound specifically to H4. It is conceivable that this difference may be due to post-translational modification of p46 and p48 from Sf9 and human cells. Alternatively, it is possible that the histone-binding specificity of p46 and p48 may be affected by the 15-amino-acid amino-terminal peptide remaining fused to the proteins following their expression in *E. coli* as GST fusion proteins and subsequent processing with thrombin. On the other hand, given that the Hat1 holoenzyme bound weakly to H2A and also acetylated the H2A (see below), p46 in the native Hat1 holoenzyme may contribute to H2A binding and acetylation.

Figure 2



The human Hat1 holoenzyme consists of two subunits, Hat1 and p46. (a) Scheme for immunoaffinity purification of the Hat1 holoenzyme and its polypeptide subunits from human cells. (b) Glycerol gradient sedimentation of the Hat1 holoenzyme purified from human 293 cells. The gradient was fractionated into 16 samples which were assayed for HAT activity (upper panel) and subjected to electrophoresis through an SDS–10% polyacrylamide gel stained with Coomassie blue (lower panel). The location of protein size markers sedimented in a parallel glycerol gradient is shown at the top: 46 kDa is ovalbumin, 68 kDa albumin, 160 kDa aldolase and 240 kDa catalase. (c) Peak fractions from the glycerol gradient were immunoblotted and probed with anti-Hat1N antibodies, a monoclonal antibody that recognizes both p46 and p48 (15G12) and a monoclonal antibody specific for p48 (11G10). Recombinant p46 and p48 purified from *E. coli* (lanes 1,2) were run as size markers alongside the Hat1 holoenzyme (lane 3 in each panel). The immunoreactive lower molecular weight species are carboxy-terminal truncations of p46 and p48 expressed in *E. coli*.

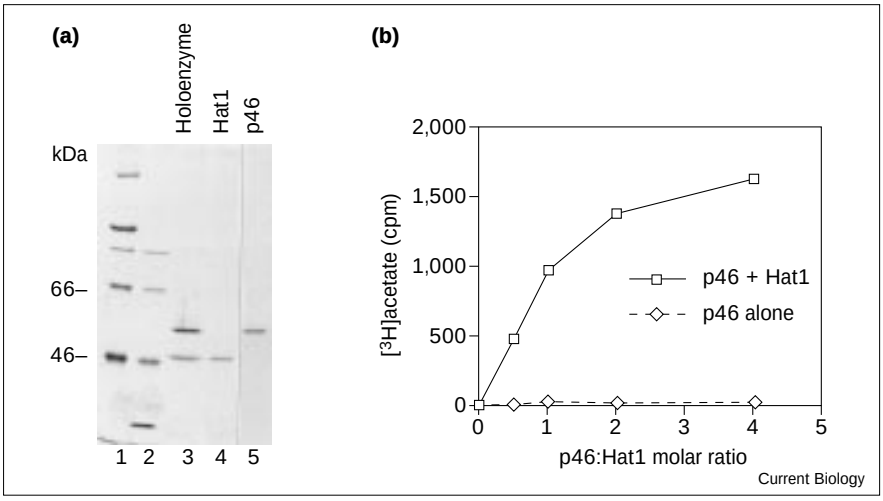
The human Hat1 holoenzyme is a B-type HAT that acetylates H2A at Lys5 and H4 at Lys5 and Lys12

Using purified histones as substrates, the human Hat1 holoenzyme acetylated H2A and H4 but did not acetylate H2B or H3 (Figure 5a, lanes 5,6; Figure 5b, lanes 3,4). This was true whether H2A and H4 were present as monomeric proteins or were associated into H2A·H2B dimers or $H3_2{\cdot}H4_2$ tetramers (data not shown). Acetylation of H4 was more efficient than acetylation of H2A, roughly reflecting the greater affinity of the Hat1 holoenzyme for H4 than for H2A (Figure 4, top). In addition, acetylation of H4, but not of H2A, was detectable when an equimolar mixture of H2A·H2B dimers and $H3_2{\cdot}H4_2$ tetramers was used as the substrate (data not shown), arguing that the Hat1 holoenzyme preferentially modified H4 rather than H2A.

The Hat1 holoenzyme did not acetylate core histones that are part of oligonucleosomes purified from human cells (Figure 5a, lane 7). Upon long exposure of the gel to film, weak acetylation of nucleosomal H4 could be detected (data not shown), but this could be due to acetylation of H4 near the ends of an oligonucleosome. The inability of the Hat1 holoenzyme to acetylate nucleosomal histones may be due to steric hindrance by either H2A·H2B dimers or DNA. To test whether DNA was sufficient to block acetylation, recombinant core histone tetramers (which are efficiently acetylated by the Hat1 holoenzyme; Figure 5a, lane 5) were reconstituted onto covalently closed circular DNA by salt gradient dialysis. As shown in Figure 5a (lane 8), the presence of DNA completely prevented acetylation of the core histone tetramers. This was not due to non-specific inhibition of the enzyme by DNA, because addition of random hexanucleotides (to the same amount as the DNA in Figure 5a, lane 8) had no inhibitory effect on the enzyme (data not shown). We attribute this lack of inhibition to the fact that these short oligonucleotides are too small to impede the interaction between Hat1 and the histones. The inability of the human Hat1 holoenzyme to acetylate nucleosomal histones was also observed with the yeast enzyme [13] and is consistent with Hat1 being a B-type acetyltransferase — that is, an enzyme that acts on newly synthesized histones prior to their incorporation into chromosomes.

