Timing of the Appearance of Macronuclear-specific Histone Variant hv1 and Gene Expression in Developing New Macronuclei of *Tetrahymena thermophila*

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ABSTRACT Vegetative cells of the ciliated protozoan *Tetrahymena thermophila* contain a transcriptionally active macronucleus and a transcriptionally inactive micronucleus. Earlier studies (Allis, C. D., C. V. C. Glover, J. K. Bowen, and M. A. Gorovsky, 1980, *Cell,* 20:609–617; and Allis, C. D., Y. S. Ziegler, M. A. Gorovsky, and J. B. Olmsted, 1982, *Cell,* 31:131–136) demonstrated the existence of a macronuclear-specific histone variant, hv1, which is enriched in small punctate regions in nucleoli of several mammalian cell lines. These observations suggest that this histone variant is highly conserved in evolution and may be associated with actively transcribed sequences.

Despite large differences in structure and function during vegetative growth, macro- and micronuclei are related. During conjugation, the sexual phase of the life cycle in *Tetrahymena,* postzygotic division products of micronuclei give rise to new micro- and macronuclei, while the old macronucleus moves to the posterior of each cell and is eliminated. In this study using antiserum specific for hv1, we determined by indirect immunofluorescence the time during conjugation at which hv1 first appears in the developing new macronuclei.

In growing, starved, and young mating cells (2–5 h after mixing opposite mating types), only macronuclei are detected with affinity-purified antibodies against hv1. Newly formed macronuclei are either not stained or only weakly stained in cells in which the old macronucleus is located in the center of the cell. However, new macronuclei are clearly observed in cells in which the old macronucleus has moved to the posterior of the cell (~8 h). During later stages of conjugation (10–16 h), the intensity of hv1 staining in new macronuclei increases with time corresponding to the increasing DNA content of these nuclei. Disappearance of detectable hv1 from old macronuclei begins nearly 1 h after these nuclei reach the posterior cytoplasm (~9–10 h) and is sometimes complete before these nuclei are eliminated from the cells.

Autoradiography of cells labeled for brief periods with [^{3}H]uridine shows that new macronuclei begin to synthesize RNA very soon after the second postzygotic division (~8 h). During stages when hv1 is clearly detected in new macronuclei, anlagen are active in RNA synthesis. RNA synthesis in old macronuclei ceases very close to the time when RNA synthesis begins in new macronuclei. Thus, the addition of hv1 coincides closely with the transformation of a transcriptionally inactive germinal nucleus into that of a transcriptionally active somatic nucleus. We suspect that addition of hv1 plays a fundamental role in macronuclear differentiation and the ability of these cells to carry out transcriptional activity.

Chromatin of most eucaryotic cells contains five major types of histone: H1, H2A, H2B, H3, and H4. However, it is clear that the histone complement of many organisms is more heterogeneous than once believed. Primary sequence variants (or subtypes) have been described within all of the histone classes except H4 (for review see reference 1). Despite ample

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documentation for the existence of histone subtypes, the role that these proteins play in modulating chromatin structure/function (if any) is not clear. The occurrence of precisely staged switches in the synthesis and deposition of specific histone variants during development (4, 11, 14) and the demonstration that these changes affect chromatin structure (17) suggest that histone variants may play an important role in the differentiation of chromatin structure and function.

In vegetative cells of the ciliated protozoan *Tetrahymena thermophila*, macro- and micronuclei represent fully differentiated nuclei which carry out different functions for the cell (7). The transcriptionally active macronucleus supports the cell during vegetative growth and yet is eliminated from the cell every sexual generation. Genetic continuity is provided by the transcriptionally inactive micronucleus. In support of the idea that histone variants are involved in the differentiation of chromatin structure, we have demonstrated that macronuclei from *Tetrahymena* contain two relatively minor histone variants, hv1 and hv2, which are missing from micronuclei (1). Preliminary characterization with one of these variants, hv1, indicates that it is not a simple sequence variant of any of the major macronuclear histones. However, several lines of evidence (detergent binding properties, patterns of secondary modification, and amino acid composition) suggest that hv1 is more like H2A than any other histone. Polyclonal antiserum raised against hv1 reacts strongly with itself following immunoblotting, but fails to cross-react with any other macronuclear histone, including H2A (3).

Since all transcriptional activity resides within the macronucleus in vegetatively growing *Tetrahymena*, it is reasonable to suggest that hv1 may play a role in gene activity. Immunofluorescent studies using antiserum specific for hv1 have recently demonstrated that determinants recognized by these antibodies are enriched in nucleolar chromatin in several different mammalian cell lines (3). Thus hv1 seems to represent a highly conserved histone variant associated with active chromatin.

Despite significant differences in structure and function, macro- and micronuclei are related. During conjugation, the sexual phase of the life cycle in *Tetrahymena*, micronuclei undergo meiosis, exchange, and fertilization to produce diploid zygotic nuclei. Postzygotic division products of these nuclei then give rise to new macro- and micronuclei. Since new macronuclei (anlagen) originate from micronuclei during conjugation, this is probably the time when macronuclei acquire the properties that distinguish them from micronuclei (4). At some point during the differentiation of new macronuclei, genes must begin to be expressed, allowing these nuclei to now support the needs of the cell. Since several lines of evidence suggest that hv1 is associated with transcriptionally active chromatin (see above), we sought to determine precisely when during conjugation hv1 first appears in developing new macronuclei and to correlate the time of its appearance with the onset of gene expression in these nuclei.

The results presented here demonstrate that hv1 is added to new macronuclei very soon after these nuclei are morphologically distinct from micronuclei (8–9 h) and continues to be added throughout subsequent stages of macronuclear anlagen development. During these stages, new anlagen are first found to synthesize RNA. Thus, the addition of this macronuclear-specific histone variant to a previously inactive germinal nucleus coincides closely with its transformation into that of a transcriptionally active somatic nucleus.

MATERIALS AND METHODS

Cell Culturing and Labeling Conditions

STRAINS: The strains of *Tetrahymena thermophila* used for conjugation were CU 399 (Chx/Chx [cy-S] VI) and CU 401 (Mpr/Mpr [6-mp-S] VII). These genetically marked strains were generously provided by P. Bruns (Cornell University).

