H2A.Z Functions To Regulate Progression through the Cell Cycle†

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Histone H2A variants are highly conserved proteins found ubiquitously in nature and thought to perform specialized functions in the cell. Studies in yeast on the histone H2A variant H2A.Z have shown a role for this protein in transcription as well as chromosome segregation. Our studies have focused on understanding the role of H2A.Z during cell cycle progression. We found that Δh2a cells were delayed in DNA replication and progression through the cell cycle. Furthermore, cells lacking H2A.Z required the S-phase checkpoint pathway for survival. We also found that H2A.Z localized to the promoters of cyclin genes, and cells lacking H2A.Z were delayed in the induction of these cyclin genes. Several different models are proposed to explain these observations.

The packaging of DNA into chromatin affects processes such as transcription, replication, and genome integrity (22). DNA replication initiates at specific origins of replication, and the utilization of an origin is influenced by its chromatin structure (87): nucleosomes positioned over an origin interfere with its ability to initiate replication (72), while the presence of appropriately positioned nucleosomes located adjacent to origins facilitates replication initiation (48). Furthermore, origins located in or adjacent to telomeric heterochromatin replicate late due to the presence of the chromatin-associated Sir proteins (12, 52, 63, 73). Chromatin can modulate the repair of DNA by affecting accessibility to damaged sites (62, 83). Repair from damage is intimately associated with chromatin structure, with transcriptionally active chromatin being more efficiently repaired than inactive chromatin (33, 75).

Chromatin also affects cell cycle progression. Histones in chromatin lacking N-terminal acetylation sites activate a DNA damage checkpoint and undergo a G2/M delay (53), as do mutants in the NuA3 and SAGA histone acetyltransferase complexes (35, 93), while mutations in the NuA4 histone acetyltransferase complex cause a complete G2/M arrest (10, 35, 45).

In addition to their role in replication and repair, histones and nucleosomes play a direct role in regulating gene expression, including the transcription of genes that are required for normal cell cycle progression (13). The periodic expression of cell cycle genes requires the function of chromatin modifying (SAGA) and remodeling (Swi/Snf) factors that alter the chromatin structure of the promoters of these genes at specific stages of the cell cycle (14, 15, 39, 40).

In addition to the role of the major histones in these processes, there are specific histone variants that affect individual processes such as genome integrity, transcription, or chromosome segregation (37). The histone H3 variant H3.3 is primarily involved in transcriptional regulation whereas cenH3 is vital for chromosome segregation, and the H2A variant H2A.X is directly involved in DNA repair and genome integrity. Histone H2A.Z is a highly conserved histone H2A variant that plays a role in chromosome segregation, genomic integrity, and gene regulation (1, 11, 17, 38, 41, 44, 46, 50, 55, 56, 64, 67, 76). In Drosophila melanogaster, a single variant called H2AvD has sequence characteristics of both H2A.X and H2A.Z and is involved in DNA repair as well as gene regulation (43, 76).

We explored the role of histone H2A.Z in cell cycle progression in yeast and found that mutants in H2A.Z had defects in progression through S phase and were delayed in origin firing. In our analysis of these phenotypes, we found that H2A.Z was also required for the efficient and timely transcriptional induction of cell cycle-regulatory proteins, which may explain the delay in replication initiation. Defects in replication initiation have been shown to cause DNA damage (29, 31, 71), and consistent with the role of H2A.Z in the initiation of replication, we found that in the absence of H2A.Z, the S-phase checkpoint was essential for survival, suggesting a connection between this histone variant and the maintenance of genomic integrity.

MATERIALS AND METHODS

Yeast strains. Yeast strains and their genotypes are listed in Table 1. Details on strain construction are available upon request. Standard yeast media and methods were used.

For cell cycle synchronization and release, log-phase cells were treated with alpha factor (0.02 μg/ml) for 3 h at 25°C or 2 h at 30°C, washed, and released into YPD (70a) with 0.1 mg/ml pronase.

Protein lysate preparation and immunoblotting protocols followed were essentially as previously described, except that glass beads were used to disrupt cells instead of dry ice (30).

Total RNA was isolated by the method in reference 69, and cDNA was prepared using the reverse transcription-PCR (RT-PCR) kit from Invitrogen. Replication timing experiments were performed as outlined previously (23). Samples for chromatin immunoprecipitation (ChIP) were prepared as described previously (17), with a minor modification that cells were disrupted using glass beads instead of lyticase.

Primer sequences for all PCR amplicons are available upon request.

