Proteolytic Processing of H1-like Histones in Chromatin: A Physiologically and Developmentally Regulated Event in *Tetrahymena* Micronuclei

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ABSTRACT Micronuclei isolated from growing cells of *Tetrahymena thermophila* contain three H1-like polypeptides α, β, and γ. Micronuclei isolated from young conjugating cells (3–7 h) also contain a larger molecular weight polypeptide, X, which is being actively synthesized and deposited into these nuclei (Allis, C. D., and J. C. Wiggins, 1984, *Dev. Biol.*, 101:282–294). Pulse-chase experiments (with growing and conjugating cells) suggested that X is a precursor to α and that α is further processed to γ and a previously undescribed and relatively minor species, δ. These precursor-product relationships were supported by cross-reactivity with polyclonal antibodies raised against α and peptide mapping. While β consistently became labeled under chase conditions (both in growing and mating cells), it was not clear whether it is part of the in vivo processing event(s) which interrelates X, α, γ, and δ. β was not recognized by α antibodies. Despite this uncertainty, these results suggest that proteolytic processing serves to generate significant changes in the complement of H1-like histones present in this nucleus.

The chromatin of most eucaryotic cells contains five major histone classes: H1, H2A, H2B, H3, and H4. Of these, the inner histones (H2A, H2B, H3, and H4) play a fundamental and well-documented role in generating a highly conserved nucleosome core particle structure (14, 20, 23, 27) and presumably for this reason, the primary structure of these molecules has been rigidly maintained throughout evolution (20). The fifth variety of histone, H1 (or H1-like polypeptides), is known to associate with the linker region of chromatin (36) and therein is thought to play a yet undefined role in the higher-order packaging of the nucleosomal thin filament (29, 32). The primary structure of H1 histones is also much more variable (both between species and within different tissues of a given species) than that of the nucleosomal histones (19, 21). Extreme examples of this variability are seen in sperm (for a review see reference 34), fowl erythrocyte nuclei (30, 34), and the micronucleus of *Tetrahymena* (see below and reference 4), where entirely different H1 molecules are found associated with the chromatin at various stages of development. Interestingly, the chromatin in all three of the above cases is both highly condensed and transcriptionally inactive.

Vegetative cells of the ciliated protozoan, *Tetrahymena thermophila*, contain both a transcriptionally active, amitotically dividing macronucleus and a transcriptionally inactive, mitotically dividing micronucleus. Thus, these nuclei represent an ideal system to study how similar genetic information is maintained in different structural and functional states (15). As might be expected, the major forms of inner histones (H2A, H2B, H3, and H4) in macro- and micronuclei are similar if not identical (references 6, 9, and 16 and M. A. Gorovsky and J. K. Bowen, unpublished observations). Perhaps the most striking difference in histone composition between macro- and micronuclei of vegetative cells is seen with the linker-associated histones of the H1 class (4, 6). Macronuclei contain a typical H1 which has been shown to be missing from micronuclei by biochemical (17) and immunofluorescent (22) analyses. Micronuclei, on the other hand, contain three unique polypeptides, α, β, and γ, which do not have typical H1 solubility properties (C. D. Allis, R. L. Allen, and M. A. Gorovsky, unpublished observations),

but which are associated with linker regions of micronuclear chromatin (4). None of these polypeptides is detected in acid extracts from macronuclei (4). Like macronuclear H1, α, β, and γ are all phosphorylated (5) and lysine-rich (C. D. Allis and M. A. Gorovsky, unpublished observations).

Despite gross differences in structure and function, macro- and micronuclei are related. During the sexual phase of the life cycle, conjugation, micronuclei undergo meiosis, exchange, fertilization, and postzygotic division to give rise to new macro- and micronuclei. Recently, patterns of histone synthesis and deposition were examined in various classes of nuclei isolated from starved and conjugating cells (6). In this study, unusual behavior was observed among the micronuclear-specific H1-like molecules, α, β, and γ, which can be summarized as follows: (*a*) During starvation and early periods of conjugation (before the differentiation of new macro- and micronuclei), the amount of preexisting α, β, and γ in micronuclear acid extracts decreases (by staining) such that with time all become barely detectable. (*b*) During the same period a new complement of α (not β or γ) accumulates in micronuclear chromatin through active synthesis and deposition. (*c*) During this same time interval, a previously undescribed large molecular weight polypeptide(s) (labeled "X"; see reference 6, Fig. 2, *g* and *h*) also accumulates in micronuclear chromatin (again through active synthesis and deposition). (*d*) Acid extracts prepared from micronuclei soon after the beginning of new macronuclear development (10 h; reference 6, Fig. 3*a*) contain unusually high amounts of α (by staining); β and γ are still not detected at this stage. (*e*) During later stages of nuclear differentiation (14–16 h), β and γ reappear in micronuclear acid extracts (by staining) and the amount of α is reduced from that observed at 10 h (reference 6, Fig. 4*a*). Interestingly, β and γ are not being synthesized at this time.

From the above data, it was suggested that a precursor-product relationship could exist between the micronuclear H1-like molecules, α, β, and γ (6). Furthermore, it was suspected that the larger molecular weight polypeptide, X, could be part of this proteolytic processing series. In this report, we have directly examined the possibility that a precursor-product relationship exists between the H1-like molecules of *Tetrahymena* micronuclei. We present evidence that a physiologically and developmentally regulated proteolytic processing event(s) does interrelate most if not all of the micronuclear H1-like histones. While physiologically regulated, proteolytic processing of H3 histone was first demonstrated in *Tetrahymena* micronuclei (1), these results provide the first indication that a similar mechanism(s) may operate with H1-like histones as part of the pathway of micronuclear differentiation. Thus, proteolytic processing of nucleosomal and linker-associated histones exists as a potent mechanism to generate histone diversity in this system.

MATERIALS AND METHODS

Cells and Culture Conditions: Genetically marked strains of *Tetrahymena thermophila*, Cu399 (Chx/Chx[cy-s]VI) and Cu401 (Mpr/Mpr[6-mp-5]VII) were used in all experiments reported here. These were kindly provided by P. Bruns (Cornell University). Cells were grown axenically in 1% enriched proteose peptone as previously described (18). All matings were performed in 10 mM Tris (pH 7.4) according to Bruns and Brussard (12) as modified by Allis and Dennison (2). All cultures were maintained at 30°C.

Isotopic Labeling and Chase Conditions: Growing cells (300,000–400,000 cells/ml) were pelleted from food, resuspended in 10 mM Tris (pH 7.4), and labeled for 30 min with [^{3}H]lysine (2 μCi/ml, 50 Ci/mmol). An aliquot of these pulse-labeled cells was removed and micronuclei were isolated (pulse). The remainder of the pulse-labeled cells was removed, washed once with 10 mM Tris containing 10,000-fold excess of nonradioactive lysine, and resuspended in enriched proteose peptone which also contained excess cold lysine (chase). These manipulations effectively stop further incorporation of the isotope into trichloroacetic acid (TCA)[1]-insoluble material (1).

