Tetrahymena Contain Two Distinct and Unusual High Mobility Group (HMG)-Like Proteins

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Abstract. Previous studies have described the existence of high mobility group (HMG)-like proteins in macronuclei of the ciliated protozoan, *Tetrahymena thermophila* (Hamana, K., and K. Iwai, 1979, *J. Biochem. [Tokyo]*, 69:1097–1111; Levy-Wilson, B., M. S. Denker, and E. Ito, 1983, *Biochemistry*, 22:1715–1721). In this report, two of these proteins, LG-1 and LG-2, have been further characterized. Polyclonal antibodies raised against LG-1 and LG-2 fail to cross react with each other or any other macronuclear polypeptide in immunoblotting analyses. As well, LG-1 and LG-2 antibodies do not react with calf thymus, chicken, or yeast HMG proteins. Consistent with these results, a 47 amino-terminal sequence of LG-1 has been determined that shows limited homology to both calf thymus HMGs 1 and 2 and HMGs 14 and 17. Two internal sequences of V8 protease-generated peptides from LG-2 have been determined, and these do not share any homology to the LG-1 sequence or any other sequenced HMG proteins. Comparison of the partial sequences of LG-1 and LG-2 with the complete amino acid sequence of the *Tetrahymena* histone H1 (Wu, M., C. D. Allis, R. Richman, R. G. Cook, and M. A. Gorovsky, 1986, *Proc. Natl. Acad. Sci. USA*, 83:8674–8678) rules out the possibility that LG-1 and LG-2 are proteolytically derived from H1, the other major macronuclear perchloric acid–soluble protein. Interestingly, however, both LG-1 and LG-2 are efficiently extracted from macronuclei by elutive intercalation (Schröter, H., G. Maier, H. Ponsting, and A. Nordheim, 1985, *Embo (Eur. Mol. Biol. Organ.) J.*, 4:3867–3872), suggesting that both may share yet undetermined properties with HMGs 14 and 17 of higher eukaryotes.

Examination of the pattern of LG-1 and LG-2 synthesis during the sexual phase of the life cycle, conjugation, demonstrates that the synthesis of LG-1 and LG-2 is coordinately increased from basal levels during the differentiation of new macronuclei (7–13 h), suggesting that both of these proteins play a role in determining a macronuclear phenotype. However, a specific induction of LG-2 synthesis is detected in early stages of conjugation (meiotic prophase, 1–4 h), leading to maximal synthesis of LG-2 at 3 h. Interestingly, the early induction of LG-2 synthesis closely parallels the hyperphosphorylation of histone H1. Taken together, these data suggest that LG-1 and LG-2 are not strongly related to each other or to higher eukaryotic HMG proteins. The synthetic data support the idea that these proteins may have different functions.

THE high mobility group (HMG)[1] proteins compose the most thoroughly studied class of nonhistone chromosomal proteins. This family of relatively low molecular weight proteins (mol wt <30,000) can be extracted from chromatin by low salt or with dilute acids, and characteristically contain a large number of basic and acidic amino acids distributed in a polar fashion (Johns, 1982). In higher eukaryotes two pairs of HMG proteins have been distinguished based on differences in molecular weight, amino acid composition, and primary sequence. HMG 1 and HMG 2 are relatively large polypeptides (mol wt 25–29,000) while the second pair, HMG 14 and HMG 17, are somewhat smaller (mol wt 7–14,000) (Johns, 1982). Chromatin-associated proteins with solubility properties and amino acid compositions similar to calf thymus HMG proteins have been isolated from members of all four eukaryotic kingdoms (Mays, 1982). Due to the lack of amino acid sequence information and the susceptibility of HMG proteins to proteolysis (Johns, 1982), the relationship of these proteins to each other and to the higher eukaryotic HMG proteins is often not clear.

Interest in the HMG proteins arose after various investigators determined that HMGs 14 and 17 were associated with transcriptionally active chromatin (Weisbrod and Weintraub, 1979; Weisbrod et al., 1980; Levy-Wilson et al., 1979; Davie and Saunders, 1981). These results, however, remain controversial (for review see Reeves, 1984). Furthermore, a clear role for HMGs 1 and 2 has not been determined. Various studies have suggested functions for HMGs 1 and 2 in

1. *Abbreviation used in this paper*: HMG, high mobility group.

transcription or replication; at this time these results also remain inconclusive (see Enick and Bustin, 1985).