CULTURE CONDITIONS: Cells were grown axenically in 1% enriched proteose-peptone as previously described (8). When cultures reached approximately 400,000 cells/ml, cells were pelleted from growth media at 250 *g* for 2 min (at room temperature), washed once with 10 mM Tris, pH 7.4, and resuspended in 10 mM Tris at half the original cell density. Cells were typically grown and starved in a flask 10 times larger than the volume of the culture (without shaking). To induce conjugation, equal numbers of starved cells (typically starved for 24 h) of opposite mating types (i.e., CU 399 and CU 401) were mixed again in a flask 10 times larger than the volume of mating cells. Pair formation usually began ½ h after mixing with kinetics similar to those previously published (5, 12). Usually 85–90% of the cells ultimately paired in these cultures. Starved cells (24 h in 10 mM Tris) were used in some experiments. All cell cultures (growing, starved, or mating) were maintained at 30°C.

Immunofluorescence Microscopy

FIXATION OF CELLS: All cells were stained by the indirect method. In preliminary experiments with conjugating cells, cells were fixed for immunomicroscopy as previously described (9). However, these procedures led to considerable cell lysis especially with mating cells (see text). After numerous experiments with various other fixation protocols, the following procedure was developed which was utilized in all of the experiments reported here. 10 μl of fixative (two parts saturated mercuric chloride to one part 95% ethanol) was added directly to 3 ml of cells (starved or mating cells in 10 mM Tris), hand mixed, and incubated for 5 min at room temperature. Cells were then gently pelleted (250 *g* for 2 min), resuspended in absolute methanol (3–6 ml), repelleted, and resuspended again in methanol (1 ml). Cells resuspended in methanol were dropped directly onto coverslips, air dried for 30 min, and stored overnight at 4°C in PBS (9).

INDIRECT IMMUNOSTAINING AND ADDITIONAL STAINING WITH ETHIDIUM BROMIDE: Cells were then incubated with immune or preimmune serum (appropriately diluted in PBS according to the titer of the immune serum), followed by incubation with fluorescein-conjugated goat anti-rabbit serum (Miles Laboratories Inc., Elkhart, IN). All intervening washes were in PBS. An extension of this protocol was devised to allow detection of micronuclei in cells stained with hv1 antibodies (micronuclei do not contain hv1). Here, cells reacted with first and second antibodies as described above were stained additionally with a dilute solution of ethidium bromide (1 μg/ml ethidium bromide in PBS). This concentration of ethidium bromide allows detection of micronuclei under ultraviolet light (micronuclei are reddish-orange) without interfering with the yellow-green fluorescence imparted to macronuclei (or new macronuclei) by the prior reaction with hv1 antibodies. In fact, we observed intermediate shades of these two colors in new macronuclei as they differentiated from micronuclei. Thus, the transition from red to yellow-green color allowed us to accurately follow the addition of hv1 in new macronuclei during early stages of anlagen differentiation (see text for details).

AFFINITY PURIFICATION OF hv1 ANTIBODIES: In some cases, hv1 antibodies were affinity purified from paper blots essentially according to the procedures of Olmsted (15). For this work, hv1 protein was blotted to nitrocellulose paper (Millipore Corp., Bedford MA; 0.45 μm, type HA). After incubation with unfractionated anti-hv1 serum, blots were washed extensively before bound antibodies were released from the paper by treatment with 5 M sodium iodide. After dialysis against PBS, hv1 antibodies were concentrated, titered, and used on cells prepared as described above. Characterization of the anti-hv1 serum used in these studies has been described previously (3).

Isotopic Labeling and Autoradiography

Mating cells were labeled for 5 min with [^{3}H]uridine (10 μCi/ml; 50 Ci/mmol) before being fixed and spread onto slides according to Karrer (10). Slides were processed for autoradiography as previously described (18) except that slides were incubated twice in 0.02% iodine in 70% ethanol (M.-C. Yao, personal communication) before being dipped in Kodak NTB-2 emulsion. After appropriate exposure and development, cells were stained through the emulsion with Giemsa (10).

RESULTS

Fixation of Tetrahymena for Immunofluorescence

To determine the timing of hv1 addition to developing new macronuclei of *Tetrahymena*, we chose to apply the technique of indirect immunofluorescence (using antibodies specific for hv1; reference 3) to conjugating cells. This approach has the following important advantages over studies performed using standard biochemical analyses (4). First, cells can be staged precisely using various morphological criteria (size, number, and position of nuclei). Second, immunofluorescence microscopy allows questions to be asked of specific nuclei within defined cytoplasmic locations of the cell. During conjugation in *Tetrahymena* (as well as in other developmental systems), specific patterns of nuclear differentiation occur in accordance with the position of nuclei in the cytoplasm of the mating cell (13, 16). For example, following the second postzygotic division, anterior micronuclei differentiate into new macronuclei while posterior micronuclei remain micronuclei. Thus, it is important to study biochemical events that occur not only in specific classes of isolated nuclei (2, 3), but also in nuclei within defined positions of the cell.

Indirect immunofluorescence has already been utilized in several studies with starved *Tetrahymena* (3, 9). However, the procedures used to "fix" the cells in these studies do not (at least in our hands) adequately maintain the integrity of either starved or conjugating cells (D. Wenkert and C. D. Allis, unpublished observations). Often cell lysis occurs causing macro- and micronuclei to be released from the cells. This situation is obviously undesirable since all information concerning the position of specific nuclei is then lost. For this reason, we sought to develop a fixation protocol that would maintain cellular integrity and still fulfill all of the requirements necessary for immunofluorescence. To carry out these initial studies, polyclonal antiserum raised against total acid-soluble proteins extracted from macronuclei was utilized. When this serum (termed "anti-whole histone") was characterized by immunoblotting, strong reactivity was observed over all major types of macronuclear histone (especially H2A and H2B, data not shown). Since many of these histones are common to both macro- and micronuclei (for example, H2A, H2B, H3, and H4), antibodies in this serum would be expected to react with all types of nuclei present in growing, starved, or mating cells.

Fig. 1 shows typical results obtained when conjugating cells (5, 9, and 15 h after opposite mating types are mixed) are fixed (according to the methods described in Materials and Methods) and stained with anti-whole histone antibodies by the indirect immunofluorescence method. When examined by phase-contrast optics (*a* and *c*), numerous intact pairs are observed that display reasonable morphology. During early stages of macronuclear anlagen development (9 h), new macronuclei are easily recognizable as large paired phase-dark nuclei (reference 2 and the short arrows labeled *AN* in Fig. 1 *c*). This property of new macronuclei is not as apparent in cells after exconjugation (Fig. 1 *e*).

