

Histone H1 Is Required for Proper Regulation of Pyruvate Decarboxylase Gene Expression in *Neurospora crassa*

H. Diego Folco,¹ Michael Freitag,² Ana Ramón,³ Esteban D. Temporini,⁴† María E. Alvarez,⁴ Irene García,³ Claudio Scazzocchio,³ Eric U. Selker,² and Alberto L. Rosa^{1*}

Instituto de Investigación Médica Mercedes y Martín Ferreyra, INIMEC-CONICET,¹ and Departamento de Química Biológica, Facultad de Ciencias Químicas, Universidad Nacional de Córdoba,⁴ 5016 Córdoba, Argentina; Institute of Molecular Biology, University of Oregon, Eugene, Oregon 97403-1229²; and Institut de Génétique et Microbiologie, Université de Paris-Sud, UMR 8621, 91405 Orsay Cedex, France³

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We show that *Neurospora crassa* has a single histone H1 gene, *hH1*, which encodes a typical linker histone with highly basic N- and C-terminal tails and a central globular domain. A green fluorescent protein-tagged histone H1 chimeric protein was localized exclusively to nuclei. Mutation of *hH1* by repeat-induced point mutation (RIP) did not result in detectable defects in morphology, DNA methylation, mutagen sensitivity, DNA repair, fertility, RIP, chromosome pairing, or chromosome segregation. Nevertheless, *hH1* mutants had mycelial elongation rates that were lower than normal on all tested carbon sources. This slow linear growth phenotype, however, was less evident on medium containing ethanol. The pyruvate decarboxylase gene, *cfp*, was abnormally derepressed in *hH1* mutants on ethanol-containing medium. This derepression was also found when an ectopically integrated fusion of the *cfp* gene promoter to the reporter gene *hph* was analyzed. Thus, *Neurospora* histone H1 is required for the proper regulation of *cfp*, a gene with a key role in the respiratory-fermentative pathway.

Eukaryotic DNA is compacted into chromatin, a dynamic nucleoprotein complex organized into nucleosomes (31). Each nucleosome contains an octameric core of four conserved histones, H2A, H2B, H3, and H4, around which ~146 bp of DNA are wrapped (35). The N-terminal tails of core histones are thought to interact with nuclear proteins and to be involved in internucleosomal interactions that can lead to chromatin condensation (36, 71). Posttranslational modifications of histone tails have been shown to be involved in various aspects of gene regulation, suggesting the existence of a “histone code” specifying gene silencing, activation, and other processes (6, 26). Chromatin of most eukaryotes includes less conserved “linker histones,” most notably, histone H1 (28, 29, 63). Histone H1 has been proposed to play a role in creating and/or maintaining the higher-order structure of chromatin (52). In contrast to core histones, linker histones appear rather mobile and in fact are not permanently associated with chromatin (33, 44). The results of in vitro studies have suggested that H1 participates in the organization of nucleosomes (62), restricts the translational mobility of nucleosomes (48, 66), and can inhibit transcription (70). Interestingly, H1 is enriched in nuclease-resistant and inactive chromatin (69) and in regions of DNA that are methylated (3). Additional clues to the possible functions of H1 have come from in vivo studies. In higher eukaryotes, which have multiple linker histone genes subject to developmental regulation (30), some H1 variants seem to be dispens-

able (13, 60). In tobacco, H1 plays roles in development and male meiosis (49). An H1 variant is essential for the development of the germ line in *Caenorhabditis elegans* (25). Changes in H1 stoichiometry modulate core histone acetylation in cultured mammalian cells (18).

The functions of H1 have been studied most extensively for *Tetrahymena* and fungi. Both *Tetrahymena thermophila* and *Saccharomyces cerevisiae* have single genes for linker histones with unconventional structures (5, 72). The *Tetrahymena* gene encodes a lysine- and alanine-rich protein that lacks the globular domain, whereas the yeast protein consists almost entirely of two globular domains without a long C-terminal tail. Deletion of the linker histone gene did not lead to gross phenotypic changes in either organism. Nevertheless, the *Tetrahymena* linker histone mutant showed increased nuclear size (57) as well as abnormal activation of some genes and repression of others (58). Disruption of the yeast H1-like gene caused decreases in the steady-state levels of a substantial number of unrelated mRNAs (22).

Filamentous fungi have canonical H1 linker histones. *Asco-bolus immersus* contains a single H1 gene encoding a typical tripartite H1 protein with a charged amino-terminal domain, a globular winged helix domain, and a charged alanine- and lysine-rich carboxy-terminal domain (4). *Asco-bolus* strains in which the H1 gene was silenced by methylation showed increased sensitivity of chromatin to micrococcal nuclease (MNase), global DNA hypermethylation, and a shortened “life span” (4). These phenotypes are consistent with important roles of H1 in global chromatin function and gene regulation (2). The dispensability of the equivalent gene in *Aspergillus nidulans* (*hhoA*) (53), however, challenged the idea that canonical H1 proteins are essential in eukaryotes. We chose *Neurospora crassa* to further define the functions of linker

* Corresponding author. Mailing address: Instituto de Investigación Médica Mercedes y Martín Ferreyra, INIMEC-CONICET, Friuli 2434, 5016 Córdoba, Argentina. Phone: (54)(351)468-1465. Fax: (54)(351)469-5163. E-mail: arosa@immf.uncor.edu.

† Present address: Department of Plant Pathology, University of Arizona, Tucson, AZ 85721.

TABLE 1. *N. crassa* strains used in this study

Strain	Genotype ^a	Source
74-OR23-1A	<i>mat A</i>	FGSC 987
74-OR8-1a	<i>mat a</i>	FGSC 988
N228	<i>mat a</i> ; <i>al-1</i> ⁺ ; <i>al-1::hph</i>	This study
N623	<i>mat A his-3</i>	FGSC 6103
N1264	<i>mat A</i> ; <i>nic-3 wc-1 arg-10</i>	Reference 32
N1445	<i>mat a his-3</i> ; <i>am</i> ¹³² <i>inl</i>	Reference 43
N1815	<i>mat A</i> ; <i>hH1</i> ^{RIP1}	This study
N1817	<i>mat A</i> ; <i>hH1</i> ^{RIP2}	This study
DF3	<i>mat a</i> ; <i>hH1</i> ^{RIP1}	This study
HH3	<i>mat A his-3</i> ; <i>hH1</i> ^{RIP1}	This study
HH28	<i>mat A</i> ; <i>his-3</i>	This study

^a RIP designations indicate that the *hH1* gene contained RIP mutations.

histones. This fungus exhibits DNA methylation (32, 61), repeat-induced point mutation (RIP) (55), and a fully characterized set of core histones (21); in addition, as in *A. immersus*, nucleosomal repeats are slightly longer than in *Aspergillus* and yeast cells (53).

Here we report the cloning and characterization of the *hH1* gene of *N. crassa*. This gene encodes a typical histone H1 protein. We constructed viable *hH1* null mutants, demonstrating that *hH1* is not essential in *Neurospora*. We found, however, that H1 mutants grew more slowly than wild-type strains on the various tested carbon sources, and we observed misregulation of the *cfp* (cellular filament polypeptide) gene (1), which encodes *Neurospora* pyruvate decarboxylase. Our results indicate that histone H1 is required for the proper regulation of *cfp* expression.

