MOLECULAR AND CELLULAR BIOLOGY, Feb. 1988, p. 945–954
0270-7306/88/020945-10$02.00/0

Vol. 8, No. 2

Comparison of the Structure and Cell Cycle Expression of mRNAs Encoded by Two Histone H3-H4 Loci in *Saccharomyces cerevisiae*

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Received 6 July 1987/Accepted 20 November 1987

The haploid genome of *Saccharomyces cerevisiae* contains two nonallelic sets of histone H3 and H4 gene pairs, termed the copy I and copy II loci. The structures of the mRNA transcripts from each of these four genes were examined by nuclease protection and primer extension mapping. For each gene, several species of mRNAs were identified that differed in the lengths of their 5′ and 3′ untranslated regions. The cell cycle accumulation pattern of the H3 and H4 mRNAs was determined in cells from early-exponential-growth cultures fractionated by centrifugal elutriation. The RNA transcripts from all four genes were regulated with the cell division cycle, and transcripts from the nonallelic gene copies showed tight temporal coordination. Cell cycle regulation did not depend on selection of a particular histone mRNA transcript since the ratio of the multiple species from each gene remained the same across the division cycle. Quantitative measurements showed significant differences in the amounts of mRNA expressed from the two nonallelic gene sets. The mRNAs from the copy II H3 and H4 genes were five to seven times more abundant than the mRNAs from the copy I genes. There was no dosage compensation in the steady-state levels of mRNA when either set of genes was deleted. In particular, there was no increase in the amount of copy I H3 or H4 transcripts in cells in which the high-abundance copy II genes were deleted.

For most classes of histone, gene expression is regulated in a cell-cycle-dependent fashion, with the maximal amount of histone RNA and protein synthesis occurring in late G_1 or early S phase (28, 37). The replacement or basal histone gene variants (49) and avian histone H5 (7) are notable exceptions to this rule. The cell cycle regulation of histone mRNA expression has been investigated in several systems and is likely to involve changes in the rate of gene transcription (14, 17, 32), changes in mRNA stability (15, 17, 40), and structural features of the mRNA transcripts and their templates (4, 25).

The core histone genes of budding yeast, *Saccharomyces cerevisiae*, are organized as duplicate H2A-H2B and H3-H4 gene pairs (43). The four nonallelic chromosomal gene sets are unlinked and, at each locus, the paired genes are divergently transcribed (19, 45). The DNA sequences of analogous genes are highly conserved within the coding regions, but the 5′ and 3′ untranslated regions are divergent. The yeast core histone genes show a typical cell-division-cycle-dependent pattern of expression. Both the rate of synthesis of new histone (23, 24) and the appearance of histone mRNA are periodic in the division cycle (20). There appear to be several mechanisms regulating expression of the genes, and both transcriptional and posttranscriptional controls have been proposed for the H2A and H2B genes (18, 26, 32, 33).

In this paper, we report the structure and cell cycle pattern of accumulation of the yeast histone H3 and H4 genes. These experiments addressed several aspects of H3 and H4 gene expression. First, we determined the structures of the H3 and H4 mRNA transcripts from each of the genes. Second, we assayed the cell cycle pattern of accumulation of the transcripts and compared the time course of their expression from each of the nonallelic gene sets. Finally, we determined the effect of deleting either H3-H4 gene locus on the level of expression of the remaining genes. The results of these experiments lead to the suggestion that the two *S. cerevisiae* histone H3-H4 loci are regulated independently at the level of mRNA expression.

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MATERIALS AND METHODS

Strains and plasmids. Plasmids containing the yeast histone gene fragments have been described previously (45). The specific gene probes were made by subcloning restriction fragments of these plasmids into either M13mp8 or M13mp9. Recombinant M13 bacteriophage were grown in *Escherichia coli* JM101 [Δ(*lac-pro*) *thi supE* F′ *traD36 proAB lacI*^{q} Δ*lacZM15*] (29). The cloned probe fragments were verified by DNA sequence analysis (36). The yeast strain YP3 was a diploid homozygous for the markers *ura3-52*, *ade2-101*, and *lys2-801* (21). The histone H3-H4 deletion strains were derived from YP3 and are described elsewhere (M. M. Smith and V. B. Stirling, J. Cell Biol., in press). The mutant strain MSY164 was a diploid homozygous for the deletion of the copy I H3-H4 locus, while MSY165 was a diploid homozygous for the deletion of the copy II H3-H4 locus. These strains were also homozygous for the markers *ura3-52*, *ade2-101*, and *lys2-801*.

S1 analysis. Total RNA was prepared from either asynchronous cultures in early log phase (4×10^6 to 8×10^6 cells per ml) or elutriator fractions. Cells were suspended in 1 ml of a 1% diethylpyrocarbonate–0.1 M sodium acetate–0.01 M EDTA (pH 5.1) buffer and broken either by vortexing the mixture with 1 g of 470-μm glass beads (50) or by use of an Eaton press (9). Sodium lauryl sulfate was then added to a final concentration of 0.5%, and the RNA was deproteinized by phenol extraction at 50°C, followed by a series of room temperature extractions.

Probes for S1 analysis of the 5′ ends of the messages were

made by 5'-end labeling the appropriate restriction fragment with T4 polynucleotide kinase (2) and isolating single-stranded probes on strand separation gels (27). Alternatively, probes which protected the 5' or 3' ends of the transcripts were produced by in vitro DNA synthesis of radiolabeled single-stranded DNA from an M13 template. For these reactions, the M13 sequencing primer was extended in the presence of Klenow polymerase, an excess of cold dGTP, dCTP, and TTP, and a known and limiting amount of dATP and [α-^{32}P]dATP to provide a probe of known specific activity. After digestion with a restriction enzyme that cleaved at a unique site in the M13 linker region downstream of the insert, the single-stranded uniformly labeled probe was isolated by electrophoresis on an 8 M urea–6% polyacrylamide gel.

The primed synthesis probes incorporated more radioactive label, were easily purified from the unlabeled complementary strand, and were generally more sensitive than single end-labeled probes. The 5' ends of the mRNAs were mapped by S1 analysis by using both end-labeled probes and uniformly labeled probes, as well as by primer extension assays. The 3' ends were only mapped by using the uniformly labeled probes; however, the results were consistent with those obtained by using a variety of different fragments (45; data not shown).

