Replication-dependent and independent regulation of HMG expression during the cell cycle and conjugation in *Tetrahymena*

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ABSTRACT

Two abundant high-mobility-group (HMG)-like proteins, HMG B and HMG C, exist in the ciliated protozoan, *Tetrahymena thermophila*. Of these, HMG C is specific to transcriptionally active macronuclei, while HMG B is found in macronuclei and in transcriptionally inactive micronuclei [1]. Using Northern and *in situ* analyses, we show that the genes encoding HMG B and HMG C are not expressed uniformly throughout the vegetative cycle or during the sexual process, conjugation. Elevated expression of both genes is observed during macronuclear S phase of the vegetative cycle and during endoreplication of developing new macronuclei in later stages of conjugation. Interruption of any of these macronuclear DNA replications by aphidicolin leads to a rapid drop in the message levels of HMG B and HMG C. These results resemble what is typically observed for replication-dependent nucleosomal histones and differ from the apparent lack of cell cycle regulation observed for HMG genes in vertebrates. A specific-induction of HMG B mRNA is also observed early in conjugation and during this interval, inhibition of micronuclear DNA synthesis by aphidicolin does not affect the message level of HMG B. Thus, during conjugation, expression of HMG B shows both replication-dependent and independent regulation. Results similar to these with HMG B are obtained with histone H4II gene, a gene which is also expressed during micro- and macronuclear S phases during the vegetative cycle. These results demonstrate surprising complexity in the expression of HMG genes in *Tetrahymena* and lend support to the hypothesis that cell cycle regulation plays an important role in directing HMG-like proteins to the appropriate nucleus [2]. Interestingly, expression of neither HMG gene is perfectly synchronized with that of histone H4II gene during the developmental program suggesting that important differences exist between vegetatively growing (cell cycle control) and conjugating (developmental control) cells.

INTRODUCTION

Of all of the cell cycle-regulated genes, those encoding histones are the best-studied. Replication-dependent histone mRNAs accumulate to significant levels during S phase of the cell cycle coincident with the peak of DNA replication. This coupling between histone gene expression and DNA replication is believed to occur through a combination of transcriptional regulation and posttranscriptional changes in RNA stability [3, 4]. Much less is known concerning high-mobility-group (HMG) proteins, abundant chromatin-associated nonhistone proteins. Although HMG proteins and their genes have been isolated and characterized from a wide variety of vertebrate and nonvertebrate sources [5], the cellular function(s) of this rapidly expanding class of nonhistone chromosomal proteins [6, 7] and the expression profile of the genes encoding them are only beginning to be defined.

In contrast to histones, HMG gene expression does not appear to be cell cycle-regulated. In vertebrates, three major subgroups of HMG proteins have been identified based upon biochemical properties, HMG 1/2, HMG 14/17, and HMG I/Y [5], and in general, expression of these genes remains constant throughout most of the cell cycle. Unlike nucleosomal histones, only modest changes in HMG protein and/or mRNA levels have been reported when cells enter S phase or when DNA synthesis is inhibited [8, 9]. Although vertebrate HMG proteins do not seem to exhibit strong cell cycle regulation, correlations with cellular differentiation have been observed [10, 11], and thus, it appears that in higher cells, HMG gene expression is regulated differently from that of replication-dependent nucleosomal histones.

Abundant HMG-like proteins have been identified in several lower eukaryotes including yeast [12], *Tetrahymena* [13, 14], and *Drosophila* [15], however, the precise relationship of these proteins to the well-characterized vertebrate HMG proteins is not clear. In *Tetrahymena*, two abundant HMG-like proteins, HMG B and HMG C, are present in macronuclei [13, 14], suggesting that both polypeptides may function in the packaging of transcriptionally competent chromatin. However, recent studies have shown that HMG B, but not HMG C, is also located in transcriptionally inactive micronuclei during periods of micronuclear DNA replication [1]. Thus, it is unlikely that HMG

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B functions exclusively in the establishment of active chromatin and we have suggested that HMG B plays a more general role in modulating chromatin structure to accommodate a wide range of biological processes including transcription, replication, and recombination.