When the identical cells are examined by fluorescence microscopy (Fig. 1, *b*, *d*, and *f*), intense nuclear staining is observed. (This staining is not observed with preimmune serum or second antibody alone, data not shown.) All types of nuclei from all stages of development (macronuclei, micronuclei, anlagen, and old macronuclei) are stained with this antiserum. We conclude from these results that the procedures we have developed (see Materials and Methods) adequately maintain cellular and nuclear integrity and morphology and still leave all nuclei freely permeable to antibodies.

Timing of hv1 Addition to Developing New Macronuclei in Conjugating Tetrahymena

Recently, indirect immunofluorescence using antibodies specific for hv1 was used to substantiate the macronuclear-specific nature of this variant in starved and growing cells (3). We have now applied indirect immunofluorescence utilizing our new fixation protocol to examine the distribution of hv1 in conjugating cells. The results presented in Fig. 2 demonstrate that as expected only macronuclei are detected with anti-hv1 serum in either starved or 5-h, mating cells. Furthermore, this staining reaction is not observed with the preimmune serum from this rabbit (Fig. 2, *e* and *f*). Unlike the staining reaction with anti-whole histone serum (Fig. 1), micronuclei are not detected with anti-hv1 serum.

To determine the timing with which hv1 is added to the chromatin of developing new macronuclei, we fixed mating cells at various time points during conjugation and stained them by indirect immunofluorescence using affinity-purified hv1 antibodies (antibodies were affinity purified by the procedures outlined by Olmsted [15]). By 8 h of conjugation (Fig. 3), there are numerous examples of paired cells which show no obvious signs of having begun macronuclear anlagen differentiation under phase-contrast optics (see the unlabeled paired cells in Fig. 3 *c*). However, pairs are also observed that do contain examples of slightly swollen anteriorally located macronuclear anlagen (see the labeled cells in Fig. 3, *a* and *c*). In either case, the single large macronuclei are easily detected by fluorescence microscopy in each member of these pairs (Fig. 3, *b* and *d*), but the young anlagen (labeled *AN*) contain little if any determinants recognizable by hv1 antibodies. The inserts shown in Fig. 3 (*c* and *d*) illustrate a pair with weakly staining anterior anlagen in cells in which the old macronucleus is large and centrally located. While several examples of this type of cell were observed (2/24), our results suggest that the amount of hv1 present in new macronuclei at this stage of development is extremely low.

In slightly older pairs, again at 8 h, new macronuclei are somewhat larger and are consistently phase dark. In these cells the new anlagen occupy a more central position within the cell and the former macronucleus is now situated in the posterior tip of each cell (see examples in Fig. 3, *e* and *g*). In these cells, developing new macronuclei are clearly stained by hv1 antibodies (right-hand pair in Fig. 3*f*). As the old macronuclei decrease in size during their elimination from the cells, staining of new macronuclei increases (left-hand pair in Fig. 3, *e* and *f* and three pairs in Fig. 3, *g* and *h* which increase in age from left to right). Interestingly, it is often the case that one old macronucleus is easily detectable by anti-hv1 staining while the other (in the opposite member of the pair) appears to no longer contain determinants recognizable by these antibodies. We suspect that the elimination of hv1 molecules from each of the old macronuclei of a pair does not necessarily occur simultaneously.

Since these studies utilize antibodies raised against a histone variant specific to macronuclei, we did not detect micronuclei by fluorescence microscopy. While this is the expected result from studies with hv1 antibodies, our inability to detect micronuclei (by phase-contrast or fluorescence microscopy)