Quantitative PCR. Quantitative PCR with radionuclides was performed on cDNA for RT-PCR analysis and on DNA from ChIP reactions. Reaction volumes were typically 50 μl and contained 20 mM Tris-HCl, pH 8.4, 50 mM KCl, 1.5 mM MgCl2, 0.2 mM of each deoxynucleotide triphosphate, 0.2 μM of each primer, and 0.034 μM of each [α-32P]dATP and [α-32P]dCTP and 1 U of Plat-
**TABLE 1. Strains used in this study**

| Strain | Genotype |
|--------|----------|
| W303-1a | MATa ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 |
| JRY4012 | MATa lys2a:HisG W303-1a |
| BUY683 | MATa lys2a:HisG htz1Δ:kanMX W303-1a |
| BUY679 | MATa lys2a:HisG htz1Δ:kanMX W303-1a |
| ROY1944 | MATa lys2a:HisG bar1Δ::LEU2 W303-1a |
| ROY1945 | MATa lys2a:HisG bar1Δ::LEU2 W303-1a |
| BUY828 | MATa lys2a::TRP1 htz1Δ:kanMX W303-1a |
| BUY830 | MATa lys2a::TRP1 htz1Δ:kanMX W303-1a |
| ROY2245 | MATa ade2-1 bar1Δ::LEU2 DAM::LEU2 |
| BUY979 | MATa ade2-1 bar1Δ::LEU2 DAM::LEU2 |
| ROY2814 | MATa lys2a::HisG bar1Δ::LEU2 htz1Δ:kanMX |
| ROY2975 | MATa lys2a::HisG bar1Δ::LEU2 URA3::ORC1-HA W303-1a |
| ROY2980 | MATa lys2a::HisG bar1Δ::LEU2 TRP1::HA-CDC45 W303-1a |
| ROY3236 | MATa ade2-1 RAD5 snf1Δ::HIS3 mec1Δ::TRP1 |
| ROY1903 | MATa ade2-1 RAD5 snf1Δ::HIS3 mec1Δ::TRP1 |
| ROY2253 | MATa ade2-1 RAD5? snf1Δ::HIS3 mec1Δ::TRP1 W303-1a |
| ROY2427 | MATa RAD5Δ CAN1 tel1Δ::kanMX W303-1a |
| ROY2490 | MATa RAD5Δ? CAN1Δ? lys2Δ::HisG tel1Δ::URA3 htz1Δ::URA3 W303-1a |
| ROY2360 | MATa ade2-1 meclΔ::TRP1 W303-1a |
| ROY2454 | MATa meclΔ::TRP1 htz1Δ::kanMX W303-1a |
| ROY2161 | MATa lys2a::HisG rad9Δ::URA3 W303-1a |
| ROY2159 | MATa rad9Δ::URA3 htz1Δ::kanMX W303-1a |
| ROY2397 | MATa ade2-1 chk1Δ::HIS3 W303-1a |
| ROY2459 | MAT7 ade2Δ lys2Δ::HIS3 htz1Δ::kanMX W303-1a |
| ROY2169 | MATa lys2a::HisG rad17Δ::LEU2 W303-1a |
| ROY2171 | MATa lys2a::HisG rad17Δ::LEU2 htz1Δ::kanMX W303-1a |
| ROY2917 | MATa lys2a::HisG mec1Δ::KAN-HIS3 W303-1a |
| ROY2954 | MATa lys2a::HisG mec1Δ::KAN-HIS3 htz1Δ::kanMX W303-1a |
| ROY1963 | MATa lys2a::HisG htz1Δ::URA3 W303-1a |
| ROY2206 | MATa pol2-2::LEU2 htz1Δ::kanMX |
| ROY2208 | MATa ade2-1 pol2-2::LEU2 htz1Δ::kanMX |
| ROY2210 | MATa ade2-1 pol2-2::LEU2 htz1Δ::kanMX |
| ROY2248 | MATa ade2-1 pol2-2::LEU2 htz1Δ::kanMX |
| ROY2252 | MATa ade2-1 pol2-2::LEU2 htz1Δ::kanMX |
| ROY2803 | MATa ade2-1 rds5Δ::LEU2 W303-1a |
| ROY2532 | MATa ade2-1 rds5Δ::LEU2 W303-1a |
| ROY2535 | MATa ade2-1 rds5Δ::LEU2 W303-1a |
| ROY3307 | MATa ade2-1 swr1Δ::matMX W303-1a |
| ROY3340 | MATa ade2-1 swr1Δ::matMX W303-1a |
| ROY3341 | MATa ade2-1 swr1Δ::matMX W303-1a |
| ROY3757 | MATa lys2a bar1Δ::LEU2 SAC1-3xHA::HIS htz1Δ::kanMX W303-1a |
| ROY3759 | MATa ade2-1 bar1Δ::LEU2 DAM::LEU2 arn680Δ::HIS arn690Δ::TRP1 W303-1a |
| ROY3760 | MATa ade2-1 bar1Δ::LEU2 DAM::LEU2 arn680Δ::HIS arn690Δ::TRP1 W303-1a |