Mating cells (200,000 cells/ml) were labeled directly in Tris with [^{3}H]lysine (2 μCi/ml, 50 Ci/mmol) for the times indicated in the appropriate figure legends. In chase experiments, an aliquot of pulse-labeled cells was pelleted, washed once with Tris containing 10,000-fold excess cold lysine, and resuspended in Tris containing excess lysine. These manipulations stop further incorporation of the isotope into TCA-insoluble material and do not seem to affect the timing or synchrony of the stages of conjugation itself (7).

Nucleus Isolation and Histone Extraction: Micronucleus isolations from growing cells were performed as previously described (18). From mating cells, micronuclei were isolated according to Allis and Dennison (2) using only differential sedimentation to obtain a micronucleus-enriched preparation. Although there is some variation in the degree of macronuclear contamination in various micronuclear preparations (see the variation in the amount of macronuclear H1 in various preparations), interpretation of the results presented here is not affected since X, α, β, γ, δ, and H3^F are unique to micronuclear chromatin (references 4 and 6 and this report). Histones were extracted from micronuclei as previously described (4) taking all precautions to avoid artifactural protein losses. Where indicated (see figure legends), histone from unlabeled micronuclei was added as a carrier in the TCA precipitations.

Gel Electrophoresis: First- (Triton-acid-urea or acid-urea) and second-dimension (SDS) gels used in this report have been described in detail elsewhere (1, 3, 4). Gels were stained with Coomassie Blue, photographed, and where appropriate, fluorographed according to Bonner and Laskey (11) and Laskey and Mills (25).

In Vitro Iodination of Proteins with Bolton-Hunter Reagent: Acid extracts from micronuclei were precipitated with TCA, washed, and dried as described previously (4). These were dissolved directly in reaction buffer (0.125 M sodium borate buffer, pH 8.5, containing 1% SDS). Typically, acid extracts from 3–6 × 10^8 micronuclei were dissolved in 20 μl of reaction buffer. Meanwhile 200 μCi of Bolton-Hunter reagent ([^{125}I-3-[4-hydroxyphenyl]-propionic acid *N*-hydroxy-succinamide ester; reference 10) was removed and allowed to air-dry. The dissolved histone in reaction buffer was then added directly to the dried reagent and allowed to react on ice for 30 min. Afterward, stop buffer (0.125 sodium borate buffer, pH 8.5, containing 0.2 M glycine) was added (140 μl of stop buffer per mCi of Bolton-Hunter reagent). After 15 min on ice the mixture was made 20% TCA and the precipitate was collected and washed as previously described (4). Preliminary experiments (R. L. Allen and C. D. Allis, unpublished observations) demonstrated that iodinated histone (prepared as described above) electrophoresed with an identical mobility to that of unlabeled histone in our first- and second-dimension gels. Furthermore, iodinated histones produced identical peptide maps to that of in vivo [^{3}H] lysine-labeled material.

Cleavage with Proteases: Individual stained spots or bands ([^{125}I]-labeled, see above) were cut from appropriate one- or two-dimensional gels and cleaved with proteases according to a modification of the technique of Cleveland et al. (13). Gel pieces containing iodinated histones of interest were added to the wells of our standard SDS second-dimension gel (4) along with 20 μg (per well) of calf thymus histone (unlabeled and dissolved in Cleveland digestion buffer: 0.125 M Tris, pH 6.8/0.5% SDS/10% glycerol; reference 13) to act as a buffer against uncontrolled proteolysis (due to variation in amount of labeled histone substrate). Following this, an overlay of 5 μl of Cleveland digestion buffer containing 0.05 (low), 0.5 (medium), or 5 (high) μg per lane of *Staphylococcus aureus* V8 protease (or chymotrypsin at 10-fold lower concentrations) was added to appropriate lanes followed by an overlay of Laemmli electrophoresis buffer (24). Electrophoresis was carried out for 30 min at 150 V, stopped for 30 min and resumed for 1,800 additional V-h. Gels were stained and fluorographed as described above.

Immunization: Two-dimensional gel-purified α (~20 μg) was dissolved in H_2O, emulsified by sonication with an equal volume of complete Freund's adjuvant and injected subcutaneously into a rabbit. The immunization and bleeding schedule was that used previously to obtain antibodies against *Tetrahymena* tubulin (35) and *Tetrahymena* histone hv1 (8). Sera were aliquoted and stored at −80°C. In all experiments, preimmune serum for controls was obtained from the same animal immunized with α.

Immunoblotting: The specificities of preimmune and immune sera were assayed by immunoblotting analyses. Micronuclear acid-soluble proteins (from growing or 5-h mating cells) were fractionated on one- and two-dimentional gels and transferred electrophoretically to either derivatized diazotized

[1] *Abbreviation used in this paper:* TCA, trichloroacetic acid.

paper blots (31) or nitrocellulose (33). Blots were then incubated with sera (1/20–1/50 dilution, see figure legends) at 37°C overnight. After washes, diazotized paper blots were incubated with iodinated protein A (200,000 cpm/ml, 2 h at 37°C) and autoradiographed. Nitrocellulose blots were incubated with peroxidase-conjugated goat anti-rabbit serum (1/2,000 dilution), and reacted with 4-chloro-1-napthol.

For some experiments, antibodies were affinity purified by elution from nitrocellulose blots (28). Elution was carried out as described in (37).

RESULTS

Incorporation of Radioactive Amino Acids into X, α, β, γ, and δ under Pulse-Chase Conditions

GROWING CELLS: Previous results (see the introduction for a summary and reference 6 for more details) suggested that a precursor-product relationship could exist between the micronuclear H1-like molecules α, β, and γ. To investigate this possibility directly, we examined the synthesis of these histones by labeling cells with [^{3}H]lysine, preparing micronuclei, and examining the two-dimensional gel profile (Triton-acid-urea by SDS) of acid-soluble proteins by both staining and fluorography. Fig. 1, *a* and *b* illustrates the results obtained when growing cells were removed from food, resuspended in Tris (10 mM, pH 7.4), and labeled for 30 (pulse) with [^{3}H]lysine. Although α, β, and γ were easily observed by staining (Fig. 1 *a*), the corresponding fluorograph (Fig. 1 *b*) shows that only α was intensively labeled; β and γ were

