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# Localization and Expression of mRNA for a Macronuclear-Specific Histone H2A Variant (hv1) during the Cell Cycle and Conjugation of *Tetrahymena thermophila*

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**hv1 is a histone H2A variant found in the transcriptionally active *Tetrahymena* macronucleus but not in the transcriptionally inert micronucleus. This, along with a number of other lines of evidence, suggests that hv1 is associated with active genes. We have used a cDNA clone as a probe to study hv1 mRNA accumulation throughout the cell cycle and during conjugation. In situ hybridization to glutaraldehyde-fixed growing cells, whose position in the cell cycle was determined by size and morphology, showed that hv1 message is present throughout the cell cycle. The message was uniformly distributed in these vegetative cells. Compared with four other *Tetrahymena* histone genes studied to date (S.-M. Yu, S. Horowitz, and M. A. Gorovsky, Genes Dev., 1: 683, 1987; M. Wu, C. D. Allis, and M. A. Gorovsky, Proc. Natl. Acad. Sci. USA 85:2205, 1988), hv1 mRNA is the only one that does not show a pattern of accumulation during the cell cycle that could explain the nuclear localization of its encoded protein. Thus, either hv1 or some molecule with which it associates contains a macronuclear-specific targeting sequence or there exists a cell cycle-regulated event that restricts its translation to the macronuclear S phase. In situ hybridization to conjugating cells revealed that hv1 message amounts increase just prior to macronuclear development and decline precipitously after the cells separate. The hv1 message showed no marked subcellular localization and is, therefore, unlikely to play a role in the cytoplasmic determination known to occur during macronuclear development.**

A histone H2A variant in the ciliated protozoan, *Tetrahymena thermophila*, called hv1 has been shown to possess a high degree of evolutionary conservation with a family of H2A variants in higher organisms, referred to as H2A.F or H2A.Z (3, 34). hv1 is specific to the transcriptionally active *Tetrahymena* macronucleus and is absent from the transcriptionally inert micronucleus, leading to the suggestion that it is associated with transcriptionally active chromatin (2). This hypothesis has been supported by experiments which show the following. (i) hv1-specific antibodies stain nucleoli in mammalian cells, presumably in the highly transcriptionally active rDNA chromatin (4). (ii) hv1-specific antibodies stain developmentally puffed regions in *Drosophila* polytene chromosomes (S. C. R. Elgin, V. Dietrich, E. K. Steiner, J. B. Olmsted, C. D. Allis, and M. A. Gorovsky, submitted for publication). (iii) hv1 appears in developing macronuclear anlagen at approximately the same time that new transcription begins (33). Gabrielli et al. (12) have detected the minor variant MI ( = H2A.Z) only in the transcribing fraction of mouse cell chromatin, and Ridsdale and Davies (29) have shown that levels of H2A.Z are significantly elevated in the transcriptionally active polynucleosomes of chicken erythrocytes, again indicating a relationship between these H2A variants and transcriptionally active genes.

In the sea urchin, H2A.F/Z mRNA is found in all embryonic stages and adult tissues tested (10), in contrast to the developmental class switching shown for the major H2As (25). Wu and Bonner (37) have shown that in Chinese hamster ovary cells, H2A.Z is expressed at a basal level throughout the cell cycle while the major histones H2A.1 and H2A.2 are expressed in a DNA synthesis phase-dependent fashion. These patterns of expression suggest that variants have a function in the cell that differs from that of the major H2As. This hypothesis is strongly supported by sequence comparisons of major and variant H2As in chickens, sea urchins, and *T. thermophila*, which indicate that the variants are more similar to each other than to the major H2As and thus, have evolved even more slowly than the major H2As (34).

The *Tetrahymena* life cycle consists of a vegetative growth phase and a sexual conjugation phase, which can be induced by mixing different mating types under starvation conditions. During vegetative growth, the DNA synthesis phases for macro- and micronuclei are nonoverlapping (20, 26, 35). Wu et al. (36) have demonstrated that the mRNAs coding for macronuclear- or micronuclear-specific linker histones are present in vegetative cells only during the S period of the nucleus to which the protein is localized and have suggested that these histones become localized to a given nucleus because they are synthesized and available to be deposited on newly synthesized DNA only during a specific S phase. In conjugation, micronuclei undergo meiosis, exchange, fertilization, and two postzygotic divisions to form four new micronucleuslike nuclei in each paired cell. The second postzygotic division is aligned in the anterior-posterior axis. The two new nuclei in the anterior of the cell differentiate into macronuclei; the two in the posterior become micronuclei (27). Nanney (24) has shown that positioning of the nuclei in these different regions of the cell is important for determining nuclear development.