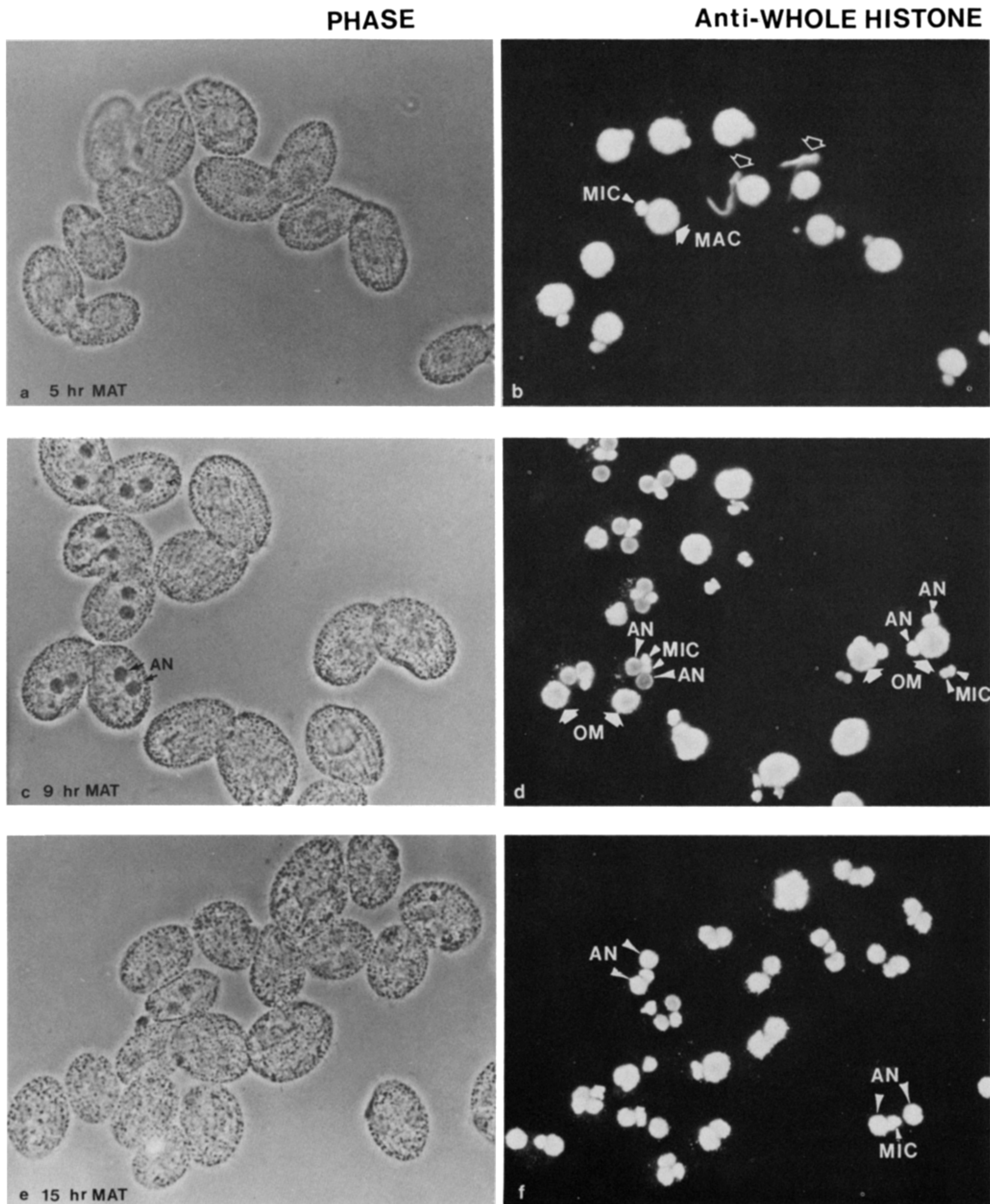

FIGURE 1 At various time points following the initiation of conjugation, 5 h (*a* and *b*), 9 h (*c* and *d*), and 15 h (*e* and *f*), mating cells were fixed, reacted with first (1:1,000 dilution of "anti-whole histone" antiserum in PBS) and second antiserum, and examined by phase-contrast (*left*) and fluorescence (*right*) microscopy. Depending upon the stage of conjugation, micronuclei (*MIC*), macronuclei (*MAC*), developing new macronuclei (*AN*, anlagen), or old macronuclei (*OM*) are easily detected with anti-whole histone antibodies. The open arrows in *b* point to micronuclei in the crescent stage of meiotic prophase. × 400.

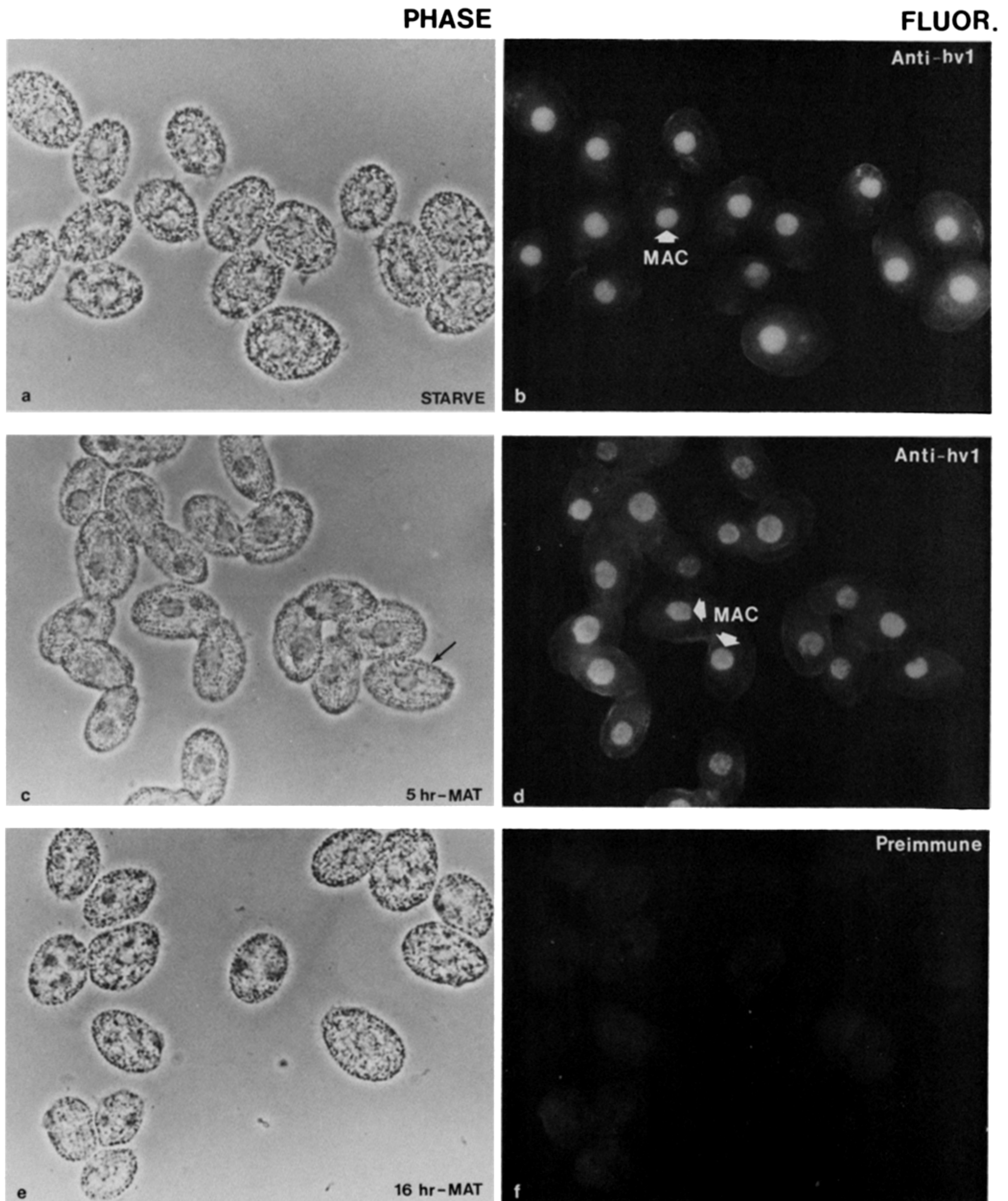

FIGURE 2 Starved (*a* and *b*) or 5-h mating cells (*c* and *d*) were fixed, incubated with first (1:7 dilution of unfractionated anti-hv1 serum in PBS) and second antiserum, and examined by phase-contrast (*left*) and fluorescence (*right*) microscopy. In these cells, as expected, only macronuclei (*MAC*) are detected with hv1 antibodies. The arrow in *c* points to an unpaired nonmating cell within the population. As a control, 16-h mating cells (*e* and *f*) were treated similarly, but were incubated with preimmune serum. Here, only cytoplasmic background fluorescence is observed. Similar results with preimmune serum were obtained with cells at all other time points analyzed. × 400.

