Identification of a Replication-independent Replacement Histone H3 in the Basidiomycete *Ustilago maydis*

Verma Anju, Tamas Kapros, and Jakob H. Waterborg

From the Division of Cell Biology and Biophysics, School of Biological Sciences, University of Missouri-Kansas City, Kansas City, Missouri 64110

*Ustilago maydis* is a haploid basidiomycete with single genes for two distinct histone H3 variants. The solitary U1 gene codes for H3.1, predicted to be a replication-independent replacement histone. The U2 gene is paired with histone H4 and produces a putative replication-coupled H3.2 variant. These predictions were evaluated experimentally. U2 was confirmed to be highly expressed in the S phase and had reduced expression in hydroxyurea, and H3.2 protein was not incorporated into transcribed chromatin of stationary phase cells. Constitutive expression of U1 during growth produced ~25% of H3 as H3.1 protein, more highly acetylated than H3.2. The level of H3.1 increased when cell proliferation slowed, a hallmark of replacement histones. Half of new H3.1 incorporated into highly acetylated chromatin was lost with a half-life of 2.5 h, the fastest rate of replacement H3 turnover reported to date. This response reflects the characteristic incorporation of replacement H3 into transcribed chromatin, subject to continued nucleosome displacement and a loss of H3 as in animals and plants. Although the two H3 variants are functionally distinct, neither appears to be essential for vegetative growth. KO gene disruption transformants of the U1 and U2 loci produced viable cell lines. The structural and functional similarities of the *Ustilago* replication-coupled and replication-independent H3 variants with those in animals, in plants, and in ciliates are remarkable because these distinct histone H3 pairs of variants arose independently in each of these clades and in basidiomycetes.

Core histones provide the packaging proteins for DNA in eukaryotic cells. During the S phase when genomic DNA is duplicated, replication-coupled expression of histone genes provides new protein to assemble newly replicated DNA. Histone chaperones assist in the creation of new nucleosomes to maintain a stable, compacted, repressed state of chromatin. Within this context, regulatory proteins modulate chromatin environments to facilitate access to the DNA for gene transcription. The components and processes that allow RNA polymerases to transcribe a chromatin template, such as epigenetic modifications of DNA and histones, are being identified and intensely studied. The processes of nucleosome displacement from DNA by transcribing RNA polymerases and of nucleosome reassembly from available histones remain poorly understood.

In research going back decades, it was observed that the composition of nucleosomes across transcribed gene regions, identified in part by high levels of histone acetylation, changed over time. Replication-coupled (RC) histone H3 variants like H3.2 in birds were replaced by histone H3.3, a constitutively expressed form of animal histone H3. This histone is now known as a replacement histone or as a replication-independent (RI) H3 variant (1, 2). Specialized chaperones such as HIRA and Daxx selectively bind these RI H3 proteins at a small region, residues 87–90, which is uniquely different between RI and RC forms (3, 4). In the S phase, RC and RI variants are both present for replication associated chaperones to assemble nucleosomes. Outside of the S phase, the transcribed genes are only repackaged using new RI H3 proteins. The basis for this selectivity is found in one or more of the following reasons: outside of the S phase, RC H3 proteins are not synthesized, histone H3 molecules from displaced nucleosomes cannot be reused in nucleosome assembly, and transcription-associated chaperones like HIRA use only RI H3 sequence variants.

Many of these processes have been identified and studied in Saccharomyces cerevisiae as a simple model system. However, this yeast makes only a single H3 protein from two histone H3 genes. This H3 is produced in a replication-coupled pattern of expression with significant H3 protein availability outside of the S phase. It acts like an animal H3.3 in that it is used by HIRA (4). In Schizosaccharomyces pombe, a single H3 protein is produced, partly from two RC H3 genes and partly from two constitutive ones (5). Neither model organism can be used to study the contribution of histone H3 variants in replicative and replacement nucleosome assembly.

Study of the functions provided by RC and RI H3 variants in animals and in plants is limited because the multiplicity of genes of both types prevents effective use of gene knock-out and replacement approaches. In each case, the duplication of the ancestral histone H3 and the structural and functional differentiation into replicative RC and replacement RI H3 variants arose independently (6–8). Gorovsky and co-workers (7) have used the diploid protist Tetrahymena as a model system. In ciliates, distinct RC and RI H3 genes have arisen independently from their appearance in animals and in plants (6–8). In Tet-

---

* This work was supported by Missouri Life Sciences Research Board Award 13254 (to J. H. W.).

† To whom correspondence should be addressed: UMKC, 414 BSB, 5007 Rockhill Rd., Kansas City, MO 64110-2499. Tel.: 816-235-2591; Fax: 816-235-1503; E-mail: WaterborgJ@umkc.edu.

This is an Open Access article under the CC BY license.
rahymena with 2 RC and 2 RI loci, complete knock-out of both RC or both RI loci produces viable vegetative cells. In RC knockouts, replication results in dramatically reduced cell proliferation rates and hypoploidy, likely because of insufficient nucleosome formation using only RI H3 histone. Proficient growth required up-regulation of RI loci. Conversely, RI knockouts are viable but fail to produce viable spores (9). The validity of these results for animals or plants has not been confirmed.

Rahymena with 2 RC and 2 RI loci, complete knock-out of both RC or both RI loci produces viable vegetative cells. In RC knockouts, replication results in dramatically reduced cell proliferation rates and hypoploidy, likely because of insufficient nucleosome formation using only RI H3 histone. Proficient growth required up-regulation of RI loci. Conversely, RI knockouts are viable but fail to produce viable spores (9). The validity of these results for animals or plants has not been confirmed.