We have used in situ hybridization of hv1 probes to growing and conjugating cells to address three questions about the expression of hv1 mRNA and the specific localization of hv1 to the *Tetrahymena* macronucleus. First, what is the cell cycle regulation of the hv1 message; is it present only during macronuclear S phase and not during micronuclear S phase? Second, are hv1 message amounts regulated during conjugation? Third, is there any subcellular localiza-

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tion of hv1 message in conjugating cells that could contribute to the localized determinants that cause macronuclear differentiation?

## MATERIALS AND METHODS

**Cells and culture conditions.** *T. thermophila* (strains Cu 427 and Cu 428) were grown axenically in enriched proteose peptone at 28°C as described previously (15). Cells in logarithmic growth were used at a density of $\sim 2 \times 10^5$ cells per ml. Starved cells were obtained by washing and resuspending a log-phase culture in 10 mM Tris (pH 7.4) and shaking at 28°C for at least 24 h. Conjugation was carried out essentially as described previously (19) by mixing equal numbers of starved cells of strain Cu 427 (mating type VI) and Cu 428 (mating type VII).

**Probe preparation.** hv1 cDNA containing 417 base pairs of coding sequence and 249 bp of 3'-flanking sequence (34) was subcloned into the Promega transcription plasmid pGEM-2. $^3$H-labeled single-stranded RNA probes were prepared with the Amersham SP6 transcription kit according to the instructions of the manufacturer. Antisense probes, 1,167 bases in length and containing all of the inserted hv1 sequences, were generated by digesting the recombinant vector with *Sph*I (by the recommendations of the manufacturer) and transcribing with T7 polymerase. Sense probes were made by digesting with *Hin*dIII and transcribing with SP6 polymerase, producing a 721-base transcript. RNA probes sizes were adjusted to a mass average of approximately 150 bases by limited alkaline hydrolysis (8). Probes had a specific activity of $\sim 5 \times 10^7$ cpm/μg and were used at a concentration which was one-third of the saturation value.

**Cell preparation for in situ hybridization.** Growing and conjugating cells were fixed in glutaraldehyde as described previously (38). Samples from conjugating cells were taken at 3, 5, 7, 9, 12, 15, and 24 h after mixing. Pretreatment of cells for in situ hybridization was carried out as described by Yu et al. (39), who modified the procedure published by Angerer and Angerer (5).

**In situ hybridization and cell cycle analysis.** In situ hybridization was carried out as described by Cox et al. (8) at 45°C in the following buffer: 50% formamide, 0.3 M NaCl, 10 mM Tris (pH 8.0), 1 mM EDTA, 1× Denhardt (0.02% Ficoll, 0.02% polyvinylpyrrolidone 360, 0.02% bovine serum albumin), 500 μg of yeast tRNA per ml, and 10% dextran sulfate. This stringency of hybridization is similar to that used for previously published Northern (RNA) blots with hv1 probe (34), which showed hybridization to a single message, and also to other Northern blots performed with hv1 RNA probes which showed a single band (data not shown). Autoradiography was completed as described previously (5). Sense RNA hybridization was used to correct for nonspecific background. Cells were stained for 15 min with 1% Giemsa stain (Sigma Chemical Co.) in 10 mM phosphate buffer (pH 6.5). Grain counts were taken from a video monitor connected to an Olympus BH-2 microscope equipped with an Ikegami ITC-48 surveillance television camera. Cell cycle analysis was carried out as described by Yu et al. (39). Briefly, dividing cells, which accounted for about 15% of the total, were identified morphologically and divided into four stages. Position in the cell cycle of the remaining 85% of the cells was estimated by size. Outlines of ~200 randomly selected cells were traced on transparent sheets laid over the television screen, and grains were counted in each. Areas of the cells were measured with a model 4956 Tektronix digitizer. The cells were then ranked in order according to size. Fourteen intervals of cells were positioned in the cell cycle as 15% plus the cumulative fraction of cells per interval × 85%. For cells that could be put into morphological stages (i.e., dividing cells and conjugating cells), grains were counted in 5 to 15 cells of each stage, and the average number of grains in each cell type was calculated. The cell cycle analysis was performed twice. The average of the two experiments was determined, and the standard error of the average was calculated from the standard errors of data points from each experiment as described previously (23). The percent dividing cells in each stage was averaged from both experiments and the grain numbers were normalized so that both experiments had the same total number of grains.