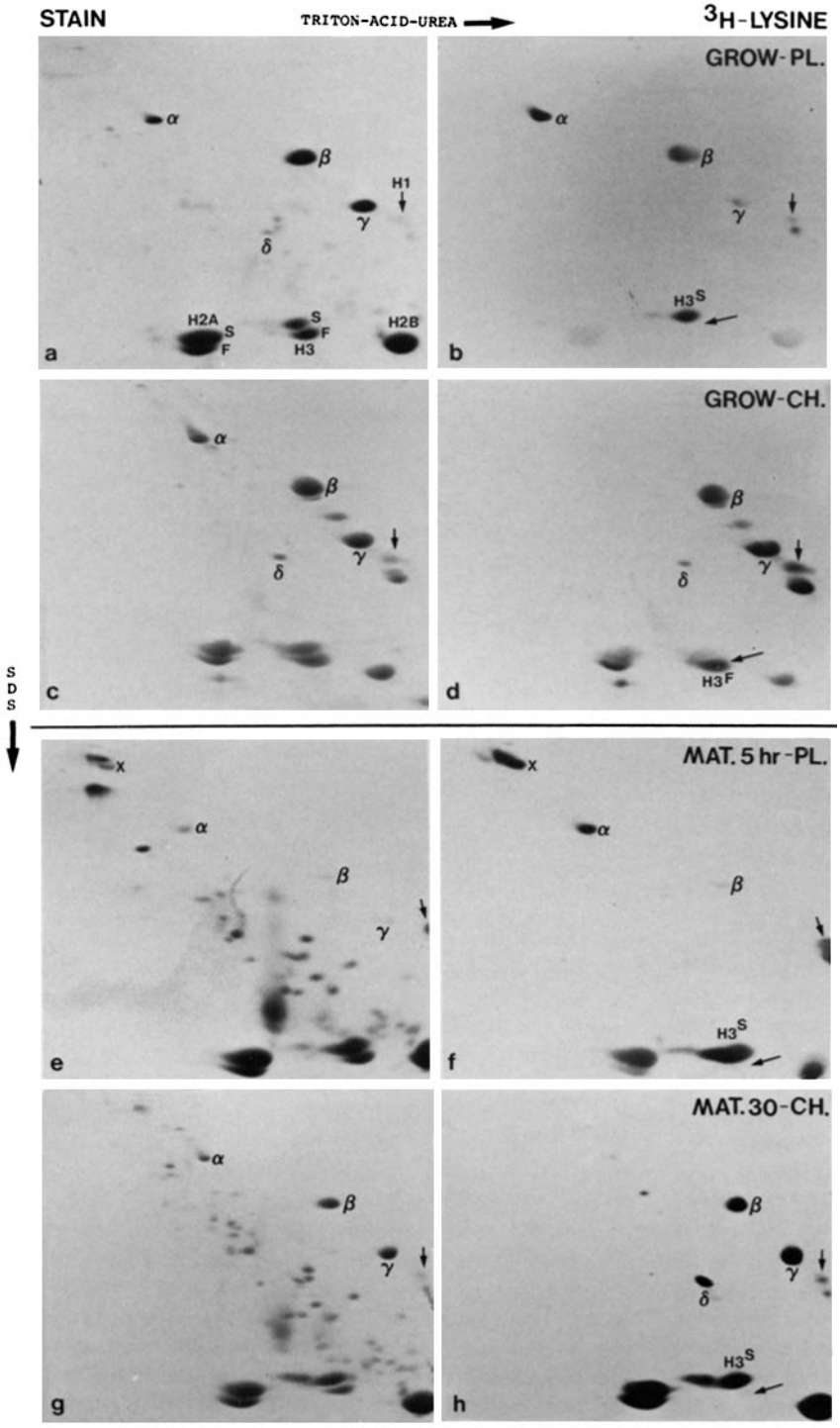

FIGURE 1 Distribution of radioactivity among micronuclear H1-like histones under pulse-chase conditions in growing and conjugation cells. Growing cells (300,000–400,000 cells/ml) were labeled for 30 min with [^{3}H]lysine (pulse: *a* and *b*). An aliquot of these cells was allowed to grow under chase conditions (see Materials and Methods) for two cell generations (chase: *c* and *d*). Similarly, mating cells (~88% paired) were labeled for 30 min at 5 h of conjugation (pulse: *e* and *f*). An aliquot of these cells was allowed to complete conjugation (30 h in Tris) under chase conditions (chase: *g* and *h*). Acid extracts prepared from these micronuclei were electrophoresed in parallel two-dimensional gels (Triton-acid-urea by SDS) and analyzed by staining (*a*, *c*, *e*, and *g*) and fluorography (*b*, *d*, *f*, and *h*). Downward-pointing arrows denote macronuclear type H1 which likely results from variable macronuclear contamination within these micronuclear preparations. Long arrows in *b*, *d*, *f*, and *h* point to the position of micronuclear-specific H3^F. We have typically observed disproportionately high amounts of H3^S (relative to the other nucleosomal histones) after this labeling procedure, but not when labeling is performed directly in food. The reason for this difference is not clear, but it has been observed in earlier studies (1). Note that more uniform labeling of nucleosomal histones is observed with conjugating cells (*f* and *h*).

labeled at intermediate and low levels, respectively. This fluorograph also reveals preferential labeling of $H3^S$ (as compared with $H3^F$) which has been demonstrated to be a precursor to $H3^F$ (1).

The observation that α is preferentially labeled under pulse conditions is suggestive of a precursor-product relationship that could exist between α, β, and γ. To test this possibility further, an aliquot of the pulse-labeled cells (shown in Fig. 1, *a* and *b*) was pelleted from the labeling buffer, washed once with Tris containing enough nonradioactive lysine to suppress further incorporation of the tritiated isotope into protein (1, 7), and returned to food (which also contained excess nonradioactive lysine) for two cell generations. Micronuclei were then prepared from these chased cells and their acid extracts were analyzed in parallel two-dimensional gels (Fig. 1, *c* and *d*). While α, β, and γ were again easily observed in the staining pattern (Fig. 1 *c*), the corresponding fluorograph (Fig. 1 *d*) shows that β and γ were both strongly labeled in chased cells. Unlike pulsed cells (Fig. 1 *b*), however, little if any label was observed in α. The fluorograph shown in Fig. 1 *d* also contains a relatively minor polypeptide (labeled δ) which became labeled under chase conditions (it is unlabeled in the pulsed cells, see Fig. 1, *a* and *b*). Finally, the fluorograph shown in Fig. 1 *d* demonstrates that the conversion of pulse label from $H3^S$ into $H3^F$ occurred during the two-generation chase of the experiment. This is expected since proteolytic processing of $H3^S$ into $H3^F$ has been shown to be a cell cycle–dependent event in growing cells (1).