Single-stranded probes generated by either 5'-end labeling or in vitro DNA synthesis were hybridized to 1 to 10 μg of total RNA in a buffer containing 0.3 M NaCl, 0.01 M Tris (pH 7.5), and 0.1 mM EDTA. The samples were boiled in sealed capillaries and then transferred to a 60°C water bath for 4 h. After hybridization, the samples were expelled into 0.2 ml of S1 nuclease digestion buffer (38) and digested with 1,000 U of S1 nuclease (Boehringer Mannheim Biochemicals) at 37°C for 60 min. The reaction was stopped by the addition of 1 ml of cold 95% ethanol. After ethanol precipitation, samples were analyzed by electrophoresis on an 8 M urea–6% polyacrylamide gel.

Primer extension. The oligonucleotides for primer extension were made by using a SAM II synthesizer (Biosearch). The primers were 5'-end labeled by T4 polynucleotide kinase (2), phenol extracted, and ethanol precipitated overnight. The labeled primers were hybridized to total yeast RNA in Eppendorf tubes at 65°C for 3 to 5 min in a buffer containing 50 mM Tris hydrochloride (pH 8.3), 150 mM KCl, 0.5 mM EDTA, 1 mM dithiothreitol, 7 mM $MgCl_2$, and 0.5 mM each deoxynucleotide triphosphate (8). The samples were then chilled on ice, and 5 to 10 U of avian myeloblastosis virus reverse transcriptase (Life Sciences, Inc.) was added. Reactions were allowed to proceed at 42°C for 60 to 90 min. The samples were then ethanol precipitated and separated by electrophoresis on an 8 M urea–6% polyacrylamide gel.

Quantitative assays of histone mRNA. Quantitative measurements of histone H3 and H4 mRNA species were performed on cells from early-log-phase cultures (4×10^6 to 8×10^6 cells per ml) by using copy-specific, uniformly labeled 3' hybridization probes. The probes were synthesized as described above. The radioactivity per picomole of each probe was calculated from its DNA sequence and the specific radioactivity of the nucleotide precursors used in the synthetic reaction. Corrections for the fractional part of the mRNA and probe molecules protected from S1 digestion were calculated from the known transcript maps and the DNA sequences of each.

Several controls were used to verify that equivalent amounts of RNA were compared for the levels of histone mRNAs. First, the concentrations of all of the purified RNA samples were determined from their A_{260}. Second, the RNA samples were separated by electrophoresis on 1% agarose gels, stained with ethidium bromide, and photographed. The relative amounts of RNA were then estimated by soft-laser densitometry of the rRNA species in each lane. Finally, in some cases Northern (RNA blot) transfers of the RNA gels were hybridized with probes for the histone genes, the *SMT1* gene, and the *ura3-52* gene. The results of these three estimates of the RNA concentrations were in good agreement.

In the titration reactions, a constant amount of 3'-end single-stranded cDNA probe was hybridized in solution to increasing amounts of total yeast RNA. The reactions were treated with S1 nuclease and analyzed by electrophoresis on denaturing polyacrylamide gels as described above. Autoradiographs of the titrations were scanned by soft-laser densitometry to determine the extent of hybridization at each concentration. The maximum hybridization at infinite RNA was calculated from linear regression analysis of double-reciprocal plots of the hybridization density versus the amount of yeast RNA. This value was used to convert the relative hybridization densities to absolute mass amounts based on the known input of picomoles of probe and the fraction of the probe complementary to the mRNA. The fraction of total yeast RNA that was histone mRNA was calculated from the initial linear portion of the titration curve at limiting total RNA.

Northern analysis. Total RNA was separated by electrophoresis through 2% methyl mercury agarose gels as described previously (1). After electrophoresis, the gel was stained with ethidium bromide to ascertain the integrity of the RNA. The gel was then electroblotted overnight onto Nytran membranes (Schleicher & Schuell, Inc.) in a Transblot apparatus (Bio-Rad Laboratories) in accordance with the instructions of the manufacturers. All DNA fragments used for probes in Northern analysis were subcloned into M13mp8 or M13mp9 and labeled by incorporating [α-^{32}P]dATP from a primer that hybridized downstream of the inserts (22). The probe was then added to the prehybridized filter and allowed to hybridize for 24 to 36 h. Hybridization was detected by autoradiography of the filters at −72°C by using X-RP X-ray film (Eastman Kodak Co.) and intensifying screens.

Cell cycle separation. Cell cycle separation was performed by centrifugal elutriation of yeast cultures in early-log-phase growth as described previously (13). Cultures were grown in synthetic defined medium (39) to a density of 0.5×10^7 to 1×10^7 cells per ml, pelleted by centrifugation, washed in ice-cold deionized water, and sonicated for 15 to 20 s with the microtip of a Sonicator Cell Disruptor (model W-375; Heat Systems-Ultrasonics, Inc.). The cells were then loaded into a JE-6B elutriator rotor (Beckman Instruments, Inc.), which was held at 3,000 rpm and 4°C. Fractions of 150 ml were collected at increments of 1 or 2 ml/min. For analysis of cell cycle separation, samples from elutriator fractions were stained with mithramycin (41), generously provided by Pfizer Research. The nuclear morphologies of cells of each fraction were then scored under a fluorescence microscope (Leitz/Opto-Metric Div. of E. Leitz, Inc.). Elutriator fractions were also characterized with respect to the size of cells in each fraction by use of a model ZM particle counter (Coulter Electronics, Inc.) and a model 256 Channelyzer.

DNA sequences. The DNA sequences of the *S. cerevisiae* histone H3 and H4 genes have been reported previously (44) and are available in the GenBank genetic sequence database (3) as accession numbers X00724 and X00725. Additional

DNA sequence information, to include the 3′ end of the copy II H4 gene, has also been recently reported (5).

RESULTS

H3 and H4 RNA structure. The organizations of the two nonallelic histone H3-H4 loci are shown in Fig. 1. We will refer to the copy I histone H3 gene as *HHT1* and the copy I histone H4 gene as *HHF1*. Similarly, the copy II genes will be referred to as *HHT2* and *HHF2*. To better understand the pattern of expression of the histone H3 and H4 mRNAs, we refined the transcription maps of the genes. The approximate termini of the H3 and H4 mRNAs were reported previously based on S1 nuclease assays of mRNA from wild-type yeast cells (44). However, these results were ambiguous because of cross-hybridization between the coding sequences of the duplicate nonallelic gene copies; it was impossible to rule out major transcript initiation sites close to the coding DNA sequences. We have now investigated the structures of the mRNAs in greater detail by using S1 assays with probes specific for each gene copy and primer extension assays. In these assays, RNA from yeast strains lacking one or the other of the H3-H4 gene sets were used to identify copy-specific RNA termini (Smith and Stirling, in press).