**Analysis of DNA synthesis phases.** A procedure was developed to analyze the macro- and micronuclear DNA synthesis patterns throughout the cell cycle in a manner similar to that used for analyzing message amounts. Logarithmically growing *Tetrahymena* cells were pulse-labeled for 20 min with [*methyl*-$^3$H]thymidine (20 μCi/ml) (84.1 Ci/mmol; Dupont NEN Research Products) and then fixed immediately by washing the cells quickly in 10 mM Tris (pH 7.4) and then placing the cells in ice-cold 5% trichloroacetic acid (TCA) for 15 min. It was necessary to treat the cells first with TCA because glutaraldehyde fixation made it impossible to wash away unincorporated nucleotides. Cells were then washed twice with cold 5% TCA and then washed briefly with 1% glutaraldehyde. Cells were fixed with 1% glutaraldehyde on ice for 1 h to preserve the cell morphology and washed twice in ice-cold $H_2O$ for 10 min, twice in 50% ethanol for 15 min, and twice in 70% ethanol for 15 min. Cell suspension samples (5 μl), resuspended at a final concentration of $10^6$ cells per ml in 70% ethanol, were dropped onto poly-L-lysine-treated glass microscope slides, dried, and prepared for autoradiography as described previously (5). A cell cycle analysis similar to that used for in situ hybridization was carried out, except that instead of counting grains, 220 cells were simply scored as plus or minus for DNA synthesis in macro- and micronuclei.

## RESULTS

**hv1 message accumulation throughout the cell cycle.** A method has been developed previously (39) to study the accumulation of specific mRNAs at different times in the *Tetrahymena* cell cycle. Because of difficulties in synchronizing growing *Tetrahymena* cells, this procedure is based on the technique of in situ hybridization. In this analysis, the positions of dividing cells in the cell cycle can be determined on the basis of their morphology, and micronuclear S phase is known to occur in recently divided micronuclei during and just after cell division (20, 35, 36). The positions of nondividing cells in the cell cycle are determined by size. This practice assumes that cells grow continuously throughout the cell cycle, which appears to be the case (39).

Glutaraldehyde-fixed, logarithmically growing *Tetrahymena* cells were hybridized in situ to $^3$H-labeled, antisense hv1 RNA probes. Both dividing cells and interphase cells were clearly labeled (Fig. 1), indicating that, unlike the mRNAs for macronuclear H1, hv1 messages are not found only during macronuclear S phase. To quantitate the level of hv1 mRNA throughout the cell cycle, Giemsa-stained, autoradiographed cells were sized or placed in stages and the grains in each cell were counted. Similar to the findings of Yu et al. (39), the dividing cells occupied about 15% of the cell cycle; 6.7% had dividing micronuclei, 3.3% had divided