CONJUGATING CELLS: Similar pulse-chase experiments were carried out with conjugating cells to investigate whether a precursor-product relationship could exist between a recently described large molecular weight polypeptide, X, (which is actively synthesized and deposited into micronuclei of young conjugants; reference 6) and α, β, or γ. As expected from recent studies monitoring histone synthesis and deposition in conjugating cells (6), X and α were intensely synthesized and deposited into micronuclei at 5 h of conjugation (Fig. 1, *e* and *f*). Little if any β or γ was detected in these nuclei by staining or fluorography. However, when an aliquot of these pulse-labeled mating cells was pelleted, washed with Tris containing excess nonradioactive lysine, and allowed to complete conjugation under chase conditions (up to 30 h in Tris), the results shown in Fig. 1 *h* were obtained. As with cells chased under growth conditions, β, γ, and δ were strongly labeled. Little if any label was observed in X or α.

The results presented in Fig. 1, *e–h* do not allow us to determine when the processing event(s) first occurs during the mating process since only extreme time points are shown. To investigate this question in more detail, we labeled mating cells at 6 h of conjugation with [^{3}H]lysine for 30 min and then removed an aliquot for an immediate micronucleus isolation (pulse, Fig. 2 *a*). The remainder of the culture was then chased as before to 10 h (Fig. 2 *b*), 13 h (Fig. 2 *c*), or 17 h of conjugation (Fig. 2 *d*). As before (Fig. 1, *e–h*), X and α were actively synthesized and deposited into micronuclei from early stages of conjugation (6 h, Fig. 2 *a*). This is before the time when micronuclei give rise to either new micro- or macronuclei. However, when these cells were chased to 10 h (a stage containing relatively young new macro- and micronuclei, (Fig. 2 *b*), most of the label was observed over α. By this stage much of the previous pulse label in X was gone except for a poorly resolved series of three spots (which are slightly larger than the spot labeled *X* in Fig. 2 *b*). Little if any label was evident in β, γ, or δ at 10 h.

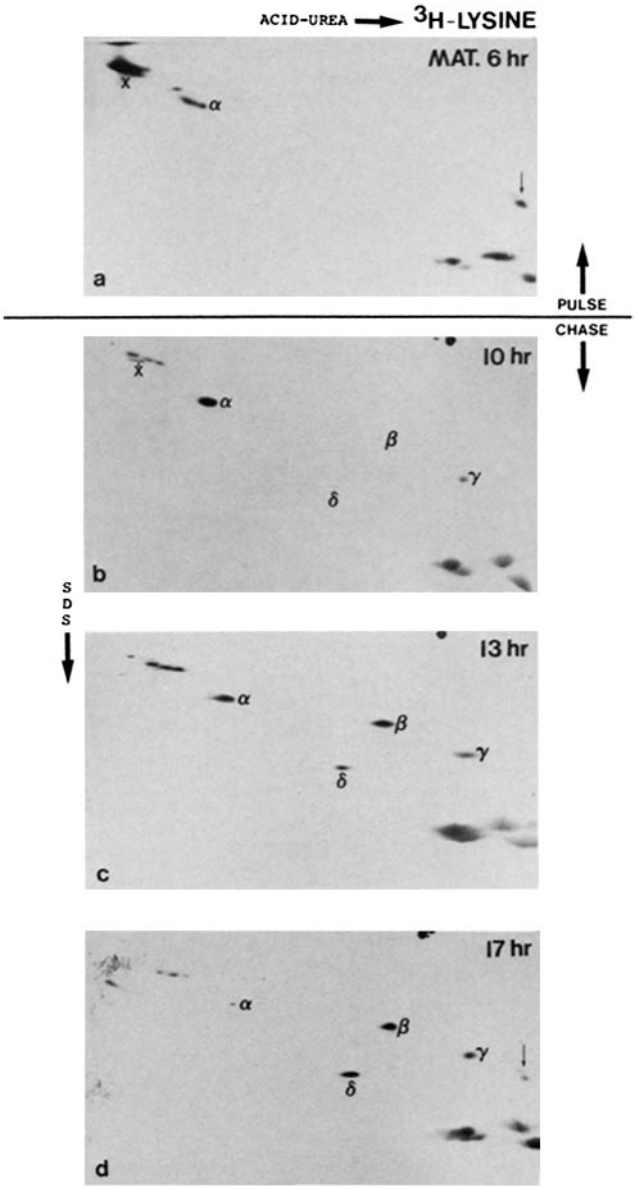

FIGURE 2 Distribution of pulse-chase radioactivity among micronuclear H1-like histones during various time points of conjugation. Mating cells (~90% paired) were pulse labeled for 30 min at 6 h of conjugation with [^{3}H]lysine (*a*). An aliquot of these pulse-labeled cells was then allowed to continue conjugation (under chase conditions) to 10 (*b*), 13 (*c*), or 17 (*d*) h. Micronuclei were isolated from all time points and acid extracted. After parallel two-dimensional gel electrophoresis (acid-urea by SDS), acid extracts were analyzed by fluorography. In all cases, carrier micronuclear histone (unlabeled) was added to each sample before TCA precipitation and for this reason the staining profiles for each time point are not shown. As in Fig. 1, variable amounts of macronuclear H1 are shown with downward-pointing arrows. All time points were derived from the same mating.

By 13 h of conjugation, new micronuclei are likely to be committed to the pathway of micronuclear differentiation inasmuch as many hours have elapsed since the second postzygotic division (which occurs at 7 h, see reference 26 for a description of stages in conjugation). It is noteworthy that at 13 h significant amounts of label were observed in α, β, γ, and δ. Little if any label was observed in the X region of the gel except for the previously mentioned higher molecular

weight series of spots. Thus, significant amounts of label accumulate in β, γ, and δ for the first time between 10 and 13 h of conjugation.

Similar results to those shown at 13 h of conjugation (Fig. 2*c*) were obtained at 17 h (Fig. 2*d*) except that the amount of label in α was reduced. Significant amounts of label were observed over β, γ, and δ. Taken together, the data present in Figs. 1 and 2 suggest that in mating cells a precursor-product relationship exists between X and α during early stages of conjugation (5–10 h) and between α and potentially β, γ, or δ during later stages of mating (10–17 h).

Timing of Synthesis and Deposition of X into Micronuclei during Conjugation

At 10 h of conjugation significant amounts of α are present (by staining) in micronuclear chromatin (6). If indeed X is a precursor to α, it is reasonable to suspect that X is synthesized and processed into α throughout a large fraction of conjugation preceding 10 h. Furthermore, one would also expect to see label preferentially distributed over X (as compared with α) in a short labeling interval (pulse). When cells were *pulsed* for 10 min with [^{3}H]lysine at hour intervals between 3 and 8 h of conjugation, it was clear that large quantities of X were synthesized and deposited into micronuclei from 4 to 7 h (data not shown). X was also preferentially labeled during a 10-min pulse, even though α accumulated by stain during this interval of conjugation (presumably due to the ongoing processing of newly synthesized X into α). These data strongly support the idea that X is a precursor to α.