Ahmad and Henikoff (10) had suggested that the basidiomycete Cryptococcus might contain a replacement H3 variant. Genome sequencing of the related corn smut Ustilago maydis revealed that this haploid organism has two distinct H3 genes. One is paired with the single histone H4 gene, an organization often seen for RC histones, and the other one is a solitary gene, like animal H3.3, with a replacement-like variant sequence at residues 89 and 90 (11) (see Fig. 1). We have evaluated the expression and stability of the Ustilago histone H3 proteins. We have concluded that the solitary H3 gene indeed has all the functional characteristics of a replacement RI H3 variant and that the H4-paired H3 gene is an RC variant. The similarities of the Ustilago H3 variants in gene structural organization, in the differences of the polypeptides produced, in the selectivity of incorporation into replicative and transcription linked nucleosomes, and in protein stability with the RC and RI H3 variants in plants and in animals is remarkable. It provides insight in the conserved nature of nucleosome assembly processes because the RC-RI H3 divergences arose hundreds of millions of years apart, each time at the ancestral root of what have become broad clades of diverse, multicellular eukaryotes (6).

We have begun to exploit the possibilities for homologous gene replacement in U. maydis (12) and complementation by plasmid-based histone H3 mutants to evaluate the contribution of these two H3 variants in nucleosome assembly during transcription and replication. The viability of strains with KO U1 or U2 loci, coding for the RI H3.1 and RC H3.2 variants, respectively, is reported.

**EXPERIMENTAL PROCEDURES**

**Culture of Ustilago—**U. maydis 521, strain 9021 obtained from the Fungal Genetics Stock Center (University of Missouri-Kansas City, Kansas City, MO) and defined as WT, was grown in synthetic dextrose (SD) medium (6.7 g of Difco yeast nitrogen base without amino acids (Benton-Dickinson, Sparks, MD) with 20 g of glucose/liter) at 30 °C on 2% agar or in liquid culture at 150 rpm with typical experimental use at 107 cells/ml. Cell density was determined by hemocytometer counting. Stationary phase conditions developed for WT cells above 2 × 109 cells/ml in SD medium. Labeling in vivo with [3H]acetic acid (20 Ci/mmol; MP Biomedicals, Irvine, CA) was performed at 4 mCi/liter for 5 min, unless specified otherwise, after 10 min of preincubation at 10 μg/ml cycloheximide (Sigma), added from 2 mg/ml stock in ethanol. Labeling with [4,5-3H]lysine (60 Ci/mmol; MP Biomedicals) was performed at 0.4 mCi/liter for 30 min, unless specified otherwise, and with [35S]methionine (540 Ci/mmol; MP Biomedicals) at 0.1 mCi/liter for 20 min. Cell cycle progression was arrested in the S phase by the addition of 10% (w/v) hydroxyurea to 1 mg/ml (13) in 1-liter log cultures at 2.2 × 106 cells/ml for 90 min. The cells were released from the block by washing with preconditioned SD medium. Tritiated lysine (50 μCi) was added to each culture during the last 30 min prior to cell collection after the culture was concentrated to 250 ml by centrifugation (5 min, 800 × g). The distribution of cells across the G1, S, and G2/M phases was determined by flow cytometry as described (13) in a FACS Calibur 877 (Benton-Dickinson) with ModFit LT (Verity Software) data analysis.

**Replacement Histone H3—**Histones were extracted and purified from cell pellets essentially as described (14). Cells from 0.25 to 2.0 liters of culture were collected by centrifugation (5 min, 800 × g) and resuspended into two to four pellet volumes of 40% GuCl in KPi, (40% guanidine HCl, 0.05 m KH2PO4, 0.05 m K2HPO4, adjusted by KOH to pH 6.8, with 1.4 mM 2-mercaptoethanol). The cells were sonicated on ice in aliquots of 15 ml for 5 min twice with a Branson Sonifier 400 with medium tip at 25% initial output with cooling break, which resulted in 90% cell breakage, clarified (10 min, 30,000 × g), incubated on ice for 15 min at 0.25 × HCl, clarified (30 min, 30,000 × g), diluted with 0.1 m KPi, pH 6.8, to the refractive index of 5% GuCl in KPi, adjusted to 1.4 m 2-mercaptoethanol, and incubated overnight under rocking with 1 ml of settled BioRex70 resin (200–400 mesh; Bio-Rad) per extract from 2 × 1010 cells, an experimentally optimized ratio for the Ustilago procedure. After repeated washing by 1 × g settling of the resin from 5% GuCl in KPi until the supernatant was clear, resin was placed in a small column and washed with 8 volumes of 5% GuCl in KPi, and histones were eluted by 10 volumes 40% GuCl in KPi, Histones were dialyzed in 3,500 molecular weight cut-off Spectra/Por 3 dialysis membranes (Spectrum Labs, Rancho Dominguez, CA) thrice against 100 volumes of 2.5% acetic acid with 1.4 mM 2-mercaptoethanol and recovered by lyophilization. Histone H3 was purified by reversed phase HPLC on Zorbax Protein-Plus columns (0.4 × 25 cm, New England Nuclear) as described (14). Crude histones were dissolved in 0.25 ml of 8 m urea in 1 m acetic acid and injected in the column, equilibrated at 1 ml/min with 40% acetonitrile (Fisher) in 0.1% TFA (Sigma), washed for 2 min in this solvent, and eluted by a gradient from 40 to 55% acetonitrile in 0.1% TFA over 45 min. The two histone H3 variants co-eluted 43 min after injection, as monitored by absorbance at 214 nm. They were identified by acid urea Triton (AUT) gel electrophoresis of lyophilized column fractions based on protein size and characteristic affinity for Triton X-100 (15, 16). Semi-preparative purification of histone H3 used Vydac C4 columns (214TP510, 1.0 × 25 cm; Grace, Deerfield, IL) with the same elution conditions at 3 ml/min.