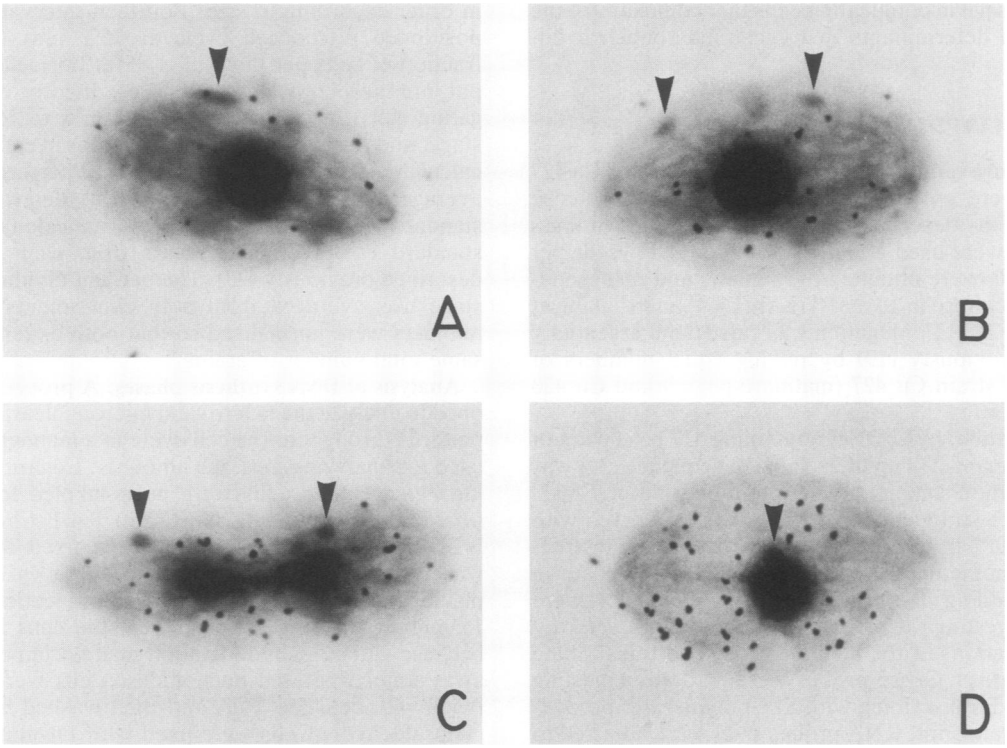


FIG. 1. In situ hybridization of hv1 RNA probes to growing *Tetrahymena* cells. Autoradiographic exposures were for 11 days. Photographs were taken on Kodak Panatomic-X film with an Olympus C-35AD-2 camera on an Olympus BH-2 microscope. (A) Dividing micronucleus; (B) divided micronucleus; (C) dividing macronucleus; (D) nondividing cell. Micronuclei are indicated by arrowheads.

micronuclei, 2.6% were in macronuclear division, and 1.9% were in cytokinesis. The nondividing cells were ranked in order by size and divided into 14 intervals of 15 cells each to occupy the remaining 85% of the cycle. Figure 2A shows the results of two independent analyses plotted as the average number of grains per cell in each interval versus percent cell cycle. The results show that hv1 message was present throughout the cell cycle, with a small increase in message amounts in dividing cells (i.e., during micronuclear S phase) and a larger increase later in the cell cycle.

The large peak of hv1 accumulation appeared to coincide with a period in late macronuclear S phase or in early macronuclear $G_2$ when compared with the results obtained by Yu et al. (39) for *Tetrahymena* histone H4 genes. In order to positively localize the position of macronuclear S phase in our analysis, we studied DNA synthesis throughout the cell cycle in a manner similar to that used to study message amounts. Logarithmically growing *Tetrahymena* cells were pulse-labeled with [$^3$H]thymidine for 20 min, fixed in 5% TCA, washed with TCA to remove unincorporated nucleotides, and then fixed in glutaraldehyde to obtain morphology similar to that seen for in situ-hybridized, glutaraldehyde-fixed cells. Cells were positioned in the cell cycle, as described for in situ hybridization, and scored as positively or negatively labeled for macro- and micronuclear DNA synthesis. Figure 2B shows the results of this analysis graphed as percent labeled cells in each interval versus percent cell cycle. These data demonstrate that macro- and micronuclear S phases are distinct, supporting the legitimacy of this method of analyzing the cell cycle. A comparison of the data from hv1 message amounts (Fig. 2A) and DNA synthesis (Fig. 2B) throughout the cell cycle indicates that the peaks of hv1 message accumulation correspond with micronuclear S and the end of macronuclear S.

**hv1 message accumulation in conjugation.** *Tetrahymena* cells were fixed in glutaraldehyde at various times after the start of conjugation and hybridized in situ to $^3$H-labeled hv1 RNA probes. Cells were placed into 12 stages of conjugation by morphological criteria, and grains were counted in each cell. Figure 3 shows the results from a number of representative pairs of conjugating cells. Figure 4 shows a graph of the average number of grains per cell in each of these stages. Early in conjugation, the amount of hv1 messenger RNA per cell was very low. There was a slight increase in the message level during the prezygotic micronuclear divisions and then a very dramatic increase during the second postzygotic division, which occurs just prior to macronuclear anlagen development. The peak of accumulation coincided with the first stages of anlagen development. The amount of hv1 message per cell began to decline in the later stages of macronuclear development and continued declining as the two conjugating cells separated. The message amount was dramatically decreased in exconjugants which have degraded one of their two micronuclei. At the peak of message accumulation, there was approximately 40 times more hv1 mRNA than seen in cells just beginning or ending conjugation.