To determine which amino acids of H2A and H4 were acetylated, recombinant $H3_2{\cdot}H4_2$ tetramers or H2A·H2B dimers were purified from *E. coli* and used as substrates, because these histones were completely non-acetylated when expressed in bacteria [22]. The recombinant histones were acetylated with the Hat1 holoenzyme and [^{3}H]acetyl-coenzyme A and were then analyzed by electrophoresis in acetic acid–urea gels. Only one radiolabeled species was obtained for H2A, whereas two labeled species were observed for H4 (Figure 5b, lanes 3,4). The acetylated H4 species co-migrated with mono-acetylated and diacetylated H4 in acetic acid–urea gels. This suggested

Figure 3



The p46 subunit stimulates the activity of the human Hat1 catalytic subunit. (a) Silver-stained SDS–10% polyacrylamide gel of the Hat1 holoenzyme (lane 3), the Hat1 catalytic subunit (lane 4) and the p46 subunit (lane 5) purified from human 293 cells. Lanes 1 and 2 contain molecular weight markers. (b) HAT activity (determined using a phosphocellulose filter binding assay) was assayed with increasing amounts of either p46 alone (0, 0.5, 1, 2 and 4 pmol p46) or p46 in the presence of a constant amount of Hat1 catalytic subunit (1 pmol Hat1 and 0, 0.5, 1, 2 and 4 pmol p46).

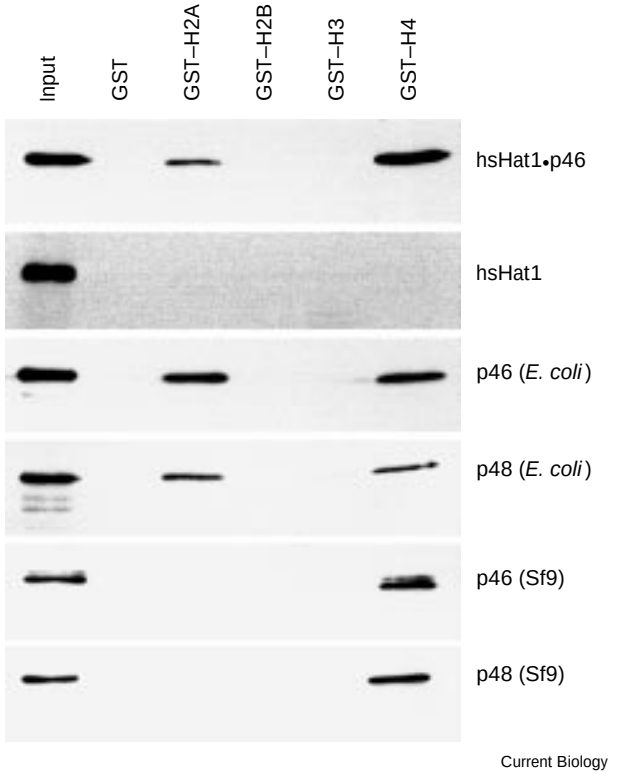
that Hat1 could acetylate a single lysine residue in H2A, whereas up to two lysines could be modified in H4. The identity of the modified sites was revealed by amino-terminal sequencing of [^{3}H]acetylated recombinant H2A and H4, which was readily achieved with histones expressed in *E. coli* because, unlike H2A and H4 purified from human cells, they were not blocked by α-amino group acetylation of the amino-terminal serine residue [22]. Upon Edman degradation, a single peak of radioactivity was released from H2A corresponding to Lys5 acetylation, whereas two approximately equal peaks of radioactivity at Lys5 and Lys12 were produced from H4 (Figure 5c). In addition, in the case of the H4 sample, a large peak of radioactivity also eluted during the first cycle of Edman degradation. This peak of radioactivity was reproducible and was both enzyme- and histone-dependent; it could not, however, be attributed to acetylation of either the amino or the hydroxyl group of the H4 amino-terminal serine (α-amino group acetylation would prevent Edman degradation and no radiolabeled O-acetylserine was released during the first Edman degradation cycle; data not shown). The source of this peak of radioactivity is currently unclear. Interestingly, the three sites modified by human Hat1 in H2A and H4 have a common amino-acid sequence motif surrounding the acetylated lysine that fits the consensus sequence GXGKXG (in single-letter amino-acid code, where X is any amino acid) previously established for the *S. cerevisiae* Hat1 enzyme [13]. Thus, the substrate specificity of the human Hat1 holoenzyme *in vitro* recapitulates the Lys5, Lys12 pattern of diacetylation characteristic of newly synthesized H4. Moreover, the substrate specificity of the human Hat1 holoenzyme is very similar to that of its yeast ortholog, the only difference being that human Hat1 acetylates both Lys5 and Lys12 with approximately equal efficiency, whereas the yeast enzyme acetylates Lys12 more efficiently than Lys5 [13].

Binding of p46 and p48 to amino-acid residues within helix 1 of the histone-fold domain of H4

Trypsin digestion removes at least residues 1–10 from H2A and residues 1–17 from H4 [23], which include sequences surrounding the sites of acetylation within the amino-terminal domains of H2A and H4. To determine whether these sequences were necessary for binding to p46, co-precipitation assays were performed with either intact or trypsinized histones and p46 expressed in *E. coli* as a GST fusion protein. As illustrated in Figure 6a, both intact and trypsinized $H3_2{\cdot}H4_2$ tetramers or H2A·H2B dimers almost quantitatively bound to GST–p46 (which, in this experiment, was present in a roughly equimolar amount to the histones). We obtained identical results with GST–p48 (data not shown). This experiment demonstrated that sequences immediately surrounding the sites of acetylation were not necessary for binding to p46 or p48. As might be anticipated from the fact that DNA inhibits histone acetylation by the Hat1 holoenzyme (Figure 5a), p46 and p48 did not bind to either oligonucleosomes or $H3_2{\cdot}H4_2$ tetramers assembled onto circular DNA (data not shown).