**RESULTS**

**Genetic analysis between htz1Δ and replication genes.**

We have previously demonstrated that H2A.Z and the N-terminal tail of histone H4 have an overlapping role required for viability in yeast (17). Given that the N-terminal tail of H4 is important for both transcription and cell cycle progression (32, 53), we investigated whether H2A.Z, in addition to its role in transcription, also affects cell cycle progression. As a first step in determining whether it has a role outside of transcription, we investigated genetic interactions between HTZ1 and mutations in genes involved in chromatin assembly, chromosome structure, replication, repair, cell cycle progression, and checkpoint control. We systematically crossed htz1Δ strains with either deletions or conditional mutations in nearly 90 genes (see Table S1 in the supplemental material). Visual examination of the double mutants after tetrad dissection for significant growth defects identified several genes involved in replication and checkpoint control to be synthetically lethal or to cause extreme sickness in combination with htz1Δ. Genetic interactions were observed between htz1Δ and conditional mutants in orc5-1, orc2-1, mri1-1 (Fig. 1A), sld5-1, psf1-1, or mec3-1 (data not shown). Interestingly, mutations in the DNA helicases snr2Δ, mmn3Δ, and sgs1Δ (data not shown); DNA polymerases; the cyclins clb2Δ, clb3Δ, clb5Δ, or clb6Δ; or the kinase cdc7-1 had no synthetic phenotypes when combined with htz1Δ. Since Clb5p and Clb6p are redundant for replication initiation (70), we tested the phenotype of a clb5Δ clb6Δ htz1Δ triple mutant and observed a severe growth defect in this strain (Fig. 1A).
Our genetic interaction data reveal synthetic phenotypes between proteins required for replication and mutations in Htz1p. These genetic interactions can be understood based on the functions of the interacting genes. The origin recognition complex (ORC) (including Orc2p and Orc5p) binds to replication origins and is required for initiation of replication and the S-phase checkpoint (7, 71, 86). The GINS (Go, Ichi, Ni, and San) complex (77) (including Sld5p and Paf1p) is required...
for the establishment of replication forks and progression of the forks from the origins, while the MCM (minichromosome maintenance) complex (92) (including Mcm3p) is believed to be a DNA helicase that binds to the ORC at origins prior to initiation while replication initiation is regulated by the Cdc28/ cyclin-dependent modification of various complexes (18). Thus, proteins that function in replication had genetic interactions with this histone variant.

The S-phase checkpoint pathway was essential in the absence of H2A.Z. We showed that H7ZI genetically interacted with genes involved in replication, and mutants in replication proteins have previously been shown to be synthetically lethal with checkpoint mutants (9, 31, 74). We therefore asked whether cells lacking H2A.Z also had genetic interactions with checkpoint proteins. We generated and analyzed double mutants between htz1Δ and mutations in genes involved in checkpoint control (reviewed in reference 54). We systematically crossed htz1Δ strains with either deletions or conditional mutations in the genes known to be involved in DNA damage, S-phase, and spindle checkpoint control (Fig. 1B). We identified several genes involved in replication checkpoint control to be synthetically lethal or to cause extreme sickness in combination with htz1Δ. We identified rad53-1 and pol2-12 to be lethal with htz1Δ, while mrc1-1 (Fig. 1D) and asf1Δ (data not shown) cells were synthetically sick. The systematic genetic analysis with H2A.Z has also identified Mrc1p to be synthetically sick with H2A.Z (81). The synthetic lethality between htz1Δ and rad53-1 or pol2-12 was confirmed by plasmid-shuffle experiments (Fig. 1C). The interaction between htz1Δ and pol2-12 was allele specific, as pol2-18 was not lethal with htz1Δ (data not shown). The pol2-12 allele of DNA polymerase epsilon is defective in the S-phase checkpoint (59), while pol2-18 is a replication-defective allele of this gene (6). The synthetic interactions with Rad53p, Pol2p, Mrc1p, and Asf1p reflect a requirement for the checkpoint in cell survival when replication is defective.

Compared with the replication checkpoint, mutations in the DNA damage checkpoint genes did not have any synthetic growth defects in the absence of Htz1p (Fig. 1D), suggesting that the defect was being generated during replication in these mutant cells.

The S-phase checkpoint is functional in htz1Δ cells. Since H2A.Z mutants had genetic interactions with the S-phase checkpoint genes, we inquired whether these cells had a fully functional S-phase checkpoint pathway. Yeast cells grown in the presence of a sublethal dose of hydroxyurea (HU; 0.2 M) initiate replication but block cell cycle progression in S phase via the S-phase checkpoint (60), and we investigated whether htz1Δ cells were able to arrest in S phase, since this checkpoint was necessary for the viability of htz1Δ cells. Wild-type and mutant cells were first synchronized in the G1 phase of the cell cycle with α factor and then released into medium containing HU. Wild-type cells shifted to HU-containing media arrest in the S phase of the cell cycle with short mitotic spindles, a single nucleus, and large buds (Fig. 2A and B). Identically treated htz1Δ cells showed that these were also proficient in activating the checkpoint because they also arrested in the S phase of the cell cycle (as monitored by flow cytometry) with short spindles, a single nucleus, and large buds (Fig. 2A and B).

Activation of the S-phase checkpoint also results in the phosphorylation of checkpoint proteins such as Rad53p. We also investigated the Rad53p phosphorylation response of htz1Δ cells in the presence of HU. Wild-type and mutant cells arrested with α factor were released into various concentrations of HU (0 to 25 mM) for 30 or 60 min, and the phosphorylation of Rad53p was monitored as an index of checkpoint activation (Fig. 2C). We found that Rad53p was phosphorylated in the mutant within 30 min of exposure to low concentrations of HU, while this was seen only after 60 min in wild-type cells, suggesting that cells lacking H2A.Z were hypersensitive to activation of the checkpoint. The hypersensitivity may be related to the observation that cells exposed to small doses of DNA damage more rapidly activate the checkpoint pathway to additional damage (61). When cells are exposed to HU, early origins of replication fire but the firing of late origins is delayed. To determine if the firing of late origins in HU was also delayed in htz1Δ mutants, we used an assay that detects replication intermediates generated at origins (68). Normally, ARS305 is an early firing origin whose activation is not affected by HU, whereas ARS801 is a late origin that does not fire in the presence of HU within 120 min (2). Cells synchronized in α factor were released into YPD with 0.2 M HU and harvested at 30-min intervals, and the appearance of replication intermediates was analyzed (Fig. 2D). The ARS305 replication intermediates were clearly visible in wild-type and htz1Δ cells, while ARS801, a late origin, failed to fire in wild-type and htz1Δ cells in the presence of HU within the first 120 min.