**Subcellular localization of hv1 mRNA.** Concentration of specific mRNAs to specific subcellular locations has been described in differentiated cells (18) and in the eggs of both insects and vertebrates (11, 21, 28). No specific subcellular localization of hv1 mRNA was detected either in vegetative cells (Fig. 1) or at any stage of conjugation (Fig. 3).

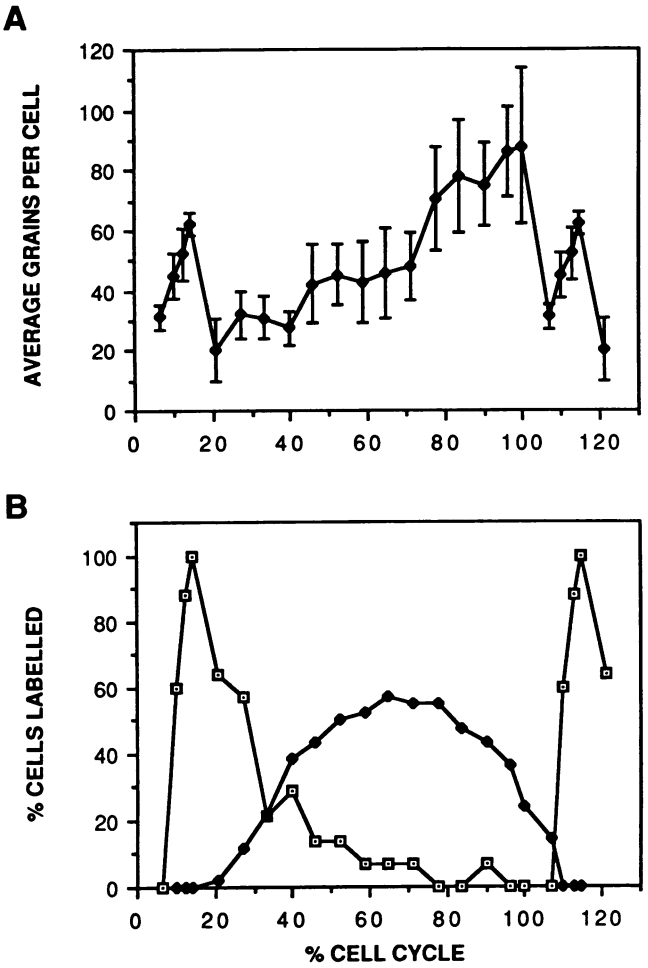


FIG. 2. Regulation of hv1 message amounts throughout the cell cycle. (A) Results from in situ hybridization of hv1 RNA probes to growing *Tetrahymena* cells. The average of two experiments is shown, with standard errors represented by error bars. (B) Results from [$^3$H]thymidine pulse-labeling of growing cells on the same cell cycle scale as hv1 message accumulation. Percentage of cells in each interval with labeled micronuclei (⊡) or macronuclei (◆) are shown.

## DISCUSSION

Histone variants have been divided into three classes based on their expression characteristics (30). Synthesis of the predominant, replication-dependent variants is induced with the onset of DNA synthesis and is repressed at its end. Replication-independent (or basal or replacement) histone variants are expressed throughout the cell cycle and in nondividing cells. Tissue-specific histone types have also been described, particularly in the sperm of many organisms. A dramatic case of the uncoupling of histone and DNA synthesis has been shown in some oocytes in which large stores of histone mRNA and protein are accumulated (for a review, see reference 32). Except in early development, periodic changes in the synthesis of histones are paralleled by similar fluctuations in the amount of mRNA encoding them. This regulation has been shown to involve both transcriptional and posttranscriptional factors (17, 31).