Vegetative cells of the ciliated protozoan *Tetrahymena thermophila* provide a simple system to study both the structure and possible function of eukaryotic HMG proteins. Each cell contains two distinct nuclei: a transcriptionally active macronucleus which governs the phenotype of the cell and a micronucleus which is a transcriptionally inactive germline nucleus (Gorovsky, 1973). If HMG-like proteins in *Tetrahymena*, like HMGs 14 and 17 in higher eukaryotes, are associated with transcriptionally active chromatin, one would expect to find these proteins enriched in macronuclei (where ~40% of genome has been shown to be transcribed; Gorovsky, 1985) and depleted or missing from micronuclei. To date four polypeptides isolated from *Tetrahymena* macronuclei have been classified as being HMG-like proteins based upon solubility properties, electrophoretic mobilities, and amino acid compositions (Hamana and Iwai, 1979; Levy-Wilson et al., 1983). Two of these proteins, LG-1 and LG-2 (HMG C and HMG B of Levy-Wilson et al., 1983), have been compared with calf thymus HMG proteins on the basis of electrophoretic mobility and amino acid composition (Hamana and Iwai, 1979; Levy-Wilson et al., 1983), but these reports have been inconclusive. For example, the mobility of LG-1 in SDS gels is similar to that of the smaller HMG proteins; however, its amino acid composition more closely resembles that of HMGs 1 and 2. Conversely, the mobility of LG-2 in SDS gels is more similar to that of the larger HMG proteins; however, its composition, which is lower in hydrophobic content and contains more alanine and glycine than LG-1, more closely resembles that of HMGs 14 and 17. In this report we have extended these studies by using both polyclonal antibodies and microsequencing to investigate the relationship (if any) of LG-1 and LG-2 to each other and to HMGs 1, 2, 14, and 17. Our results suggest both at the level of primary sequence and immunological cross-reactivity that these two *Tetrahymena* HMG-like proteins are distinct from each other and differ considerably from the higher eukaryotic HMG proteins.

Materials and Methods

Cell Culture and Labeling

Genetically marked strains of *Tetrahymena thermophila*, CU 427 (Mpr/Mpr-[6-mp-s]VI) and CU 428 (Chx/Chx[cy-s]VII), were used in all experiments reported here. These were kindly provided by P. Bruns (Cornell University, Ithaca, NY). Cells were grown axenically in 1% (wt/vol) enriched proteose peptone as described (Gorovsky et al., 1975). All matings were performed in 10 mM Tris-HCl (pH 7.4) according to Bruns and Brussard (1974) as modified by Allis and Dennison (1982). All cultures were maintained at 30°C. Growing cells were labeled with ^{32}P-orthophosphate (5 μCi/ml) or [^{3}H]sodium acetate (5 μCi/ml, 4.5 Ci/mmol) as previously described (Allis and Gorovsky, 1981; Allis et al., 1985). In some cases cells were pretreated with cycloheximide according to the procedure of Vavra et al. (1982) before labeling with [^{3}H]sodium acetate. Mating cells were labeled with [^{3}H]lysine (1 μCi/ml, 46 Ci/mmol) as described in Allis et al. (1985).

Isolation of Macronuclei

Macronuclei were isolated from growing, starved, and mating cells by the methods of Gorovsky et al. (1975) and Allis and Dennison (1982) with the following changes: spermidine was omitted from all buffers and 1 mM PMSF, 10 mM sodium butyrate, and 10 mM iodoacetamide were added to the nucleus isolation buffer (medium A).

Acid Extraction and Precipitation of Nuclear Proteins

Fresh nuclei were washed once in 0.25 M sucrose:10 mM Tris:3 mM CaCl:1 mM MgCl:1 mM PMSF (pH 7.5), resuspended in 0.4 N H_2SO_4 at a concentration of ~2 × 10^5 nuclei/μl, shaken for 1 h at 4°C, and then centrifuged at 15,000 *g* for 15 min. The supernatant (S1) was made 5% in PCA, incubated 15 min on ice, and centrifuged at 15,000 *g* for 15 min to pellet the PCA-insoluble material. The supernatant (S2) was made 20% in TCA, incubated 15 min on ice, and centrifuged at 15,000 *g* for 15 min to pellet the PCA-soluble material. PCA and TCA pellets were washed once in acidified acetone, once in acetone, and dried. In cases where total macronuclear acid-soluble protein was examined, the 0.4 N H_2SO_4 supernatant (S1) was precipitated directly with TCA.