All of these results suggested that htz1Δ cells, exposed to HU, triggered the appropriate checkpoint to block cell cycle progression.

Removal of HU and restoration of nucleotide pools results in the resumption of DNA synthesis from stalled forks and new forks from late firing origins (80). If htz1Δ cells were defective in recovery from DNA damage during S-phase arrest, their viability should be reduced after exposure to HU. The wild type and H2A.Z mutants were maintained for various lengths of time (up to 8 h) in 0.2 M HU and then plated onto YPD plates lacking HU to determine the viability of those cells (Fig. 2E). These analyses revealed that htz1Δ cells retained their viability to the same extent as wild-type cells in HU, indicating that the S-phase checkpoint that monitored completion of replication functioned properly to arrest these cells until the HU-induced replication stress had subsided.

Timing of replication was delayed in htz1Δ cells. The genetic interactions observed with htz1Δ mutants suggested that this histone variant might play a role in replication. As a first step in determining if this was indeed the case, we measured the replication timing of several different loci in wild-type and mutant cells at 30°C (27).

Consistent with previously published data (23), in wild-type cells a region adjacent to the ARS1 locus replicated early in S phase (35 min after release from α factor), and a region adjacent to the HMR locus replicated later (45 min after release from α factor) (Fig. 3A), and the difference in replication timing between these two loci was approximately 10 min. In cells lacking H2A.Z, ARS1 and HMR both replicated 15 min later than in the wild type. These results suggested that H2A.Z might be influencing the timing of replication from origins. Notably, H2A.Z loss led to a delay in the entire replication program, and unlike other determinants that affect the differ-
ential firing of origins during replication (4, 20, 26), this mutation did not cause the early and late replication program to be disrupted. The profiles also suggested that in the mutant, the regions examined took longer to replicate since the peaks were significantly broader at all loci examined. This could be due to slower fork progression or a consequence of a loss of synchrony in replication initiation. To distinguish between the two, we measured fork progression rates of a replication fork that initiated from ARS607 and replicated a ~75-kbp stretch of DNA (79). The time of replication of three EcoRI restriction fragments (2.8 kb, 2.1 kb, and 1.7 kb long) located ~4.5 kb, ~39 kb, and ~63 kb from ARS607 was determined and plotted as a function of distance, and the data demonstrated that the rate of fork progression was not significantly different in the mutant compared to the wild-type cells (Fig. 3B). These results indicated that fork progression per se was not affected
in the mutant and that the defect was likely in replication initiation.

Cdc45p release from origins was affected in htz1Δ cells. One of the steps in replication initiation is the association of Cdc45p with origins (4, 5, 94), which then migrates with the replication fork as replication commences. Thus, the association of Cdc45 with, and its release from, origins are good indicators of replication initiation. We therefore investigated the loading and release of Cdc45p at early and late origins in wild-type cells and htz1Δ mutants by ChIP. In wild-type cells grown at 15°C, we found that Cdc45p was loaded at ARS305 (an early origin) at 75 min while it was present at ARS501 (a late origin) at 135 min (Fig. 3C). The difference in time of replication in Fig. 3C compared to that in Fig. 3A is due to the fact that cells were grown at 15°C. Since Cdc45p is a component of the replication fork, upon replication initiation it moves with the fork and occupancy at the origin sequence is lost. Compared to wild-type cells, loading of Cdc45p at both origins was not dramatically delayed in htz1Δ cells. While there was no major delay in the binding of Cdc45p to the origin, in the mutant the residence time of Cdc45p at the origin increased by an additional 30 min before release from the origin (Fig. 3C). Our results would argue either that replication was initiating asynchronously in the mutant population or replication initiation or events soon thereafter were delayed. These two scenarios are consistent with the data showing that regions of the genome took longer to replicate in the mutant.