Characterization of Antiserum against α

To determine whether immune serum raised against two-dimensional gel-purified α (see Materials and Methods) contained antibodies to α, acid-soluble proteins from micronuclei prepared from growing (Fig. 3, *a* and *b*) and 5-h-mating (Fig. 3, *c* and *d*) cells were subjected to two-dimensional gel elec-

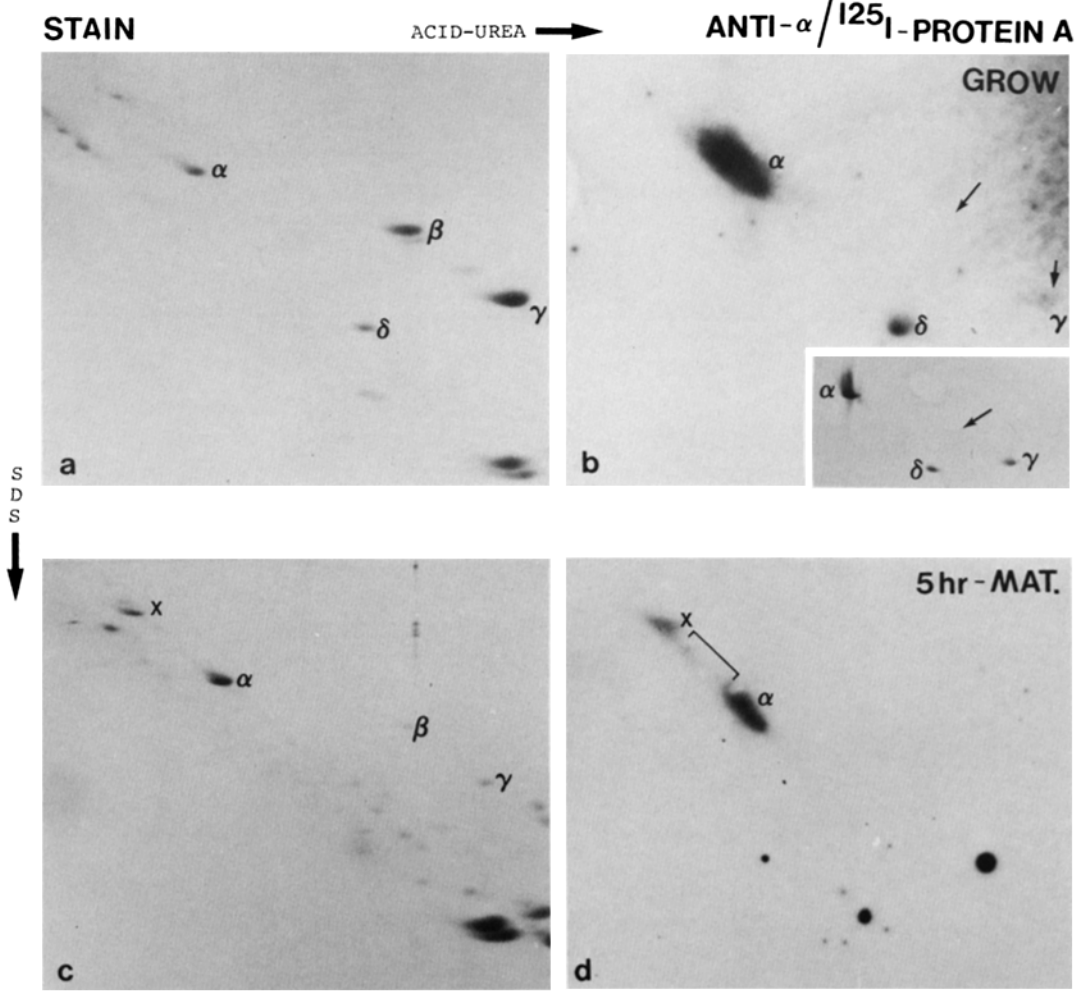

FIGURE 3 Analysis of α antiserum by immunoblotting. Parallel two-dimensional gels (acid-urea by SDS) of acid-soluble proteins from micronuclei of growing (*a* and *b*) or 5-h mating (*c* and *d*) cells were either stained directly (*a* and *c*) or used for electrophoretic transfer to diazotized paper blots (*b* and *d*) as described in the Materials and Methods. Shown in *b* and *d* are immunoautoradiographs after sequential incubation with α antiserum (1/20 dilution) and ^{125}I-protein A (see Materials and Methods and text for details). The extremely regular dark spots seen in *d* (which are unlabeled) presumably represent staining artifacts from the protein A incubation. These do not correspond to proteins in the corresponding stained gel profiles (*a* and *c*). No reactions to α, δ, X, or γ were observed with preimmune serum (1/20 dilution). Shown in the inset of Fig. 2*b* is a peroxidase-staining reaction (α antiserum – 1/50 dilution) of micronuclear histone from growing cells following transfer to nitrocellulose. This blot shows the strongest reaction we have ever observed of α antibodies to γ. Most often, only weak reactions are observed. In all cases, reactivity of α antibodies to β (see downward-pointing arrows in Fig. 2*b*) has never been observed.

trophoresis and analyzed by immunoblotting. In both micronuclear preparations, a major spot of radioactivity (^{125}I-protein A label) was observed corresponding to α, as might be expected. Immunoautoradiographs of micronuclei prepared from growing cells (Fig. 3*b*) consistently showed as well strong radioactivity to a spot that corresponds to δ. While faint reactivity is typically observed over γ in these immunoblots (Fig. 3*b*, inset), reactivity to β has never been observed.

The results obtained from immunoblots of micronuclei prepared from 5-h mating cells (Fig. 3*d*) clearly demonstrate that X polypeptide also reacts positively with α antibodies. This is not unexpected since our results from pulse-chase experiments suggest that X is a precursor to α. The results presented in Fig. 3 have also been verified using blot-purified α antibodies (using one-dimensional SDS gels). Here, strong reactivity was observed to X, α, and δ polypeptides (data not shown). These data suggest that X and δ share antigenic determinants with α. A possible explanation for the failure to produce strong reactions to γ and β is presented below.

Peptide Mapping

To characterize micronuclear H1-like histones further, we used the technique of Cleveland et al. (13) to map the partial proteolytic digestion products of individual protein spots (or bands) cut from one- or two-dimensional gels. To avoid confusion with enzyme and carrier protein which is included in each lane (see Materials and Methods) and to increase the sensitivity with which resulting peptides are detected, we iodinated histones in vitro with Bolton-Hunter reagent (10) before proteolytic cleavage. Preliminary experiments indicated that no differences in peptide maps were obtained between proteins labeled in vitro with [^{125}I]Bolton and Hunter reagent and those labeled in vivo with [^{3}H]lysine (R. L. Allen and C. D. Allis, unpublished observations).