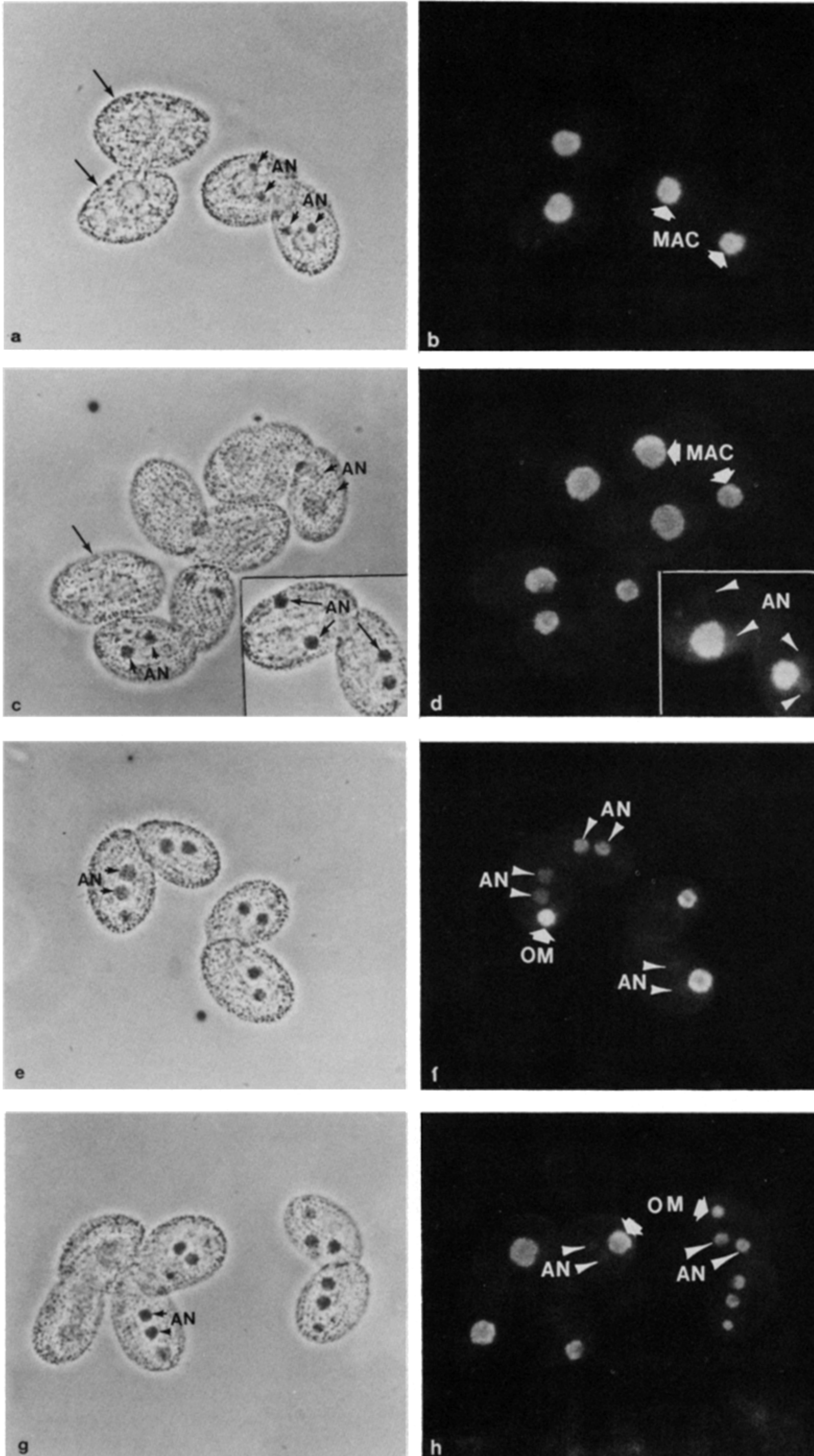

FIGURE 3 Mating cells at 8 h of conjugation were fixed, incubated with first (affinity-purified hv1 antibodies, see Materials and Methods) and second antibodies, and examined by phase-contrast (*left*) and fluorescence (*right*) microscopy. Long arrows in *a* and *c* point to nonmating cells. *MAC*, macronuclei; *AN*, anlagen; *OM*, old macronuclei. Note that changes in the position and size of the old macronucleus can be closely correlated with the onset and increase of hv1 staining in new macronuclei (see text for details). × 400.

makes precise staging of mating cells difficult before stages when anlagen are apparent. For this reason, we sought to stain nuclei (after reaction with first and second antibodies) with a dilute solution of ethidium bromide (a fluorescent DNA-binding dye). In preliminary experiments a concentration of ethidium bromide was determined that would stain micronuclei reddish-orange (see small arrowheads, Fig. 4) without interfering with the yellow-green fluorescence imparted to macronuclei (large arrow, Fig. 4) by the earlier reaction with hv1 antibodies.

Fig. 4 shows several examples of mating cells taken from 8 h of conjugation. These cells were treated identically to those shown in Fig. 3 except for the additional stain with ethidium bromide. In these examples, micronuclei are easily detected allowing us to precisely stage the cells (for example, Fig. 4*A* shows a cell that has completed the second postzygotic division). In cells that contain large centrally located yellow-green macronuclei (indicating the presence of hv1 in these nuclei), micronuclei and young anlagen have a reddish hue suggesting that hv1 is not present. In Fig. 4*B*, however, two pairs are