Surprisingly, mRNA for the macronuclear-specific histone H2A variant hv1 of *T. thermophila* is easily detectable during both the macro- and micronuclear DNA synthesis phases. Although the amount of hv1 message may increase with the S phases, the increase is small compared with the four previously studied *Tetrahymena* histone messages whose accumulation is clearly coupled to DNA replication (36, 39) and the message is also present in cells not synthesizing DNA. hv1 mRNA accumulation, then, appears to be most like that expected of a DNA replication-independent histone, i.e., present throughout the cell cycle, but may also exhibit a small amount of replication-dependent expression. H2A variants in other systems are synthesized at basal levels throughout the cell cycle (37) or throughout development (10). This consistent pattern of regulation, along with a remarkable evolutionary conservation (34), is strong evidence that these H2A variants must have an important function in the cell, different from the major histones. As noted previously, that function is likely to be involved in transcriptional activation.

The increase in hv1 mRNA also differs from that of other histone messages known to accumulate during macronuclear S period inasmuch as it occurs only during the latter part of the S period, rather than paralleling the bulk of DNA synthesis. If hv1 were indeed associated with active genes, one might have expected the hv1 mRNA peak to occur early in macronuclear S, since there is evidence in other systems that active genes are early replicating (13, 14, 16). However, Charret (7) has shown that nucleolar DNA (presumably rDNA coding for rRNAs) is late replicating in *Tetrahymena* cells, and hv1-specific antibodies have been shown to stain nucleoli of mammalian cells (4). Thus, the higher level of hv1 message accumulation in late S phase may reflect a special need for hv1 protein in nucleolar chromatin. It is unlikely that hv1 is associated exclusively with nucleolar chromatin, since there is much more hv1 protein associated with nucleosomes in the cell (about 15 to 20% of the amount of a major histone [2]) than would be needed for nucleolar chromatin (making up 3% of the total DNA; C. Giri and M. Gorovsky, unpublished observations).

Wu et al. (36) have proposed a model for the targeting of histone proteins to specific nuclei in *T. thermophila*, based on coordination of their synthesis with the nonoverlapping periods of DNA replication in macro- and micronuclei. They have shown that mRNA encoding H1, a macronuclear-specific protein, is present only during macronuclear S phase and that messages encoding the micronuclear-specific linker histones are found only during micronuclear S. Similarly, messages from both of the two H4 genes (encoding identical proteins) accumulate markedly during macronuclear S, while messages from one of the genes accumulate again during micronuclear S (39). These proteins, then, may use a mechanism of regulated, timely synthesis to determine their nuclear localization; i.e., they are only synthesized at the time a specific set of newly replicated DNA is available. hv1 is a macronuclear-specific histone. Therefore, it was expected that hv1 mRNA accumulation would be linked to macronuclear S phase. However, hv1 message is present during micronuclear, as well as macronuclear, S phase.

Formally, four possibilities can explain the macronuclear localization of hv1 in the absence of coordinated expression of the hv1 gene with macronuclear DNA replication. First, hv1 synthesis could be restricted to a compartment of the cell favoring macronuclear localization. Since macro- and micronuclei lie in close proximity for most of the cell cycle, such localization would have to be closely restricted to the macronuclear envelope or occur when the micronucleus moves away from the macronucleus prior to cell division. No such localization was observed during any stage of the cell cycle in our studies. A second possibility is that the hv1