In this study we have used *in situ* hybridization and Northern analyses to investigate the cell cycle and developmental expression of HMG B and HMG C genes in vegetatively growing and conjugating *Tetrahymena*. Using these methods we observe a surprisingly complex pattern of HMG gene expression. Unlike HMG gene expression in multicellular eukaryotes, the expression of HMG B and HMG C genes is coupled to DNA replication in macronuclei during the vegetative cycle and in developing new macronuclei during conjugation. During these intervals, inhibition of macronuclear DNA synthesis by aphidicolin results in a rapid drop in the message levels of HMG B and HMG C. However, during early stages of conjugation, the message level of HMG B is specifically induced and inhibition of micronuclear DNA synthesis has no effect on the message level of HMG B. Thus, expression of the HMG B gene is complex exhibiting both replication-dependent and independent regulation. We suspect that these differences reflect differences in regulation during the cell cycle and the developmental program, respectively.

MATERIALS AND METHODS

Cell culture and strains

Genetically marked strains of *Tetrahymena thermophila*, CU 427 (Mpr/Mpr[6-mp-s]VI) and CU 428 (Chx/Chx[cy-s]VII), were used in all experiments reported here. They were kindly provided by P. Bruns (Cornell University, Ithaca, NY). Cells were grown axenically in 1% (wt/vol) enriched Proteose Peptone as described by Gorovsky et al. [16]. Cells were typically starved overnight (16 to 18 hr) at 30°C in 10 mM Tris (pH 7.4). All matings were performed in 10 mM Tris-HCl (pH 7.4) according to Bruns and Brussard [17], as modified by Allis and Dennison [18]. All cultures were maintained at 30°C.

Aphidicolin treatment and labeling with ³H-thymidine

Aphidicolin (Sigma, St. Louis, MO) in various concentrations ranging from 0.2 to 25.0 µg/ml was prepared in dimethylsulfoxide (DMSO, Fisher Scientific, Pittsburgh, PA). Cells were pretreated with aphidicolin for 5 min before labeling with ³H-thymidine (80 Ci/mmol, 2 to 10 µCi/ml) for 30 min. Incorporation of the label into acid-insoluble DNA was monitored by spotting 100 µl of cells in duplicate onto Whatman filters which were then air dried, washed twice in cold 10% trichloroacetic acid (TCA) followed by two washes in cold 95% ethanol, air dried and subjected to liquid scintillation counting. Dose response experiments indicated that a significant fraction (80 to 95%) of ³H-thymidine incorporated during the 30 min labeling interval was inhibited at an aphidicolin concentration of 1 µg/ml relative to minus aphidicolin (DMSO only) controls. Since higher concentrations of aphidicolin provided only a modest increase in the percent inhibition, 1 µg/ml aphidicolin was used in all experiments reported here.

For conventional ³H-thymidine autoradiography, growing or conjugating cells were pretreated with aphidicolin (1 µg/ml) or DMSO for 5 min before pulse labeling the cells for 15 min with ³H-thymidine (10 to 20 µCi/ml). Cells were then fixed, washed

with cold 5% TCA to remove unincorporated nucleotides, dropped onto microscope slides (10⁶ cells/ml) and processed for autoradiography as described previously [19].

Probe preparation

Antisense or sense RNA probes corresponding to 184 bp of coding sequence of the HMG B gene [14], was synthesized using a BglII-EcoRV fragment of HMG B gene in bluscript vector (Stratagene, La Jolla, CA). The plasmid DNA was linearized with XbaI or EcoRV and was synthesized from either T₃ or T₇ promoters using ³H-UTP (42 Ci/mmol) and ³H-CTP (26 Ci/mmol). For antisense or sense HMG C RNA probe synthesis, a BamHI-HindIII fragment of containing 270 bp coding sequence of the HMG C gene [14] in the same vector was digested by HindIII or BamHI and RNA was synthesized from the T₇ or T₃ promoters. In some experiments, probes were synthesized using ³³P-UTP (1,000–3,000 Ci/mmol). For *in situ* analyses, RNA probes had a specific activity of 1×10⁸ dpm/µg for ³H and 3×10⁹ dpm/µg for ³³P and were used at a concentration which was one-third and one-thirtieth of the saturation value for ³H and ³³P probes, respectively.