The probes used to map the 3′ ends of the H3 and H4 transcripts are shown in Fig. 1. The sequences flanking the H3 and H4 coding regions are divergent and provide copy-specific probes that hybridize to the 3′ untranslated regions of the mRNAs. The probe for the *HHT1* gene was a 751-base-pair (bp) *Sau*3AI fragment that began 1 base before the TAG termination codon and spanned the end of the mRNA. The *HHT2* 3′-end probe was also a *Sau*3AI fragment, 374 bases in length, that started 1 base upstream of the stop codon. Both H4 probes began at sites downstream from the end of the coding region. The *HHF1* probe was a 555-bp

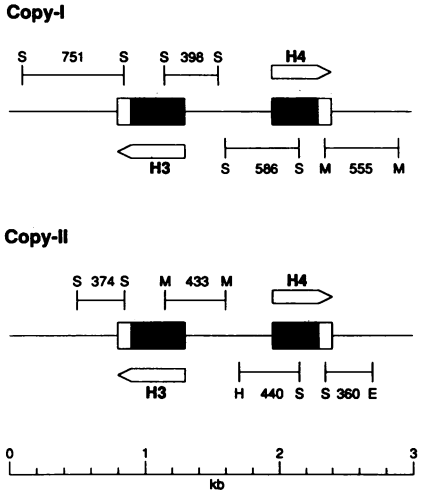

FIG. 1. DNA fragments used to assay histone H3 and H4 mRNAs. The locations of the restriction fragments subcloned to probe the histone mRNAs are shown for the two nonallelic H3-H4 loci. The transcribed histone H3 and H4 sequences are indicated by the boxes and the direction of transcription is shown by the arrows. The regions of homology between the coding sequences are shaded, and the divergent 3′ noncoding transcribed sequences are open. The 5′ noncoding transcribed sequences are too short to illustrate. The positions of the cloned probes and their sizes in base pairs are shown above the H3 genes and below the H4 genes. Restriction enzyme sites: S, *Sau*3AI; M, *Msp*I; H, *Hin*dIII; E, *Eco*RI. kb, Kilobases.

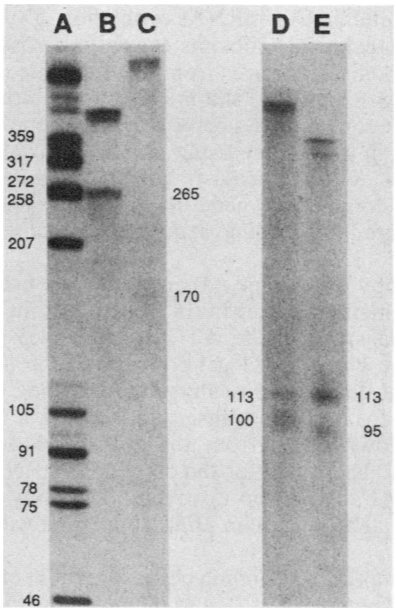

FIG. 2. Mapping of histone H3 and H4 mRNA 3′ ends. The 3′ ends of the H3 and H4 mRNAs were mapped by nuclease S1 protection as described in Materials and Methods. The labeled DNA probes used in these experiments were the fragments spanning the 3′ end of each gene, as shown in Fig. 1. Lane A contains an end-labeled *Sau*3AI digest of pBR322 DNA to provide size markers for the gel. The sizes (in bases) of some of these marker fragments are indicated to the left of the lane. The results for the histone H3 genes are shown in lane B, for the copy II H3 gene (*HHT2*), and lane C, for the copy I H3 gene (*HHT1*). The *HHT1* mRNA protected a fragment of about 170 bases, while the *HHT2* mRNA protected a fragment of about 265 bases. The results for the histone H4 genes are shown in lane D, for the copy I H4 gene (*HHF1*), and lane E, for the copy-II H4 gene (*HHF2*). The *HHF1* mRNA protected two bands of about 100 and 113 bases, and the *HHF2* mRNA protected two fragments of approximately 95 and 113 bases. The upper bands in lanes B through E at about 374, 751, 555, and 360 bases are from residual undigested full-length probes.

*Msp*I fragment that began 63 bases downstream of the end of the coding region. The *HHF2* probe was a 360-bp *Sau*3AI-*Eco*RI fragment that started 68 bases downstream from the stop codon.

The results of S1 nuclease assays with these probes are presented in Fig. 2, and the positions to which they map are summarized in Fig. 5B. Both H3 genes had a single region of 3′-end formation located approximately 170 bases past the H3 stop codon for the copy I gene and about 265 bases downstream of the stop codon for the copy II gene. In contrast, both H4 mRNAs had multiple regions of 3′-end formation. For the copy I H4 gene, these were centered at 163 and 176 bases past the stop codon. The two S1-protected bands corresponding to these termini appeared to be of equal intensity. The copy II H4 messages had termini mapping about 163 and 181 bases past the stop codon. Although not visible in Fig. 2, additional minor species were also detected that corresponded to termini at positions 186 (*HHF1*) and 190 (*HHF2*).

The 5′ ends of the H3 and H4 mRNAs were determined by S1 nuclease and primer extension assays. The probes used for S1 mapping of the 5′ termini are shown in Fig. 1. Unlike the 3′-end probes, these probes overlapped the coding regions of the genes and were therefore not specific for a single copy. These particular fragments were chosen to

permit the detection of mRNAs with short 5′ untranslated regions. The technical problems caused by cross-hybridization of the coding sequences from the duplicate copies were solved by also examining the mRNAs from strains deleted for either the copy I or the copy II H3-H4 gene pair (Smith and Stirling, in press). By using strains with mRNAs from only one gene set, protected fragments representing the 5′ ends of one copy of the gene could be distinguished from those generated by coding sequence homology with the other copy.

For the copy I H3 gene experiments, we used a 398-bp *Sau*3AI fragment that began 185 bases within the coding sequence and spanned the ATG codon and 5′ end of the mRNAs. The copy II H3 gene probe was a 433-bp *Msp*I fragment that began 98 bases inside the coding region. The probes used for both H4 transcripts began at a *Sau*3AI site 156 bases downstream from the ATG codon within the coding DNA. The probe for the copy I H4 mRNA extended to the *Sau*3AI site 586 bp upstream. The copy II H4 gene probe ended at an upstream *Hin*dIII site and was 440 bases long.