Fig. 4 shows a comparison of the peptides typically produced from β (lanes *1–3*), X (lanes *4–6*), α (lanes *7–9*), δ (lanes *10–12*), and γ (lanes *13–15*) with various amounts of V8. Upon V8 digestion, a tightly spaced ladder of partial peptides was produced from X (bracket labeled *A* + *A′*), α (*A*), and γ (*A′*). The fact that the A peptides from α and the A′ peptides from γ consistently appeared as unstable, similarly spaced "ladders" of partial peptides suggests that these portions of the two proteins may be similar (at least with respect to V8 cleavage sites). A direct test of this possibility is presented below (see Fig. 5).

It is also apparent from the V8 digestion profile that X and α contain peptides (*B* and *C*, Fig. 4) which were *not* detected in γ. Digestion kinetics suggest that the B peptide from α is unstable and may give rise to the limit peptides seen in the C region of the gel. If α (or a portion of α) is the precursor to γ (as our pulse-chase experiments suggest), it seems reasonable to suggest that the B-C V8 peptides (which are unique to α) represent the part of α which is *not* preserved in γ by the in vivo proteolytic cleavage (see below). B and C peptides were

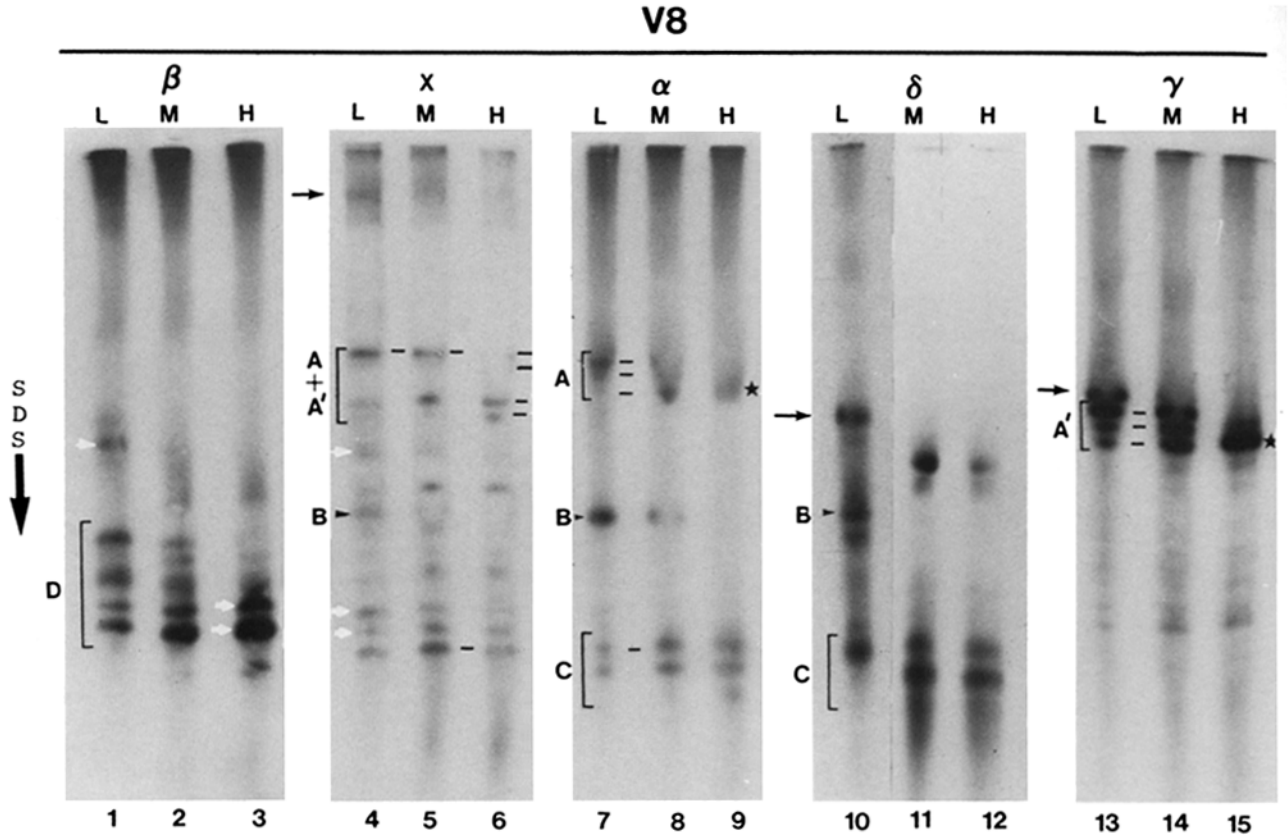

FIGURE 4 Partial proteolytic peptide map of α, β, γ, X, and δ following digestion with V8 protease. In vitro iodinated histones (β, lanes *1–3*; X, lanes *4–6*; α, lanes *7–9*; δ, lanes *10–12*; and γ, lanes *13–15*) were digested with various amounts of V8 protease (see Materials and Methods for details). Digestion products were electrophoresed in an SDS gel and analyzed by fluorography. (See the text for a description of the peptides labeled *A*, *A′*, *B*, *C*, and *D*). Rightward-pointing long arrows indicate the position of intact X, δ, and γ. Short white arrows in the peptide maps of β and X point to peptides which may be shared in these two polypeptides. In some of our other V8 maps of X (lanes *4–6*), the B peptide has been very evident at low enzyme concentrations.

also readily apparent in the V8 map of δ (lanes *10–12*). This suggests that δ may be produced from α during the same in vivo proteolytic processing event that generates γ from α. In other words, V8 peptide maps of α, γ, and δ (Fig. 4) strongly suggest that α is comprised of both a γ "half" (which does not contain the B and C peptides) and a δ "half" (which does contain the B and C peptides).

Surprisingly, digestion of β with V8 proteases consistently produced a series of partial peptides (bracket labeled *D*, Fig. 4) which are not readily apparent in digests of α or γ. Two limit peptides are consistently produced (Fig. 4, white arrows) which migrate more slowly than the C peptides from α. When β is digested with chymotrypsin (data not shown), a peptide map is generated which seems somewhat similar to that produced from α and γ. Nonetheless, two independent lines of evidence, V8 peptide mapping and cross-reactivity with α antibodies (Fig. 3), suggest that β is not closely related to α or γ. It is perhaps significant that V8 peptides that are diagnostic for β (Fig. 4, white arrows within the *D* bracket) are observed in V8 map of X (see Discussion).

Peptide Map of the V8 Limit Peptide A (from α) and A′ (from γ)

Results presented in Fig. 4 suggest that α and γ (and X) contain a V8 limit peptide (A star and A′ star, respectively) which may be similar in both polypeptides (based on their partial proteolytic behavior with V8 protease). To directly examine this possibility, in vitro iodinated α and γ were first digested with high levels of V8 protease. The A and A′ V8 limit peptides (Fig. 4, star) were then excised and redigested with various levels of chymotrypsin (Fig. 5). Essentially identical chymotryptic maps were produced from these V8 peptides which strongly suggests that the A and A′ V8 peptides from α and γ, respectively, are similar in primary sequence.