In order to map more precisely the region(s) of H4 required for recognition by p46 and p48, a series of carboxy-terminal deletion mutants lacking the various secondary structure elements of H4 (Figure 6b) were expressed as GST fusion proteins and purified from *E. coli*. Equal amounts of each GST–H4 protein were used in pull-down assays with p46 (identical results were obtained with p48). $H4_{1–41,}$ a small peptide containing only the amino-terminal domain and helix 1 within the histone-fold domain of H4, bound to p46 as efficiently as longer H4 mutants and even full-length H4 (Figure 6c, lanes 1–6). In contrast, $H4_{1–34}$ and $H4_{35–102}$, which lack the distal and proximal portion, respectively, of helix 1, did

Figure 4



The p46 and p48 polypeptides bind directly to H2A and H4, and p46 is necessary for stable binding of the Hat1 holoenzyme to core histones. Pull-down assays were performed with purified proteins (Hat1·p46, Hat1, p46 or p48) and either GST beads or GST–histone beads (GST fused to H2A, H2B, H3 and H4). Recombinant p46 and p48 proteins from either *E. coli* or Sf9 cells were used. After extensive washes of the glutathione–Sepharose beads in buffer A300, the polypeptides that remained bound to GST–histones were detected by SDS–PAGE and western blotting. In each panel, the input lane contains the equivalent of half of the protein used in each pull-down assay.

not bind to p46 (Figure 6c, lanes 7,8). Therefore, amino-acid residues within helix 1 (residues 31–40) in the histone-fold domain of H4 were critical for binding to p46. To find out whether sequences in the amino-terminal domain of H4 were also important, a series of amino-terminal deletions were assayed. Both $H4_{8–41}$ (which lacks the Lys5 acetylation site) and $H4_{15–41}$ (which lacks both the Lys5 and Lys12 acetylation sites) bound to p46 as efficiently as $H4_{1–41}$, which has an intact amino-terminal domain (Figure 6c, lanes 9–13). $H4_{21–41}$ and $H4_{28-41}$, however, bound to p46 less strongly (Figure 6c, lanes 14,15). Although this might indicate that p46 made contacts with the distal portion of the amino-terminal domain of H4 (residues 16–30), we cannot rule out the possibility that p46 binding was occluded when H4 helix 1 was located near the GST moiety, especially in the $H4_{28–41}$ mutant in which helix 1 was immediately next to the GST moiety. In addition, even if p46 made contacts with the amino-terminal domain of H4, this interaction was not sufficient for high-affinity binding to p46, because the $H4_{1–34}$ mutant did not bind under our assay conditions (Figure 6c, lane 7).

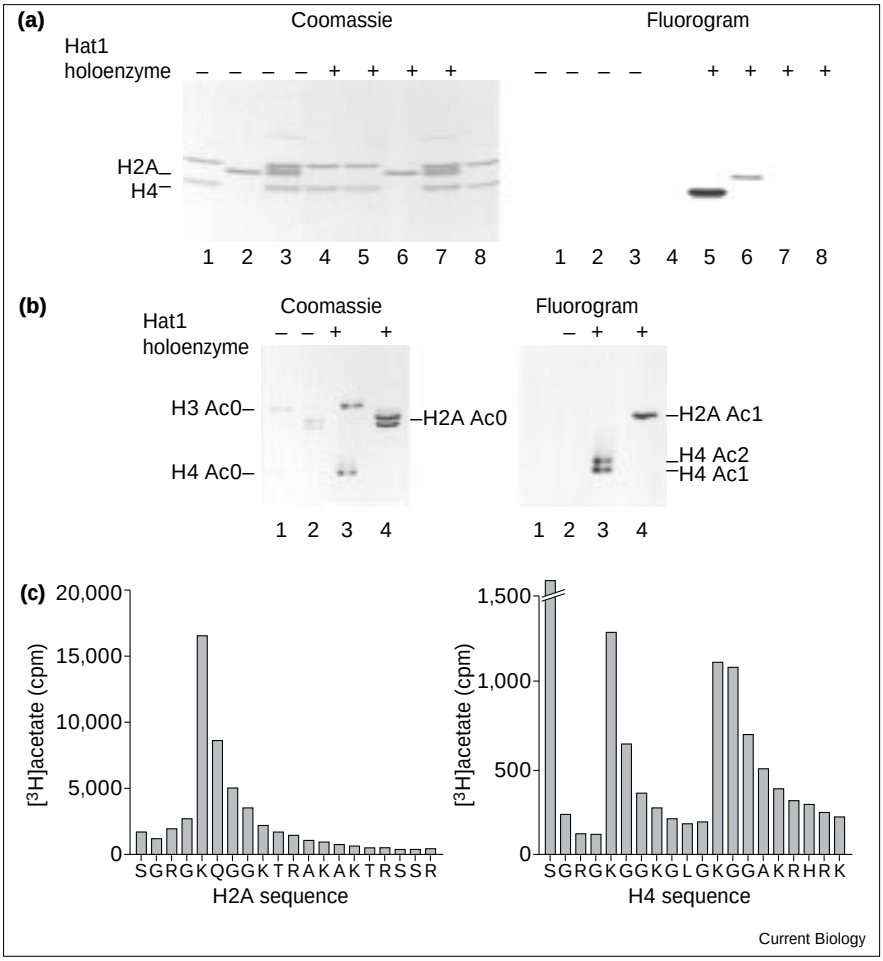
The four types of core histone share the common polypeptide backbone structural motif known as the histone fold [24]. Because the Hat1 holoenzyme also bound to and acetylated H2A (Figures 4,5a), we determined whether H2A binding by p46 required the region corresponding to helix 1 of H4. This was indeed the case: p46 bound to $H2A_{1–38}$ (H2A amino-terminal domain plus helix 1) as efficiently as to intact H2A (Figure 6d, lanes 5,6) but did not bind to a mutant protein encompassing only the amino-terminal domain, $H2A_{1–29}$ (Figure 6d, lane 4). We do not know whether the α-helical character of this region is required for binding. Interestingly, however, H2A and H4 share a significant degree of amino-acid sequence similarity in helix 1 of their histone folds, whereas the corresponding α helices of H3 and H2B are somewhat less similar (Figure 6e). It seems likely, therefore, that specific amino-acid residues within helix 1 in the histone-fold domains of H2A and H4 are critical determinants of p46 and p48 binding.