Extraction of Total Cell PCA-soluble Protein

2 × 10^7 cells in either 10 mM Tris pH 7.4 (starved or conjugating) or enriched proteose peptone (vegetative) were pelleted at 2,500 *g* for 5 min at 4°C. The cell pellet was resuspended in 1 ml of 0.25 M sucrose:10 mM Tris:3 mM $CaCl_2$:1 mM $MgCl_2$:1 mM PMSF that had been made 5% in PCA. The cell suspension was sonicated briefly (~30 s) and centrifuged at 1,400 *g* for 5 min at 4°C. The supernatant was made 20% in TCA, incubated on ice for 10 min, and centrifuged at 15,000 *g* for 15 min at 4°C to pellet the PCA-soluble material. The pellet was washed once in acidified acetone, once in acetone, and dried.

Elutive Intercalation

Macronuclei (1 × 10^7) were washed into buffer E (see Schröter et al., 1985) before being resuspended in 200 μl of 0, 5, or 10 mM eithidium bromide in buffer E as described by Schröter et al. (1985). After a 30-min extraction at 4°C, nuclei were pelleted and the supernatant was converted to 20% TCA. Precipitated proteins were collected, washed, and dried as described above. Pilot experiments with the X2 buffer described by Schröter et al. (1985) suggested that macronuclei were not stable in this low ionic strength buffer. The effects of other intercalating drugs or buffer conditions were not investigated.

Isolation of Calf Thymus HMG Proteins

Calf thymus HMGs 1, 2, 14, and 17 were isolated from 5% PCA extracts of total thymus tissue by differential acetone precipitation as described by Nicolas and Goodwin (1982).

Gel Electrophoresis

First- (acid/urea or SDS) and second-dimension SDS gels used in this report have been described previously (Allis et al., 1979; 1980*a*, *b*). Gels were stained with Coomassie Blue, photographed, and, where appropriate, processed for fluorography.

Column Chromatography

Histone H1, LG-1, and LG-2 were purified from PCA extracts of macronuclei by Bio-Gel P-60 (Bio-Rad Laboratories, Richmond, CA) column chromatography (1.5 × 100 cm) as described by Hamana and Iwai (1971). Fractions were examined by SDS gel electrophoresis, pooled, dialyzed exhaustively against water, and lyophilized. Pure protein was resuspended in water and used for microsequencing.

Peptide Mapping and Preparative Cleavage of LG-2

Individual bands were cut from first- or second-dimension SDS gels and digested with *Staphylococcus aureus* V8 protease by the method of Cleveland et al. (1977). Gel pieces containing the protein of interest were placed in standard SDS second-dimension gel lanes and overlaid with 5 μl of Cleveland digestion buffer (Cleveland et al., 1977) containing 0.05 (low), 0.5 (medium), or 5 (high) μg per lane of protease followed by an overlay of Laemmli electrophoresis buffer (Laemmli, 1970). Electrophoresis was carried out for 30 min at 150 V, stopped for 30 min, and resumed for 1,800 V-h. Gels were stained as above. For preparative V8 cleavage of LG-2, V8 protease (0.2 mg/ml in Cleveland digestion buffer) was layered on top of a preparative SDS gel. A lightly stained 13-cm gel strip containing LG-2 was then placed on top of the V8 solution and overlaid with Laemmli electrophoresis buffer. Electrophoresis was then carried out as described above.