Cell preparation for *in situ* hybridization

Growing cells were fixed in glutaraldehyde as described previously [20]. In some experiments cells were incubated with bromodeoxyuridine (BrdU, Boehringer Mannheim, Indianapolis, IN) at 100 µg/ml for 15 min prior to fixation. Pretreatment of the cells for *in situ* hybridization was carried out as described by Yu et al. [21] except that a higher concentration of proteinase K (80 µg/ml, Boehringer Mannheim) was used in analyses with BrdU antibodies (see below).

In situ hybridization and cell cycle analyses

Cell morphology and cell size. *In situ* hybridization was carried out as described by Cox et al., [22] at 50°C in the following buffer: 0.3 M NaCl, 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 50% formamide, 1×Denhardt's solution, 0.5 mg/ml yeast tRNA, 10% dextran sulfate. The stringency of hybridization is similar to previously published Northern blots [23] which showed hybridization to a single message for HMG B and HMG C. Autoradiography was completed as described previously [21]. Cells were stained through the emulsion for 15 to 30 min with 1% Giemsa stain (Fisher Scientific) in 10 mM phosphate buffer (pH 6.5). Grain counts were taken from photographic prints using sense probe hybridization to correct for nonspecific background. Cell cycle analyses were carried out as described by Yu et al. [21] and White and Gorovsky [24]. Briefly, dividing cells which accounted for 10 to 15% of the total vegetative population were identified using morphological criteria (see Results). Approximate position in the cell cycle of the remaining non-dividing cells was estimated by cell size.

Staining with BrdU antibodies. More accurate identification of macronuclear S phase in non-dividing cells was achieved by combining *in situ* analyses with immunofluorescence using fluorescein-conjugated anti-BrdU antibodies (Boehringer Mannheim). Cells were labeled with BrdU before being fixed and processed for *in situ* analyses as described above except that a DNA denaturing step (dilute acid—0.1 N HCl for 5 min) was included prior to incubation with the antibody and hybridization of the probe. Following acid denaturation, cells were quickly neutralized with 0.1 M borate buffer (pH 8.0) before incubation

with antibody; antibody dilution and incubation times were as specified by the manufacturer. In these analyses, micro- or macronuclear DNA replications, indicated by nuclei staining positively with the anti-BrdU antibodies, and grain densities resulting from *in situ* autoradiography, could be evaluated in the same cells. We noticed, however, that staining of micro- or macronuclei with anti-BrdU antibodies was not as strong following the steps for hybridization and autoradiography, and therefore, cells were routinely photographed for BrdU staining immediately following incubation with the antibody. Identical cells were then relocated and rephotographed following *in situ* hybridization and autoradiography. Some shrinkage of the cells occurs after incubation with the antibody and therefore, cell size is not exactly the same in photographs taken before and after *in situ* hybridization.

Isolation of RNA and Northern analyses

Standard methods were used for RNA isolation and Northern analyses [23]. Growing, starved, and conjugating cells at different developmental stages were treated with aphidicolin or DMSO for 25 to 30 min prior to the isolation of total RNA. Parallel agarose gels containing 1% formaldehyde were transferred to zeta-probe membranes (Bio Rad, Richmond, CA) followed by UV-crosslinking (Stratagene). Blots were then probed with random primed HMG B, HMG C, histone H4II [21] and α -tubulin genes ([25]; Bowen and Gorovsky, personal communication) (Boehringer Mannheim Random Primed DNA Labeling Kit) having a specific activity of $1-2 \times 10^9$ dpm/ μ g. Hybridization conditions were identical to Schulman et al. [23] which detect only one message for HMG B and HMG C. Quantitation of HMG B and HMG C hybridization signals was achieved by densitometric scanning of slot blots containing a dilution series of each sample. Evenness of RNA loads was verified by staining the gels with ethidium bromide prior to transfer.