Given that p46 stimulated acetylation of H4 by the Hat1 catalytic subunit (Figure 3b), it seemed likely that a strong binding site in H4 for p46 would enhance the activity of the Hat1 holoenzyme. This was indeed the case, because the holoenzyme acetylated the $H4_{1–41}$ mutant (amino-terminal domain plus helix 1) about five-fold more efficiently than the amino-terminal domain alone ($H4_{1–34}$ in Figure 6f). This relatively small effect is probably due to additional contacts between either p46 or Hat1 and the amino-terminal domain of H4 which, although transient, may enable the holoenzyme to acetylate the $H4_{1–34}$ mutant relatively efficiently.

Hat1 and p46 are located in the nucleus of S-phase cells

Because the bulk of histone synthesis is restricted to S phase of the cell cycle, B-type HATs such as Hat1 are likely to be required mainly during this period. In order to determine the intracellular localization of the Hat1 protein in S-phase cells, a construct for transient expression of Hat1 tagged with the FLAG epitope was transfected into asynchronously growing COS1 cells and the transfected cells were pulse-labeled with bromodeoxyuridine (BrdU) to label S-phase cells specifically. On the basis of biochemical fractionation experiments, it has been proposed that B-type HATs are located in the cytoplasm [13,25,26]. Unexpectedly, the FLAG-epitope-tagged Hat1 polypeptide (red in Figure 7) was clearly located in the nucleus of S-phase cells (green in Figure 7). A similar result was also obtained using a construct expressing FLAG-epitope-tagged p46 (data not

Figure 5



The Hat1 holoenzyme purified from human 293 cells is a B-type acetyltransferase that modifies H2A at Lys5 and H4 at Lys5 and Lys12. **(a)** The Hat1 holoenzyme was used to acetylate various histone substrates in the presence of [^{3}H]acetyl-coenzyme A and the reaction products were resolved by SDS–PAGE. The substrates used were recombinant $H3_2{\cdot}H4_2$ tetramers (lanes 1,5), recombinant H2A (lanes 2,6), oligonucleosomes lacking histone H1 (lanes 3,7) and recombinant $H3_2{\cdot}H4_2$ tetramers assembled onto plasmid DNA (lanes 4,8). **(b)** HAT assays were performed with recombinant $H3_2{\cdot}H4_2$ tetramers (lanes 1,3) or H2A·H2B dimers (lanes 2,4). The reaction products were analyzed by electrophoresis through an acetic acid–urea–polyacrylamide gel. Histone species with zero, one, or two acetyl groups are indicated by Ac0, Ac1 and Ac2, respectively. **(c)** The products of HAT assays performed as described in (a) were subjected to amino-terminal sequencing and the amount of radioactivity released during each Edman degradation cycle determined. As indicated by the truncated bar, a large peak of radioactivity (4,400 cpm) was released during cycle 1 of the H4 sequencing reaction.

shown). In some transfected S-phase cells a weak cytoplasmic signal could also be detected, but nuclear staining was always predominant (data not shown). Thus, although this result did not rule out the possibility that some Hat1 holoenzyme may be in the cytoplasm, it clearly showed that a substantial portion of the enzyme was in the nucleus during S phase.

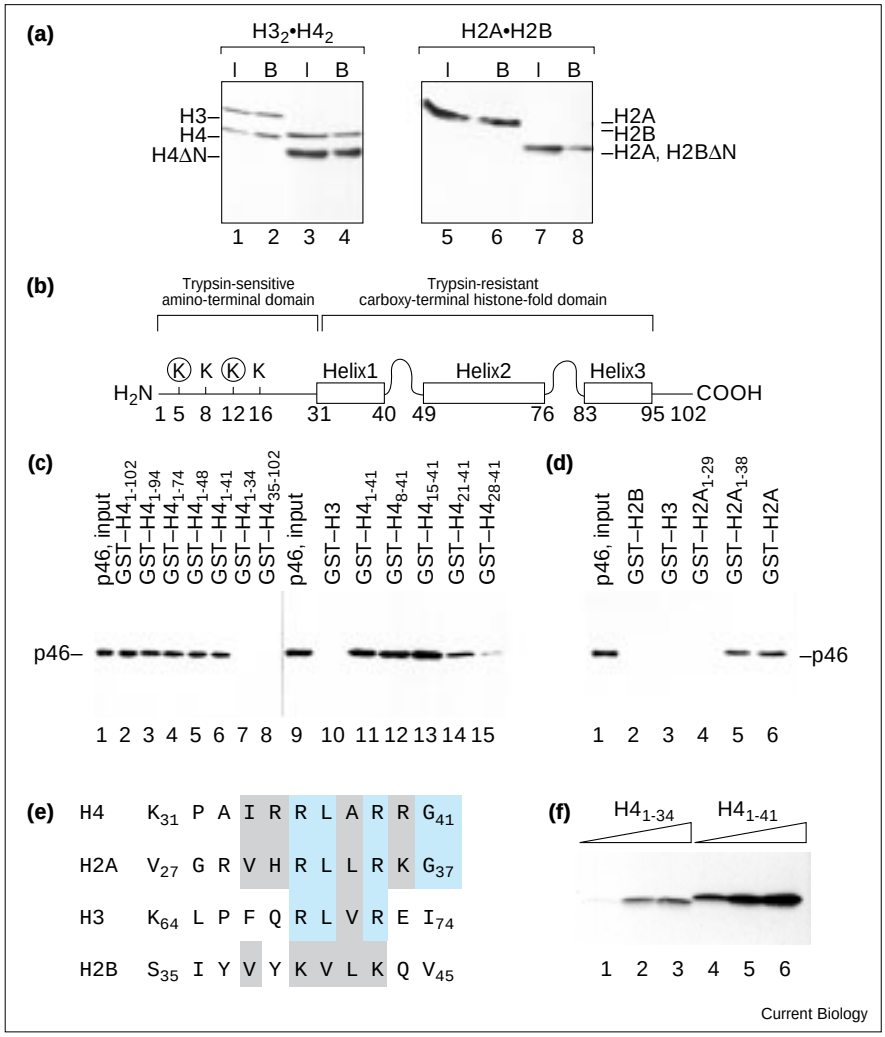
In primate cells, there is evidence suggesting that the large amounts of Hat1 present in cytosolic extracts, such as the S100 extract used here to purify the holoenzyme, is due to the extraction of a soluble nuclear enzyme during biochemical fractionation. For instance, both endogenous Hat1 and the FLAG–Hat1 protein, which are clearly nuclear as judged by immunofluorescence (Figure 7 and data not shown), are found predominantly in cytosolic extracts when biochemical fractionation is performed by hypotonic swelling and Dounce homogenization, although, in both cases, detectable amounts of Hat1 remain in nuclear extracts (data not shown). In addition, affinity-purified antibodies directed against the endogenous Hat1 polypeptide predominantly stain the nucleus of HeLa cells (data not shown).