MATERIALS AND METHODS

Strains and genetic methods. Standard *Neurospora* methods were used (10). The *N. crassa* strains used in this work are described in Table 1. Strain N228 carries a duplication of the *al-1* gene and was kindly provided by C. Staben (University of Kentucky). Strains N1445 and N1264 were described previously (32, 43). Strains HH3 (*hH1*^{RIP} *his-3*) and HH28 (*his-3*) were obtained by a cross between DF3 (*mat a hH1*^{RIP1}) and a *his-3* mutant (FGSC 6103). Strains used to measure RIP and linear growth were constructed for this study as detailed in Tables 2 and 3, respectively.

Nucleic acid manipulations. *N. crassa* genomic DNA was prepared and analyzed as previously described (20, 37). For Southern blot analyses, 0.5 to 1.0 µg of DNA was used. Total mycelial RNA was purified by a method used for plant tissues (68). Northern analyses were performed as described previously (37). The

following probes were used for Southern and Northern hybridizations: a 1.2-kb *EcoRI hH1* fragment from pDF2 (see below), a 1.7-kb *BamHI-EcoRI hH1* fragment from pMF233 (see below), 0.6-kb *HindIII* and 0.2-kb *HindIII-EcoRI hH1* fragments from pDF1 (see below), a 537-bp PCR fragment (positions +460 to +997) and a 338-bp PCR fragment (positions -348 to -10) from *cfp*, a 0.9-kb *BamHI-EcoRI* Ψ₆₃ (*Fsr-63*; 5S RNA pseudogene) fragment from pPG22 (43), an 0.8-kb *BamHI* ζ-η (*Fsr-33*; 5S RNA pseudogene) fragment from pVM152 (43), a 2.7-kb *HindIII his-3* fragment from pBM60 (38), and a 1.1-kb *BamHI-ClaI hph* fragment from pCSN44. For methylation analyses, genomic DNA was digested with methylation-insensitive *DpnII* or its methylation-sensitive isoschizomer *Sau3AI*. The *his-3* probe was used as a control probe to verify that digestions were complete.

Protein purification and electrophoresis. Protein purification and electrophoresis were carried out as described previously (53).

Cloning of the *hH1* gene. Genomic DNA (*N. crassa* FGSC 987) and DNA purified from plate stocks of phage λZAPII from a mycelial cDNA *N. crassa* library (47) were used as templates in PCR experiments with oligonucleotides NC1 (5'-ACCTTTTGCACGCCCTT-3') and HDF1 (5'-AGTGGTATCGTTC CAAGACGG-3'). PCR products of 1.7 kb (genomic DNA) and 1.2 kb (cDNA) were obtained, cloned into the p-GEM T Easy vector (Promega) (plasmids pDF1 and pDF2, respectively), and sequenced.

Because the *hH1* gene is not available in *Neurospora* cosmid libraries, a genomic library of wild-type *N. crassa* strain FGSC 987 was constructed in the λDASHII vector by using genomic DNA partially digested with *Sau3AI* (10 to 18 kb) and an in vitro packaging kit (Gigapack II Gold; Stratagene). A positive clone containing the whole *hH1* gene was isolated by screening with the 0.2-kb *HindIII-EcoRI* genomic fragment from pDF1. Oligonucleotide primer H1rt (5'-GGATGTGGTGTGGTTTTAGG-3') was used to sequence a 250-bp region upstream of the ATG start translation codon. 5' Rapid amplification of cDNA ends (RACE) was carried out with a 5'/3' Race kit (Boehringer Mannheim, Mannheim, Germany) and oligonucleotides NC2 (5'-TTCTTGGCAAGCTTG GTGC-3') and NC4 (5'-CTTGTGAAGAGCGAGTC-3'). The *hH1* gene was mapped by restriction fragment length polymorphism (RFLP) analysis (42) with the 0.6-kb *HindIII* genomic fragment from pDF1 as a probe.

EGFP fusion constructs. To generate histone H1 fusions to enhanced green fluorescent protein (EGFP), the *BamHI-BglII* fragment from plasmid pFA6a-GFP(S65T)-kanMX6 (34) was inserted into *BamHI*-digested pBM60 (38) to yield pMF255. The GFP (S65T) gene was replaced by a PCR-amplified EGFP gene (19), and the fragment was inserted into *BamHI-EcoRI*-digested pMF255, yielding pMF267. The *Neurospora ccg-1* promoter (*Pccg-1*) and 5' untranslated region of the transcript directly preceding the *ccg-1* ATG (nucleotides 738 to 1659) were PCR amplified and inserted into *NotI-XbaI*-digested pMF267, yielding pMF272. PCR-amplified *Neurospora hH1* was inserted into *BamHI-PacI*-digested pMF272 to yield pMF280. Both pMF272 and pMF280 were targeted to the *his-3* locus of N623 (38), yielding strains N2261 and N2276, respectively. EGFP imaging was performed as previously described (15).

Generating *hH1* mutants by RIP. A 1.7-kb *BamHI-EcoRI* PCR fragment from *hH1* DNA cloned into pBM61 (pMF233) was targeted to the *his-3* locus (38). Transformants were crossed to strain N1264, and random ascospores were isolated 28 days after fertilization. Strain N1264 is *mat A* and carries *nic-3*, which was determined by RFLP analysis (see above) to map closely to *hH1* on LG VIII. Therefore, selection for growth on minimal medium and screening for *mat*

TABLE 2. Lack of effect of disruption of *hH1* on RIP mutations

Type	Cross ^a	Relevant genotype	% RIP frequency (mean ± SD) ^b	No. of progeny analyzed
1	FGSC 987 × N228	(+) × <i>al-1</i> ⁺ / <i>al-1</i> ⁺	47 ± 9	1,786
2	N1815 × N228	<i>hH1</i> ^{RIP} × <i>al-1</i> ⁺ / <i>al-1</i> ⁺	77 ± 6	2,023
3	<i>s-wt</i> × N228	(+) × <i>al-1</i> ⁺ / <i>al-1</i> ⁺	66 ± 6	6,348
4	<i>s-hH1</i> × N228	<i>hH1</i> ^{RIP} × <i>al-1</i> ⁺ / <i>al-1</i> ⁺	57 ± 6	4,045
5	<i>d-hH1</i> × FGSC 987	<i>hH1</i> ^{RIP} ; <i>al-1</i> ⁺ / <i>al-1</i> ⁺ × (+)	69 ± 14	3,067
6	<i>d-hH1</i> × N1815	<i>hH1</i> ^{RIP} ; <i>al-1</i> ⁺ / <i>al-1</i> ⁺ × <i>hH1</i> ^{RIP}	73 ± 11	2,870
7	<i>d-wt</i> × FGSC 987	<i>al-1</i> ⁺ / <i>al-1</i> ⁺ × (+)	73 ± 6	4,823
8	<i>d-wt</i> × N1815	<i>al-1</i> ⁺ / <i>al-1</i> ⁺ × <i>hH1</i> ^{RIP}	74 ± 7	4,288

^a Strain N228 has an ectopic duplication of the wild-type *al-1*⁺ gene, and FGSC 987 is a wild-type (+) strain. A total of 11 wild-type (*s-wt*) and 7 *hH1*^{RIP} (*s-hH1*) progeny were isolated from a cross between N1815 and FGSC 988. Crosses of *s-wt* and *s-hH1* strains with N228 (type 3 and 4 crosses) were designed to test linkage of the *hH1*^{RIP} allele with increased RIP frequencies observed in crosses of N1815 and N228 (compare type 1 and 2 crosses). Duplications of *al-1*⁺ in wild-type (*d-wt*) or *hH1*^{RIP} (*d-hH1*) progeny were obtained by crossing N1815 and N228 and selecting progeny whose *al-1*⁺ copies had survived RIP. Eight *d-hH1* and seven *d-wt* strains were crossed with wild-type or *hH1*^{RIP} strains (type 5 to 8 crosses).