**Polyacrylamide Gel Analysis—**AUT gel electrophoresis was performed in 15- or 30-cm-long gels with Triton X-100 (Bio-Rad) at 6 mm, a concentration optimized for Ustilago histone H3 variant separation, as described (15, 16). Proteins were quantitated by densitometry of gels, stained with Coomassie Brilliant Blue R-250, and destained in 7% acetic acid, 20% methanol. Specific radioactivity was determined by densitometry of fluorographic exposures exactly as described (17, 18).
Replacement Histone H3 in Ustilago

qRT-PCR Quantitation of H3 Gene Transcripts—Total RNA was isolated with TRIzol reagent (Invitrogen) using 0.4–0.6 mm glass beads for cell breakage for 3 min in a Mini-Beadbeater-16 (Biospec Products, Bartlesville, OK). Genomic DNA was digested with DNase I. The qRT-PCR procedure with separate cDNA synthesis step was performed according to the manufacturer’s instructions using the Superscript III Platinum two-step qRT-PCR kit with SYBR Green (Invitrogen) in an Applied Biosystems 7500 thermal cycler (Invitrogen). *Ustilago* peptidyl-prolyl isomerase (ppi) (GenBank NW_101009, locus UM03726.1) was used as a constitutively expressed reference gene (19, 20). Primers used for the amplification of histone genes (see Fig. 1A) U1 (U1F and U1R), U2 (U2F and U2R), and H4 (H4F and H4R) and for the ppi gene (ppiF and ppiR) are listed in Table 1. Histone mRNA qRT-PCR levels were quantitated as 2−ΔΔct, relative to ppi levels, following the manufacturer’s instructions.

Transformation of *Ustilago*—Transformation of *U. maydis* was modified from published procedures (12, 21) as follows. Cells (5 × 10⁶), subcultured under continuous logarithmic growth in SD medium for at least 4 days, were collected at 10⁵ cells/ml culture density by centrifugation for 5 min at 1100 g, gently washed with 30 ml of SCS (20 mM sodium citrate, pH 5.8, 1 M sorbitol), and resuspended in 1 ml SCS. Protoplasts were produced by adding 2 ml of 128 mg/ml Vinoflow FCE (Novo, Applied Biosystems) and incubating for 10 min. A solution of 0.5 ml of 40% (w/v) PEG 1 M sorbitol, and resuspended in 1 ml SCS. Protoplasts were collected by centrifugation for 10 min at 1100 g, washed twice with 1 ml of SCS and once with 1 ml of STC (1 M sorbitol, 10 mM Tris-HCl, pH 7.5, 100 mM CaCl₂), and resuspended in 1 ml of ice-cold STC. A mixture of 5 μl of transforming DNA at 1 mg/ml in STC and 1 μl of 15 mg/ml heparin (Sigma) in STC was added to protoplasts (10⁶ in 0.05 ml) on ice and incubated for 10 min. A solution of 0.5 ml of 40% (w/v) PEG 4000 (Sigma) in STC was added and incubation on ice was continued for 15 min followed by the addition of 0.5 ml STC. Protoplasts were centrifuged for 5 min at 1100 × g, and the pellet was resuspended in 0.2 ml of STC. Aliquots (0.02 ml) were plated on 1% SD agar containing 1 M sorbitol and 2 μg/ml carboxin and grown at 30 °C. Transformants were collected after 4–5 days. Carboxin (CBX) (5,6-dihydro-2-methyl-1,4-oxathiine-3-carboxanilide; Vitavax) and CBX resistance plasmid pGR3 were kind gifts from S. Gold (Athens, GA). Used without linearization as a transformation reference, pGR3 yielded thousands of transformants per plate in this procedure.

Transformation Constructs—Genomic DNA was isolated by cetyl trimethylammonium bromide (22), as modified (23), by the addition of 1% cetyl trimethylammonium bromide (Sigma) in 0.1 M Tris·HCl, pH 7.5, 0.7 mM NaCl, 10 mM EDTA, 14 mM 2-mercaptoethanol to pelleted cells, vigorous vortexing for 3 min with 0.4–0.6-mm glass beads, incubation for 45 min at 65 °C, the addition of 1 volume of chloroform:isoamyl alcohol (24:1, v/v), centrifugation for 5 min at 12,000 × g, DNA precipitation from the aqueous phase with 0.9 M ammonium acetate in 2-propanol, and a wash of the DNA with 70% ethanol.

For the U1 KO construct, a 863-bp U1 left border (LB) sequence was amplified by PCR in a MJ Research PTC-225 PCR cycler (GMI, Ramsey, MN) using primer pair U1KOLBF and U1KOLBR, and a 1043-bp U1 right border (RB) sequence by primer pair U2KORBF and U2KORBR. The transformation construct was assembled by ligation of EcoRI- and SalI-digested plasmid pGEM-4Z (Promega, Madison, WI) to Sacl- and SfiI-digested U1 LB, the CBX cassette excised by SfiI from pMF1-c (12), kindly provided by M. Feldbrügge (Düsseldorf, Germany), and the Sacl- and EcoRI-digested U1 RB. The construct was linearized by Xbal or SmaI for use in transformation through double-strand DNA repair-based homologous recombination (24). The equivalent U2 KO transformation construct was ligated using HindIII- and Sall-restricted pGEM-4Z, the Sall- and SfiI-digested U1 LB, the CBX cassette excised by SfiI from pMF1-c (12), kindly provided by M. Feldbrügge (Düsseldorf, Germany), and the SfiI- and EcoRI-digested U1 RB. The construct was linearized by Xbal or SmaI for use in transformation. Constructs were verified by restriction digestion and sequencing.

CBX-resistant colonies were subcultured into liquid SD at 3 μg/ml carboxin. Genomic DNA was prepared, and homologous recombination at the targeted U1 and U2 loci was analyzed by PCR using a variety of primer pairs that uniquely recognized sequences within the transforming constructs, the carboxin resistance cassette, and sequences inside and beyond the border sequences. These primer sequences will be provided upon request. Two U1KO transformation experiments produced 65 CBX-resistant clones that grew in liquid SD and con-

TABLE 1

| Primer name | Primer sequence | PCR* |
|-------------|-----------------|------|
| U1F         | AGCTTCTCAGTCTGTGTGATT | 344 (235) |
| U1R         | GTTTAGGTAAGCAATATGGGTGAT | 292 (175) |
| U2F         | TCCGACACAGTTAACCTCTCTGCTG | 362 (260) |
| U2R         | AGCTTCTCCGACACACCCGAC | 267 (267) |
| H4F         | AAAGGCGCCAGGTCCTGCGT | 863 |
| H4R         | TTGGATGCTGCTAAACACCTCGACG | 1043 |
| ppiF        | TGGTGTGGTACGCTGGTGCAGTCAT | 1020 |
| ppiR        | CGAAGCTCGTGTGAAGACAC | 1015 |