Inspection of the amino-acid sequences of both subunits of the Hat1 holoenzyme for motifs conforming to the known nuclear localization signals [27] revealed only one putative bipartite signal in the Hat1 subunit, which is not conserved in *S. cerevisiae* Hat1 (underlined in Figure 1b). Deletion of this putative nuclear localization signal had no effect on the intranuclear localization of Hat1, however (data not shown). Thus, perhaps Hat1 or p46 harbors a cryptic nuclear localization signal. Alternatively, the enzyme might enter the nucleus via its association with an unidentified polypeptide, or may enter during nuclear envelope breakdown at mitosis.

Discussion

We show here that human cells contain a two-subunit B-type histone acetyltransferase holoenzyme. The catalytic subunit, Hat1, is homologous to yeast Hat1p [13], and the core-histone-binding subunit, p46, is a member of a

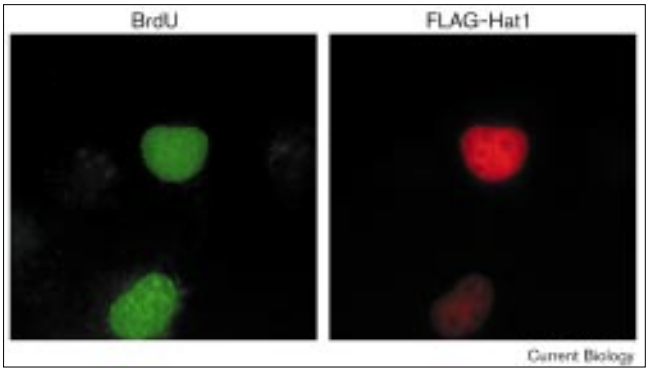
Figure 6



Amino-acid residues within the first α helix of the histone-fold domains of H2A and H4 are critical for high-affinity binding to p46. **(a)** Pull-down assays were performed with intact (lanes 1,2,5,6) or trypsinized (ΔN; lanes 3,4,7,8) $H3_2{\cdot}H4_2$ tetramers or H2A·H2B dimers and GST–p46 purified from *E. coli*. Lanes labeled I contain half of the input histones. After extensive washes of the glutathione–Sepharose beads in buffer A300, the histones that remained bound to GST–p46 (lanes labeled B) were detected by western blotting with anti-histone antibodies. **(b)** The secondary structure of histone H4 based upon the crystal structures of the histone octamer and the nucleosome core [24,37]. The four acetylatable lysines in the amino-terminal domain of H4 are indicated. The two lysines acetylated by Hat1 are circled. The residues bordering each structural element of the H4 protein are numbered. **(c)** Pull-down assays were performed with p46 (purified from Sf9 cells) and GST fusion proteins encoding deletion mutants of the H4 protein (the numbers indicate the amino- and carboxy-terminal amino acids of each mutant). After extensive washes of the glutathione–Sepharose beads in buffer A200, the p46 protein that remained bound to GST–histones was detected by western blotting. Input lanes contain the equivalent of half of the input protein used in each pull-down assay. **(d)** Pull-down assays were performed with p46 (purified from *E. coli*) and GST fusion proteins encoding deletion mutants of the H2A protein ($H2A_{1–29}$ encodes the amino-terminal domain only; $H2A_{1–38}$ encodes the amino-terminal domain plus helix 1 of H2A). **(e)** Amino-acid sequence similarities in helix 1 of the histone-fold domains of human core histones. Identical amino-acid residues are shaded blue, while similar residues are shaded gray. Note that Pro32 in H4 and Pro66 in H3 are part of helix 1 in the crystal structure of the nucleosome core [37]. Helix 1 of H3 extends to Phe78; only the portion similar to helix 1 in the other core histones is shown here. **(f)** Helix 1 enhances acetylation of the amino-terminal domain of H4 by the Hat1 holoenzyme. Equal amounts of peptides $H4_{1–34}$ (amino-terminal domain; lanes 1–3; 1.4, 2.8 and 5.6 pmol, respectively) and $H4_{1–41}$ (amino-terminal domain plus helix 1; lanes 4–6; 1.4, 2.8 and 5.6 pmol, respectively) were acetylated *in vitro* with [^{3}H]acetyl-coenzyme A and Hat1 holoenzyme (0.35 pmol). The reaction products were resolved by electrophoresis through an SDS–tricine–polyacrylamide gel and detected by fluorography.

highly conserved subfamily of proteins containing WD-repeat motifs, which are found in several proteins that bind to and modify histones [5,13,17,18]. The Hat1 holoenzyme described here is likely to be the same as an enzyme that was previously partially purified from HeLa cells [25], because it has the same substrate specificity (for Lys5 and Lys12 of H4), a similar native molecular weight of approximately 100 kDa and a catalytic subunit of similar apparent molecular weight in SDS–polyacrylamide gels — the 46 kDa Hat1 polypeptide. The human enzyme is also strikingly similar in both polypeptide composition and substrate specificity to the *S. cerevisiae* Hat1p·Hat2p holoenzyme [13]. In addition, an enzyme with a very similar polypeptide composition and substrate specificity was recently purified from maize embryos [28]. Interestingly, the activity of the maize enzyme is closely linked to DNA synthesis during embryo germination [28]. Yeast and human Hat1, as well as related HATs from other species [29], modify H2A *in vitro*. This conservation suggests that H2A acetylation may be physiologically important, although its role is currently not known. Unlike the case of H3 and H4, acetylation of newly synthesized H2A or H2B in human cells is not prominent, if it occurs at all [30], suggesting that acetylation of H2A probably occurs at times other than its synthesis.