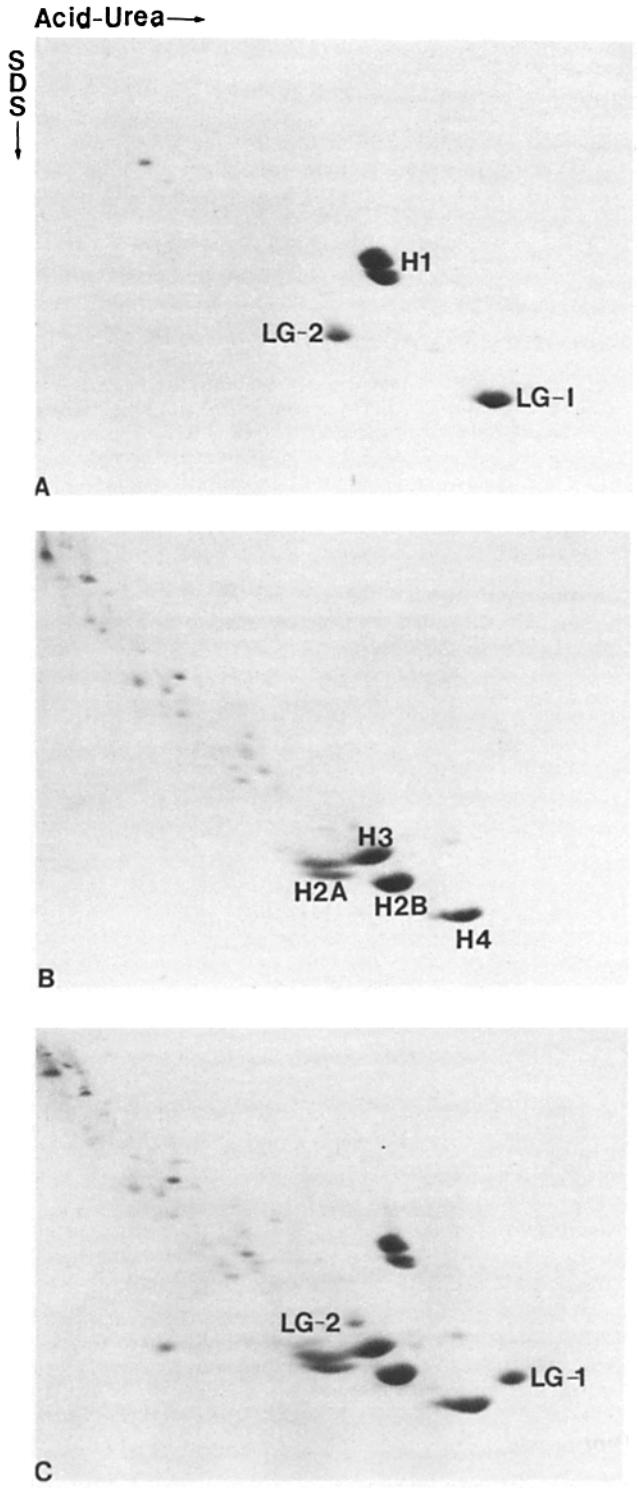

Figure 1. Two-dimensional gel analysis of macronuclear PCA-soluble and PCA-insoluble protein. Two-dimensional gel analysis (acid–urea by SDS) of (*A*) macronuclear 5% PCA–soluble protein, (*B*) macronuclear 5% PCA–insoluble protein, and (*C*) macronuclear total acid–soluble protein.

Elution and Recovery of Proteins

Individual stained protein bands were cut from one- or two-dimensional gels after being equilibrated with 62.5 mM Tris (pH 6.8). In some cases gel bands were stored at 4°C in the above solution in the presence of 1% (vol/vol) β-mercaptoethanol. Proteins were then electroeluted by the method of Hunkapiller et al. (1983). Eluted protein was dried in a speedvac concentrator, redissolved in 50–100 μl of water, and precipitated with 10 vol of ethanol (2 h at −80°C followed by an overnight incubation at −20°C). The final dried precipitate was resuspended in 0.1% SDS/0.1 M ammonium bicarbonate.

Amino Acid Sequencing

Microsequencing of column-purified or electroeluted protein was carried out as previously described (Allis et al., 1986) except that the PTH-derivatives were identified by HPLC using a PTH analyzer (model 120A; Applied Biosystems, Inc., Foster City, CA).

Immunoblotting

Proteins were separated on one-dimensional 15% SDS gels (Laemmli, 1970) and transferred electrophoretically to nitrocellulose. Blots were then blocked and incubated with antisera (1:200) for 6 h at 37°C. After washes, nitrocellulose blots were incubated with peroxidase-conjugated goat anti-rabbit serum (1/2,000 dilution) for 2 h and reacted with 4-chloro-1-naphthol. The efficiency of transfer was estimated by staining parallel blots with Amido black.

Results

Isolation of LG-1 and LG-2

The *Tetrahymena* HMG-like proteins LG-1 and LG-2 (HMG C and HMG B of Levy-Wilson et al., 1983) can be isolated in large quantities from purified macronuclei along with histone H1 due to their selective solubility in 5% perchloric acid (Fig. 1 *A*). Like other HMGs, LG-1 and LG-2 (and H1) can also be extracted from macronuclei with 0.35 M NaCl and are soluble in 2% TCA (data not shown). The copurification of LG-1 and LG-2 with histone H1 and the low recovery of these HMG-like proteins in acid extracts from macronuclei isolated by the method of Gorovsky et al. (1975) suggested the possibility that LG-1 and LG-2 may be proteolytic breakdown products of histone H1 (see Gorovsky et al., 1974). Fig. 1, *A* and *C*, however, indicate that significant quantities of LG-1 and LG-2 can be found in acid extracts from macronuclei isolated by the method of Gorovsky et al. (1975), provided that spermidine is omitted from the nucleus isolation buffer and care is taken to avoid proteolysis (see Materials and Methods).