* PCR product in bp from DNA templates and, in parentheses, from RNA/cDNA templates.
continued growth when selection was removed. Eight strains contained KO recombination events at the U1 locus and likely were heterokaryons. Clone 47 was identified as a homokaryon U1 knock-out containing the CBX cassette with error-free homologous recombination in LB and RB sequences, as confirmed by sequencing. Multiple confirmed U2 knock-out strains were obtained from recombinant heterokaryon strains that contained both wild-type and knocked out U2 loci in varying ratios, as measured by PCR analysis (data not shown). Continued growth when selection was removed. Eight strains contained KO recombination events at the U1 locus and likely were heterokaryons. Clone 47 was identified as a homokaryon U1 knock-out containing the CBX cassette with error-free homologous recombination in LB and RB sequences, as confirmed by sequencing. Multiple confirmed U2 knock-out strains were obtained from recombinant heterokaryon strains that contained both wild-type and knocked out U2 loci in varying ratios, as measured by PCR analysis (data not shown). Continued culture under selection produced stable, slow growing homo-}

**RESULTS**

*Identification of Ustilago H3 Variants—* Sequencing of the genome of the haploid basidiomycete *U. maydis* revealed the existence of two single-copy histone H3 genes (11): one on chromosome 11 as a solitary histone, named U1 (GenBank AACP01000135.1, locus UM03916.1) and one, named U2, in a divergently transcribed gene pair with histone H4 on chromosome 6 (GenBank AACP01000090.1, locus UM02709.1) (Fig. 1A). These histone forms differ only in a few amino acids, including residues 89–90 (Fig. 1B). This region defines the replacement character of animal histone H3.3 (4, 25), that interacts with the specific replication-independent histone chaperones HIRA and Daxx (3) and that exists in the independently evolved replacement H3 variants of higher plants (8, 26–28) and ciliates (7). The characteristic sequence at residues 87–90 was the basis for the suggestion that the basidiomycete *Cryptococcus* might also contain this type of replacement histone H3 (10). We chose to use *U. maydis* as the model system (29) to evaluate the existence of a replacement histone H3.2 (Fig. 2B) based on the single methionine in the mature protein (Fig. 1B). This indirectly identified U1 as the gene that produces histone H3.1 (Fig. 1A).

**Relative Abundance of H3 Variants—** The relative amounts of the two histone H3 variants varied with culture conditions. Continuous subculture to maintain logarithmic growth resulted in cells with up to 80% of histone H3 in the H3.2 form (*lanes A and C* in Fig. 2C and *lane 0 h* in Fig. 3A). When cultures were allowed to reach near stationary phase (2 × 10^7 cells/ml), the relative amount of H3.1 protein increased to more than 30%, whereas continued culture into the full stationary phase, when cell budding ceases, cell walls thicken, and cell density stabilizes, produces cultures with up to 50% H3.1 protein (*lane E* in Fig. 2B and Fig. 3). Such a gradual replacement of proliferation associated protein by another form, named a replacement variant (25), which preferentially occurs across transcribed gene regions (31), was one of the first indications for the existence of replacement H3 histone variants (1, 2). The changes in H3 variant abundance support the notion that H3.2 is a replication-coupled (RC) variant and H3.1 a replication-independent (RI) form.

**Histone Acetylation of H3 Variants—** The loss of nucleosomes across transcribed chromatin regions followed by the preferential use of replacement H3 variant forms to create new nucleosomes predicts that a replacement H3 form will show raised acetylation levels (26, 32). H3.1 was acetylated 10–30% higher than H3.2 under all growth conditions, as measured in stained AUT gels. H3.1 had 1.2–1.6 acetylated lysines (AcK) per molecule during logarithmic growth (*lanes A and C* in Fig. 2B and Fig. 3). Such a gradual replacement of proliferation associated protein by another form, named a replacement variant (25), which preferentially occurs across transcribed gene regions (31), was one of the first indications for the existence of replacement H3 histone variants (1, 2). The changes in H3 variant abundance support the notion that H3.2 is a replication-coupled (RC) variant and H3.1 a replication-independent (RI) form.

**Relative Abundance of H3 Variants—** The relative amounts of the two histone H3 variants varied with culture conditions. Continuous subculture to maintain logarithmic growth resulted in cells with up to 80% of histone H3 in the H3.2 form (*lanes A and C* in Fig. 2C and *lane 0 h* in Fig. 3A). When cultures were allowed to reach near stationary phase (2 × 10^7 cells/ml), the relative amount of H3.1 protein increased to more than 30%, whereas continued culture into the full stationary phase, when cell budding ceases, cell walls thicken, and cell density stabilizes, produces cultures with up to 50% H3.1 protein (*lane E* in Fig. 2B and Fig. 3). Such a gradual replacement of proliferation associated protein by another form, named a replacement variant (25), which preferentially occurs across transcribed gene regions (31), was one of the first indications for the existence of replacement H3 histone variants (1, 2). The changes in H3 variant abundance support the notion that H3.2 is a replication-coupled (RC) variant and H3.1 a replication-independent (RI) form.

**Histone Acetylation of H3 Variants—** The loss of nucleosomes across transcribed chromatin regions followed by the preferential use of replacement H3 variant forms to create new nucleosomes predicts that a replacement H3 form will show raised acetylation levels (26, 32). H3.1 was acetylated 10–30% higher than H3.2 under all growth conditions, as measured in stained AUT gels. H3.1 had 1.2–1.6 acetylated lysines (AcK) per molecule during logarithmic growth (*lanes A and C* in Fig. 2C and *lane 0 h* in Fig. 3A). When cultures were allowed to reach near stationary phase (2 × 10^7 cells/ml), the relative amount of H3.1 protein increased to more than 30%, whereas continued culture into the full stationary phase, when cell budding ceases, cell walls thicken, and cell density stabilizes, produces cultures with up to 50% H3.1 protein (*lane E* in Fig. 2B and Fig. 3). Such a gradual replacement of proliferation associated protein by another form, named a replacement variant (25), which preferentially occurs across transcribed gene regions (31), was one of the first indications for the existence of replacement H3 histone variants (1, 2). The changes in H3 variant abundance support the notion that H3.2 is a replication-coupled (RC) variant and H3.1 a replication-independent (RI) form.
Replacement Histone H3 in Ustilago