Figure 7



The Hat1 polypeptide is located in the nucleus during S phase in COS1 cells. COS1 cells were electroporated with a construct for transient expression of FLAG-epitope-tagged Hat1 and pulse-labeled with BrdU ~36 h post-transfection. The FLAG–Hat1 protein was detected with monoclonal M2 antibody against the FLAG epitope, followed by a Texas red-conjugated anti-mouse immunoglobulin secondary antibody. BrdU incorporation into DNA was detected using a fluorescein-conjugated anti-BrdU monoclonal antibody.

The Hat1 holoenzyme has the appropriate substrate specificity for a B-type HAT; these are enzymes dedicated to acetylation of newly synthesized histones. It was therefore surprising to find that most of the Hat1 and p46 polypeptides are located in the nucleus of S-phase cells. Although a number of previous reports describe B-type HATs as cytoplasmic [13,25,26,28], these studies were all based upon biochemical fractionation experiments and may have been affected by the fact that Hat1 is readily extractable from nuclei. It is formally possible that Hat1 may have an unknown function to play in the nucleus, but because the enzyme cannot acetylate nucleosomal histones, we think this is unlikely. Our results do not rule out the possibility that newly synthesized H4 may be acetylated prior to its entry into the nucleus. For instance, the Hat1 holoenzyme may be rapidly co-transported into the nucleus along with a complex of newly synthesized H3 and H4. Interestingly, a potential homolog of Hat1 has been identified in *Tetrahymena* which acetylates H4 at Lys4 and Lys11 (which correspond to Lys5 and Lys12 in human H4). On the basis of biochemical fractionation, this enzyme is present both in the cytoplasm and micronuclei, but not in macronuclei [31]. Conceivably, the intracellular localization of Hat1 may be regulated according to cell-cycle stage or cell type.

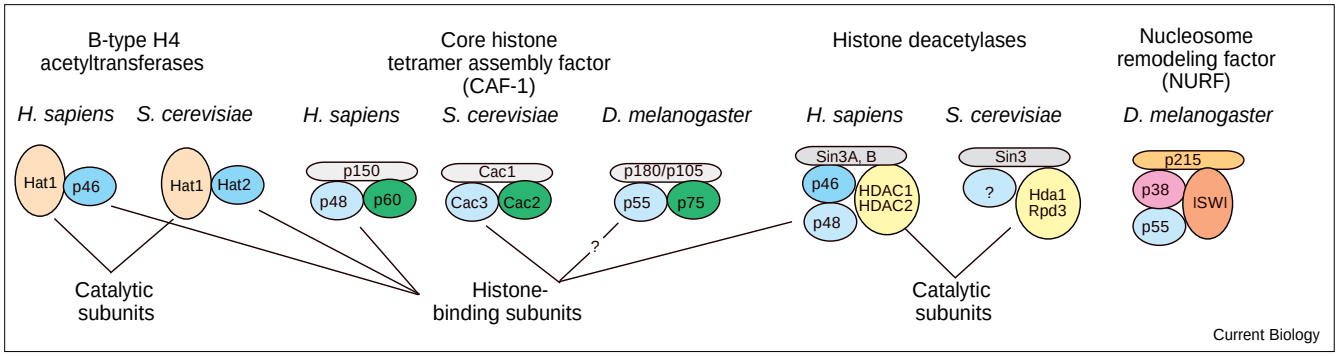
The ability of p46 to bind selectively to H2A and H4 (both of which are substrates of the Hat1 holoenzyme) and not to H2B or H3 provides a first level of substrate specificity to the Hat1 holoenzyme. A second level of specificity lies in the ability of this enzyme to selectively acetylate lysine residues which conform to the GXGKXG consensus motif [13]. The recognition of the sequence immediately surrounding the lysine to be acetylated could be mediated by the Hat1 catalytic subunit or may involve additional contacts between the p46 subunit and the amino-terminal domains of H2A and H4.

The identification of p46 as a subunit of the Hat1 holoenzyme in human cells reveals that p46/p48 family members are integral subunits of three types of protein known to be involved in the *de novo* nucleosome assembly pathway, namely the B-type histone H4 acetyltransferase, the replication-dependent core histone tetramer assembly factor CAF-1 and the histone deacetylases (Figure 8). As illustrated in Figure 8, a remarkably similar situation exists in *S. cerevisiae*, although it is not known whether Cac3p (or Hat2p) is a subunit of any of the five known histone deacetylases of *S. cerevisiae* [19].

In spite of their high degree of amino-acid sequence similarity, the p46 and p48 polypeptides are biochemically distinct in that p46 is found uniquely in the Hat1 enzyme, whereas only p48 is associated with CAF-1 (Figure 8). As explained in the Background, there is both genetic and biochemical evidence that the two corresponding homologs to p46 and p48 in *S. cerevisiae*, Hat2p and Cac3p (Figure 8), are also functionally distinct. Although the two human proteins bind to core histones with identical specificity, the existence of this functional disparity between p46 and p48 in yeast and human cells is intriguing. It is currently unclear, however, whether the existence of two distinct core-histone-binding proteins will extend to organisms other than yeast and human. For instance, in spite of extensive efforts, only a single analog of p46 and p48, known as p55 [32], has thus far been identified in *Drosophila*. The p55 polypetide is a subunit of *Drosophila* CAF-1, associates with acetyltransferases and deacetylases in cell extracts [32,33] and, interestingly, is also an integral subunit of the NURF protein (Figure 8), an ATP-dependent nucleosome remodeling factor. NURF alters histone–DNA interactions and is thought to play a role in enhancing the accessibility of transcription factors to their DNA-binding sites in chromatin. Although p55 is associated with histones in cell extracts, the recombinant protein has not yet been shown to bind directly to core histones [33]. By analogy with human p46 and p48, however, it seems likely that p55 will also be found to bind to histone H4 directly.