V8 Peptide Mapping followed by Reaction with α Antibodies

Immunoblotting with intact micronuclear histones suggests that X and δ contain determinants that are easily recognized by α antibodies (Fig. 3). Only a weak positive reaction was obtained with γ. This is somewhat surprising since our peptide mapping data (Figs. 5–7) demonstrate that a part of α (A V8 peptides) was conserved in γ (A′ V8 peptides). To investigate which V8 peptides from X, α, and δ react with α antibodies, purified histones were digested with V8 protease (without carrier protein), transferred to nitrocellulose, and incubated with α antibodies. Fig. 6 shows a peroxidase-color reaction of such an immunoblot. Reactivity was consistently observed over the B and C peptides from X, α, and δ, while little if any reactivity was found over the A and A′ peptides from α and γ, respectively. No reaction has ever been observed with any of the V8 peptides produced from β. Since V8 cleavage of γ does not produce B and C peptides (Fig. 4), and since α antibodies seem to preferentially recognize determinants in these peptides, it is likely that this explains the inability of γ to be well recognized with α antibodies.

DISCUSSION

The results presented here demonstrate that several of the H1-like micronuclear histones are related by a physiologically and developmentally regulated proteolytic processing event(s). Included in this category are X, α, γ, and δ. Three independent lines of evidence suggest that α is derived from yet a larger precursor, X: First, X is actively synthesized and deposited into micronuclei of young mating cells after pulse labeling, and this pulse label is converted to α under chase conditions (Fig. 2). Second, polyclonal antibodies raised against α cross-react with X (Fig. 3). Finally, a comparison of the V8 peptide maps of X and α reveals shared diagnostic peptides between the two histones (Fig. 4). Curiously, X is only observed (by stain or label, Fig. 1) in micronuclei isolated from mating cells. Our failure to observe X in micronuclei from growing cells is likely a result of altered processing kinetics (X to α) in growing and starved mating cells. We have made several attempts to label X in growing cells by reducing the labeling time to brief (5 min) pulses. We have not observed any significant label in X (M. Zapp and C. D.

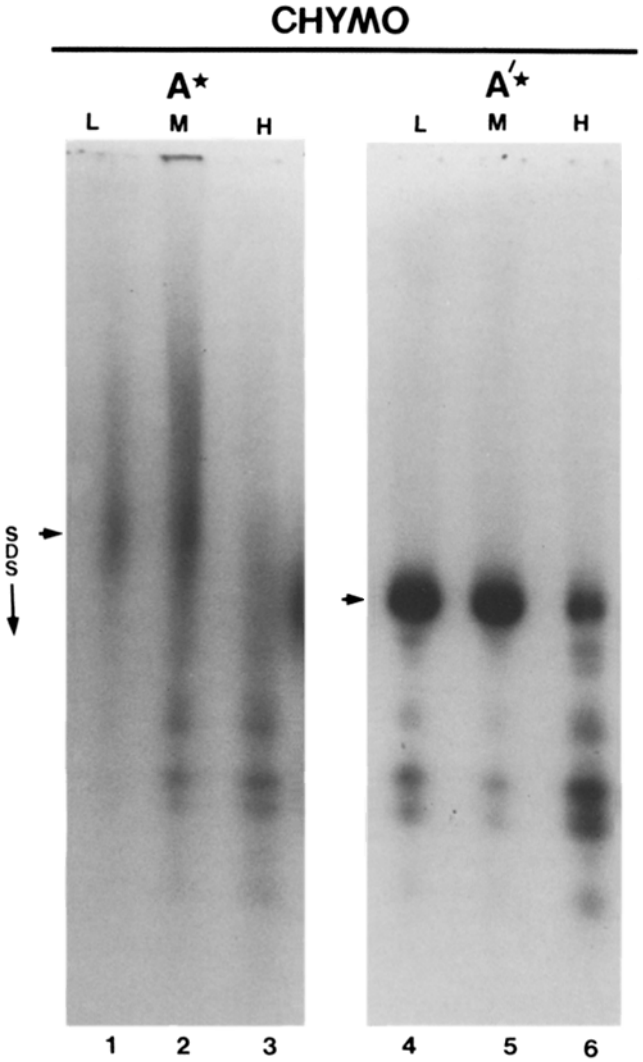

FIGURE 5 Partial proteolytic peptide map of V8 fragments A* (from α) and A′* (from γ) following redigestion with chymotrypsin. In vitro iodinated α and γ was first digested with high levels of V8 protease. Digestion products were displayed on an SDS gel and the A* and A′* peptide from α and γ, respectively, was localized by staining and autoradiography. These stable V8 peptides were then excised from the gel and redigested with various amounts of chymotrypsin (see Materials and Methods and text for details). Again, digestion products were displayed on an SDS gel and analyzed by fluorography. Rightward-pointing arrows indicate the position of intact A and A′ star V8 peptides.

Allis, unpublished observation) which suggests that X is rapidly processed into α in growing cells (assuming that α is derived from X in growing cells as it is in mating cells).

Data presented here also suggest that α is further processed into two "halves", one of which is γ and the other, δ. Estimates of molecular weight of α, γ, and δ are consistent with this possibility ($\alpha \cong 40{,}000$, $\gamma \cong 22{,}000$, $\delta = 20{,}000$, C. D. Allis and R. Richman, unpublished observations) as is the fact that V8 peptide maps of δ and γ roughly "sum" to give the V8 map of α (Fig. 4). Furthermore, both α and δ are highly reactive with α antibodies (Fig. 3) as are the principal V8 peptides they share in common (*B* and *C*, Fig. 6). While γ is not strongly reactive with α antibodies, it does not contain the B and C V8 peptides shared by α and δ. Thus, it is reasonable to suggest that the polyclonal antibodies raised against α and used in these studies were directed primarily at that portion of α which gives rise to δ (and not γ).

Since δ is never present in acid extracts from micronuclei in amounts that approximate γ (by staining), we suspect that δ turns over with significantly different kinetics from that of γ. (γ is a relatively major species in acid extracts from micronuclei of growing cells [4]). While the exact function of the H1-like histones is not clear, earlier work has demonstrated that α, β, and γ were present in linker regions of micronuclear chromatin (4). Interestingly, reexamination of that work (reference 4, Fig. 5*B*) has revealed the presence of δ in that chromatin fraction as well. Whether δ plays a structural and/or functional role in micronuclear chromatin or is simply the nonfunctional cleavage product derived from the processing event that generates γ from α is not clear.