and C in Fig. 2C) is subject to very rapid acetylation at up to six sites with an average of 3.4 tritiated AcK/H3.1 (lane B in Fig. 2C). Turnover is so rapid that labeling for 20 min results in a much reduced specific radioactivity, even without applying a chase protocol. The same situation has been observed and analyzed in *Chlamydomonas* (17). The loss of acetate label caused by turnover (lane D in Fig. 2C) is consistent with a half-life of less than 4–5 min. For histone variant H3.2, acetylation levels are lower. Pulse labeling produces 2.4 tritiated AcK/H3.2 (lane B in Fig. 2C) in bulk H3.2 with 1.4 AcK/molecule (lanes A and C in Fig. 2C). Turnover appears a bit slower with a half-life of 5–6 min (data not shown). These rates of histone acetylation turnover are consistent with rates at which N-terminal lysines are acetylated and rapidly deacetylated in transcriptionally active chromatin of other species (33).

**Histone Synthesis of H3 Variants**—The rise in H3.2 abundance in growing cultures suggested a coupling between H3.2 synthesis and the S phase when newly replicated DNA is packaged into chromatin. Conversely the rise in H3.1 abundance when cell proliferation ceases (Fig. 3A) predicts the absence of such a relationship and suggests cell cycle-independent histone synthesis. Such a constitutive pattern is typical for replacement H3 histones in animals (4), plants (34), and ciliates (9). This prediction was tested experimentally by RT-PCR analysis of U1 and U2 transcript levels and by measuring histone H3 variant protein synthesis rates in hydroxyurea-synchronized *U. maydis* cultures (13). Early log phase cultures were treated for 90 min with 1 mg/ml hydroxyurea to arrest cells in the S phase (13) and released into conditioned SD medium, and histones were purified after 30 min of pulse labeling for histone synthesis with tritiated lysine. Such a single block procedure produced a reasonable degree of synchrony in logarithmically growing cultures, as determined by flow cytometry across the first

FIGURE 2. Separation, identification, and post-translational modification of H3 histones. A, separation of H3 variants, co-eluted from reversed phase HPLC, in an acid urea (AU) gradient gel with Triton X-100 from 0 to 10 mM where H3 variants co-electrophorese under AU conditions at 0 mM Triton. The slant in electrophoretic mobility across the gradient, marked by methylene blue at the discontinuous running front, by nonhistone proteins in this partially purified HPLC H3 pool and by two guidance lines, results from the glycerol gradient used to stabilize the gradient gel (16). The characteristic high affinity for Triton of core histone H3 is modulated by differences of exposed side chains within the histone fold of the H3 variants (Fig. 1B) (48, 49). The broken line marks the optimal concentration of 6 mM Triton X-100 in 8 M urea and 1 M acetic acid for the separation of the H3 variants of *U. maydis*. B, pattern of H3 variants in AUT gels, pulse-labeled *in vivo* with [35S]methionine for 20 min at 0.1 mCi/liter (lanes A and B) or with [3H]lysine for 30 min at 0.4 mCi/liter (lanes C and D) or chased for 24 h in fresh SD medium into stationary phase (lanes E and F). Coomassie-stained gel lanes are marked by c, and 33-day fluorographic exposures are marked by f. Nonacetylated and hexa-acetylated H3 species are marked by 0 and 6, respectively. C, pattern of histone H3 variants in logarithmically growing cultures, incubated with 4 mCi/liter [3H]acetate for 5 min (lanes A and B) or 20 min (lanes C and D). Coomassie-stained gel lanes are marked by c, and 18-day fluorographic exposures are marked by f. Nonacetylated through hexa-acetylated H3 species are marked by numbers 0–6, respectively.
Replacement Histone H3 in Ustilago

FIGURE 3. Changes in H3 variant abundance and steady state acetylation. A, histone H3 variants were isolated from mid-log phase cultures (0.4 × 10^7 cells/ml), marked as 0 h (log), and after 2, 6, and 12.5 h continued culture into stationary phase (stat). B, the increased abundance of H3.1, expressed as a percentage of total histone H3 (solid line), during continued growth coincides with reduced levels of histone H3 acetylation (broken line) and cessation of cell budding.

few hours after release from the block (Fig. 4G). A similar degree of synchrony was observed after a double hydroxyurea block protocol (data not shown).

Lysine pulse labeling showed that most label was observed across the tail of the histone H3 peak in reversed phase HPLC (Fig. 4A). At this position a late-eluting peak was seen in S phase-enriched cultures (Fig. 4, A and D). Subsequent analysis (Fig. 5) showed that newly synthesized histone H3 of *U. maydis* elutes as a discrete entity later than the bulk H3 form into which it is converted over time. For the experiment presented in Fig. 4, both new and bulk histone H3 protein was pooled together and analyzed on AUT gel by Coomassie staining (Fig. 4B) and fluorography (Fig. 4C). The peak incorporation of label at 1 and 3 h (Fig. 4A) was predominantly found in H3.2 (Fig. 4C). The specific radioactivity of H3.2 at the time of the second S phase (Fig. 4G) was reduced in prominence because the same amount of tritiated lysine was supplied to twice the number of cells (Fig. 4D). The replication-coupled pattern of H3.2 gene expression was confirmed by qRT-PCR analysis of H3.2 U2 gene transcript levels. The H3.2 expression pattern was like that of the single *U. maydis* H4 gene (Fig. 4F). The slight rise in H3.1 synthesis, detected by labeling at the height of the first S phase after release from hydroxyurea (Fig. 4E), was not seen in H3.1 transcript levels (Fig. 4F). These results identify H3.1, as predicted, as a replication-independent H3 variant. The increased incorporation of constitutively transcribed H3.1 during the S phase is likely to reflect co-incorporation with H3.2 protein by chaperones without H3 sequence specificity at a time when large amounts of newly replicated DNA are available for assembly into chromatin. This identified the plant RI H3 as a replacement histone variant (26). For the *Ustilago* system, this prediction was evaluated and experimentally confirmed for histone H3.1 as follows.