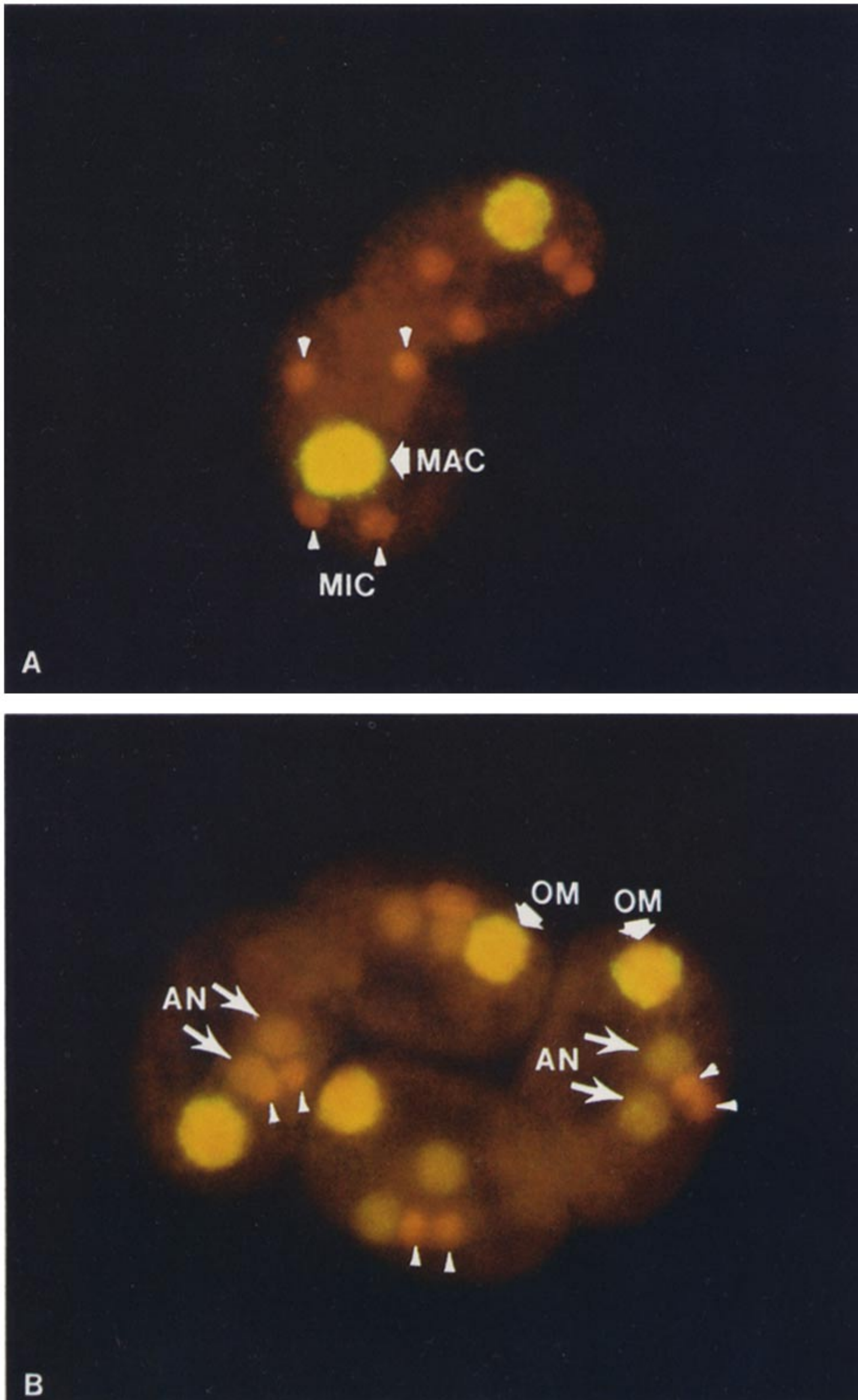

FIGURE 4 Mating cells (all at 8 h) were incubated with first (anti-hv1 serum, affinity purified) and second antibodies as in Fig. 3. In this case, however, cells were subsequently stained with a dilute solution of ethidium bromide (see Materials and Methods) to permit detection of micronuclei (red staining) by fluorescence microscopy. Therefore, yellow-green nuclei represent nuclei that are stained by antibodies against hv1; red nuclei are those that do not react with hv1 antibodies. Shown in *A* is a mating cell that has just completed the second postzygotic division. This cell contains a centrally located yellow-green macronucleus and reddish micronuclei. In *B* two pairs are shown, each of which contains a large old macronucleus (*OM*) located in the posterior of each cell. Note the new macronuclei (arrows labeled *AN*) contain a shade of yellow-green (especially the right-hand pair), suggesting that detectable amounts of hv1 have been added to the new macronuclei by this time (see text for details). × 900.

shown where the old macronuclei (labeled *OM*) are still large, but have moved to the posterior of each cell. In these cells, new macronuclei are predominantly yellow-green (especially in the right-hand pair), suggesting that significant amounts of hv1 have been added to the new macronuclei by this time.

Finally, we have monitored the intensity of hv1 staining in developing new anlagen during subsequent time points of conjugation. Shown in Fig. 5 are the staining reactions of 10- (*a* and *b*), 12- (*c* and *d*), 14- (*e* and *f*), and 16-h (*g* and *h*) mating cells with affinity-purified hv1 antibodies. While new macronuclei are easily detected by fluorescence microscopy in 10-h mating cells (Fig. 5 *b*), the staining intensity increases significantly between 10 and 12 h of conjugation (compare Fig. 5, *b* and *d*). By the time a significant fraction of the cells has undergone exconjugation (see the unpaired cells with two anlagen at 14 h, Fig. 5, *e* and *f*), old macronuclei are difficult to detect by either phase-contrast or fluorescence microscopy. (These nuclei are also not stained with ethidium bromide.) By 14 and 16 h of conjugation (a time when new macronuclei have a ploidy of approximately 8C, reference 2), examples of anlagen can be found (see labeled anlagen in Fig. 5, *f* and *h*) which are stained to an extent comparable to macronuclei in nonmating cells (nonmating cells in Fig. 5, *a* and *b*). Therefore, we conclude that addition of hv1 to the chromatin of developing macronuclei continues while the DNA of these nuclei is endoreplicated to at least 8C. (More advanced stages of macronuclear differentiation require that exconjugants are returned to food [2], which was not done in these studies.)

Onset of RNA Synthesis in Developing New Macronuclei

To determine the timing with which RNA synthesis begins in new macronuclei, we chose an experimental approach that would enable us to make comparisons to the results obtained with hv1. Like immunofluorescence microscopy, autoradiography allows accurate staging of the cells and analyses to be made of specific nuclei within the cell. Therefore, mating cells were pulse labeled with [^{3}H]uridine during various stages of macronuclear anlagen development (Fig. 6, all cells at 8 h of conjugation) and processed for autoradiography.

As expected, large centrally located macronuclei actively carry out RNA synthesis in all stages that precede the development of new macronuclei (see the nonmating cells in Fig. 6*A*, the mating cells in *B* and *C*, and the left-hand pair in *D*). However, when macronuclei move to the posterior of mating cells and become densely stained with Giemsa (e.g., the pair in Fig. 6 *F*), we typically observe little if any RNA synthesis in these nuclei. In cells that have just completed the second postzygotic division (pairs in Fig. 6, *B* and *C* and left-hand pairs in *D* and *E*), examples of slightly swollen anterior new macronuclei (arrows labeled *AN*) can be found that are not yet active in RNA synthesis (Fig. 6 *B*, and left-hand pair in *D* and *E*). However, several examples are found (3/35) where young anterior anlagen have begun RNA synthesis (Fig. 6 *C*). In these cells the old macronuclei are still located within the center of each cell and are still active in RNA synthesis. It is interesting to note that RNA synthesis can occur in both old and new macronuclei at the same time.