To examine any possible relationship between histone H1, LG-1, and LG-2, these proteins were excised from one-dimensional SDS gels and subjected to partial proteolytic mapping with V8 protease (see Materials and Methods). As can be seen from Fig. 2, the V8 peptide map of H1 (lanes *1–4*) is significantly different from that of LG-2 (lanes *5–8*) and LG-1 (lanes *9–12*). This result suggests that LG-1 and LG-2 are not H1 degradation products. The V8 maps of LG-1 and LG-2, however, are remarkably similar and contain several peptides that seem to co-migrate in SDS gels (see *arrows* in Fig. 2, lanes *6*, *7*, *11*, and *12*). Interestingly, a light digestion of LG-2 with V8 protease produces a peptide with an SDS gel mobility very similar to that of intact LG-1 (see *star* at lane *6*). HMG proteins are known to be very susceptible to proteolysis (Johns, 1982) and the similarities in the peptide maps of LG-1 and LG-2 shown in Fig. 2 suggests the possibility that LG-1 may be derived from LG-2 by proteolysis or that both LG-1 and LG-2 are derived from a common precursor.

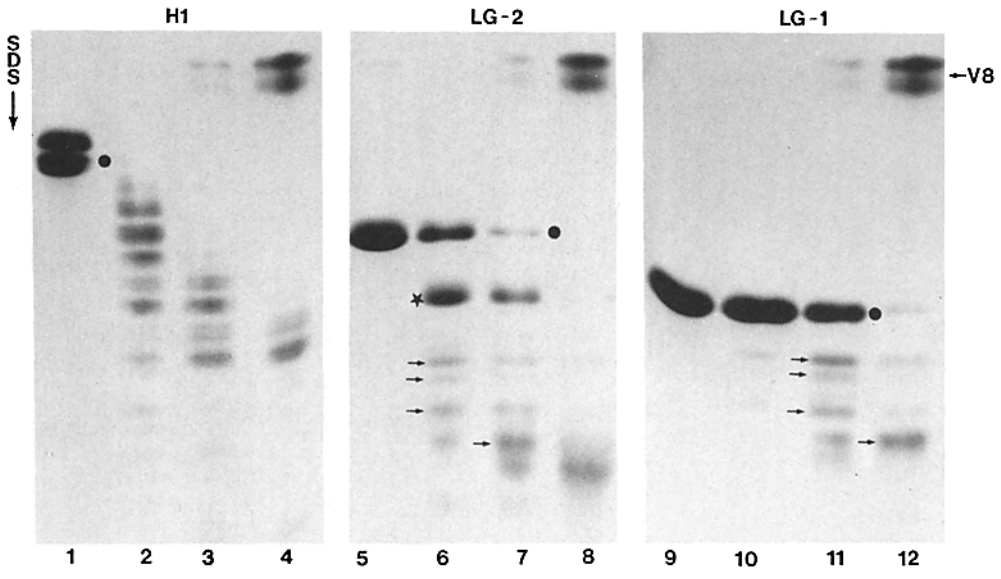

Figure 2. Partial proteolytic peptide map of H1, LG-2, and LG-1 after digestion with V8 protease. SDS gel pieces of H1 (lanes *1–4*), LG-2 (lanes *5–8*), and LG-1 (lanes *9–12*) were digested with increasing concentrations of V8 protease. Lanes *1*, *5*, and *9*, untreated; lanes *2*, *6*, and *10*, low concentration of protease; lanes *3*, *7*, and *11*, medium; and lanes *4*, *8*, and *12*, high (see Materials and Methods). The resulting peptides were then resolved on a 22% SDS gel. Solid circles denote the positions of intact H1, LG-2, and LG-1. Star in lane *6* indicates the position of a peptide derived from LG-2 that migrates close to the position of intact LG-1 (lane *9*). Small arrows in lanes *6*, *7*, *11*, and *12* indicate the positions of similar migrating peptides found in the maps of both LG-2 and LG-1. The leftward facing arrow at the top right points to the position of intact V8 protease. All samples shown were subjected to electrophoresis in the same polyacrylamide gel; spaces between some of the lanes were included for clarity.