Primer extension analyses

To map the 5' end of transcripts from HMG B or H4II mRNAs, primer extension using reverse transcriptase of avian myeloblastosis virus (AMV) was carried out as described by Ghosh et al. [26]. The synthetic primers complementary to the +10 to +34 of the HMG B gene and -30 to -6 of the H4II gene was 32 P 5' end labeled with T_4 polynucleotide kinase and 32 P- γ -ATP and annealed to 20 μ g of total RNA from growing or 1 hr, 4 hr, and 13 hr conjugating cells. Hybridization was carried out in 16 μ l of buffer containing 0.3 M NaCl, 10 mM Tris-HCl (pH 7.5), and 1 mM EDTA (pH 8.0) at 70°C for one hour. After hybridization, the reverse transcription reaction was conducted in a final volume of 50 μ l of buffer, consisting of 50 mM Tris-HCl (pH 8.0), 10 mM dithiothreitol, 80 mM KCl, 6 mM $MgCl_2$, 0.5 mM each deoxynucleoside triphosphate, 50 μ g/ml actinomycin D, and 20 U of AMV reverse transcriptase (Boehringer Mannheim) at 45°C for 1.5 hr. The extended products were ethanol precipitated and electrophoresed on 10% polyacrylamide-8M urea gels.

RESULTS

HMG B and HMG C message accumulation throughout the vegetative cell cycle

The *Tetrahymena* vegetative cell cycle is unusual from that of most eukaryotic cells in as much as two nuclei, germinal micronuclei and somatic macronuclei, replicate DNA and divide

Figure 1. Nonoverlapping micro- and macronuclear DNA synthesis is inhibited by aphidicolin in growing *Tetrahymena*. Shown for orientation purposes in (A) is a cartoon of various stages in the vegetative cell cycle of *Tetrahymena*. Cell-cycle stages are determined by a combination of morphological features such as micronuclear division, macronuclear division, cytokinesis and cell size. In order, stages shown are: round micronucleus not adjacent to the macronucleus, I; elongated dividing micronucleus not adjacent to the macronucleus, II; divided macronucleus and cell in cytokinesis, III; newly divided daughter cells, IV; small, V; medium, VI; and large, VII nondividing (interphase) cells each with a round micronucleus adjacent to a macronucleus. Solid-filled micronuclei (stages III and IV) and macronuclei (stage VI) represent cells in micro- and macronuclear S phases, respectively (see [2, 21, 27] for details). Shown in (B) are representative examples of vegetative cells preincubated for 5 min with (+Aphid. at 1 μ g/ml in DMSO) or without (-Aphid., DMSO only) aphidicolin before labeling with 3 H-thymidine (10 μ Ci/ml) for 15 min. Cells were fixed, prepared for autoradiography and stained with 4, 6-diamidino-2-phenylindole (DAPI) as described [19]. Although only a few cells of each type are shown, no examples of positively labeled micro- or macronuclei were observed in any of the aphidicolin-treated cells. Exposure times were identical for each set of cells.

Figure 2. *In situ* hybridization analyses of HMG B and HMG C gene expression in vegetative cells. Antisense ^3H -RNA probes for messages coding HMG B (a and c) or HMG C (b and d) were hybridized to cells from a logarithmically growing culture of *Tetrahymena* (200,000 cells/ml). Examples of labeled (black arrows) and unlabeled nondividing cells are shown (stages V–VII, Fig. 1A) as well as several dividing cells (black open arrows; stage III, Fig. 1A). Inserts in a and b denote longer exposures of dividing cells showing a low, basal level of HMG B and HMG C mRNA in comparison to the overexposed interphase cells known to be in macronuclear S phase. Nonspecific background levels are also shown for sense RNA controls of HMG B and HMG C (e and f, respectively). Exposure times equal 3 weeks.

at nonoverlapping points in the cell cycle (see Fig. 1A and [27]). Because of difficulties in synchronizing large numbers of *Tetrahymena*, the technique of *in situ* hybridization was used to determine whether HMG B and HMG C gene expression is coupled to micro- and/or macronuclear DNA synthesis. In these analyses, the position of dividing cells in the cell cycle is precisely determined on the basis of morphology. Micronuclear S phase occurs in newly divided micronuclei during cytokinesis (stage III, Fig. 1A) and continues in small daughter cells just after cell

division (stage IV, Fig. 1A, [21, 27, 28]). In contrast, the position of nondividing (interphase) cells in the cell cycle can be estimated by cell size since cells grow continuously throughout the cell cycle (compare stages V–VII, Fig. 1A). It has been determined that macronuclear S phase occurs in medium size, nondividing cells (see stage VI, Fig. 1A, [21]). Examples of cells in micro- and macronuclear S are shown in the lefthand panel of Figure 1B. If expression of HMG B and/or HMG C genes in *Tetrahymena* is coupled to micro- and/or macronuclear DNA synthesis, we