^b Because all crosses included a parent with a single *al-1* gene, the RIP frequency was calculated as (number of white colonies/total number of colonies) × 200.

A increased the likelihood of isolating strains with a single mutated *hH1* copy at the native locus. We isolated 43 prototrophs, 27 of which were *mat A* and 22 of which showed evidence of RIP mutations. The mutated alleles of strains N1815 (*hH1*^{RIP1}), N1817 (*hH1*^{RIP2}), N1816 (*hH1*^{RIP3}), and N1819 (*hH1*^{RIP4}) were amplified by PCR with *Pfu* DNA polymerase. Products from at least five reactions per strain were pooled prior to sequencing.

Construction of the ectopic *cfp-hph* reporter locus. Plasmid pDF12 was generated by ligation of the 2.7-kb *ApaI*-*SacI* fragment from pA3, which carries *hph* under the control of the *cfp* promoter and the *Aspergillus nidulans* *trpC* terminator (E. D. Temporini and A. L. Rosa, unpublished data), into *ApaI*-*SmaI*-digested pBM61 (38). Control plasmid pDF85, with a *trpC* promoter instead of the *cfp* promoter, was constructed by ligating a 2.4-kb *ApaI*-*NotI* fragment from pCSN44 into *ApaI*-*NotI*-digested pBM61. These constructs were targeted to *his-3* of strains HH3 and HH28, and correct integrations were confirmed by Southern analysis.

MNase digestion of chromatin. Chromatin structure was analyzed by a modification of a method described for *A. nidulans* (17). Briefly, *N. crassa* was grown at 30°C in Vogel's minimal medium with 2% sucrose for 16 h. Ethanol (2%) or glucose (2%) was added, and mycelia were harvested 4 h later and used immediately or freeze-dried. Frozen and powdered mycelia were suspended (100 mg/ml) in a buffer containing 250 mM sucrose, 60 mM KCl, 15 mM NaCl, 0.05 mM CaCl₂, 3.0 mM MgCl₂, 0.5 mM dithiothreitol, and 15 mM Tris-HCl (pH 7.5), and aliquots were treated with various amounts of MNase for 5 min at 30°C. DNA was purified by phenol extraction and ethanol precipitation, digested with either *EcoRI* or *BglII*, and subjected to Southern blot analysis and probing with the 537-bp PCR fragment (+460 to +997) or the 338-bp PCR fragment (−348 to −10) from *cfp*, respectively.

Sensitivity to MMS, UV, and DMSO. *Neurospora* conidia were plated at a density of 500 conidia/plate on Vogel's minimal medium containing 1.5% sorbose, 0.1% glucose, and 0.1% fructose and supplemented with methyl methanesulfonate (MMS) at 0.005, 0.015, or 0.030% (vol/vol). Colonies were counted at 48 and 72 h (24). UV sensitivity was tested as described previously (23). The UV dose was 25 J min^{−1}, and exposure times were 0, 8, 16, 20, and 24 min. For dimethyl sulfoxide (DMSO) assays, equal numbers of conidia (~500) were plated on petri dishes containing Vogel's minimal medium with 4.5% DMSO. Colonies were counted after 72 h at 30°C, and morphology was noted.

Measurement of linear growth rates with Race tubes. Linear growth rates were determined at 30°C with glass Race tubes (40 cm long, 12 mm in diameter) (10). Ten microliters of a conidial suspension (~10⁴/ml) was inoculated at one end, and growth was monitored for 4 days at intervals of 8 to 12 h.

Measurement of RIP frequencies. Crosses were carried out on Westergaard-Mitchell medium (10) after the "female" strain had grown for 5 days at 25°C in the dark. Random ascospores were harvested, heat activated, and plated on Vogel's minimal medium containing 2% sucrose and 0.0075% Tergitol (Sigma) (59). White (RIP-mutated) and orange (wild-type) colonies were scored by visual inspection.

Microscopic examination of vegetative and sexual tissues. Asci and chromosomes were visualized by staining with hematoxylin-ferric acetate or acriflavine (50). The number, size, and shape of nuclei (i.e., mycelia and conidia) were determined after staining (Hoechst 33258 or 4',6'-diamidino-2-phenylindole [DAPI] dyes) and visualization under a fluorescence microscope. Imaging of EGFP was carried out as described previously (15). Strains were grown overnight at room temperature in a thin layer of Vogel's minimal medium without a carbon source on microscope slides to induce the *ccg-1* promoter.

Nucleotide sequence accession number. The GenBank accession number for the sequence reported in this article is AY124883.

RESULTS

Isolation of the *hH1* gene from *N. crassa*. We identified a histone H1 cDNA clone in a *Neurospora* expressed sequence tag (EST) database (b7 h12ne; <http://www.genome.ou.edu>) and used it to design specific primers to isolate the *hH1* gene from *Neurospora* genomic DNA and from a mycelial cDNA library (47) by PCR. We isolated and sequenced a 1.7-kb genomic fragment as well as a 1.2-kb cDNA fragment. Because of uncertainties regarding the N-terminal region of the predicted protein and the promoter region, the entire *hH1* gene was cloned from a λDASH *N. crassa* genomic library (Fig. 1A). We mapped *hH1* to the left arm of LG VII between 5:5A and

00003 by RFLP analysis (42) and confirmed by additional Southern analyses that *hH1* is the only histone H1 gene in *N. crassa* (data not shown). This conclusion was further supported by BLAST searches of the >98% completed *Neurospora* genome sequence (<http://www-genome.wi.mit.edu/annotation/fungi/neurospora/>; release 3: 02.12.2002).

Alignment of genomic and cDNA sequences allowed us to determine the position of four introns. The G+C content of the *hH1* open reading frame (61.2%) is significantly higher than that of the introns (46.4%), consistent with the previously described codon bias for constitutively expressed genes of *N. crassa* (12). We determined the major transcription start sites by RACE experiments (Fig. 1A) and identified a consensus TATA box (8). Northern blot analyses of total RNA extracted from vegetative mycelium revealed a single 1.4-kb *hH1* transcript. RNA levels were similar when mycelia were grown on sucrose, glucose, ethanol, or ethanol-glucose media (data not shown).

The sequence surrounding the most likely initiation codon of *hH1*, CCATCACCATGCC (initiation codon in italic type), fits the consensus translation start site sequence for *Neurospora* (7, 12) (Fig. 1A). Conceptual translation of the *hH1* open reading frame yields a protein of 236 amino acids with a predicted molecular mass of 24.2 kDa and high contents of alanine (26.3%) and lysine (23.3%). This predicted protein shows strong similarity to H1 from various organisms, suggesting that *hH1* encodes a bona fide histone H1. Sequence alignments revealed that residues 36 to 112 of *Neurospora* H1 correspond to the globular winged helix domain, the most conserved region of linker histones (Fig. 1B). Moreover, the *Neurospora* H1 protein shows the characteristic three-domain structure of metazoan histone H1 proteins: (i) an N-terminal region of 35 amino acids, (ii) a globular region of 77 amino acids, and (iii) a positively charged C-terminal region of 124 amino acids. The predicted isoelectric point (10.2) may account for our observation that H1 migrates as a protein of 32 kDa (see below), a behavior shared by its homologs from *A. nidulans* (53) and *A. immersus* (4).