Origin firing was delayed in cells lacking Htz1p. We further examined the replication delay in htz1Δ cells by analyzing replication origin firing and fork progression using two-dimensional agarose gel electrophoresis (2D gels) (8). We released G1-synchronized cells into S phase at 15°C and analyzed replication structures at the early-origin ARS305. In wild-type cells, bubble structures indicative of origin firing were first observed at 60 min after release; the abundance of initiation bubbles declined significantly by 75 min and were almost gone by 90 min (Fig. 3D). In htz1Δ cells, initiation bubbles also were first observed at 60 min. However, in contrast to wild-type cells, the signal was significantly weaker in the htz1Δ cells at 60 min, and initiation events at ARS305 occurred over a much broader period, lasting until about 120 min. Thus, replication initiation events appear to occur asynchronously throughout the population of htz1Δ cells, with most cells being delayed in ARS305 initiation. Nevertheless, replication initiation remained highly efficient in htz1Δ cells because virtually no fork structures were detected. The 2D gels also suggest that fork movement within the vicinity of the origin is normal, as there is no accumulation of smaller bubble structures or any evidence of fork pausing. Analysis of a natural pause site (the tRNA gene, SUP6) also suggests there is no defect in replication fork pausing or progression through natural pause sites in htz1Δ cells. These data are consistent with the analyses of replication timing (Fig. 3A) and Cdc45 chromatin association (Fig. 3C) and suggest that cells lacking H2A.Z have a lengthened S phase.

H2A.Z localization at early and late origins. We therefore determined if there was any correlation between the presence of the variant at origins and origin function. If H2A.Z was present in the vicinity of origins, then it might influence origin firing and S-phase progression by affecting the local chromatin structure. We mapped H2A.Z at all known origins of replication on chromosome VI using quantitative ChIP (Fig. 3E). We determined that H2A.Z was present in various amounts at different origins. We next compared the levels of H2A.Z at various origins to their time of replication and the usage of these origins (25, 91). Surprisingly, we did not find any correlation between H2A.Z occupancy at origins and replication timing or usage of those origins.

H2A.Z was required for normal cell cycle progression. Cells lacking H2A.Z showed a delay or asynchrony in replication initiation that could be the culmination of a delay in the entire program that is normally initiated at Start. We therefore investigated the kinetics of cell cycle progression in cells lacking H2A.Z by FACS analysis. Wild-type and mutant cells were initially synchronized in the G1 phase of the cell cycle with α factor and then released into YPD medium. Cells were harvested at 10-min intervals, and their DNA content was analyzed by flow cytometry (Fig. 4). The analysis revealed that cells lacking this histone variant progressed more slowly through the cell cycle, with an approximate delay of 10 min compared to wild-type cells (compare the wild type to the mutant at 40 min and 50 min after release from α factor). These results suggested that in the absence of H2A.Z cell cycle progression was delayed, presumably due to the longer S

FIG. 3. Timing of replication. A. Loci were delayed in the timing of their replication in htz1Δ cells. Alpha factor-arrested wild-type and mutant cells stably and constitutively expressing DNA adenine methylase (ROY2245 and ROY3444) were released into YPD at 30°C, and time points were collected every 5 min. Genomic DNA isolated from these samples was digested with DpnI and EcoRI and analyzed by DNA blots using probes that hybridized adjacent to ARS1 and HMR. The y axis represents the amounts of hemimethylated (newly replicated) DNA that hybridized to the probes. B. Fork progression rate on chromosome VI. Wild-type and mutant cells (ROY3739 and ROY3760) were treated and analyzed as described in the legend to Fig. 6A, except that the genomic DNA was digested with DpnI and EcoRI and the blots were probed with probes that hybridized to EcoRI fragments located −4.5 kb (probe 2.8), −39 kb (probe 2.1), and −63 kb (probe 1.7) from ARS607. The time of replication for each probe was calculated by determining the peak from the replication profiles, and these values were plotted against distance from ARS607. C. The kinetics of loading of HA-Cdc45p at ARS305 and ARS501. Alpha factor-arrested wild-type (ROY2980) and htz1Δ (ROY3236) strains carrying HA-Cdc45p were released in YPD at 15°C, and time points collected every 15 min were subjected to quantitative ChIP analysis. ChIP experiments were performed using the HA.11 monoclonal antibody. The y axis represents the ratio of immunoprecipitate (IP)/input DNA for each sample. The wild-type and mutant profiles are depicted as dashed and solid lines, respectively. D. Initiation of replication occurred over a longer duration in htz1Δ cells. Alpha factor-arrested wild-type (ROY1945) and htz1Δ (ROY3442) strains were released in YPD at 15°C, and time points were collected every 15 min. Replication intermediates were resolved by 2D gel electrophoresis, and the gels were blotted and probed with a probe specific to ARS305 and SUP6. E. H2A.Z occupancy near origins. Quantitative ChIP analysis of HA-H2A.Z localization at all known origins on chromosome VI (ChrVI) in ROY2987 cells. The time of replication (Trep) and the usage of each replication are shown below the x axis and are derived from references 25 and 91.


S phase was lengthened in *htz1Δ* mutants. The commitment to cell division versus growth occurs at the end of G1 at an execution point called Start. Cells initiate budding and spindle pole body duplication and commit to initiating DNA replication (58). Progression through Start can therefore be measured by bud emergence and the degradation of the Cdk inhibitor Sic1p (57, 84). We initially measured the kinetics of bud emergence in α factor-arrested cells that were released into fresh YPD medium at 15°C. Samples were collected every 15 min and were monitored for bud emergence by microscopy. There was a striking lag in bud emergence in the mutant (Fig. 5A). Since bud emergence is a subjective assay, we also examined the degradation of Sic1p-3xHA in α factor-arrested cells released into fresh medium by immunoblotting (Fig. 5B). The kinetics of Sic1p degradation was delayed by 15 min in cells lacking H2A.Z (Fig. 5B). Our loading controls (histone H3) indicate that the differences in Sic1p observed on our protein blots were not due to differences in loading but reflect differences in the kinetics of degradation of Sic1p. Our data suggest that at a minimum, H2A.Z was directly or indirectly regulating events at Start rather than solely affecting replication.