We took advantage of the novel observation that lysine pulse label was observed primarily in a peak of new H3 (nH3), which eluted 2 min after the main H3 (mH3) peak. The pulse label was shortened from 30 to 15 min, and the HPLC sampling rates were doubled (Fig. 5A). During the chase period, the label shifted from the nH3 HPLC peak with 22 ± 4% (n = 8) of the absorbance in H3 (Fig. 5A) to the mH3 peak with a half-life of ~30 min (Fig. 5D). The H3 specific radioactivity in HPLC fractions showed that 20% of label was lost in 2.5 h (Fig. 5D).

Pooled m and n fractions were run in parallel on AUT gels, stained (Fig. 5B) and fluorographed (Fig. 5C), and the specific radioactivity of each variant was determined. New protein (nH3) for both variants matured to the mH3 peak with a half-life of 30 min and appeared complete by 90–120 min (Fig. 5, E and F). The specific labeling of H3.2 was stable for at least 2.5 h (Fig. 5F). In contrast, H3.1 was clearly subject to protein turnover with a 50% loss in 2.5 h (Fig. 5E). In these cell cultures with a doubling rate of 1.7 h, this is ~1.5 cell cycles. The pattern of specific radioactivity was fitted to linear decay patterns (Fig. 5, D and F). Data precision was insufficient to determine whether turnover was linear or, as expected, exponential.

Histone H3 Synthesis under Nonproliferative Conditions—Log cultures were treated for 90 min with hydroxyurea as before (Fig. 4) to stop cell proliferation. The cells were labeled for 30 min in fresh SD medium with tritiated lysine, and a pulse-labeled aliquot was collected. To other aliquots, hydroxyurea was added to 1 mg/ml to block cell cycle progression for an additional 2, 4, 6, or 10 h. Culture density was monitored during this chase period, and cell numbers remained unchanged. The specific labeling of H3.1 and H3.2 variants was determined by densitometry of stained and fluorographed AUT gels. The specific radioactivity of H3.2 remained unchanged throughout the experiment. The labeling of H3.1 decayed exponentially with a...
half-life of 3.5–4 h (data not shown). This result confirmed that new H3.1 is subject to extensive turnover both in cycling and noncycling cells.

The replication-independent pattern of H3.1 was confirmed by measuring H3.1 synthesis when hydroxyurea inhibited DNA replication (HU lanes in Fig. 6, A and B) and in stationary cultures when cell proliferation had ceased (Stat lanes in Fig. 6, A and B, and Fig. 6, E). In hydroxyurea, labeling of new H3.1 and H3.2 was reduced 5–10-fold (HU lanes in Fig. 6B, 14 and 22%, respectively) relative to growing cells (Log lanes in Fig. 6B). In stationary cultures, labeling of RI H3.1 was little affected (compare Stat-n and HU-n lanes in Fig. 6B). In contrast, labeling of
new H3.2 dropped to less than 1% (compare Stat-n and Log-n lanes in Fig. 6B). The reduction of H3.1 labeling in nonproliferative cultures is likely to reflect loss of replicative nucleosome assembly sites, whereas transcription linked sites persisted. In contrast, reduction of replication decreases and cessation of cell proliferation eliminates the replication-coupled synthesis and deposition of H3.2.

Taken together, these experiments demonstrate that H3.1 is a constitutively expressed, and thus replication-independent (RI), histone H3 with all functional characteristics that are seen in animal RI H3.3 and the equivalent plant replacement H3 variants. In contrast, the prominent pattern of S phase enhanced expression of H3.2, which was as strongly replication-coupled as the histone H4 gene, and cessation of its synthesis when cell proliferation ceases, clearly identifies H3.2 as a replication-coupled variant.

Are RC and RI H3 Variants Required?—The genome of haploid *U. maydis* contains single RI H3.1 (U1) gene and a single RC H3.2 (U2) gene, coupled through a shared promoter with the single H4 gene (Figs. 1A and 4F). This represents a uniquely simple system to evaluate whether either H3 variant is essential. Transformation cassettes for homologous recombination at U1 and U2 loci were created based on the knock-out plasmid pMF1-c with CBX as selectable marker (12).

Homologous recombination target sequences (1 kb) were produced by PCR. The LB sequences were upstream of the TATA promoter sites of the U1 and U2 genes. The RB sequences were downstream of the 3′-UTRs of the U1 and U2 genes, as determined from available GenBank expressed sequence tag clones. A low number of stable, PCR- and sequencing-verified U1 knock-out (U1KO) and U2 knock-out (U2KO) homokaryon transformants were obtained (see “Experimental Procedures”). H3 proteins from U2KO and U1KO isolates on AUT gels showed the absence of H3.2 and of H3.1 protein in these strains, respectively (Fig. 6C). This showed that neither knock-out causes lethality and...
that vegetative growth is possible with either H3 variant protein. A similar conclusion was reached in *Tetrahymena* (9). Synthesis and acetylation of H3.1 in U2KO and of H3.2 in U1KO cells in growing and stationary cultures was indistinguishable from WT results (Fig. 6D).

Some mutant phenotypes are described below. A full phenotypic analysis of U1KO and U2KO strains will be presented elsewhere. Growth rate comparisons of WT cells (Fig. 6E), U1KO cells (Fig. 6F), and U2KO cells (Fig. 6G) revealed that cell cycles had lengthened in the KO strains (Fig. 6H). The effect was moderate, with a 25% increase in cell doubling time for the U1KO strains without RI H3.1 protein. U1KO cells were a bit smaller than WT ones with normal budding. Histone H3 protein yield was unaffected on a per cell basis in log and stationary cultures, leading to a high protein load per AUT lane when histones were extracted from the same number of U1KO and WT cells (Fig. 6C). U1KO cultures reached stationary phase at cell densities (2 to 3 \(10^7\) cells/ml) similar to WT cells (Fig. 6, compare F with E) with WT-like changes in cell wall morphologies.