An H1-EGFP fusion protein is localized in nuclei. To confirm that *Neurospora* histone H1 is localized in nuclei, we constructed translational fusions of the H1 and EGFP genes (19) and targeted these fusion constructs to the *his-3* locus. Fusion constructs with the native *hH1* promoter or the inducible *qa-2* promoter did not yield sufficient H1-EGFP for visualization. We therefore made use of the *Neurospora ccg-1* (*grg-1*) promoter (*Pccg-1*) (41) to drive the overexpression of EGFP fusion genes (see Materials and Methods). Expression patterns observed were typical for normal *ccg-1* induction; i.e., on sucrose medium, little or no expression was observed in hyphae growing on or through agar. Fusions of *Pccg-1*-H1-EGFP genes produced high levels of H1-EGFP fusion protein, localized exclusively in the nucleus (Fig. 2a to d). Control fusions of *Pccg-1*-EGFP genes produced cytoplasmic EGFP (Fig. 2e and f).

Construction of *hH1* mutants. We made use of the RIP process (55) to create *hH1* mutants. A 1.7-kb fragment of *hH1* was introduced at the *his-3* locus (LG IR) by gene replacement (38). Single-copy transformants of mating type *mat a* (LG IL) were crossed with a *mat A* strain marked on LG VII (*nic-3 wc-1 arg-10*). Progeny carrying RIP-mutated alleles at the en-

A.

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-303 ttccaggaggtcacagaggccccccccccaggtcgcaggggagacgggaggtccgtcg
-243 gggcagggcaggaagaatcagcgaatcactcgtcgccagggagacccccgctccg
-183 tatataaacacccaatcttccccctcgagcgcgaactgagcccccacatcctcctcgt
      *                               *
-123 cctttgtttcccagtcacgatccaacctcaactgaaccttttgcaagccctttc
-63 agttgtatcttcagcaagatatcctcagcctccagccctaaaccacaaccacatccatc
-3 accATGCCTCCCAAGAAGACCGAGACCAAGGCCGCGATGCCTCTGCCGCGCGCTCTCT
  1 M P P K K T E T K A A D A S A A A A P
  58 GCTCCCGCGCTGCTCCACCTCTGCTCCCAAGACCAAGCCCCAGCACCCATGCCTCT
  20 A P A A A P T S A P K T K S P S T H A S
  118 TACCTTgtaagctttacttaatcccagaagaacgcacatcttttgtaacattcaaacag
  40 Y L
  178 GACATGATCACGGACGCTATTGTGTGCGgtattttaacccccctcattatctttgttggc
  42 D M I T D A I V A
  238 gtgttttggttggtggtcgttctctggtcgggtgagcaaatgagacaattgctctcatga
  298 tgcaagcaaccaagagtcacccacaccactcaccaccctttactgtccctcagtcgtgc
  358 tgattccttctttttgcatgctacagCTCAAGGACCGCGCTGGATCTAGgtatgtgcc
  51 L K D R A G S S
  418 ttttctgcttacttttctcatttttccaccagggtttgccaagccacaccgcaaacct
  478 tgccggcccttctcaggtcgcaccgatgaccacttctcttatttccatgacgcgat
  538 gaaggatcacaacatggtgctaacgcacaccacagCCGTCGAAGCTCTCAAGAAGTATGTC
  59 R Q A L K K Y V
  598 CGTGCCAACAACACGCTCGGCAACGTCACCGACAACATGTTGACTCGCTTTCAACAAG
  67 R A N N T L G N V T D N M F D S L F N K
  658 GCCTTGAAGAACGGTGTGACACAAGGGTGTCTTTGAGCAGCCCAAGGgtatgtcaccaccc
  87 A L K N G V D K G V F E Q P K
  718 acatgacgcacatctccttctgattactctttgcatgctcacacacgatatagGTCTTC
  102 G P S
  778 CGGTGGCACCAAGCTTGCCAAGAAGGTGCTAAGCCTGCTCCCAAGAAGGCTGCTCCAA
  105 G G T K L A K K V A K P A P K K A A P K
  838 GAAGGAGACCAAGGAGAAGAAGCCCGCTGCCGCTAAGAAGGAGGGTGCCGCCAAGAAGGA
  125 K E T K E K K P A A A K K E G A A K K E
  898 GACCAAGGAGAAGAAGGCTCCTGCTGCCAAGAAGCGGCTGCCCCCAAGAAGGCTGCTGC
  145 T K E K K A P A A K K A A A P K K A A A
  958 TCCCAAGAAGGAGGTCAAGGAGAAGAAGCGGCTGCCCCCAAGAAGAAGCGCTGCTCC
  165 P K K E V K E K K A A A P K K K A A A P
  1018 TGCCGTTGCCGACAAGGAGACCGTCTCACCAAGACCAAGTCTGGCCGTGTGCCAAGAG
  185 A V A D K E T V L T K T K S G R V A K S
  1078 CACCGCTAAGCCTGCTGCCCAAGAAGCGCGCTGCTCCCAAGAAGGCTGCCCCAGCAA
  205 T A K P A A A K K A A A P K K A A A S K
  1138 GAAGGCCGAGAAGCGGAGCCGCTGCCGAGAAGGCATAAactcgttcctttgggatgat
  225 K A E K A E P A A E K A *
  1198 gtttttgtctcttttttgccgctcgtctcttctcctcctcctcctcctcctcctcctcctc
  1258 ccaccacttgccgatgtgacgggcttactcgaagggcgtttggggaatcatattgcttggg
  1318 aacacttatcaacataaggggggtttcattggtctacaagtcaacgcgttttctctt
  1378 tttcaaacatcgccacagcgcgcgggggtagcttctgtttctggttctcctcctcctcctc
  1438 cttgattcacaacactcactcattggggattgaggaggtttggatcgtttgtgctgaacgc
  1498 aacggagggtaggtttgggctacaacctatggatgatgggaaatgggaaacggggcacc
  1558 ggcggcttcgaggggtgtcgaagaagcgaaggacatacaccacaaaagccttatcttggat
  1618 actgcattgcataataatccgctcttggaacgataccactttttcttcttcaataacct