Figure 3. Both HMG B and HMG C message levels are elevated during macronuclear S phase in vegetative cells. Immunofluorescent analyses using anti-BrdU antibodies (a, c, e, g, i and k) combined with *in situ* hybridization analyses (b, d, f, h, j and l) were performed in vegetative cells pulsed *in vivo* with BrdU. Anti-BrdU antibodies detect both macronuclear S phase cells (white arrows) and micronuclear S phase cells (white open arrows) containing replicating micronuclei (denoted by double white arrowheads). Following antibody incubation and photography, *in situ* hybridization was performed with the same cells as described in Figure 2 except that both HMG B and HMG C probes were ^{33}P -labeled. Messages for HMG B and HMG C accumulate significantly in macronuclear S phase cells (see white and black arrows), but not in micronuclear S phase cells (see open white and black arrows) or other interphase (nondividing) cells which are not in macronuclear S phase.

predict that their messages would accumulate in cells with the S-phase morphologies shown in Figure 1A (nuclei in S phase are darkened).

To test this prediction, glutaraldehyde-fixed, logarithmically growing *Tetrahymena* cells were hybridized *in situ* to ^3H -labeled, antisense HMG B and HMG C RNA probes. With either HMG B (Fig. 2, a and c) or HMG C (Fig. 2, b and d) probes, labeled (marked by black arrows) and unlabeled cells are clearly observed, demonstrating that HMG B and HMG C messages are not present uniformly throughout the cell cycle. Cell cycle analyses based upon cell size measurements suggest that 50–60% of the labeled interphase cells are in a mid-size range suggesting that these cells are in macronuclear S phase. At this exposure, dividing cells (large open arrows in Fig. 2) are essentially unlabeled suggesting that message levels for HMG B and HMG C are low or nonexistent during micronuclear S phase. Essentially no grains above background were detected using sense strand, control probes (Fig. 2, e and f).

To determine more precisely whether the positively labeled nondividing cells shown in Figure 2 are in macronuclear S phase, *in situ* analyses were repeated using cells pulsed *in vivo* with BrdU and prestained with anti-BrdU antibodies. With either HMG B (Fig. 3, a through f) or HMG C (Fig. 3, g through l) probes, essentially all labeled, nondividing cells were positively identified as being in macronuclear S by staining of macronuclei, but not micronuclei, with anti-BrdU antibodies. In contrast, dividing cells containing only BrdU-positive micronuclei and interphase cells containing macro- and micronuclei were weakly labeled with both HMG B and HMG C probes. These results demonstrate that,

unlike HMG mRNAs in higher eukaryotes, messages for HMG B and HMG C accumulate significantly during macronuclear S phase and are low during other stages of the cell cycle. Since macronuclear S phase occupies approximately one quarter of the vegetative cell cycle [21] and the half lives of HMG mRNAs under these conditions are not known, these results are not surprising. Results similar to these have been obtained for genes which encode macronuclear-specific histones such as H1 [2] and H4I [21].

Recently, biochemical and immunocytological approaches have documented the presence of HMG B, but not HMG C, in micronuclei during intervals of DNA replication [1]. These results prompted us to reexamine longer exposures of our *in situ* analyses to determine if a second peak of HMG B mRNA exists during micronuclear S phase. Although the message level for HMG B (and HMG C) is elevated above background in dividing cells (see inserts to Figs. 2a and b), this is also true for other interphase cells that are not in macronuclear S phase (data not shown). These results suggest that in addition to replication-dependent expression of HMG B and HMG C genes during macronuclear S phase, a low, basal level of expression of both genes is maintained throughout the rest of the cell cycle. Presumably, this low level of HMG B mRNA is used to synthesize HMG B which is found in micronuclei during vegetative growth [1].