Although the ability of the p46/p48 family members to bind directly to core histones may be sufficient to justify their presence in the Hat1 acetyltransferase, the CAF-1 nucleosome assembly factor, the histone deacetylases and NURF, we cannot formally rule out the possibility that p46/p48 may have additional biochemical functions aside from bringing these four different types of proteins to their histone substrates. The p46 subunit is clearly important for Hat1 enzyme function because p46 strongly

Figure 8



Members of the p46/p48 family of core histone-binding proteins are involved in many aspects of histone metabolism in *S. cerevisiae*, *Drosophila* and human cells.

enhances the activity of the Hat1 catalytic subunit (Figure 3) [13]. Gene disruption in *S. cerevisiae* [14] also demonstrated that Cac3p is required for CAF-1 activity, although the telomeric silencing defect of a yeast strain lacking Cac3p (*cac3Δ*) is less prominent than that of either the *cac1Δ* or *cac2Δ* strains (strains lacking either one of the other two subunits of yeast CAF-1). The precise biochemical function of Cac3p or p48 as subunits of CAF-1 is not clear, however. This is because, in addition to p48, the p150 subunit of CAF-1 also binds directly to core histones, albeit with no apparent histone specificity, and a mutant CAF-1 protein lacking p48 binds stably to $H3_2{\cdot}H4_2$ tetramers (A.V., unpublished observations). It is possible that the other subunits of the CAF-1 protein make contacts with histone H3 or with regions of H4 other than helix 1 in the histone fold. Thus, p48 may not be absolutely necessary for CAF-1 to bind core histones. It may be that the sole function of p48 in CAF-1 is to attract the catalytic subunit of a histone deacetylase, via protein–protein interactions, to the sites of newly synthesized histone deposition. In this regard, it seems possible that at least some of the numerous genes whose transcriptional regulation is affected by disruption of the yeast *RPD3* gene [19,34,35] are activated because of a failure to deacetylate newly synthesized histones, given that the *rpd3Δ* yeast strain accumulates high levels of H4 acetylated at Lys5 and Lys12 [19].

Interestingly, a deletion that eliminates the binding site for p46 and p48 in histone H4 (that is, eliminating the amino-terminal domain and helix 1) prevents stable incorporation of this H4 mutant into *Xenopus* embryo chromatin *in vivo* [36]. A double point mutation of two conserved arginine residues within helix 1 (R35A, R36A) did not, however, prevent incorporation of this H4 mutant into chromatin [36]. Because helix 1 of H4 also contributes to heterodimerization with H3 and makes important interactions with nucleosomal DNA [37], it is unclear whether the lack of incorporation of this H4 mutant into chromatin is due to its inability to bind to p46 or to p48.

Although a number of other core-histone-binding proteins have been described in the literature [38–41], the p46 and p48 polypeptides bind to histones in a novel way. For instance, the Tup1p subunit of the general transcriptional repressor Ssn6/Tup1 is, like p46 and p48, a multi-WD-repeat polypeptide that binds to the amino termini of H3 and H4 via the so-called repression domain of the protein, which is distinct from the WD repeats [42]. In contrast, p46 and p48 bind selectively to H4 (and also to H2A, but only when the proteins are expressed in *E. coli*) and, at least for histone H4, stable binding does not require the presence of an intact amino-terminal domain or its acetylation, but is critically dependent upon amino-acid residues in helix 1 of the histone-fold domain. In addition, unlike Tup1p, stable core-histone binding by p48 seems to require a multi-WD-repeat structure, because short amino-terminal and carboxy-terminal deletions that remove single repeats in p48 strongly reduce its affinity for H2A and H4 (A.V., unpublished observations).

This mode of core-histone binding by p46 and p48 has important implications for the chromatin assembly pathway, the action of histone deacetylases and the role of H4 acetylation at Lys5 and Lys12. Several residues within helix 1 of histone H4 make important interactions with the nucleosomal DNA [37]. Hence, it seems unlikely that mature nucleosomes could form while p48 is bound to its site in histone H4 and, as a result, one would expect that CAF-1 has to be actively displaced from the sites of histone deposition. It is interesting, therefore, that CAF-1 can only give rise to regular nucleosome arrays (as judged by micrococcal nuclease digestion) in the presence of the ATP-requiring chromatin assembly and remodeling factor, ACF, in a purified system for DNA-replication-independent nucleosome assembly [43]. It seems plausible that

the requirement for ACF and ATP in this reaction may be to disrupt CAF-1–histone interactions, thereby facilitating the formation of mature histone–DNA contacts.

It is not known whether p46 or p48 stimulates the action of histone deacetylases such as HDAC1 and HDAC2 [17,18]. The rationale for the association of p46 and p48 with the deacetylase catalytic subunits is unclear because these enzymes can act upon mature chromatin, in which the binding sites for p46 and p48 are occluded by the presence of nucleosomal DNA. It may be that the role of p46 and p48 as subunits of deacetylases is confined to the *de novo* nucleosome assembly pathway, where the deacetylases are likely to act on histones prior to the formation of mature nucleosomes. Alternatively, perhaps other subunits of the deacetylases [18,19], or accessory proteins such as nucleosome remodeling factors, may assist in peeling the DNA off the histone octamer prior to p46 or p48 binding. This would suggest that the requirement for ATP-utilizing nucleosome remodeling activities to assist CAF-1 function, such as ACF [43], may extend to other proteins that contain p46 or p48 as core-histone-binding subunits, such as the histone deacetylases.

Although not essential for maintenance of transcriptional silencing [44], acetylation of H4 at Lys12 has been proposed to promote silencing of the mating-type loci in *S. cerevisiae* [45]. Because the Hat1 holoenzyme specifically acetylates newly synthesized H4 and not nucleosomal H4, this enzyme may promote the establishment of silencing in cells that were transcriptionally active at these loci, a process that is known to require passage through S phase [46]. More commonly, however, acetylation of newly synthesized histones would be expected to promote transcription by facilitating the binding of transcription factors to nucleosomal DNA [47,48]. Consequently, locus-specific regulation of histone deacetylases, or of the associated p46/p48 core-histone-binding subunits that act on newly deposited histones, may provide an additional mode of transcriptional regulation. For instance, at certain loci, the histones on both sister chromatids may either remain acetylated or be deacetylated in a concerted manner, yielding identically marked chromatin in the two daughter cells. Conversely, during particular cell divisions, access to the newly deposited and acetylated histones at specific loci might be different on each sister chromatid, yielding differentially acetylated histones in the two daughter cells. Such asymmetric inheritance could influence developmental patterning of gene expression.