The S1 bands corresponding to the 5′ termini of H3 and H4 mRNAs are shown in Fig. 3, and the positions to which they map are summarized in Fig. 5A. Multiple 5′ termini were detected for all four of the genes, and in each case one 5′ end was present in greatest abundance. The major S1-protected bands for the copy I H3 transcript (a 220-bp doublet) corresponded to a 5′ end starting about 30 bases upstream of the ATG codon. A minor species starting at approximately −10 bases was also present. The major copy II H3 mRNA (115 bp) had a shorter 5′ end that mapped to about −15 bases from the ATG. Many minor species of longer RNA could also be detected. The 5′ ends of the two H4 genes mapped to similar locations, perhaps reflecting the greater sequence homology of the DNA immediately upstream of the H4 coding region as compared with the analogous region in the H3 genes. The 5′ end of the major copy I H4 gene transcript (190 bp) mapped to a position approximately 35 bases upstream from the ATG. An additional band was detected at about −20 bases as well. The 5′ end of the major copy II H4 gene mRNA (180 bp) was located at approximately −30 bases from the ATG.

The 5′ ends of the histone mRNAs were also analyzed by primer extension with reverse transcriptase. For these experiments, the deletion strains were again essential in assigning 5′ termini to messages from one or the other of the two copies. The oligonucleotide used as the primer for the H4 messages was an 18-mer that began priming 24 bases downstream of the ATG within the coding DNA. The H3 primer was a 17-mer and primed from a site 118 bases into the coding DNA from the ATG. The results of the primer extension experiments are illustrated in Fig. 4. The positions of the primer extension fragments confirmed the 5′ ends as measured by S1 analysis and demonstrated that the 5′ ends measured by S1 analysis were not due to splice junctions. The positions of the 5′ ends, as estimated by both the S1 nuclease and primer extension experiments, are summarized in Fig. 5A.

Cell cycle expression. We addressed two aspects of the cell cycle control of histone H3 and H4 mRNA accumulation. The first of these questions was whether the timing of the increase in histone mRNAs from the duplicate gene sets is tightly coordinated. The second was whether periodic accumulation is due to preferential selection of one RNA from the multiple gene transcripts.

The regulation of the histone H3 and H4 mRNAs was examined in cells from early-logarithmic-growth cultures separated by centrifugal elutriation into cell-cycle-dependent fractions (13). Centrifugal elutriation separates cells based on their size, and, thus, small cells early in the division cycle are separated from larger cells later in the cycle. The quality of the cell cycle separation achieved by centrifugal elutriation was monitored by bud and nuclear morphology. Cells from each elutriator fraction were stained with the fluorescent dye mithramycin (48), and the age distribution was determined from a blind scoring of the stained cells. The results of these measurements were similar to those reported by Elliot and McLaughlin (10). Cell cycle separation was excellent in the earlier fractions from the elutriator but diminished as the cells became larger. However, even later fractions were largely composed of cells past the stage of nuclear division. DNA synthesis begins near the time of bud emergence, which corresponds to the 12- to 13-ml/min fractions for these cultures.

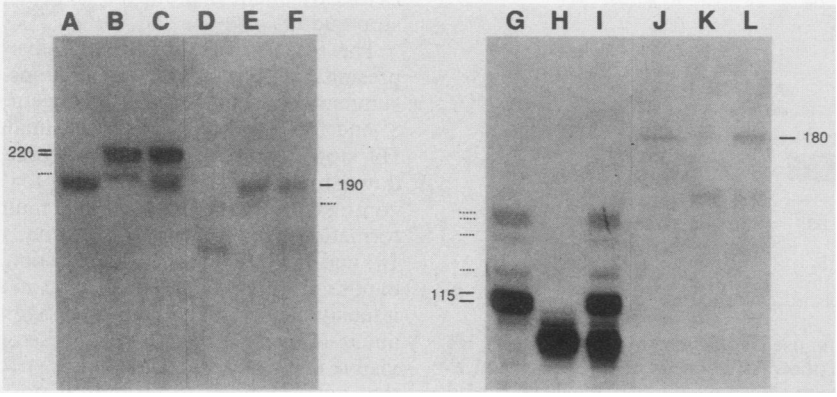

FIG. 3. Mapping of histone H3 and H4 mRNA 5′ ends. The 5′ ends of the H3 and H4 mRNAs were mapped by S1 nuclease protection as described in Materials and Methods. The 5′-end probes include part of the coding sequences of the genes and are not copy specific. Therefore, each probe was used to assay RNA from three strains: the copy I H3-H4 deletion (MSY164), the copy II H3-H4 deletion (MSY165), and the wild type (YP3). Lanes A to C illustrate the patterns observed with the copy I H3 probe and RNAs from the copy I deletion (lane A), the copy II deletion (lane B), and YP3 (lane C). Similarly, lanes D to F illustrate the pattern seen by using the copy I H4 probe, lanes G to I illustrate the pattern seen by using the copy II H3 probe, and lanes J to L illustrate the pattern seen by using the copy II H4 probe. Therefore, the copy-specific hybridization patterns are represented by lanes B, E, G, and J for the *HHT1*, *HHF1*, *HHT2*, and *HHF2* genes, respectively. ——, Major gene-specific bands with their approximate nucleotide lengths indicated; · · ·, additional minor bands.