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FIG. 1. *hH1* gene of *N. crassa*. (A) Nucleotide and predicted amino acid sequences for the *N. crassa hH1* gene. Coding regions are shown in uppercase letters, and noncoding sequences are shown in lowercase letters. Transcription start sites (asterisks) and the 3' end of the longest available EST sequence (black diamond) are indicated. The putative TATA box is shown in bold type. The sequence around the most likely ATG start codon is doubly underlined. The central globular domain of the protein is underlined. Genomic sequences flanking the *hH1* gene, from contig 3.396 (<http://www-genome.wi.mit.edu/annotation/fungi/neurospora>), are shown in italic type. The cytosine at +577 and Q60, mutated in *hH1^{RIP}* strains, are indicated in bold type. (B) Comparison of the highly conserved globular domains of histone H1 proteins from *N. crassa* (Nc); *A. fumigatus* (Af; sequence deduced from data available at <http://tigrblast.tigr.org/ufmg/>); *A. nidulans* (An; CAB72936); *Fusarium sporotrichioides* (Fs), *Magnaporthe grisea* (Mg), and *Botrytis fuckeliana* (Bf) (these three sequences deduced from ESTs available at <http://www.cogeme.ex.ac.uk>); *A. immersus* (Ai; AAF16011); *S. cerevisiae* (ScD1 and ScD2; NP_015198); *Volvox carterii* (Vc; H1-1; Q08864); tobacco (Nt; S53502); human (Hs; H1^P; XP_009973); and *Drosophila melanogaster* (Dm; P02255). Yeast H1 is unconventional because it does not contain N- or C-terminal tails but rather contains two fused globular domains (ScD1 and ScD2). The globular domains were aligned with CLUSTAL W (64). Identical residues (black) are indicated by asterisks, conservative substitutions of major groups (dark gray) are indicated by colons, and conservative substitutions of minor groups (light gray) are indicated by periods. Regions identified as α helices or β sheets in the histone H5 crystal structure are indicated.

B.

	helix I	helix II	helix III	β -hairpin					
Nc	HASYLDMITD	I VALKDRAGSSRQ-AL	KKYVRANN	TLGNVTDNMFDSLE	NKALKNGVDKGV	EEQPK	--PSGGT	KLAKK	
Af	HASYRDMIKD	ILNLKERNGSSRQVS	IKKYVLANNK	I AFASQAAFDSQ	ENKAIKAGVEKGE	FTQPK	---SGPV	KLAKK	
An	HTSYRDMIKD	ILNLKERNGSSRQ-	SIKKYVLANNK	LAPASQNAFDSQ	ENKAIKAGVEKGE	FTQPK	--TSGPV	KLAKK	
Fs	HASYQDMITD	IVNLKDRKSSRQ-	SLKKYVKANN	TL-NGTDNMFDS	LENKALKAGVDK	GVVEQPK	--PSGGT	KLAKK	
Mg	HPTYQAMITD	IINLKERNGSSRP-	OLKKYVKANN	LKGEVTD	SMFDSLENRALK	AGVDKGVVEQPK	--PSGGT	KLAKK	
Bf	HASYQDMIID	IINLKERNGSSRI-	QLKKYVKANN	KI-NAGDSMFDS	LENRALKAGV	AKVEVIMP	--SSGT	VKLAPK	
Ai	HPSYKEMITK	ITELKERNGSSRQ-	AIKKYIQSNF	KVKN----FDVQ	ENQALRRGVEK	GEVQPK	--PSGT	VKLAKK	
ScD2	SLTYKEMILK	MPQLNDGKSSRI-	VLKKYVKD	TFSSKLTSSNF	DYLENSAIKKCV	ENGELVQPK	--PSGI	IKLNKK	
ScD1	SKSYRELIIE	LTALKERKSSRP-	ALKKFTEN	KYP-IVGSASN	FDLYFNNAIKK	VEAGDEQPK	--PAGAV	KLAKK	
Vc	HPPIEMVKD	ITTLKERNGSSLP-	ALKKFTEN	KYG-KDIHDKN	FAKTL	SQVVKTFV	KGGKLV	KVKV-----SFKL---	
Nt	HPSYFEMIKD	IVTLKDKTGSSQH-	AITKFL	EDKQ--KNLPS-	NFRKLLVQL	KKLVASGKLV	KVKV-----SYKL	PAA	
Hs	HPKYSDMITVA	IQAENRAGSSRQ-	SIQKYLKSHY	---KVG-ENAD	SQKLSIKRLV	TTGVLQTK	VGASGS	FRFAKS	
Dm	HPPTQOMYDA	IKNLKERGSSLL-	AIKKYITATY	---KCD	AQKLAPFTK	KKYKLSAV	VNGKLI	QTK KGASGS	FKLSAS
	::	::	::	::	::	::	::	::	
		***	:::			*		*	

FIG. 1—Continued.

dogenous *hH1* locus were recovered as *mat A* prototrophs. Southern analysis of genomic DNAs from these strains showed evidence of DNA methylation and C:G to T:A transition mutations, both indicators of RIP (Fig. 3). Four heavily mutated *hH1^{RIP}* strains were characterized in detail. A 700-bp genomic fragment from the 5' region of the *hH1* gene was amplified by PCR and sequenced. Strains N1815 (*hH1^{RIP1}*) and N1817 (*hH1^{RIP2}*) showed the most mutations (85 and 92, respectively) and were therefore selected for further characterization. In addition to missense mutations, both strains have a CAA-to-TAA nonsense mutation at residue Q60 (Fig. 1A). Histone H1 was absent from perchloric acid (PCA) extracts obtained from vegetative tissue of strains N1815 and N1817, confirming that the *hH1* gene was disrupted (Fig. 4). No *hH1* transcript was detectable by Northern blot analyses of total RNAs isolated from these strains (data not shown). These observations support the conclusion that *N. crassa* has a single active histone H1 gene.

Absence of histone H1 does not affect DNA methylation but results in subtle changes in chromatin accessibility to MNase. Conflicting reports on the association of linker histones with methylated DNA in vivo (3, 27) and in vitro (9, 40) led us to examine effects of the *hH1^{RIP}* mutants on DNA methylation. Gross global DNA methylation was not affected, as indicated by digestion of total DNA with a methylation-sensitive restriction enzyme and staining of gels with ethidium bromide (data not shown). No localized changes in the DNA methylation levels of several known methylated regions tested (ψ 63, ζ - η , and ribosomal DNA) (39, 56) were detected in *hH1^{RIP}* mutants (Fig. 5). These findings are in stark contrast to those of studies with silenced H1 in *A. immersus*, in which the lack of H1 resulted in global hypermethylation (4).

Global chromatin structure was investigated with MNase (17). A subtle alteration in the pattern of nucleosomal DNA digestion was reproducibly found with chromatin from the *Neurospora hH1^{RIP}* mutants (Fig. 6A). Although the size of the nucleosomal repeat was similar to that in the wild type, a sharper banding pattern was observed in *hH1* chromatin. Densitometry confirmed that wild-type chromatin yielded more diffuse bands after nuclease digestion (Fig. 6B). Similar differ-

ences were observed with chromatin purified from nuclei (data not shown). These observations are consistent with the idea that in H1-depleted chromatin, linker DNA is abnormally accessible to MNase.

Histone H1 is not required during the sexual cycle. In both heterozygous and homozygous crosses, *hH1^{RIP}* mutants were fertile when used as either male or female. The development of asci and ascospores was normal, and ascospores exhibited normal morphology, viability, and germination rates. Meiotic chromosomes were stained with hematoxylin-ferric acetate and acriflavine and observed from 3 days postfertilization until the end of ascus development. Compared to controls examined in parallel and also in comparison to the results of previous studies (51), no changes in chromatin condensation or chromosome behavior were observed (data not shown).