Transcriptional induction of specific cyclin genes was delayed in *htz1Δ* mutants. One of the key events at Start is the Cdk kinase-dependent activation of transcription of genes required for G1/S progression (34), including the G1- and S-phase cyclin genes *CLN1 CLN2* and *CLB5 CLB6*, respectively (3). Previous studies on genome-wide steady-state transcript levels have suggested that H2A.Z is predominantly involved in the activation of subtelomeric genes but not cell cycle-regulated genes (55). However, our observations that events in Start might be delayed in *htz1Δ* cells and, given that bud emergence and replication initiation are temporally controlled via periodic fluctuations in the transcription of the cyclin genes (88, 89) and since H2A.Z has previously been shown to regulate the transcription of some inducible genes (1), led us to determine the effects of H2A.Z loss on the transcription of a few cell cycle-regulated genes. We chose *CLN2* and *CLB5*, and we isolated total RNA from asynchronously grown cultures and quantitated the levels of *CLN2* and *CLB5* mRNA by RT-PCR using radionuclides. These were normalized to *TUB2* mRNA, which has previously been shown to be unaffected in *htz1Δ* cells (67). Similar to previous observations (55), we observed a less than twofold decrease in the transcript levels of these genes in asynchronous cultures (Fig. 6A).

H2A.Z has been shown to affect the kinetics of induction of inducible genes (1). We therefore analyzed the induction of the *CLN2* and *CLB5* transcripts as a function of the cell cycle in wild-type and *htz1Δ* cells. Cells were arrested with α factor and released into fresh YPD medium at 16°C, and samples

FIG. 4. S phase was lengthened in *htz1Δ* mutants. FACS profiles of wild-type (ROY1945) and *htz1Δ* (ROY3442) cells through the cell cycle. Cells were arrested with α factor and released into YPD at room temperature, and samples collected every 10 min were processed for FACS analysis.
were collected every 30 min followed by quantitation of the transcripts. We analyzed the appearance of CLN2 and CLB5 transcripts after release from α factor and normalized these to the TUB2 mRNA (Fig. 6B). In wild-type cells, the levels of CLN2 and CLB5 transcripts peaked around 80 min after release from α factor, and, interestingly, the induction of both transcripts was delayed in the htz1Δ mutant. While additional experiments will be necessary to determine all of the G1/S-phase genes that are affected in htz1Δ cells, these results suggest that a delay in the transcriptional induction of genes at Start may feed into the S-phase delay seen in cells lacking H2A.Z.

**H2A.Z localized to the promoters of the CLN2 and CLB5 genes.** H2A.Z could be either directly regulating the induction of CLN2 and CLB5 transcript levels, or it may be exerting this effect indirectly. To distinguish between the two possibilities, we determined whether promoters of these genes contained H2A.Z using ChIP experiments. We mapped 3xHA-Htz1p adjacent to the CLN2, CLB5, and GIT1 promoters using an anti-HA monoclonal antibody by quantitative ChIP using quantitative PCR with SyBr Green. The data were normalized to the Tel V1R 0.5-kb locus that is depleted of this histone variant (55). We found that H2A.Z was present at both the CLN2 and CLB5 promoters (Fig. 6C), and the amount of H2A.Z at the CLN2 and CLB5 promoters was equivalent to that seen with the GIT1 promoter, where H2A.Z has previously been shown to localize (55). These data are consistent with results demonstrating that H2A.Z influenced the rapid induction of the cyclin genes.

**Overexpression of Cln2p suppresses the delay in cell cycle progression.** Expression of Cln2p is a key driver of events at Start, and overexpression of CLN2 accelerates Start (82). It has even been suggested that the G1 cyclins may be the only proteins limiting for Start (28). If the cell cycle delay in Htz1p mutants is due to a delay in the induction of G1 cyclins, then overexpression of Cln2p might alleviate the delay. We transformed wild-type and htz1Δ cells with vector alone or vector carrying Cln2p under the control of the GAL1 promoter. Cells were arrested with α factor, CLN2 was induced by a shift of cells to medium containing galactose, and 30 min after the induction cells were released into medium lacking α factor. We monitored progression through the cell cycle by measuring bud emergence. The results of this analysis are shown in Fig. 7 and indicate that overexpression of CLN2 is able to significantly alleviate the delay in budding that is observed in htz1Δ cells, demonstrating that the overexpression can bypass the htz1Δ defect. However, it is unlikely that misregulation of CLN2 is the sole cause for the defects observed in htz1Δ cells, since CLN2 overexpression suppresses an htz1 null mutant and is most likely a bypass suppressor. This would be consistent with the observation that overexpression of CLN2 also advances Start in wild-type cells (82).