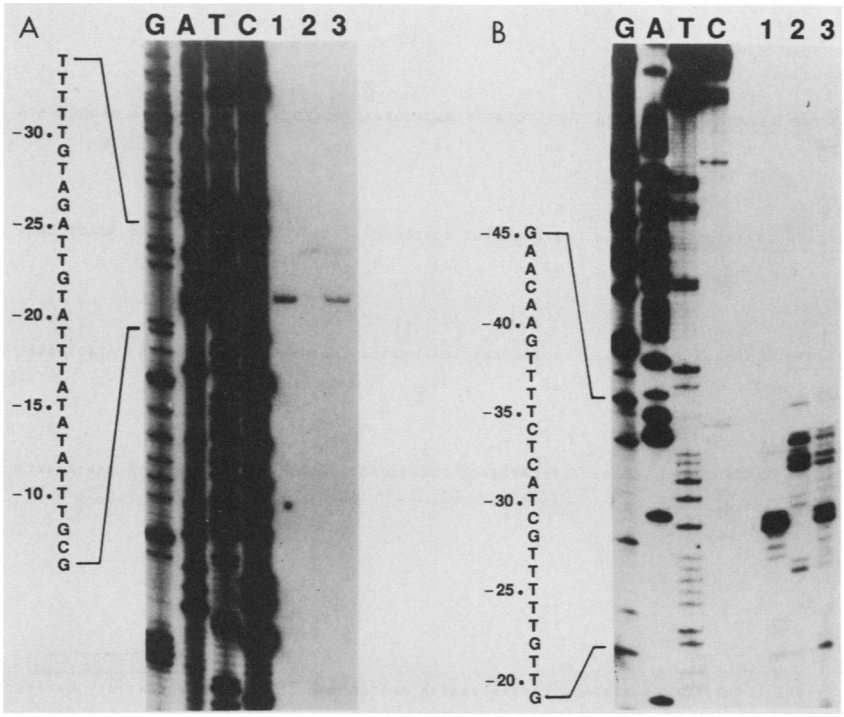

FIG. 4. Primer extension mapping of the histone H3 and H4 5′ ends. Histone H3 and H4 gene specific primers were hybridized to total RNA and extended by reverse transcriptase as described in Materials and Methods. (A) The 5′ ends of the histone H3 messages; (B) the 5′ ends of the histone H4 mRNAs. Lanes 1, RNA from the copy I H3-H4 deletion strain; lanes 2, RNA from the copy II H3-H4 deletion strain; lanes 3, RNA from the wild-type strain YP3. Products of the primer extension reactions were compared with a dideoxy sequence ladder produced by hybridizing the primer to an M13 template containing an insert of the appropriate region of the gene. The sequence ladder for panel A corresponds to the *HHT1* gene, and the ladder for panel B corresponds to the *HHF2* gene. The sequences are numbered with the ATG start codons as +1.

To monitor the timing of histone mRNA accumulation, Northern blot and nuclease S1 assays were performed on RNA from elutriation fractions of the strain YP3. The copy-specific DNA probes described above (Fig. 1) allowed us to monitor the levels of mRNA from each of the duplicate histone gene copies. For Northern experiments, total RNA from each YP3 elutriator fraction was prepared and separated by electrophoresis on methyl mercury agarose gels. The Northern transfers were first hybridized with a probe for the copy I histone genes, and the mRNA levels were recorded by autoradiography. The filters were then stripped of the original probe and rehybridized with a probe for the copy II histone mRNAs. An example of such an experiment for the histone H3 mRNAs is presented in Fig. 6A.

The RNAs from elutriation fractions were also assayed by S1 analysis by using the probes spanning the 5′ and 3′ ends of the transcripts. Using the copy-specific 3′ probes, we could monitor the accumulation of the mRNAs from each gene copy independently in the same fractionation. Figure 6B shows the cell cycle pattern of expression of the histone H4 genes from an S1 experiment using copy-specific 3′-end probes.

In both the Northern and S1 experiments, the histone H3 and H4 transcripts rose from virtually undetectable levels in the early fractions, peaked near S phase, and then gradually decreased. It should be noted that the pattern of mRNA accumulation can only be compared within each Northern or S1 nuclease experiment illustrated in Fig. 6 and not between the two experiments, since they were performed on RNA from different elutriator fractionations. Additional assays showed that the changes in the levels of H3 and H4 mRNAs from each locus were also coordinated. The lack of an abrupt decrease later in the cycle relative to the initial burst in mRNA levels is attributable to the decreased synchronization of cells in the later fractions of the elutriator. The results of these experiments established the tight temporal coordination of the steady-state levels of histone mRNA from the nonallelic duplicate gene sets. The cell cycle patterns of mRNA expression from the two loci were virtually identical at this level of resolution. The S1 nuclease assays also showed that the levels of all the multiple species of mRNA varied together throughout the cell cycle (Fig. 6; data not shown). That is, the cell cycle control of expression was not mediated by preferential expression of a particular mRNA species.

Quantitative assays of H3 and H4 mRNAs. The results presented above demonstrated the tight temporal control over the relative levels of the multiple histone H3 and H4 mRNAs from both genomic copies. However, we were also interested in whether the absolute levels of expression from the duplicate gene sets were similar. To determine whether the copy I H3 and H4 genes expressed the same amount of mRNA as the copy II H3 and H4 genes, we used the copy-specific 3′-end probes to measure the amount of mRNA from each gene present in cells from early-exponential-growth cultures. Figure 7 shows a titration in which uniformly labeled 3′-end probes were hybridized to total RNA prepared from a culture of strain YP3. In these experiments, a constant amount of probe was titrated with increasing amounts of total RNA, and the amount of histone mRNA was then estimated from the part of the curve that represented probe excess. In the examples shown in Fig. 7, the specific activities and hybridization efficiencies of the probes were almost identical. Therefore, the difference in hybridization intensities between the copy I and copy II