To study the effect of histone H1 on RIP mutations, we generated *hH1^{RIP}* strains carrying a duplication of the *al-1*

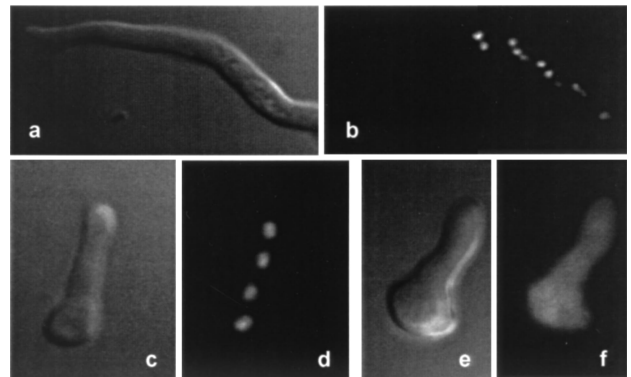


FIG. 2. EGFP-tagged H1 is localized in nuclei. Overexpressed H1-EGFP fusion protein is exclusively localized in nuclei of fully developed hyphae (a and b) and germinating conidia (c and d) of strain N2276. The nuclear localization of H1-EGFP was confirmed by staining with DAPI (data not shown). Nonfused EGFP expressed under the control of the *Pccg-1* promoter is localized in the cytoplasm (e and f) of a germinating conidium of strain N2261. a, c, and e, phase-contrast microscopy; b, d, and f, fluorescence microscopy. EGFP imaging was carried out as described previously (15).

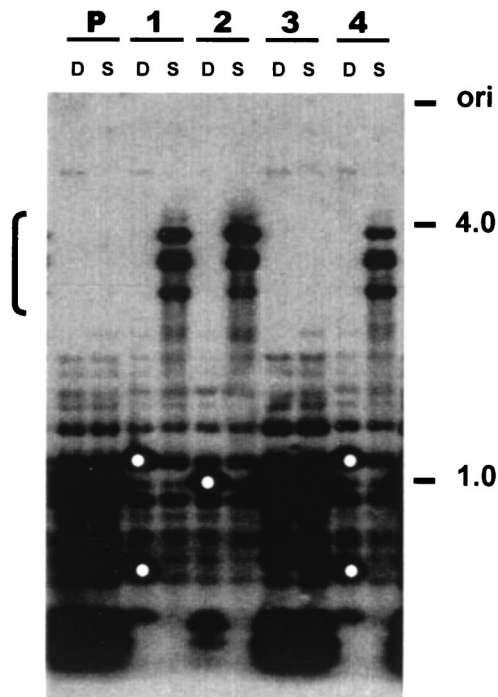


FIG. 3. Generation of *hHI* mutants by RIP. RFLPs and DNA methylation at the *hHI* locus of four RIP-mutated strains are revealed by Southern analysis. Genomic DNA from the parental strain (lanes P) with the *hHI* duplication and four prototrophic *mat A* progeny (lanes 1 to 4) were digested with either *DpnII* (D; 5-methylcytosine insensitive) or *Sau3AI* (S; 5-methylcytosine sensitive). Isolates 1, 2, and 4 carry mutated *hHI* alleles, as indicated by RFLPs in the *DpnII* lanes (white dots), and exhibit methylation, as indicated by high-molecular-weight fragments (bracket on the left) in the *Sau3AI* lanes. Isolate 3 carries two copies of *hHI* and may be free of mutations or only sparsely mutated. A 1.7-kb *hHI* fragment was used as a probe. Molecular size markers (in kilobases) are shown on the right.

gene (54) by crossing the *hHI^{RIP1}* strain (N1815) to an *al-1* duplication strain (N228). RIP frequencies in either homozygous or heterozygous crosses were equivalent to those in wild-type control crosses (Table 2).

H1 mutants exhibit a slow linear growth phenotype. Both *hHI^{RIP}* null mutants studied (N1815 and N1817) retained normal morphology in liquid or on solid minimal media (Vogel's minimal medium, PPC, and Westergaard-Mitchell medium) (10) at all temperatures tested (10 to 39°C) (data not shown). The size, shape, and distribution of nuclei in mycelia were normal. Mutants produced normal quantities of conidia (asexual spores), with the expected number of nuclei per conidium, and conidia showed normal viability. The mutants also showed normal sensitivity to MMS, UV irradiation, and DMSO. Measurements of linear growth rates, however, revealed that *hHI^{RIP}* mutants had lower mycelial elongation rates on all carbon sources tested ($P < 0.005$) (Table 3). This slow linear growth phenotype segregated with the *hHI^{RIP}* allele (data not shown). Interestingly, *hHI* mutants showed better linear growth rates on ethanol (91%) than on sucrose (85%), glucose (85%), or agar (80%) ($P < 0.001$). Based on these selective responses to different carbon sources, we hypothesized that H1

is required for the expression of one or more specific genes involved in carbon metabolism.

***cfp* is misregulated in *hHI* mutants.** The possibility that H1 plays a regulatory role in carbon metabolism was explored by studying *cfp*, the *Neurospora* gene encoding pyruvate decarboxylase (1, 20). Pyruvate decarboxylase is a key postglycolytic enzyme and converts pyruvate to acetaldehyde (65). The expression of the *cfp* gene is strongly induced by glucose and repressed by ethanol (1). Northern analyses showed that *cfp* mRNA levels were similar when wild-type and *hHI^{RIP}* mutants were grown on media containing either sucrose or glucose (Fig. 7). In contrast, no *cfp* mRNA was detected when the wild-type strain was grown under repressing conditions on media containing ethanol (1) or ethanol-glucose, but *cfp* mRNA was readily detected when *hHI^{RIP}* mutants were grown under these conditions (Fig. 7). These results suggested that histone H1 is required for the proper regulation of *cfp*.

Based on this possibility, we examined the chromatin structure of the *cfp* promoter region in the wild type and the *hHI^{RIP}* mutants under inducing and repressing conditions. Chromatin was digested with MNase, treated with *EcoRI* or *BglII*, and analyzed by Southern blotting with a *cfp* probe. Identical MNase patterns were found with chromatin from wild-type

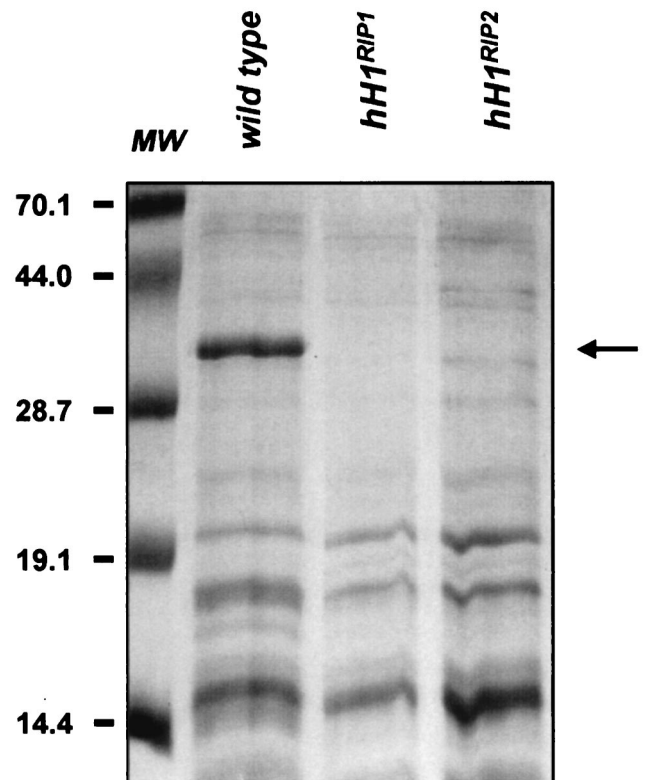


FIG. 4. Histone H1 is absent from *hHI^{RIP}* mutants. PCA-soluble protein extracts from *N. crassa* wild-type and *hHI^{RIP1}* and *hHI^{RIP2}* mutant strains were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Coomassie blue staining. *N. crassa* H1 (arrow) is readily identifiable in protein extracts from total mycelium of the wild-type strain. H1 is absent from extracts from total mycelium of the *hHI^{RIP}* mutant strains. Molecular weight (MW) markers (in thousands) are shown on the left.