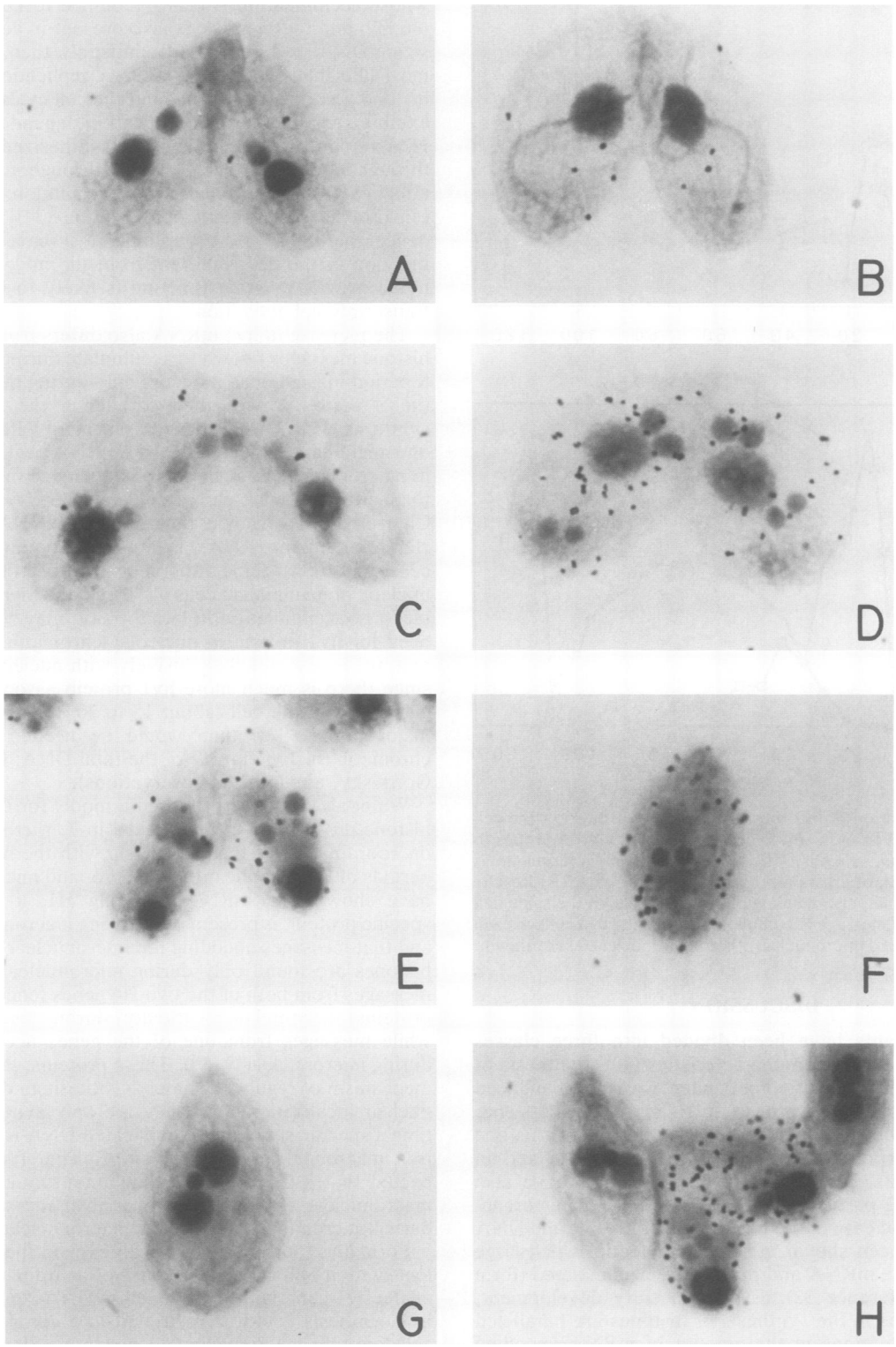


FIG. 3. In situ hybridization of hv1 RNA probes to conjugating cells. See the legend to Fig. 4 for description of stages. (A) Nuclei separate; (B) crescent; (C) exchange; (D) macronuclear development I; (E) macronuclear development II; (F) cells separate; (G) one micronucleus is degraded; (H) cells with micronucleus degraded and macronuclear development II in same field. Autoradiographic exposures were for 11 days. Photographs were taken as described in the legend to Fig. 1.