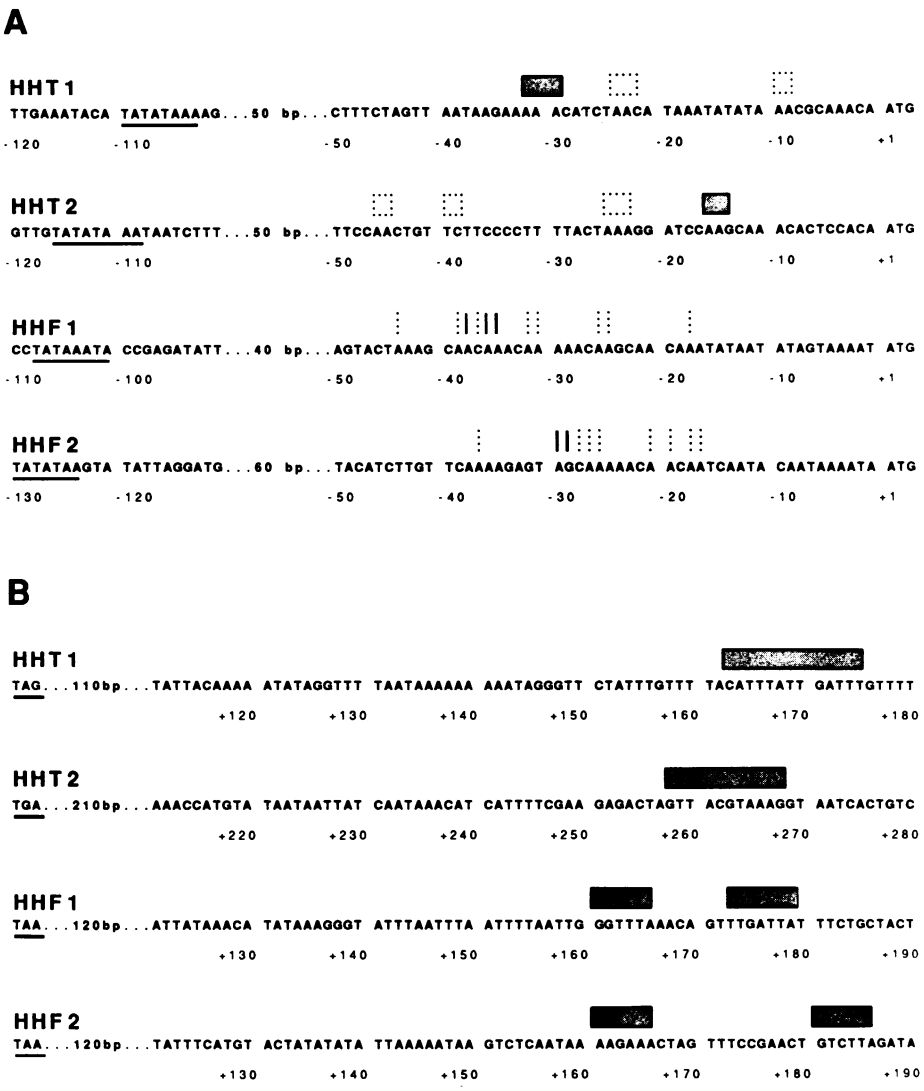

FIG. 5. Positions of histone H3 and H4 mRNA ends. The results of the nuclease S1 and primer extension mapping experiments are summarized with respect to the DNA sequences of the genes. (A) The 5′ ends of the histone mRNA transcripts. ▬, Major 5′ end positions for the H3 genes; ⋮⋮, minor transcript positions. The histone H4 mRNA 5′ ends are indicated by the lines: |, major start site; ⋮, minor start site. The TATA elements are underlined for each gene. (B) The 3′ ends of the H3 and H4 mRNAs. The major termination regions are covered by the shaded boxes. The termination codon is underlined for each gene, and the sequences are numbered beginning with the first base after this codon.

lanes reflects only the differences in the sizes of the protected fragments and in the total amounts of hybridizing RNA available. These measurements showed that the duplicate gene copies were not expressed at equivalent amounts. For both the copy I histone H3 and H4 genes, the transcripts were present at about 2 pg/μg, or 2×10^{-6} of total RNA. In contrast, the copy II transcripts were present at approximately 13 pg/μg, or 1.3×10^{-5} of total RNA. The copy II transcripts were therefore present at levels five to seven times greater than those of the copy I transcripts. If it is assumed that each cell contains approximately 0.9 pg of total RNA (47), then the average cell contains about six copies of the copy I mRNAs and 35 copies of the copy II mRNAs. Since histone mRNA expression is restricted to a short period of the division cycle, the levels of mRNA in cells actively transcribing the genes must be more abundant than this average value. Similar results were seen in the primer extension assays (Fig. 4) in which coding region oligonucleotides, homologous with both gene sets, were used to prime DNA synthesis on the H3 or H4 mRNAs. By identifying copy-specific bands in the deletion RNA (Fig. 4, lanes 1 and 2) and comparing their relative intensities in the wild-type RNA (Fig. 4, lane 3), it is clear that the copy II transcripts were significantly more abundant. It should be noted that similar comparisons of the 5′-end S1 nuclease assays cannot be made, since base mismatches between the DNA probes and mRNAs from the nonallelic genes affect hybridization kinetics and susceptibility to S1 nuclease.

Dosage compensation. The significant difference in the levels of the mRNAs from the two H3-H4 gene sets led us to investigate the importance of gene dosage and mRNA concentrations in the cell. In particular, we monitored H3-H4 RNA levels in the histone gene deletion strains. If the levels of histone H3 and H4 mRNA are limiting and critically regulated for cell function, then one would expect to observe at least a fivefold increase in the copy I transcripts in the

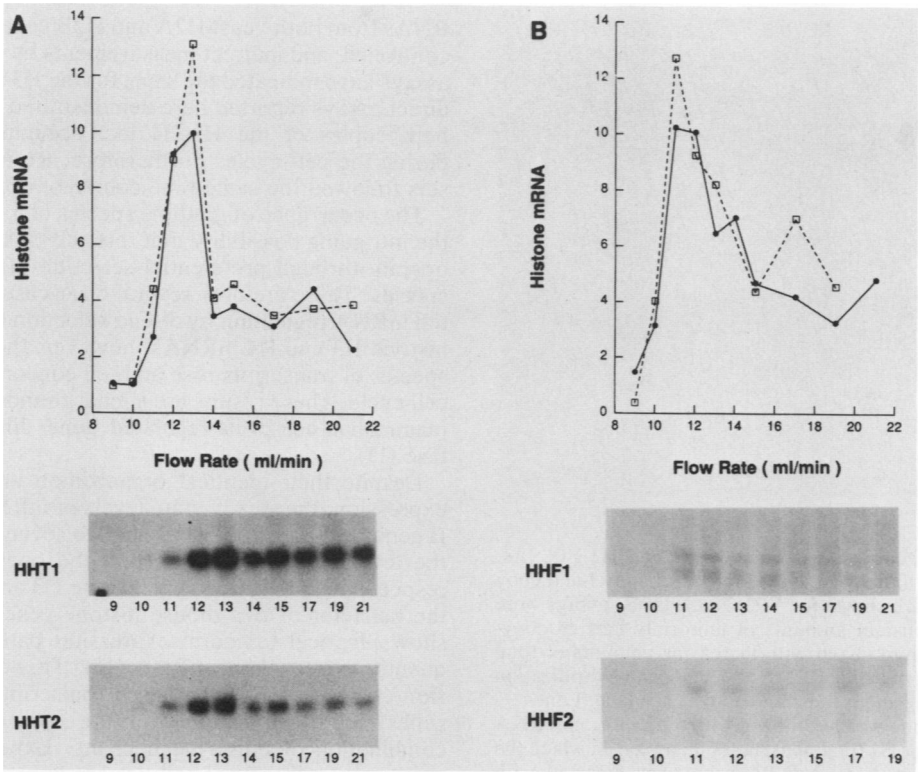

FIG. 6. Cell cycle expression of copy I and copy II histone H3-H4 genes. The levels of histone H3 and H4 mRNA species were assayed across the cell cycle in early-exponential-growth cultures fractionated by centrifugal elutriation. (A) Amounts of histone H3 mRNAs measured by Northern blot hybridizations. (B) Levels of histone H4 mRNAs measured by S1 nuclease assays. The autoradiographs of the cell cycle fractions for each gene probe are shown at the bottom of the graphs. Each lane represents hybridization of a 3′ copy-specific DNA probe with a constant amount of total RNA from cells eluted at the indicated flow rate. The autoradiographs were scanned by laser densitometry, and the relative amounts of the mRNAs are plotted versus elutriation flow rate at the top of each panel. ●, Copy I gene mRNAs; □, copy II gene mRNA levels.

copy II deletion strain. We previously showed that deletion of either H3-H4 gene set has little, if any, effect on the growth rates and cell division cycle periods of the mutant cells (Smith and Stirling, in press). The amount of message in the deletion and wild-type strains could therefore be compared directly by examining RNA made from cultures grown to the same density in the same media.