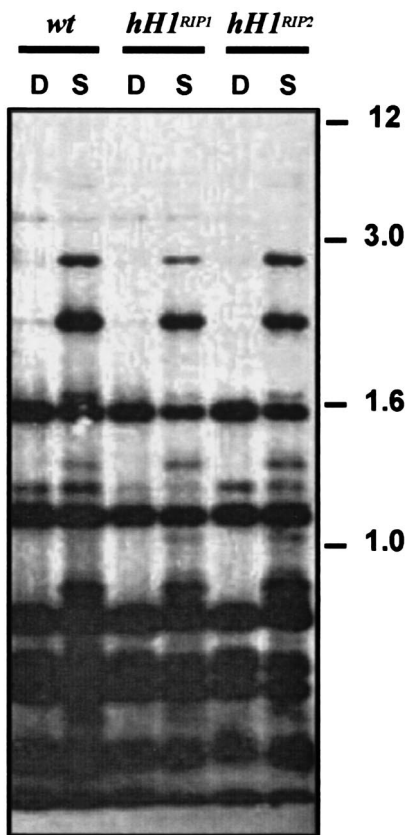


FIG. 5. Absence of histone H1 does not affect DNA methylation. For Southern blot analysis, genomic DNAs from a wild-type strain (*wt*) and two strains with *hHI^{RIP}* alleles were digested with *DpnII* (D) or *Sau3AI* (S) and probed with a ³²P-labeled ribosomal DNA repeat. Methylation patterns and relative intensities of bands were identical when wild-type and mutant strains were compared. Molecular size standards (in kilobases) are shown on the right.

TABLE 3. Linear growth rates for wild-type and *hHI^{RIP}* mutant strains

Carbon source	Growth rate (mm/h) for the following strain ^a :		<i>hHI^{RIP}</i> /wild-type ratio
	Wild type	<i>hHI^{RIP}</i>	
Sucrose	4.8 ± 0.1	4.1 ± 0.1	0.85
Glucose	4.6 ± 0.1	3.9 ± 0.1	0.85
Ethanol	3.3 ± 0.1	3.0 ± 0.1	0.91
Agar	3.0 ± 0.1	2.4 ± 0.1	0.80

^a Nine wild-type and seven *hHI^{RIP}* progeny from a cross of N1815 (*hHI^{RIP}*) and FGSC 988 (wild type) were selected at random. Linear growth rates were determined at 30°C by using Race tubes and Vogel's minimal medium (1.5% agar) supplemented with sucrose, glucose, or ethanol, each at 2%. Duplicate data were obtained for strains grown under the various growth conditions and were pooled for statistical analyses.

and *hHI^{RIP}* mutant strains grown under inducing conditions (i.e., glucose). The same pattern was observed with *hHI^{RIP}* mutant strains grown under repressing conditions, while wild-type chromatin from mycelia grown under repressing conditions (i.e., ethanol) showed subtle differences in the MNase pattern (data not shown). These results are consistent with the possibility that *cfp* is constitutively expressed in *hHI^{RIP}* mutants, as suggested by the analyses described above (Fig. 7).

We investigated whether the misregulation of *cfp* in *hHI^{RIP}* mutants was dependent on the chromosomal position and/or the integrity of the *cfp* transcriptional unit. A *cfp* promoter (*P_{cfp}*) (positions -847 to -26) was fused to the coding region of the bacterial *hph* gene, which confers resistance to hygromycin; the fusion was integrated at the *Neurospora his-3* locus. Wild-type or *hHI^{RIP}* mutant strains with a single copy of the reporter *P_{cfp}-hph* fusion gene were selected. The expression of the *P_{cfp}-hph* gene was examined in strains grown on minimal media with various carbon sources and hygromycin. As a control, we tested a fusion of the constitutive *A. nidulans trpC* promoter to *hph* (*P_{trpC}-hph*). Both wild-type and *hHI^{RIP}* cells transformed with the *P_{cfp}-hph* fusion were highly resistant to

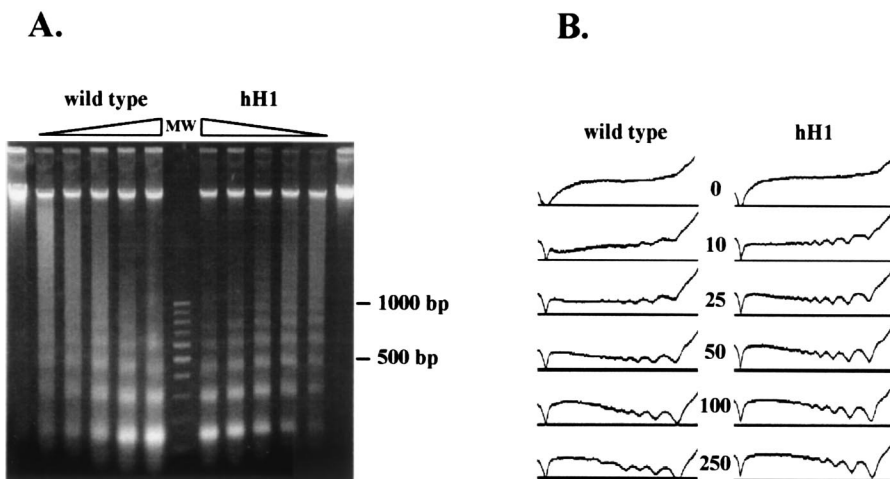


FIG. 6. Chromatin of *hHI^{RIP}* mutants is more accessible to MNase. (A) Chromatin from the wild-type strain and a strain carrying an *hHI^{RIP}* null allele (*hHI*) was obtained from mycelia and treated with various amounts of MNase (250, 100, 50, 25, 10, and 0 U/g of mycelium, indicated by the ramps). Molecular weight markers are indicated in lane MW. (B) Densitometric analysis of a gel similar to that shown in panel A. Plots were made with the program Scion Image (<http://www.scioncorp.com/>). A sharper profile of peaks and valleys was observed for *hHI* chromatin than for wild-type chromatin. Amounts of MNase in units per gram are indicated between the plots.