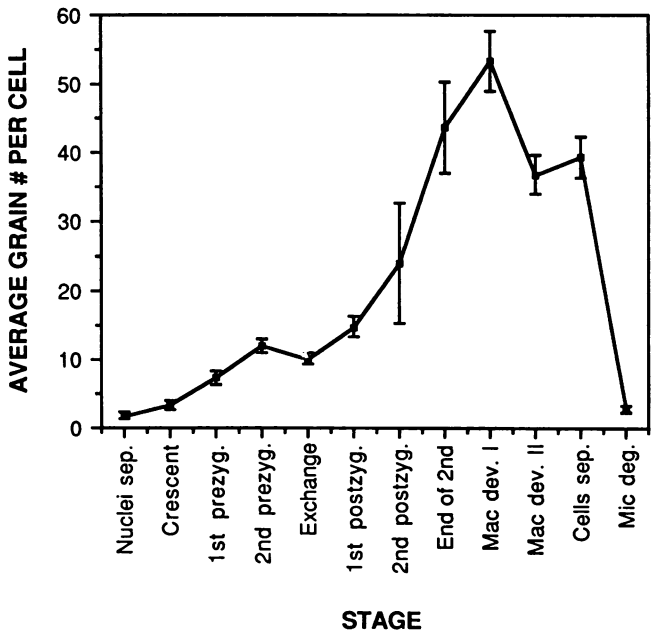


FIG. 4. hv1 message amounts in conjugation. Abbreviations of stages: Nuclei sep., micronuclei separated from macronuclei before the first prezygotic division; Crescent, micronuclear chromosomes aligned in meoitic prophase; 1st prezyg., first prezygotic micronuclear division; 2nd prezyg., second prezygotic micronuclear division; Exchange, exchange of migratory pronuclei; 1st postzyg., first postzygotic division after nuclear fusion; 2nd postzyg., second postzygotic division; End of 2nd, after the second postzygotic division, when four new nuclei are visible, but none have begun to swell; Mac dev. I, two anterior nuclei have begun to swell and develop into macronuclei; Mac dev. II, later stage of macronuclear anlagen development with large new macronuclei and small, dense old macronuclei at posterior of cell; Cells sep., separation of paired cells; Mic deg., one micronucleus in each cell degraded. Standard errors are represented by error bars.

message is selectively translated only during macronuclear S. It seems unlikely that hv1 protein synthesis is restricted to macronuclear S phase by a translational control mechanism, since little or no translational control exists for the synthesis of tubulin or histones H3 and H4 in *Tetrahymena* cells (F. J. Calzone, Ph.D. thesis, University of Rochester, Rochester, N.Y., 1982; 6). Also, to our knowledge, no example of translational regulation of histone synthesis in growing cells has been demonstrated. Another possibility is that hv1 is synthesized throughout the cell cycle, but that another molecule is required to transport hv1 specifically to the macronucleus. Such a model cannot be ruled out but seems less likely than the model we favor, which is that the hv1 protein itself has a sequence which targets it to the macronucleus. A nuclear localization signal has recently been discovered in the yeast histone H2B (22). If hv1 had such a signal, it would be particularly interesting because the signal must target the protein not just to the nucleus, but specifically to the macronucleus.

Analysis of the regulation of hv1 mRNA amounts during conjugation indicated that hv1 synthesis is largely correlated with development of the new macronucleus. Message amounts were low until the second postzygotic division, when they began to increase markedly. The hv1 mRNA level peaked during macronuclear development and remained high upon cell separation until it rapidly dropped when one of the two micronuclei was degraded. This period of high hv1 message amounts correlates with the time that macronuclear anlagen are undergoing rapid polyploidization (9). Autoradiographic studies by Allis et al. (1) have shown a 2C-to-4C endoreplication immediately following the second postzygotic division, and a 4C-to-8C endoreplication approximately 12 h after exconjugation. In our study, this second endoreplication falls at a time between cell separation and micronuclear degradation. The early accumulation of hv1 message (during the second postzygotic division) is probably a result of transcription from the old macronucleus, since new macronuclei do not begin to synthesize RNA until soon after the second postzygotic division (M. A. Gorovsky, Ph.D. thesis, University of Chicago, Chicago, Ill., 1968; 33), suggesting that hv1 mRNA synthesis is one of the late functions of the old macronucleus.

In other organisms, certain cytoplasmic mRNAs are localized in specific patterns within the cell. Mlodzik et al. (21) and Frigerio et al. (11) have demonstrated the existence of transcripts which form a concentration gradient in early development in *Drosophila melanogaster*. Rebagliati et al. (28) showed that a number of maternal mRNAs were localized either in the animal or in the vegetal hemisphere of unfertilized *Xenopus* eggs. Lawrence and Singer (18) have shown a nonrandom distribution of actin, vimentin, and tubulin mRNAs in chicken somatic cells and have suggested that localized concentrations of proteins within the cells may result from the localization of mRNAs for these proteins. We used in situ hybridization of hv1 probe to conjugating *Tetrahymena* cells to test for localization of hv1 mRNA. Our hypothesis was that, as a macronuclear-specific protein, hv1 may play a part in committing the anterior nuclei to macronuclear differentiation. Conjugating cells were analyzed for any marked subcellular localization of hv1 message; none was detected. It is unlikely, therefore, that hv1 message localization plays a role in the cytoplasmic determination known to occur during macronuclear development. The cytoplasmic determinants known to affect nuclear differentiation in conjugating *Tetrahymena* cells (24) remain to be determined.

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