Materials and methods

DNA manipulations

In order to clone the 5′ end of the *Hat1* cDNA, antisense oligonucleotide primers annealing to the 5′ end of EST cDNA clone 24027 were designed. Using these primers and a λ-arm-specific primer, several independent *Hat1* clones (as determined by sequencing across the vector–insert junction) were obtained either by PCR amplification from a λgt10 cDNA library or by 5′ RACE (rapid amplification of cDNA ends) PCR from total HeLa-cell RNA. No in-frame stop codon was found upstream of the first ATG codon (which matches the Kozak consensus sequence [49]) in the longest *Hat1* cDNA clone obtained (1568 nucleotides excluding the poly (A) tail; GenBank accession number AF030424). A description of the constructs for expression of GST–hHat1Δ1–6 and deletion mutants of H2A and H4 in *E. coli*, transient expression of FLAG-epitope-tagged p46, p48 and Hat1 in tissue culture cells and expression of p46 in baculovirus-infected Sf9 cells, is provided as supplementary material.

Immunological procedures

A rabbit antiserum against a peptide derived from the amino terminus of human Hat1 (Hat1N, acetyl-CEKFLVEYKSAVEKK-amide) was raised as described [5]. A matrix for affinity purification of antibodies that bind to the Hat1N peptide was prepared by crosslinking the peptide to Sulfo Link resin (Pierce). Anti-Hat1N antibodies were affinity-purified as described [50]. To prepare a resin for immunoaffinity purification of the Hat1 enzyme from human cells, ~1 mg pH 3-labile anti-Hat1N antibodies was crosslinked to 0.25 ml protein-A–Sepharose beads (Pharmacia) using dimethylpimelimidate [50]. Electroporation of constructs for the transient expression of FLAG-epitope-tagged Hat1 in COS1 cells was performed at 180 V (960 μF) as described [51]. Pulse-labeling with BrdU, immunofluorescence with the FLAG M2 monoclonal antibody (Kodak; 2.7 μg/ml in phosphate-buffered saline plus 1% goat serum) and data collection were performed essentially as described [52].

Protein purification

Detailed protocols for purification of the Hat1 enzyme from human 293 cells and recombinant p46 and p48 from *E. coli* and baculovirus-infected Sf9 cells can be found as supplementary material.

Histone and chromatin methods

Expression and purification of histones as GST fusions in *E. coli* was performed as described [53], except that the cells were grown at 30°C for GST–H2A, GST–H2B and GST–H3, or at 23°C for GST–H4. Purified *Xenopus* core histones (a gift from Tim Richmond and Karolin Luger, ETH Zürich) were refolded by dialysis from guanidinium-HCl [22] and reconstituted as $H3_2{\cdot}H4_2$ tetramers onto plasmid pSV011$^+$ by salt gradient dialysis.

Histone acetyltransferase (HAT) assays

Additional information concerning the dependence of the human Hat1 acetyltransferase on salt, pH and acetyl-coenzyme A is provided as supplementary material. HAT assays were performed with 0.2 μM [^{3}H]acetyl-coenzyme A (1 mCi/ml, 26.1 Ci/mmol; Andotek) and 100 μg/ml $H3_2{\cdot}H4_2$ tetramers or H2A·H2B dimers (or an equivalent amount of histones as oligonucleosomes lacking histone H1) in 25 mM Tris HCl pH $8.0_{37°C}$, 150 mM NaCl, 10% glycerol, 0.5 mM Na_2EDTA, 1 mM DTT, 5 mM sodium butyrate for 30 min at 37°C. Incorporation of [^{3}H]acetate into histones was quantitated with a phosphocellulose filter binding assay [54]. Alternatively, the histones were analyzed by electrophoresis through SDS–18% polyacrylamide gels [55], 0.9 M acetic acid–2.5 M urea–15% polyacrylamide gels [5] or SDS–16.5% acrylamide-tricine gels [56]. The gels were stained with Coomassie blue, destained and photographed, prior to fluorography with Amplify (Amersham) and exposure to film at –70°C.

GST pull-down assays

Pull-down assays were performed in 200 μl buffer A200, with ~0.1 μg of either purified p46 or p48 (2 pmol) and ~2 μg of each GST–histone (50 pmol) immobilized to glutathione–Sepharose beads. After incubation for 1 h at 4°C, the beads were washed four times with 1.5 ml of either buffer A200 or A300 and the amount of p46 or p48 remaining bound to glutathione–Sepharose beads was determined by SDS–PAGE and western blotting. Buffers A200 or A300 are 25 mM Tris-HCl pH $8.0_{4°C}$, 10% glycerol, 0.01% Nonidet-P40, 1 mM Na_2EDTA, containing either 200 mM or 300 mM NaCl.

Supplementary material

Additional methodological detail and a figure showing a *Hat1* northern blot are available with the internet version of the paper.

Acknowledgements

We thank Juan Mendez for the actin control northern blot in the supplementary material section, Robert Roeder for the constructs to express recombinant histones in *E. coli*, E. P. Bers and A. Zalensky for the anti-histone antibodies and E. Lee for the anti-p46 and anti-p48 monoclonal antibodies. Tim Richmond and Karolin Luger provided us with purified recombinant core histones and valuable data about the structure of the nucleosome prior to its publication. This work was supported by grant CA13106 from the NIH. A.V. is a postdoctoral fellow of the Jane Coffin Childs Memorial Fund for Medical Research. P.D.K. was a SmithKline Beecham Fellow of the Life Sciences Research Foundation.

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