U2KO cells without RC H3.2 grew more slowly, as measured during continuous logarithmic growth, achieved by repeated dilution of cultures (Fig. 6G) with cell doubling in 4.8 h, more than twice that of WT cells (Fig. 6H). U2KO cells were quite a bit larger than WT ones. In liquid culture, cell lengthening with septa formation and branching was observed. This mycelial-like growth was strongly enhanced in surface cultures on SD agar. Histone H3 yield on a per cell basis was 62 ± 6% (n = 6) for all strains, likely because of cell wall thickening and the resulting decreased efficiency of cell breakage by sonication. E, cell density, measured by hemocytometer, of WT cells, with subculturing and dilution with SD medium marked by broken lines. Stars mark Log and Stat lysine labeling times and cell densities. Total cell amounts were plotted in H, marked by wt, F, like E for U1KO isolate 47. G, like E for U2KO isolate 96. H, total increase in cell numbers calculated from E-G for WT, U1KO, and U2KO strains. Cell doubling times calculated from exponential curve fitting (Sigmplot) are shown.

**DISCUSSION**

It is remarkable how similar the *Ustilago* histone H3 variants are to those in animals and in plants. Every prediction made for *Ustilago*, based on the established characteristics of RI and RC H3 variants in plants and in animals, was confirmed. Evolutionary analysis has demonstrated that the structural duplication and functional divergence of RI and RC variants arose independently, hundreds of millions of years apart, in those ancestral species that gave rise to the clades of animals, plants, ciliates, and basidiomycetes (6). The RC and RI H3 variants arose 400 million years ago in the ancestral species of all multicellular metazoa. For plants, this event occurred likely more than 800 million years ago in the ancestral species of all multicellular metazoa.
Replacement Histone H3 in \textit{Ustilago}

Histone H3 Acetylation—Acetylation levels of the \textit{Ustilago} histone H3 variants, $10^{-30} \text{S}$ higher in the RI H3.1 protein than in H3.2 histone, ranged between 0.9 and 1.7 acetylated lysine per polypeptide chain, depending on culture conditions. This is slightly lower than the steady state levels of 1.8–2.1 in \textit{S. cerevisiae} (38). Lysine labeling revealed much higher levels of acetylation in newly synthesized H3 for H3.1 with $3.1 \pm 0.4$ (n = 5) and for H3.2 with $2.7 \pm 0.3$ (n = 5) acetylated lysines (Fig. 5). In each case, nonacetylated forms did not exist immediately following histone synthesis. This suggested that each nH3 polypeptide was quantitatively acetylated at one particular lysine. The level and pattern of acetylation in A. prophage graphs (Figs. 2B, 4, and 5) suggests that, in addition, N-terminal lysines 9, 14, 18, 23, and 27 are acetylated to a higher extent in new H3 than in bulk H3 protein. In yeasts, high levels of acetylation of lysine 56 have been reported (39, 40). Histone acetyltransferase Rtt109 is thought to be responsible for the transient complete acetylation of H3K56 during nucleosome assembly in replicative chromatin, during DNA repair, or associated with transcription (40–47). Support for the involvement of H3K56 acetylation comes from the increased acetylation of new H3.2 protein under conditions of replicative stress induced by hydroxyurea (lane HHL-n in Fig. 6B), as reported for \textit{S. cerevisiae} (41). Study of the sites and extent of lysine acetylation in new and matured H3 proteins of \textit{U. maydis} and \textit{S. cerevisiae}, separated by reversed phase HPLC, is in progress and will be reported elsewhere.

Are Both Histone H3 Variants of \textit{Ustilago} Essential?—The multiplicity of RC and RI H3 genes in animals and plants prevents one from addressing experimentally, by gene knock-out, the simple but important question: “Can the cell live with just one of the functionally distinct histone H3 variant forms?” Full knock-out analysis for the two RC and two RI H3 genes of diploid \textit{Tetrahymena} has been reported (9). Remarkably, neither H3 variant appeared essential under vegetative growth conditions. However, reduced availability of new H3 protein caused reduction in growth rates, whereas for as yet unresolved reasons, the RI H3 variant appears to be required to produce viable sexual progeny (9).

In haploid \textit{Ustilago}, gene disruption of the single U1 and U2 loci is relatively efficient. Following curing of the initial heterokaryons, which grew like WT cells, by continued subculture under selection, viable and stable U1KO and U2KO homokaryons were isolated that did not produce any histone H3.1 or H3.2 protein, respectively (Fig. 6C). Synthesis and acetylation of the single H3 variant appeared unaffected. In U1KO cells, the amount of H3.2 protein per cell was approximately the same as the combined amount of H3.1 and H3.2 protein per WT cell (Fig. 6C). The cell doubling time increased by ~25% (Fig. 6H), consistent with the ~25% steady state level of H3.1 protein (Figs. 2B, 3, and 6). Thus no effect was detected from the U1 gene knock-out in vegetatively growing cells other than the growth rate limitation because of the reduction in total H3 synthesis. The similar effect was seen in U2KO strains where cell doubling rates were increased more than 2-fold (Fig. 6H) when the major source of H3 protein, RC H3.2, was deleted. In \textit{Tetrahymena}, a similar reduction in growth rate was observed in RC H3 knockouts, although this effect could be partially compensated for by up-regulation of a RI H3 locus (9). Such an effect on U1 expression in U2KO strains was not detected in \textit{Ustilago}. In stationary cultures, the only difference
observed between WT and knock-out strains was an earlier browning (i.e. dying) of the KO cells. However, contrary to the expectation based on the synthesis and incorporation of H3 variant proteins in stationary cells (Fig. 6D), this effect was stronger for U2KO cells where H3.1 incorporation continued than for U1KO strains where no histone H3 protein was incorporated into the chromatin of nonproliferating cells (lane 1191–1200). Nucleosomal density differences in the chromatin of these knock-out strains are being investigated in an attempt to identify the basis for this effect.

Acknowledgments—We gratefully thank Kevin McCluskey (Fungal Genetics Stock Center, University of Missouri, Kansas City, MO), Scott Gold (University of Georgia, Athens, GA), Michael Feldbrügge (University of Düsseldorf, Düsseldorf, Germany), and Jörg Kämper and Regine Kahmann (University of Marburg, Marburg, Germany) for providing strains, plasmids, and technical and scientific advice.