Disruption of DNA synthesis by aphidicolin affects the message level of HMG B and HMG C in growing cells

Aphidicolin, a reversible inhibitor of DNA polymerase, was used to disrupt DNA synthesis to investigate further whether

expression of HMG B and/or HMG C is coupled to micro- and/or macronuclear DNA replication. Autoradiography of growing cells pulsed for 15 min with ^3H -thymidine in the presence or absence of aphidicolin (1 $\mu\text{g}/\text{ml}$) shows that micro- and macronuclear DNA synthesis is abolished in aphidicolin-treated cells under these conditions (Fig. 1B). Moreover, Northern analyses using total RNA extracted from control or aphidicolin-treated cells, demonstrate that message levels of HMG B and HMG C are reduced when DNA synthesis is blocked by aphidicolin (Fig. 4). Quantitation of the message levels in three independent experiments shows that the amount of HMG B mRNA is reduced two fold upon aphidicolin treatment while that of HMG C mRNA is reduced six fold. Blots were also reprobbed with *Tetrahymena* α -tubulin or histone H4II genes. As expected, the message level of histone H4II is reduced significantly by aphidicolin treatment while that of tubulin is unaffected.

Inhibition of DNA synthesis affects the mRNA level of HMG B and HMG C differently during conjugation

The autoradiographic experiments presented in Figure 1B show clearly that aphidicolin abolishes both micro- and macronuclear DNA replication in growing cells. However, it is not clear from the Northern analyses presented in Figure 4 whether the observed reduction in message levels of HMG B and HMG C upon aphidicolin treatment is due to the inhibition of macronuclear and/or micronuclear DNA replication (due to asynchrony in the vegetative population). To address this question more precisely, we took advantage of the fact that during the sexual process, conjugation, micronuclei and developing new macronuclei (or macronuclear anlagen) undergo several rounds of DNA replication as part of the developmental program (Fig. 5A). As expected from previously published studies [19], ^3H -thymidine autoradiography detects both micronuclear-specific (4–5 hr) and anlagen-specific (12–13 hr) DNA replication (lefthand panel of Fig. 5B). Aphidicolin treatment of conjugating cells inhibits DNA replication in both micronuclei and new macronuclei at all stages that we have examined (righthand panel of Fig. 5B).

As shown in Figure 6, HMG B and HMG C messages accumulate at distinct as well as overlapping periods of conjugation. Both HMG B and HMG C messages accumulate significantly during late stages of anlagen differentiation (13 hr) when new macronuclei endoreplicate DNA from 4C to 8C [19]. Inhibition of anlagen DNA synthesis at 13 hr reduces the message levels of HMG B and HMG C four and ten fold, respectively, relative to nontreated controls. As expected, the mRNA level of histone H4II is elevated during anlagen replication and it is reduced significantly by aphidicolin treatment. Although only the gene for HMG C is expressed during early stages of anlagen differentiation (7–10 hr), the level of HMG C mRNA is also reduced by aphidicolin during this interval.

The data presented in Figure 6 demonstrate convincingly that HMG B, HMG C, and H4II are also required at distinct periods of the developmental program. As reported earlier [23], the mRNA level of HMG B is specifically induced during early stages of conjugation (1 and 4 hr) when micronuclei enter an extended period of meiotic prophase (Fig. 6). Although micronuclei do not complete any scheduled (S-phase) DNA replications during this period, meiotic DNA (presumably, repair) synthesis occurs [19] and newly synthesized HMG B protein is deposited selectively in micronuclei during this stage [1]. Surprisingly, the

Figure 4. Inhibition of DNA synthesis by aphidicolin reduces the message level of HMG B and HMG C in vegetative cells. Northern analyses were performed using total RNA isolated from growing cells following a 25–30 min preincubation with (+Aphid.) or without (–Aphid.) 1 $\mu\text{g}/\text{ml}$ aphidicolin. Parallel blots were hybridized with probes for HMG B and HMG C; these blots were then stripped and reprobbed with α -tubulin [25] and H4II [21] gene probes, respectively. Evenness of RNA loads was verified by staining gels with ethidium bromide prior to transfer.

message level of HMG B is not affected by aphidicolin treatment during meiotic prophase. Interestingly, although the histone H4II gene is expressed later in the developmental program (4–7 hr), presumably in concert with several rounds of scheduled micronuclear DNA replication, it is also unaffected by aphidicolin in young mating cells. Thus, even though the expression of HMG B and H4II is coupled to macronuclear DNA replication during vegetative growth (Fig. 4) and to the endoreplication of new macronuclei during conjugation (Fig. 6), the expression of these genes also exhibits several features of replication-independent regulation. It is presently unclear whether these differences reflect differences in regulation between micro- and macronuclear S phases or differences in regulation between cell cycle (vegetatively growing cells) and developmental (conjugating cells) control (see the Discussion).