What also remains unclear from our study is whether or not β is part of the processing series that interrelates X, α, γ, and δ. In either growing (Fig. 1) or conjugating cells (Figs. 1 and 2) β is consistently unlabeled or weakly labeled after pulses with [^{3}H]lysine and becomes labeled under chase conditions. While these results suggest that β is derived from a precursor, partial peptides generated from β by V8 digestion (Fig. 4, *D* bracket) are not obviously similar to those seen from α, γ, or δ and cross-reactivity between β (Fig. 3) or any of its V8 peptides (Fig. 6) has never been observed with α antibodies. Given these results, one possible candidate for a precursor to β (if one exists) is X or the polypeptide(s) that migrate slightly above X in our two-dimensional gels (see the three labeled spots above X in Fig. 2). All of these species are actively synthesized and deposited into micronuclei during early periods of conjugation. It is perhaps significant that the V8 peptide map of X (Fig. 4, which could easily have been cross-contaminated with some of the larger molecular weight

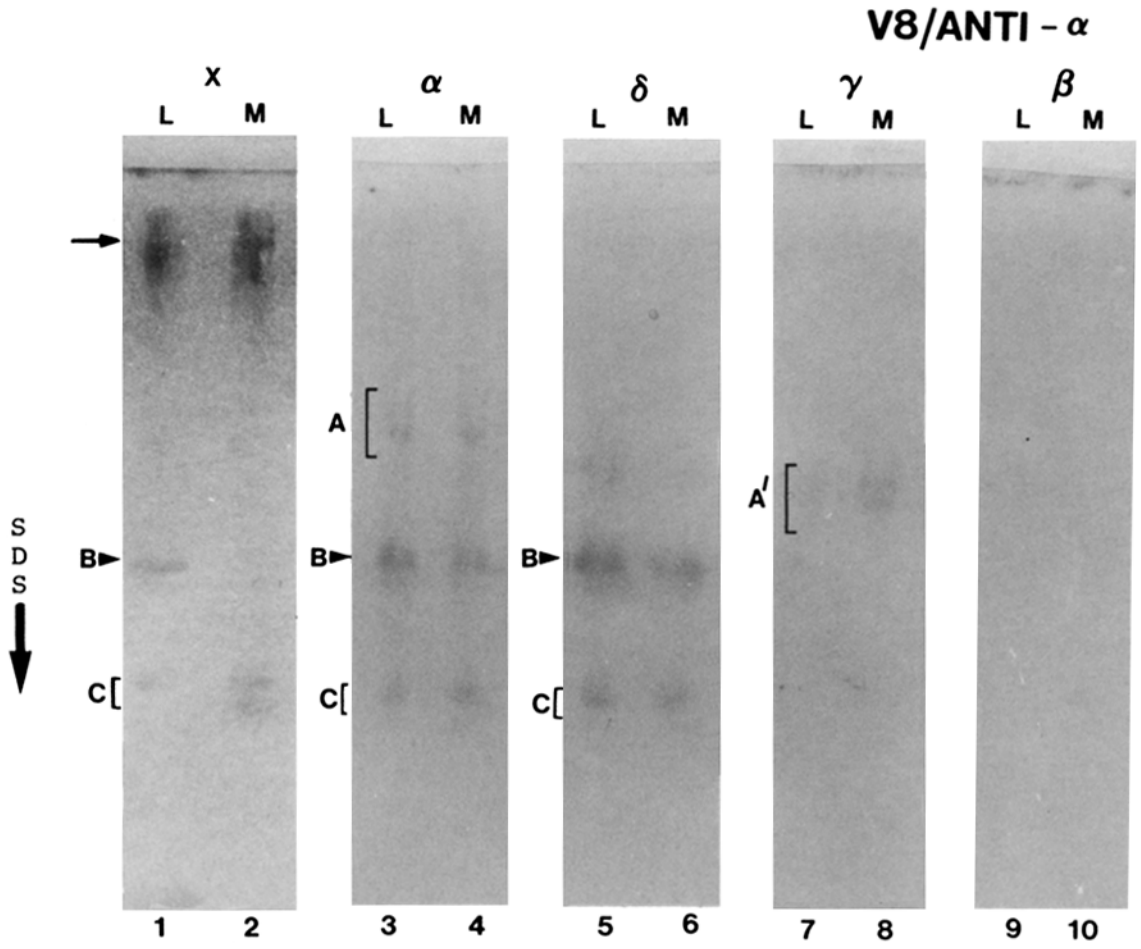

FIGURE 6 Analysis of the reactivity of V8 peptides from X, α, δ, γ, and β with α antiserum by immunoblotting. Unlabeled micronuclear histones (X, lanes *1* and *2*; α, lanes *3* and *4*; δ, lanes *5* and *6*; γ, lanes *7* and *8*; and β, lanes *9* and *10*) were digested (without carrier protein) with low (*L*) and medium (*M*) levels of V8 protease. Digestion products were electrophoresed into a standard SDS gel, transferred to nitrocellulose, and reacted with α antibodies (1/20 dilution). Antibody reactions were detected by staining with a peroxidase-color reaction (see Materials and Methods and text for details).

material) contains peptides that are diagnostic for β (white arrows in Fig. 4, lane *4*). It is, of course, possible that β is derived from a portion of X (or the higher molecular weight peptides) that is distinct from that used to generate α and therefore β would not be expected to cross-react with α antibodies. Finally, it is important to note that the studies presented here have all been carried out using isolated micronuclei. Thus, we are analyzing only those polypeptides that are synthesized and deposited into nuclei during the pulse-chase interval. If a precursor to β (or β itself) is synthesized during the pulse and retained in the cytoplasm, it will not be observed in our analyses. If it is transported into nuclei during the chase, it would appear as a radiolabeled polypeptide in our kinetic analyses. Thus, β may not be derived from a precursor polypeptide despite the reuslts of our pulse-chase experiments.

It is clear from the results presented here that a proteolytic processing mechanism(s) interrelates most (if not all) (possibly not β) of the micronuclear H1-like histones. Previous work (6) has demonstrated that preexisting micronuclear-specific histones (α, β, γ, and $H3^F$) are "cleared" from micronuclear chromatin during early time periods of conjugation before the differentiation of new macronuclei. It was reasoned that this "dedifferentiation process" may exist in *Tetrahymena* because some micronuclei must eventually differentiate into new macronuclei. β and γ (and δ) reappear in micronuclear chromatin (by stain) only after the differentiation of new macro- and new micronuclei are well underway (6). It is in this interval (between 10 and 13 h) that we observe large amounts of pulse label in α processed (under chase conditions) into γ and δ as well as the appearance of labeled β (Fig. 2). This suggests that the proteolytic processing event(s) that generates these histones (β, γ, and δ) may be activated as part of the differentiation pathway of new micronuclei. Thus, physiologically and developmentally regulated proteolytic processing of specific nucleosomal (H3; references 1 and 7) and linker associated histones (X, α, γ, δ, and possibly β; this report) plays an important (and previously unsuspected) role in generating histone diversity in *Tetrahymena* micronuclei. Presumably modulation of histone composition via this mechanism(s) has profound consequences on the structure and function of micronuclear chromatin.

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