The results of mRNA titrations with RNA prepared from early logarithmically growing cultures of the deletion strains are shown in Fig. 8. The assays (Fig. 8A and C) examined the copy II H3 and H4 mRNAs in YP3, which is wild type for its histone genes, and in a copy I deletion strain. Measurements of the autoradiographs showed that the copy I deletion strain had the same amount of copy II mRNAs as wild-type cells did. However, since the copy II mRNAs normally represent about 80% of the total H3-H4 transcripts, a detectable change in concentration was not expected. The more critical experiment was to examine the levels of copy I transcripts in the copy II deletion strain. In these cells, even a partial compensation for the loss of the bulk of the H3-H4 mRNAs would be readily detected. Remarkably, there was no detectable change in the levels of copy I H3-H4 mRNAs in the copy II deletion (Fig. 8B and D), although histone H3 and H4 mRNA concentrations were reduced to 14 to 20% of normal amounts.

DISCUSSION

From the transcript mapping experiments reported here, we conclude that each of the histone H3 and H4 genes transcribed multiple mRNAs. Each H3 gene used one major termination region, while each H4 gene used two such termination regions. All four genes encoded transcripts with multiple 5′ ends, some of which mapped within 15 to 25 bases of the initiating ATG codon. Detection of these 5′ species was impossible in previous experiments because of cross-hybridizing bands from the homologous coding regions. Other yeast genes have also been demonstrated to produce multiple transcripts (12, 35). The histone H2A and H2B genes are among those encoding multiple transcripts (30), but detailed transcription mapping has not yet been completed for these genes.

Several questions regarding the multiple transcripts remain unresolved. For example, we do not know whether all the RNAs detected in our assays represent independent transcription products or the rapid processing of a single primary transcript. A second and related question is whether the H4 mRNA set includes all possible combinations of 5′ and 3′ sequences. That is, the 5′ and 3′ RNA ends were necessarily assayed separately, and it is possible that each 5′ end is associated with only one of the two 3′-end regions. Experimental tests of these questions should be possible by examining the pattern of RNAs produced from genes in which single 3′ or 5′ ends have been specifically disrupted.

The cell cycle pattern of histone mRNA expression has now been examined for many histone genes in a variety of organisms, and, with few exceptions, transcripts have been shown to accumulate periodically near the start of S phase. Previous work by Hereford et al. (20) has shown that the

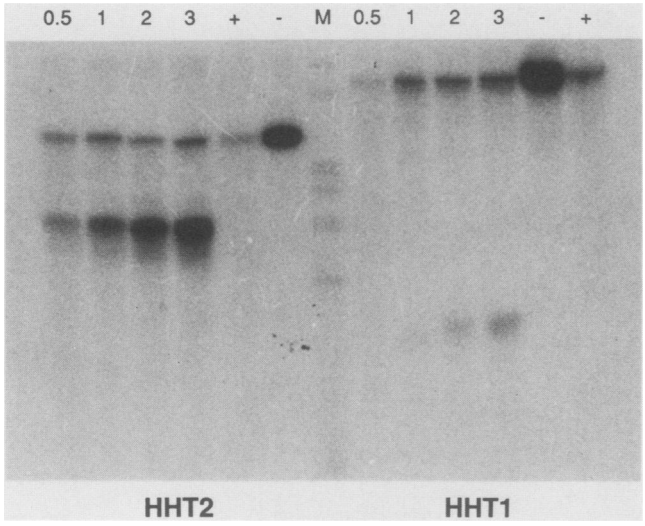

FIG. 7. Titration of copy I and copy II histone H3 mRNAs. Quantitative S1 assays to determine the amount of copy I and copy II histone H3 mRNAs are shown for YP3. Nuclease S1 assays were performed by using constant amounts of uniformly labeled copy-specific 3′-end probes hybridized with increasing amounts of total YP3 RNA. The amount of cellular RNA added to each hybridization varied from 0.5 to 3.0 μg and is indicated above each lane. Lanes −, assays in which the probe was hybridized with 5 μg of *E. coli* RNA and run without nuclease S1 treatment; lanes +, assays in which the probe also hybridized with *E. coli* RNA but was digested with nuclease S1 before electrophoresis; lane M, pBR322 *Sau*3AI marker fragments for reference. The results with the copy II H3 (*HHT2*) probe are shown in the left half of the figure, and the results for the copy I H3 (*HHT1*) probe are shown in the right half. In this experiment, the copy I and copy II H3 probes were of the same specific activity, so only the differences in sizes between the protected bands must be taken into account to determine the relative amount of each mRNA. From these calculations, the copy II histone H3 mRNA species are about five to seven times more abundant than the copy I H3 mRNAs.

RNAs from both yeast H2A and H2B gene sets are cell cycle controlled, and indirect measurements by in vitro translation assays have indicated the same for the H3 and H4 genes. The direct assays reported here demonstrated that mRNAs from both copies of the H3-H4 loci accumulated periodically during the cell cycle. Furthermore, RNAs from both gene sets followed the same time course of accumulation.

The occurrence of multiple species of 5′ and 3′ ends raised the intriguing possibility that this cell cycle regulation might operate through preferential selection of particular mRNA species. There are now several clear cases of such differential mRNA regulation by 5′-end selection (6, 34, 46). For the histone H3 and H4 mRNAs, however, the major and minor species of transcripts rose and fell concomitantly across the cell cycle. These results are similar to those obtained for the mammalian-cell-cycle-regulated gene dihydrofolate reductase (11).