REFERENCES
1. Urban, M. K., and Zweidler, A. (1983) Dev. Biol. 95, 421–428
2. Ridsdale, J. A., and Davie, J. R. (1987) Nucleic Acids Res. 15, 1081–1096
3. Lewis, P. W., Elsaesser, S. J., Noh, K. M., Stadler, S. C., and Allis, C. D. (2010) Proc. Natl. Acad. Sci. U.S.A. 107, 14075–14080
4. Elsaesser, S. J., Goldberg, A. D., and Allis, C. D. (2010)Curr. Opin. Genet. Dev. 20, 110–117
5. Takayama, Y., and Takahashi, K. (2007) Nucleic Acids Res. 35, 3223–3237
6. Waterborg, J. H. (2011) Biochem. Cell Biol., in press
7. Thatcher, T. H., MacGaffey, J., Bowen, J., Horowitz, S., Shapiro, D. L., and Robertson, A. J. (1996) Nucleic Acids Res. 22, 180–186
8. Waterborg, J. H., and Robertson, A. J. (1996) J. Mol. Evol. 43, 194–206
9. Cui, B., Liu, Y., and Gorovsky, M. A. (2006) Mol. Cell. Biol. 26, 7719–7730
10. Ahmad, K., and Henikoff, S. (2002) Mol. Cell 9, 1191–1200
11. Kämper, J., Kahmann, R., Bölder, M., Ma, L. J., Brefort, T., Saville, B. J., Banuett, F., Kronstad, J. W., Gold, S. E., Müller, O., Perlin, M. H., Wösten, H. A., de Vries, R., Ruiz-Herrera, J., Reynaga-Peña, C. G., Snetseelaar, K., McCann, M., Pérez-Martín, J., Feldbrügge, M., Basse, C. W., Steinberg, G., Ibeas, J. I., Holoman, W., Guzman, P., Farman, M., Stajich, J. E., and Kapros, T. (1994) Nucleic Acids Res. 22, 180–186
12. Waterborg, J. H., and Robertson, A. J. (1996) J. Mol. Evol. 43, 194–206
13. Cui, B., Liu, Y., and Gorovsky, M. A. (2006) Mol. Cell. Biol. 26, 7719–7730
14. Ahmad, K., and Henikoff, S. (2002) Mol. Cell 9, 1191–1200
15. Kämper, J., Kahmann, R., Bölder, M., Ma, L. J., Brefort, T., Saville, B. J., Banuett, F., Kronstad, J. W., Gold, S. E., Müller, O., Perlin, M. H., Wösten, H. A., de Vries, R., Ruiz-Herrera, J., Reynaga-Peña, C. G., Snetseelaar, K., McCann, M., Pérez-Martín, J., Feldbrügge, M., Basse, C. W., Steinberg, G., Ibeas, J. I., Holoman, W., Guzman, P., Farman, M., Stajich, J. E., and Kapros, T. (1994) Nucleic Acids Res. 22, 180–186
16. Waterborg, J. H., and Robertson, A. J. (1996) J. Mol. Evol. 43, 194–206
17. Ridsdale, J. A., and Davie, J. R. (1988) Nucleic Acids Res. 16, 5915–5926
18. Waterborg, J. H., and Kapros, T. (2002) Biochem. Cell Biol. 80, 279–293
19. Waterborg, J. H. (2002) Biochem. Cell Biol. 80, 363–378
20. Kapros, T., Robertson, A. J., and Waterborg, J. H. (1995) Plant Mol. Biol. 28, 901–914
21. Holoman, W. K., Schirawski, J., and Holloway, R. (2008) FunGenet. Biol. 45, (Suppl. 1) S31–S39
22. Holoman, W. K., Schirawski, J., and Holloway, R. (2007) Trends Microbiol. 15, 525–529
23. Ruiz-Herrera, J., Leon, C. G., Guevara-Olvera, L., and Carabez-Trejo, A. (1995) Microbiology 141, 695–703
24. Waterborg, J. H. (2000) J. Biol. Chem. 275, 13007–13011
25. Garcia, B. A., Hake, S. B., Diaz, R. L., Kauer, M., Morris, S. A., Recht, J., Shabanowitz, J., Mishra, N., Strahl, B. D., Allis, C. D., and Hunt, D. F. (2007) J. Biol. Chem. 282, 7641–7655
26. Adkins, M. W., Carson, J. J., English, C. M., Ramey, C. J., and Tyler, J. K. (2007) J. Biol. Chem. 282, 1334–1340
27. Minard, L. V., Williams, J. S., Walker, A. C., and Schultz, M. C. (2011) J. Biol. Chem. 286, 7082–7092
28. Tang, Y., Holbert, M. A., Delgoffe, N., Wurtele, H., Guillemette, B., Meeth, K., Yuan, H., Drogaris, P., Lee, E. H., Durette, C., Thibault, P., Verreault, A., Cole, P. A., and Marmorstein, R. (2011) Structure 19, 221–231
29. Aslam, A., and Logie, C. (2010) PLoS One 5, e10851
30. Masumoto, H., Hawke, D., Kobayashi, R., and Verreault, A. (2005) Nature 436, 294–298
31. Thaminy, S., Newcomb, B., Kim, J., Gathbonton, T., Foss, E., Simon, J., and Bedalov, A. (2007) J. Biol. Chem. 282, 37805–37814
32. Agez, M., Chene, M., Gueisson, R., van Heijenoort, C., Thuret, J. Y., Mann, C., and Ochsbein, F. (2007) Structure 15, 191–199
33. Xhemalce, B., Miller, K. M., Driscoll, R., Masumoto, H., Jackson, S. P., Kouzairides, T., Verreault, A., and Arcangioli, B. (2007) J. Biol. Chem. 282, 15040–15047
34. Luger, K., Mader, A. W., Richmond, R. K., Sargent, D. F., and Richmond, T. J. (1997) Nature 389, 251–260
35. Waterborg, J. H. (1990) Electrophoresis 11, 638–641