At 8 h of conjugation, there are numerous examples of pairs containing old macronuclei in the posterior cytoplasm (right-hand pairs in Fig. 6, *D* and *E* and the pair in *F*). In all of these cells, new macronuclei are actively engaged in RNA synthesis. Since new macronuclei are also stained with hv1 antibodies at this time (when old macronuclei are in the posterior of each cell, Figs. 3 and 4), we suspect anlagen that are active in RNA synthesis contain some hv1 as part of their histone complement (see Discussion).

DISCUSSION

In this report, antiserum specific for the macronuclear histone variant hv1 has been used to determine when hv1 appears during the differentiation of new macronuclei in conjugating cells. Our results demonstrate that hv1 is added specifically to developing new macronuclei very soon after these nuclei become morphologically distinct from micronuclei (8 h of conjugation, Figs. 3 and 4). Although hv1 can be faintly detected in anlagen of some cells in which the old macronucleus is still centrally located, it is clearly detected in all cells in which the former macronucleus is in the posterior cytoplasm. Staining intensity of new macronuclei then increases as old macronuclei are being eliminated from the cells and continues up to at least 14–16 h of conjugation (the latest time points analyzed in this study).

The results presented here confirm previously published biochemical (1) and immunological (3) data indicating that this histone variant is specific to macronuclei (or developing new macronuclei) and is not a component of micronuclear chromatin. Our data also agree well with other recent results from this laboratory concerning the timing of hv1 synthesis and deposition into new macronuclei of mating cells (4). In that study, small amounts of hv1 synthesis and deposition were detected by two-dimensional electrophoresis of acid extracts from macronuclear anlagen isolated from mating cells at 9 or 10 h of conjugation (these nuclei have a DNA ploidy of ~4C, reference 2). Significant amounts of hv1 were being synthesized and deposited into anlagen isolated from 14–16-h conjugating cells (~8C). Thus, we conclude from these biochemical and immunological data that hv1 is added throughout early stages of macronuclear anlagen differentiation, as the DNA content of these nuclei increases from 2 to 8C (8–16 h of conjugation in Tris).

While the biological function of hv1 in *Tetrahymena*, and of its analogs in other organisms, is not known, several lines of evidence suggest that this variant may be associated with active genes. If the presence of hv1 in macronuclei is associated with gene expression, the timing of RNA synthesis in new macronuclei should correlate closely with the timing of hv1 addition to its histone complement. In seeing whether this is the case, we have used autoradiography of mating cells that were briefly labeled with [^{3}H]uridine (Fig. 6). The results presented here (Fig. 6) are in agreement with those described by Gorovsky (6).

Like the addition of hv1 to the chromatin of new macronuclei, RNA synthesis begins soon after these nuclei become morphologically distinct from micronuclei (~8 h of conjugation). During stages when hv1 is positively detected in new macronuclei by immunofluorescence microscopy (old macronuclei in posterior of the cell), anlagen are actively engaged in RNA synthesis. We found several examples of young anterior anlagen synthesizing RNA in cells where the old macronuclei were still in the center of the cell (Fig. 6 *C*). There were also cases of faint hv1 staining in the anlagen of cells with centrally placed old macronuclei (insets to Fig. 3, *c* and *d*). Whether or not one of these two events of macronuclear