Despite their identical organization and time course of expression, the steady-state levels of mRNAs from the copy II genes were approximately five to seven times greater than the levels of the mRNAs from the copy I genes. In this respect, the duplicate yeast histone H3 and H4 genes mimic the behavior of two mouse histone gene clusters that also show identical temporal expression patterns but different quantitative levels of mRNA (16). This differential expression could be due to increased transcription of the copy II genes, increased stability of the copy II mRNAs, or a combination of these mechanisms. Experiments to distinguish among these possibilities are in progress.

Because strains deleted for either H3-H4 gene set have normal growth rates and cell cycle patterns, we expected to find an increase in the levels of copy I H3-H4 mRNA in the copy II deletion strain. Contrary to expectations, deletion of either locus did not result in an increase in mRNA from the remaining genes. There are several potential explanations for these observations. First, it is possible that the copy I mRNAs are translated more efficiently than the copy II

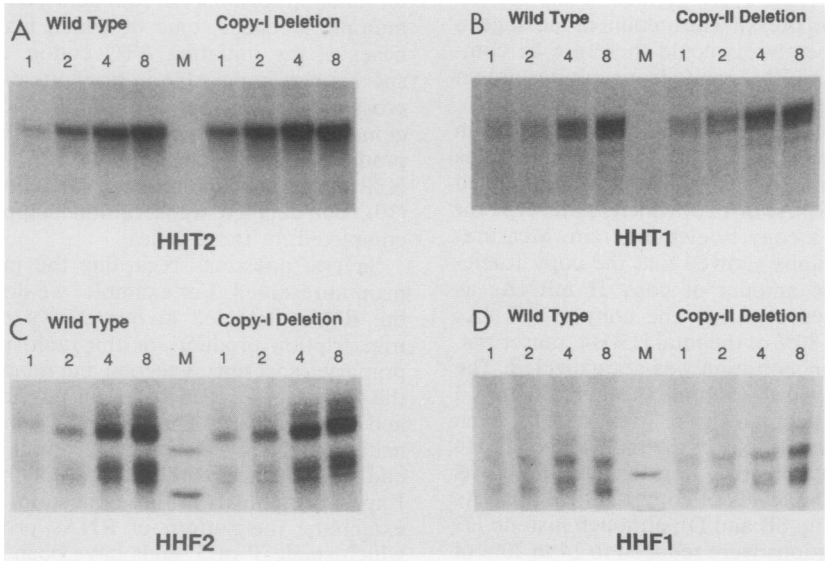

FIG. 8. Effect of histone H3-H4 gene deletions on mRNA levels. Titrations of the levels of H3 and H4 mRNAs are shown for each gene by using RNA from two strains, a strain that is wild type for its histone H3-H4 loci (YP3) and a strain in which one of the H3-H4 loci is deleted (MSY164 or MSY165). (A and B) Titrations for the H3 genes; (C and D) titrations for the H4 genes; (A and C) results for the wild-type and copy I deletion strains; (B and D) results for the wild-type and copy II deletion strains. Each probe was hybridized with 1.0 to 8.0 μg of total RNA, as indicated above the lanes in the figures. Lanes M, pBR322 marker fragments that do not necessarily fall within the portion of the gel illustrated.

mRNAs. Deletion of the copy II genes would then result in only a modest decrease in histone protein expression. Experiments to test the translational efficiency of the mRNAs are in progress. A second hypothesis for the apparent lack of dosage compensation is that the copy I genes may be expressed for a longer period of time during the division cycle. Without a decrease in transcription rate, this extended period of expression would lead to an increase in the steady-state level of mRNA. However, because of the exponential age distribution function for cells in balanced-growth culture (42), a significant increase in the length of copy I gene expression would result in only a modest increase in the average transcript levels. We have started to examine the cell cycle pattern of mRNA levels in the deletion strains. Our preliminary experiments have shown that there is no difference in the time of onset of copy I mRNA accumulation between wild-type and copy II deletion strains. Therefore, any increase in the time of expression must extend later into the division cycle. There is less resolution of cell cycle fractions in later periods of the division cycle, and at present we cannot rule out a longer period of copy I gene expression in the deletion strain. Finally, the simplest explanation for the lack of dosage compensation is that histone mRNA levels are not rate limiting for histone protein translation.

In the experiments reported here, we examined the effect of decreased gene dosage on the histone H3 and H4 genes. The effect of increased gene dosage on the H2A and H2B genes has been studied previously by Osley and Hereford (33). In their experiments, a single additional copy of the histone *HTA1-HTB1* (*TRT1*) gene set was integrated into the yeast genome, and the levels of H2A and H2B mRNA were assayed. In these strains, the steady-state levels of H2A and H2B mRNA remained the same, but the rate of transcription of the H2B genes, specifically in the duplicated locus, was increased by a factor of 2. Pulse-chase measurements showed that the mRNAs from the H2B genes in the duplicated locus were selectively degraded at twice the normal rates. This result implied that each of the H2A and H2B genes in the experimentally duplicated set were transcribed at normal rates but that their transcripts were degraded faster to maintain normal histone mRNA levels in the cell. That is, the *TRT1* genes demonstrated a copy-specific post-transcriptional dosage compensation. Moreover, when the *HTA2-HTB2* (*TRT2*) locus was deleted, the levels of *TRT1* mRNA increased by a factor of about 2, but the reverse was not true; the levels of *TRT2* gene transcripts did not increase when the *TRT1* genes were deleted (31).

It is unclear how the present study relates to the experiments with the H2A and H2B genes. If the stability of histone mRNAs can be altered to maintain normal levels of histone transcripts, some dosage compensation by the copy I H3-H4 genes would be expected in the copy II deletion. This was not detected. However, the dosage compensation reported for the H2A-H2B genes was specific for the *HTA1-HTB1* locus. Thus, both H3-H4 gene sets behave like the *HTA2-HTB2* locus. For the H3 and H4 genes, our results suggest that, despite their similarity in structure and expression, the two unlinked gene sets are independently regulated. In particular, our results indicate that the difference in the amounts of mRNA encoded by the copy I and copy II histone H3-H4 gene sets is not due to simple competition for a limiting diffusible transcription factor, since such competition would presumably be alleviated by deletion of the copy II genes. Further studies of the factors and DNA sequences required for the transcriptional and posttranscriptional regulation of the nonallelic histone H3-H4 loci should add to our understanding of the control of histone gene expression.

ACKNOWLEDGMENTS

We thank Pfizer Research for donating mithramycin and our colleagues for helpful discussions throughout the course of this work.

This work was supported by Public Health Service grant GM28920 from the National Institutes of Health. M.M.S. gratefully acknowledges the support of Faculty Research Award FRA-257 from the American Cancer Society.

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