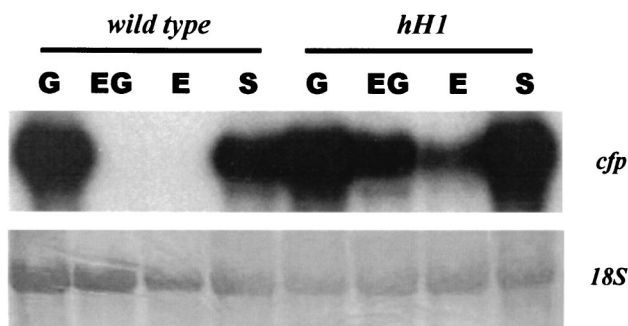


FIG. 7. The *cfp* gene is misregulated in *N. crassa* *hHI* mutants. Northern blot analyses of *cfp* mRNAs were performed with wild-type *N. crassa* and *hHI* mutant N1815 (*hHI*) of *N. crassa*. Strains were grown for 16 h at 30°C in Vogel's minimal medium containing 2% sucrose. Fresh carbon sources (G, glucose; EG, ethanol plus glucose; E, ethanol; and S, sucrose), each at 2%, were added, and mycelia were harvested 4 h later. The 18S rRNA (stained with methylene blue) is shown as a loading control.

hygromycin under inducing conditions. On ethanol-containing media, however, wild-type cells with the *Pcfp-hph* fusion were hygromycin-sensitive, whereas *hHI^{RIP}* mutants with the *Pcfp-hph* fusion were hygromycin resistant. As expected, wild-type and *hHI^{RIP}* mutant strains carrying the control *PtpC-hph* gene fusion were resistant to hygromycin on media containing either glucose or ethanol as a carbon source (data not shown). These results show that the *cfp* promoter is misregulated in *hHI^{RIP}* mutants regardless of whether it is driving the expression of the endogenous *cfp* gene or expression of a reporter gene at an ectopic location, indicating that the *cfp* promoter per se is sensitive to negative regulation by H1.

DISCUSSION

The *hHI* gene of *N. crassa* encodes a typical eukaryotic histone H1 composed of a central globular domain flanked by alanine- and lysine-rich regions. Southern blot studies, as well as analyses of available genome sequence data, indicated that *hHI* is the only gene encoding a linker histone in *N. crassa*. Linker histones from filamentous fungi are well conserved (Fig. 1B). The N-terminal tails of *Neurospora* H1 and *Ascobolus* H1 are longer (35 and 25 amino acids, respectively) than that of *A. nidulans* H1 (17 amino acids). These data are consistent with the longer nucleosomal repeats in *Neurospora* (170 ± 5 bp) (46) and *Ascobolus* (174 ± 6 bp) (53) than in *A. nidulans* (159 ± 7 bp) (17). In both *A. nidulans* and *N. crassa*, histone H1 tagged with EGFP was localized exclusively in the nucleus, consistent with an association with chromatin.

Based on intron number and distribution, *N. crassa* *hHI* seems to be more closely related to *A. nidulans* *hhoA* and a putative H1 gene from *Aspergillus fumigatus* than to the H1 gene from *A. immersus*. The positions of the first four introns of the H1 genes from *N. crassa*, *A. nidulans*, and *A. fumigatus* are identical. The position of the first intron of the *A. immersus* H1 gene is the same as that of the third intron of the *Neurospora* and *Aspergillus* H1 genes, suggesting that this is the most ancient intron in the H1 genes of filamentous fungi.

It is now clear that histone H1 and closely related linker

histones are not essential in several model organisms—*N. crassa*, *T. thermophila*, *S. cerevisiae*, *A. nidulans*, and *A. immersus*—all organisms with single genes for unusual or bona fide linker histones (4, 53, 57, 67). Interestingly, H1 mutants of these organisms exhibited different phenotypes. In *T. thermophila*, deletion of H1 genes resulted in increases in the volume of nuclei and in alterations in gene expression (57, 58). The function of the atypical H1 in *Tetrahymena* is regulated by phosphorylation (11). In *A. immersus*, silencing of the single H1 gene by MIP results in global DNA hypermethylation, a shortened life span, and hypersensitivity of chromatin to digestion with MNase (4). In the yeast *S. cerevisiae*, deletion of the atypical single *HHO1* gene has no dramatic effect on vegetative or sexual phenotypes. Freidkin and Katcoff (14) determined that *S. cerevisiae* has only approximately one HHO1p molecule per 37 nucleosomes, showing that HHO1p cannot be associated with linker DNA at all nucleosomes. Interestingly, deletion of *HHO1* in *S. cerevisiae* results in a reduction in the steady-state levels of many mRNAs, as shown by microarray analyses (22).

Mycelia from *N. crassa* *hHI* mutants have normal nuclei, and the gross structure of meiotic chromosomes does not appear to be affected during meiosis. It remains possible that the loss of H1 is compensated for by other chromatin proteins, e.g., HMG1-class proteins (45, 73), for which there are at least eight homologs in the *Neurospora* predicted proteome (M. Freitag and E. U. Selker, unpublished data). Chromatin from *hHI* mutants showed somewhat sharper bands in MNase digests, similar to what was seen for *A. immersus* H1-silenced strains (4). Perhaps linker histones from *Ascobolus* and *Neurospora* protect linker DNA from protein access, as expected from *in vitro* studies with chromatin from higher eukaryotes. No change in MNase digestion behavior was observed for *A. nidulans* chromatin depleted of H1 (53), consistent with the possibility that the protein encoded by *hhoA* represents an evolutionary variant with a more specific function. RIP was not affected in *Neurospora* *hHI* mutants. We did not observe global or localized changes in DNA methylation, in contrast to the situation for *A. immersus* (4).

The slow linear growth phenotype of *N. crassa* *hHI* mutants was dependent on the carbon source, suggesting that histone H1 may play a role in pathways associated with carbohydrate metabolism. Based on the linear growth of *hHI* mutants in media containing ethanol versus sucrose or glucose, we expected a change in the balance between respiratory and fermentative pathways. Indeed, in *hHI* mutants grown under repressing conditions, the expression of *cfp*, the gene encoding pyruvate decarboxylase, was misregulated and abnormally high *cfp* mRNA levels were detected. Moreover, the chromatin structures at the *cfp* promoter appeared to be identical under either inducing or repressing conditions in *hHI* mutants. Perhaps *Neurospora* chromatin lacking H1 fails to generate the alternative promoter structures associated with normal regulation of the *cfp* gene.

H1 may affect the expression of certain genes according to their positions in a specific chromosome "neighborhood," rather than by acting on specific regulatory sequences (16). Nevertheless, an *hph* reporter gene fused to the *cfp* promoter and introduced into an ectopic position in the *Neurospora* genome showed normal H1 dependence for gene expression.

This observation suggests that histone H1 specifically modulates *cfp* expression in *N. crassa* and that *cfp* expression is not position dependent.

Our suggestion that histone H1 participates in the expression of specific genes in *Neurospora* is consistent with previous studies showing that the depletion of H1 resulted in the induction of some genes but in the repression of others (58, 60, 67). Linker histones traditionally have been considered “stabilizers” of higher-order chromatin structure by rendering a condensed chromatin state, but it is also conceivable that histone H1 destabilizes chromatin and thus facilitates interactions of DNA with transcription factors. A recent global analysis of *S. cerevisiae* showed that several mRNAs exhibit abnormally low steady-state levels in a mutant linker histone background (22). This result indicates that histone H1 can act as a direct or an indirect “facilitator” of gene expression. In this model, the multiple modifications of core histone tails in single nucleosomes, as predicted by the “histone code” (26), may represent various states of a chromatin stabilizer. We propose that histone H1 plays a role in fine-tuning of the expression of a subset of genes involved in primary metabolism. Further studies with *Neurospora hH1* mutants and DNA microarrays will explore this hypothesis.

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