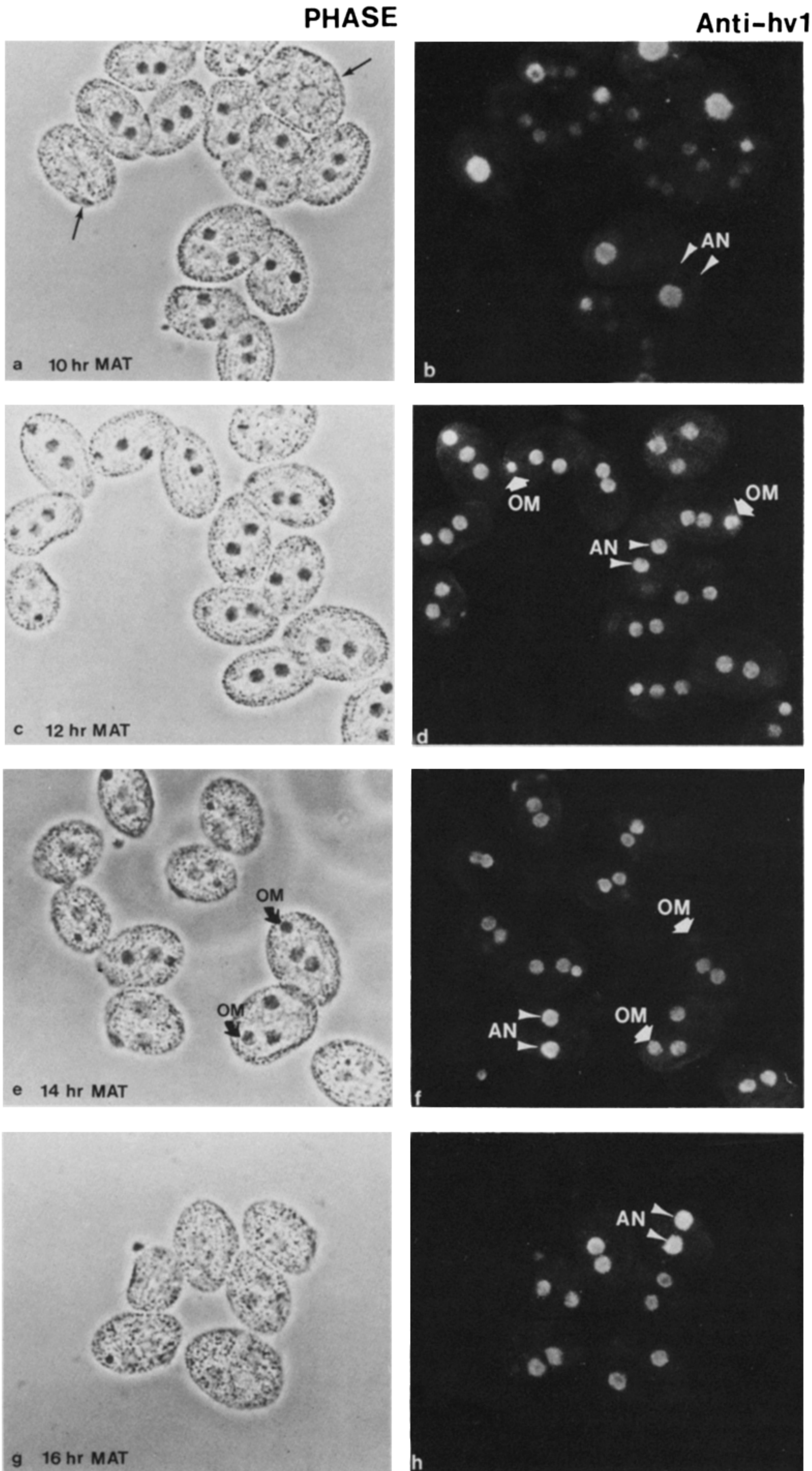

FIGURE 5 Mating cells at 10 h (*a* and *b*), 12 h (*c* and *d*), 14 h (*e* and *f*), and 16 h (*g* and *h*) were fixed, incubated with first (affinity-purified hv1 antibodies) and second antibodies, and examined by phase-contrast (*left*) and fluorescence (*right*) microscopy. Long arrows in *a* point to nonmating cells. *AN*, anlagen; *OM*, old macronuclei. Exconjugants (unpaired cells with two new macronuclei) are evident in 12-, 14- and 16-h time points. × 400.

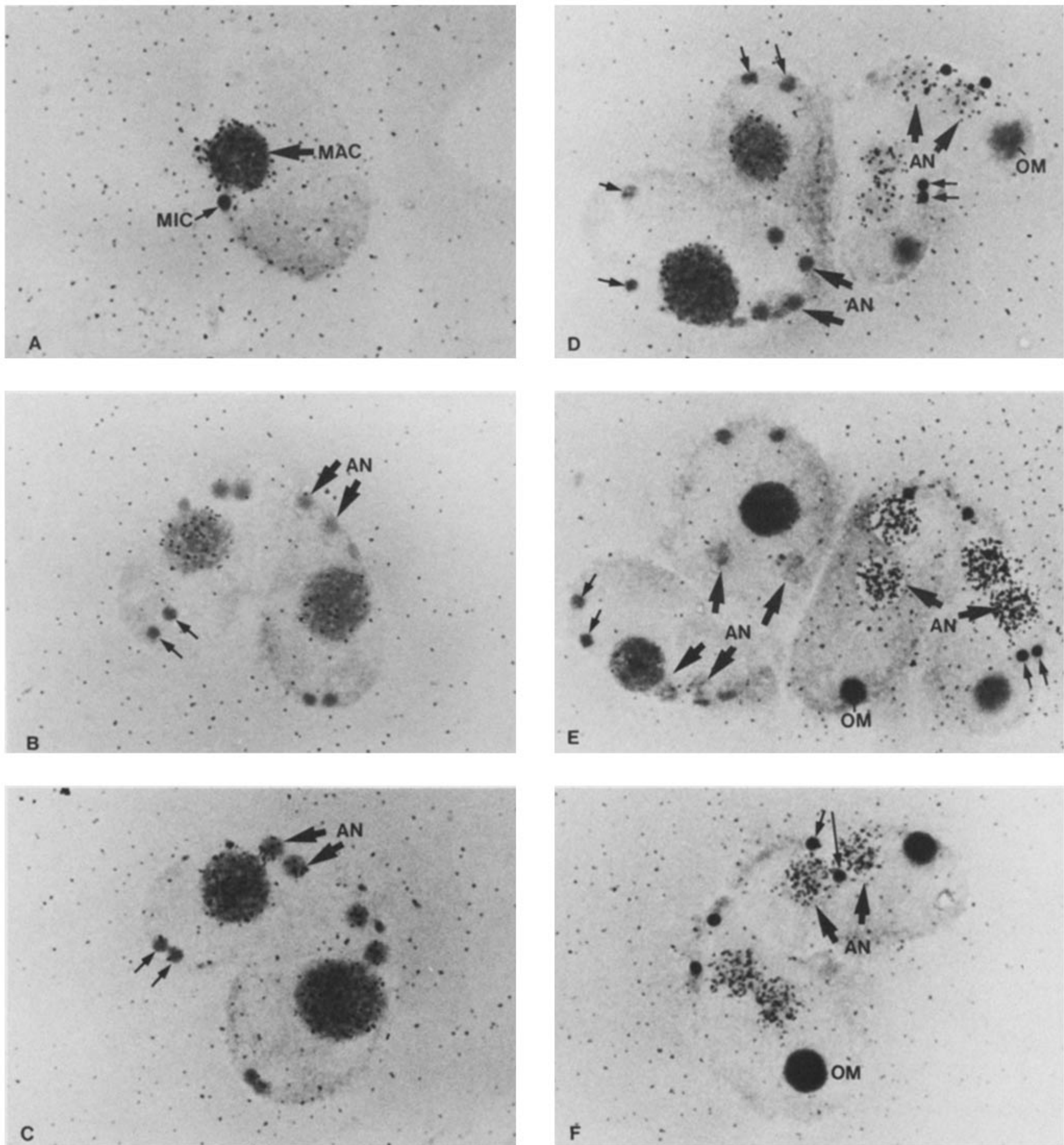

FIGURE 6 Mating cells (all at 8 h) were labeled for 5 min with [^{3}H]uridine and processed for autoradiography. A nonmating cell is shown in *A* with its macro- and micronucleus. Mating cells that have completed the second postzygotic division and have entered initial stages of macronuclear anlagen development (some examples of anlagen are indicated by arrows labeled *AN*) are shown in left-hand pairs in *B–E*. Older cells (indicated by the posterior position and condensed nature of the old macronucleus [OM]) are shown in *D* (right-hand pair), *E* (right-hand pair), and *F*. In all cells, unlabeled smaller arrows point to examples of micronuclei. Exposure time, 20 d. × 825.

differentiation (the initiation of RNA synthesis and the appearance of hv1) slightly precedes the other is difficult to determine since different methods were employed for their analysis (autoradiography and immunofluorescence microscopy). It is worth noting that the difference in time between when old macronuclei are in the center or posterior of the cell is small (15–30 min; reference 12 and C. D. Allis, unpublished observations). Therefore, we feel that the addition of hv1 closely approximates the onset of gene expression in new macronuclei.

While the exact role of hv1 in macronuclear chromatin and its relationship to transcriptional activity remain obscure, the data presented here suggest that addition of hv1 to the chromatin of new macronuclei represents a significant aspect of macronuclear differentiation in *Tetrahymena.* This finding provides strong support for the idea that histone variants play

an important role in the differentiation of chromatin structure and function.

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