**DISCUSSION**

**S-phase delay in H2A.Z mutants.** H2A.Z is known to affect transcription of genes and chromosome segregation (37). Our observations indicate that DNA replication was also affected in cells that did not contain H2A.Z, since htz1Δ cells were slow in their progression through S phase, and the timing of replication of both an early- and a late-replicating region was delayed in these cells. Furthermore, the induction of transcription of cyclin genes was affected in htz1Δ cells.

Chromatin plays an important role in replication, since nucleosomes positioned over an origin affect origin function (72) and nucleosomes located adjacent to origins facilitate replication initiation (48). Furthermore, origins localized near silenced chromatin domains are usually late replicating (52, 73), while acetylation of histones around a late origin induces it to fire early (85). Similarly, mutant phenotypes of Orc2p are suppressed by mutations in the histone acetyltransferase Sas2p (24, 36, 86). We have now shown that the histone variant H2A.Z is one additional factor that directly or indirectly influenced replication and cell cycle progression.

There are several models to explain how H2A.Z may affect S phase. Genome-wide expression analyses have suggested that H2A.Z acts as an activator and repressor of genes (38, 55), and while it does not significantly affect steady-state transcript levels of cell cycle-regulatory proteins (55 and our unpublished data), we have clearly shown that the kinetics of induction of two cell cycle-regulatory genes, CLN2 and CLB5, are affected in cells lacking Htz1p. It is therefore possible that cells lacking this variant have reduced levels of proteins involved in cell cycle progression during a specific point in the cell cycle Start. The down-regulation of these proteins, in htz1Δ cells, could therefore result in the delay in replication that we observe. The
asynchronous wild-type and \textit{htz1} primers specific for prepared from the RNA was analyzed by PCR using radionuclides and were collected, and total RNA was extracted from these cells. cDNA prepared for each time point was analyzed by PCR using radionuclides and primers specific for \textit{CLN2}, \textit{CLB5}, and \textit{TUB2}. The levels of transcript for \textit{CLN2} and \textit{CLB5} were quantitated by PhosphorImager analysis of the gels and are plotted normalized to \textit{TUB2}.

B. Alpha factor-arrested cells were released into \textit{YPD} medium at 16°C. Cells were collected at 30-min intervals, and total RNA was extracted from these cells. cDNA prepared for each time point was analyzed by PCR using radionuclides and primers specific for \textit{CLN2}, \textit{CLB5}, and \textit{TUB2}. The levels of transcript for \textit{CLN2} and \textit{CLB5} were quantitated by PhosphorImager analysis of the gels and are plotted normalized to \textit{TUB2}.

C. Localization of H2A.Z has been shown to be necessary for transcriptional induction was delayed in \textit{htz1}Δ mutants. A. Measurement of steady-state transcript levels in asynchronous wild-type and \textit{htz1}Δ strains. Wild-type (ROY1945) and \textit{htz1}Δ (ROY3442) strains were grown in \textit{YPD} medium at 16°C. Cells were collected, and total RNA was extracted from these cells. cDNA prepared from the RNA was analyzed by PCR using radionuclides and primers specific for \textit{CLN2}, \textit{CLB5}, and \textit{TUB2}. The levels of transcript for \textit{CLN2} and \textit{CLB5} were quantitated by PhosphorImager analysis of the gels and are plotted normalized to \textit{TUB2}. B. Alpha factor-arrested wild-type (ROY1945) and \textit{htz1}Δ (ROY3442) strains were released into \textit{YPD} medium at 16°C. Cells were collected at 30-min intervals, and total RNA was extracted from these cells. cDNA prepared for each time point was analyzed by PCR using radionuclides and primers specific for \textit{CLN2}, \textit{CLB5}, and \textit{TUB2}. Panel on the left are images of gels with the \textit{CLN2} and \textit{CLB5} PCR products (the time points are marked above the gel image, and “A” represents asynchronous cultures). The levels of transcript for \textit{CLN2} and \textit{CLB5} were quantitated by PhosphorImager analysis of the gels and are plotted as a function of time, normalized to \textit{TUB2}. The solid lines represent wild-type cells, and the dashed lines represent mutant cells.

FIG. 6. \textit{CLN2} and \textit{CLB5} transcription induction was delayed in \textit{htz1}Δ mutants. A. Measurement of steady-state transcript levels in asynchronous wild-type and \textit{htz1}Δ strains. Wild-type (ROY1945) and \textit{htz1}Δ (ROY3442) strains were grown in \textit{YPD} medium at 16°C. Cells were collected, and total RNA was extracted from these cells. cDNA prepared from the RNA was analyzed by PCR using radionuclides and primers specific for \textit{CLN2}, \textit{CLB5}, and \textit{TUB2}. The levels of transcript for \textit{CLN2} and \textit{CLB5} were quantitated by PhosphorImager analysis of the gels and are plotted normalized to \textit{TUB2}. B. Alpha factor-arrested wild-type (ROY1945) and \textit{htz1}Δ (ROY3442) strains were released into \textit{YPD} medium at 16°C. Cells were collected at 30-min intervals, and total RNA was extracted from these cells. cDNA prepared for each time point was analyzed by PCR using radionuclides and primers specific for \textit{CLN2}, \textit{CLB5}, and \textit{TUB2}. Panel on the left are images of gels with the \textit{CLN2} and \textit{CLB5} PCR products (the time points are marked above the gel image, and “A” represents asynchronous cultures). The levels of transcript for \textit{CLN2} and \textit{CLB5} were quantitated by PhosphorImager analysis of the gels and are plotted as a function of time, normalized to \textit{TUB2}. The solid lines represent wild-type cells, and the dashed lines represent mutant cells.