Transcription initiation sites of HMG B and H4II are identical in vegetatively growing and conjugating cells

Both HMG B and H4II show replication-dependent and independent regulation as suggested by differential sensitivity to inhibition by aphidicolin during vegetative growth and conjugation. Although the mechanism(s) underlying the complex regulation of these genes is not understood, we reasoned that a different set of transcription factors may activate HMG B and H4II genes during periods of aphidicolin sensitivity and insensitivity. As a first step towards investigating this hypothesis, primer extension assays were used to test whether different

Figure 5. Aphidicolin inhibits micro- and macronuclear DNA synthesis in conjugating *Tetrahymena*. Shown in A is a schematic representation of key stages in the sexual process, conjugation. In order, stages relevant to this study are: costimulation and pairing of starved, initiated cells, I; meiotic prophase, II; prezygotic divisions of micronuclei, III; postzygotic divisions of micronuclei leading to the establishment of anterior new macronuclei and posterior new micronuclei, IV; differentiation of new macronuclear anlagen (larger open nuclei), new micronuclei (smaller, gray-stripped nuclei) and old macronuclei (smaller, open nuclei marked with Xs), V; exconjugation followed by complete resorption of old macronuclei and one micronucleus, VI. As in Figure 1A, solid-filled nuclei represent micronuclei or developing new macronuclear anlagen known to be actively engaged in scheduled DNA replications; gray-stripped nuclei represent several unscheduled DNA replications that have been observed in micronuclei (see [19] for details). Time in hours represents the time after cells of opposite mating types are mixed (see [33] for further details). Shown in B are representative examples of 4 hr (top row) and 13 hr (bottom row) conjugating cells preincubated for 5 min with (+aphid.) or without (-aphid.) 1 μ g/ml aphidicolin before labeling with 3 H-thymidine (20 μ Ci/ml) for 15 min. Cells were analyzed as in Figure 1. No examples of positively labeled nuclei were observed in aphidicolin-treated conjugants at any stage of conjugation.

transcription initiation sites are utilized. However, the same four major transcription initiation sites of the HMG B gene (at -55, -50, -47, and -44, see [14]) were detected using RNA isolated from vegetatively growing cells and 1 hr and 13 hr conjugating cells (data not shown). Similarly, no significant difference in transcription initiation sites was observed with the H4II gene using RNA isolated from growing cells or RNA from 4 hr and 13 hr conjugating cells, and these sites agree with those previously mapped by Horowitz et al. [29]. These data suggest that at least during vegetative growth and the stages of conjugation that we have tested, different promoters are not used for the expression of HMG B and H4II.

DISCUSSION

We have analyzed the cell cycle and developmental expression of HMG genes in *Tetrahymena* using a combination of *in situ* hybridization and Northern analyses. Our results demonstrate that mRNAs for both of the two major HMG-like proteins in macronuclei, HMG B and HMG C, accumulate to significant levels during macronuclear S phase and inhibition of macronuclear DNA synthesis leads to a significant reduction in the level of both HMG B and HMG C mRNAs. Results qualitatively similar to these are obtained when a histone gene

Figure 6. Replication-dependent and independent regulation of HMG and histone message levels during conjugation. Northern analyses were performed using total RNA isolated from starved (16–18 hr in 10 mM Tris, pH 7.4; St), and 1, 4, 7, 10 and 13 hr mating cells following a 25–30 min preincubation with (+Aphid.) or without (-Aphid.) 1 μ g/ml aphidicolin. Parallel blots were hybridized with probes for HMG B and HMG C; these blots were stripped and reprobed with α -tubulin and H4II gene probes, respectively. As shown in Figure 5A micronuclear DNA replication occurs during 4–5 hr of conjugation, and replication of developing new macronuclear anlagen occurs around 7 (2C to 4C) and 13 hr (4C to 8C) of conjugation. Evenness of RNA loads was verified by ethidium bromide staining prior to transfer.

is included as a positive control which suggests, that at least in *Tetrahymena*, HMG gene expression shows some aspects of cell cycle regulation like what is typically observed for replication-dependent nucleosomal histones [4]. However, expression of each of the HMG genes responds differently to a block in macronuclear DNA synthesis. We have consistently observed that aphidicolin treatment of vegetative cells causes a greater reduction in the message level of HMG C than HMG B, but not to the extent observed for H4II mRNA (see Fig. 4). Thus, there appears to be considerable variation in the extent to which histone and HMG expression is coupled to DNA replication in *Tetrahymena*.