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Synthetic sickness that we observe between H2A.Z and replication proteins can also be explained by the possibility that reduced levels of replication proteins coupled with mutations that weaken these proteins would be expected to lead to slower growth rates.

An alternative model to explain our current observations is that the localization of H2A.Z at intergenic regions throughout the genome could conceivably create a chromatin environment that facilitates all nuclear processes, including transcription, replication, repair, and segregation. Loss of this variant might create more condensed chromatin, thereby slowing the kinetics of most nuclear processes in the cell.

A third possibility is that H2A.Z has multiple and distinct functions in the cell: its enrichment at pericentric chromatin could affect chromosome segregation (41) via interactions with kinetochore proteins; its localization in the intergenic regions at the promoters of genes could aid in the recruitment of specific transcription factors affecting activation and repression of genes (38, 55, 67); and it could also directly affect the assembly and function of replication complexes at origins, especially since origins are located in intergenic regions (90). While it is likely that H2A.Z alters the chromatin structure in intergenic regions of the genome and it is possible that it helps in recruiting the replication machinery to origins, we have analyzed the distribution of H2A.Z at all of the origins on chromosome VI and do not find any correlation between H2A.Z occupancy and replication timing or usage (25, 91). Furthermore, the binding of ORC at origins is not significantly affected in cells lacking H2A.Z (see Fig. S1 in the supplemental material).

The final possibility is that cells lacking H2A.Z have increased damage of a specific nature that is recognized by the S-phase checkpoint. We observe genetic interactions between \textit{htz1}Δ mutants and mutants in genes involved in the S-phase checkpoint but not with genes involved in the DNA damage checkpoint. This result suggested that even in the absence of any genotoxic stress, a specific kind of lesion was being induced in the absence of this histone variant, which necessitated a functional S-phase checkpoint pathway. The necessity to repair the damage in S phase could result in the delay that we observe. The S-phase checkpoint is necessary in the presence of stalled replication forks (49) or when replication initiation is impaired (21, 31, 71, 86). The misregulation of the cyclins has also been shown to activate the checkpoint, presumably due to defects in replication initiation (21, 31, 47, 78). It is therefore possible that the genetic interactions observed between \textit{htz1}Δ and the S-phase checkpoint proteins are a consequence of increased damage that needs to be repaired before cells can finish replication and progress through the cell cycle.

H2A.Z and the regulation of cyclin genes. In budding yeast, H2A.Z has been shown to be necessary for transcriptional...
activation of genes (55, 67). It is depleted from silenced regions and is elevated in the intergenic regions of inducible genes (1, 38, 42, 46, 56, 67). The absence of this variant leads to compromised recruitment of TBP and RNA polymerase II to the promoters of genes and a delay in the induction of these genes.

Interestingly, measurements of the expression of the cyclin genes in asynchronous cultures suggest that this variant played a minor role in the expression of these genes (consistent with the microarray data [55]). However, in synchronized cells traversing Start, the loss of this variant significantly affected the induction of CLN2 and CLB5 transcription. We also find that this variant was present at the promoters of these genes at a level equivalent to that observed at the GIT1 gene. The presence of this variant at the promoter of the cyclin genes and the delayed induction of these genes in the absence of H2A.Z suggest that this histone variant was required for the correct temporal activation of these genes during the cell cycle. The mechanism of regulation of the cyclin promoters by H2A.Z was likely to be similar to that observed at the GAL1 promoter (1, 67), though this needs to be further investigated.

While the histone variant is directly or indirectly required for the proper temporal regulation of the cyclin genes, at this point we are unable to determine the precise reason for the delay in progression through the cell cycle that is observed in 
htz1/Δ cells. We find that double mutants between 
htz1/Δ and various cyclins are not sick, though a triple mutant between 
clb5/Δ, clb6/Δ, and 
htz1/Δ is very sick. The 
clb5/Δ clb6/Δ double mutants are also inviable in the absence of Cln1p and Cln2p (19) or Clb3p and Clb4p (16). It is therefore possible that H2A.Z regulates multiple cyclin genes, but our genetic results suggest an even more complicated picture. Spt16 regulates transcription of the G1 cyclins, and split mutants grown at the nonpermissive temperature exhibit a decrease in the levels of 
CLN1 and 
CLN2 transcripts (51, 65). However, we do not observe any growth defect in a split double mutant (at the permissive temperature). It is therefore possible that the cell cycle phenotypes described in this work are due to the delay in the induction of cyclin genes as well as defects in the induction of other G1/S-phase genes or even other processes not related to the regulation of these genes and will need to be investigated further.

Numerous models can explain the phenotypes associated with 
htz1/Δ cells: H2A.Z affects the kinetics of induction of cell cycle-regulated genes, H2A.Z regulates global chromatin accessibility, and H2A.Z is required to repair damage at the G1/S transition. Additional experiments will be necessary to determine the role for this enigmatic variant.

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