One of the most interesting results to emerge from these studies is the observation that the abundance of RNA for neither HMG B nor HMG C is perfectly synchronized with that of histone H4II (see Fig. 6). These results suggest that HMG B and HMG C may have specialized, nonoverlapping functions during the developmental program in *Tetrahymena* and suggest further that these functions are not necessarily related to DNA replication and chromatin assembly. Along this line, we point out that the amount of HMG B mRNA in 1 to 4 hr conjugating cells is higher than expected for packaging newly replicated prezygotic and postzygotic micronuclear DNA as compared to the amount of HMG B observed during 4C–8C anlagen endoreplication (Fig. 6). Given this, it is tempting to speculate that some HMG B is synthesized and stored for assembly into macronuclear chromatin at a later stage of conjugation. However, recent experiments have shown that essentially all of the HMG B synthesized in young (1–4 hr) conjugating cells is targeted to micronuclei [1]. Since the message level of HMG C is elevated significantly during anlagen differentiation, but is low or nonexistent in young mating cells, less HMG B may be required during later stages of conjugation (assuming that HMG B and HMG C have some redundant functions).

Unlike what is typically observed when macronuclear DNA replication is inhibited during vegetatively growth (Fig. 4) or later stages of anlagen development (Fig. 6), interruption of micronuclear DNA synthesis in young mating cells, does not lead to a rapid drop in the message level of HMG B or H4II. These results suggest that at least during conjugation, the expression of HMG B and H4II genes is uncoupled from S-phase regulation altogether. Numerous studies suggest that transcriptional and posttranscriptional pathways contribute to cell cycle-regulated histone gene expression [4]. In higher cells, differential turnover of histone mRNA is widespread and is observed most dramatically when S phase is interrupted. In yeast, however, coupling of histone mRNA levels to DNA replication is due mostly, if not entirely, to transcriptional regulatory mechanisms [30]. In *Tetrahymena*, although post-transcriptional regulation has been well documented [31], it appears that most genes analyzed to date are regulated largely at the level of transcription [32]. Both HMG B [14] and H4II [29] are single copy genes and neither of which has introns; thus, differential splicing can not occur as part of the regulatory mechanism. Although HMG B and H4II have multiple 5' ends, our preliminary results show that the transcription initiation sites of HMG B and H4II are essentially the same during micro- and macronuclear S phases. Further studies are necessary to determine the level (transcriptional or post-transcriptional) at which replication-dependent and independent regulation of HMG expression occurs in *Tetrahymena*.

Finally, the question remains as to why HMG genes are at least partially cell cycle regulated in *Tetrahymena* when this level

of control is not utilized to a large extent in higher eukaryotes. Wu et al. [2] proposed a model wherein targeting of micro- or macronuclear-specific proteins to appropriate nuclei in *Tetrahymena* is provided by coupling the expression of the gene to the appropriate S phase. Of five histone genes studied to date, the message level of only one is relatively constant throughout the cell cycle; the message level of all others is tightly coupled to the appropriate micro- or macronuclear S phase (see [24] for references). The results from this study suggest that message levels for HMG B and HMG C also accumulate largely during appropriate S phases of the cell cycle and the developmental program. It seems likely then that cell cycle regulation is used in part to ensure that the correct HMG protein is synthesized and targeted to the appropriate nucleus in *Tetrahymena*. However, specialized nuclear transport systems have been described for several macronuclear-specific histones including one (histone H1) whose gene is expressed only during macronuclear S phase [24]. Therefore, we can not rule out the possibility that a mechanism like this is also being used to ensure appropriate nuclear localization of HMG proteins in this organism.

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