Elutive Intercalation

Recently Schröter and associates (1985) have described a new extraction procedure (referred to as elutive intercalation), in which HMG proteins 14 and 17 are specifically released from chicken nuclei in the presence of intercalating drugs and low ionic strength buffers. Interestingly, under appropriate conditions, their extraction procedure does not release large quantities of HMGs 1 or 2 or histone H1 and thus is reasonably specific for the smaller HMG polypeptides. When *Tetrahymena* macronuclei are subjected to elutive intercalation significant amounts of LG-1 and LG-2 are released (Fig. 3, lane *3*) with little evidence for release of any other histone or nonhistone protein (Fig. 3). Since LG-1 or LG-2 are not observed in incubations without ethidium bromide (Fig. 3, lane *2*), the data strongly suggest that release of LG-1 and LG-2 is dependent upon the presence of the intercalating drug. Thus, it would appear that the general method of elutive intercalation has now been successfully applied to a lower eukaryote. Because HMGs 14 and 17 are preferentially released from chicken nuclei by elutive intercalation (and not HMGs 1 and 2; Schröter et al., 1985), it is tempting to classify *Tetrahymena* LG-1 and LG-2 (which are also specifically released by this method) as being members of the class of smaller HMG proteins. However, based on other data presented below, we feel it best to not equate LG-1 and LG-2 to either HMG 14 or 17 at this time.

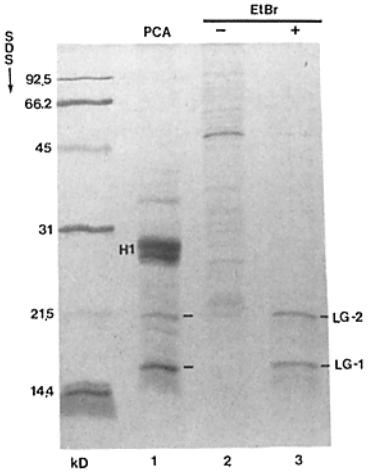

Figure 3. Selective release of LG-1 and LG-2 from macronuclei by elutive intercalation. Isolated macronuclei were washed into buffer E and subjected to elutive intercalation in the presence (+, lane *3*) or absence (–, lane *2*) of 5 mM ethidium bromide (*EtBr*) (see Materials and Methods and Schröter et al., 1985 for details). As a positive control, one aliquot of macronuclei was acid extracted as usual, generating a fraction that is soluble in 5% PCA, but insoluble in 20% TCA (lane *1*). Molecular mass markers expressed in kilodaltons.

Microsequencing

To further study the relationship between LG-1 and LG-2 and to compare these two polypeptides with HMG proteins from higher eukaryotes, LG-1 and LG-2 were purified from 5% perchloric acid extracts of macronuclei by preparative electrophoresis or gel filtration chromatography (see Materials and Methods) and subjected to automated microsequencing. Fig. 4 *A* shows a 47-residue amino-terminal sequence of LG-1 (approximately half of the molecule). This sequenced region of LG-1, like the amino-terminal halves of other sequenced HMGs, contains many basic residues (eight lysine, one arginine, and one histidine residues). Comparison of this sequence with the complete amino acid sequences of calf thymus HMGs 1, 2, 14, and 17 (see Walker, 1982, for amino acid sequences) reveals that LG-1 contains a seven-residue stretch (residues 7–13; Fig. 4 *A*, first bracket) with seven residues directly homologous to residues 28–35 of HMG 17 (six out of seven to the same region of HMG 14) and an 11-residue stretch (12–22; Fig. 4 *A*, second bracket) with 10 matches to an internal sequence found in both HMGs

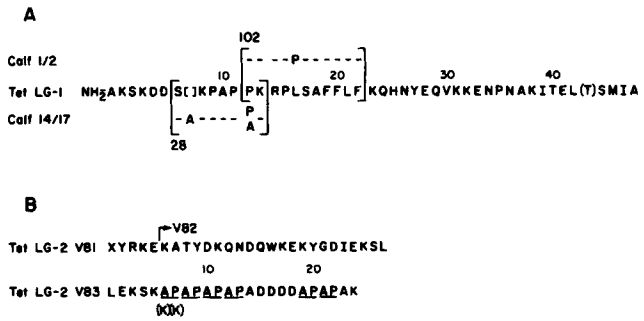

Figure 4. Partial amino acid sequences of LG-1 and LG-2. Shown in *A* is the NH_2-terminal amino acid sequence of LG-1 given in one-letter code. The large brackets contain the regions of homology between LG-1 and calf thymus HMGs 14 and 17 (lower bracket) and between LG-1 and calf thymus HMGs 1 and 2 (upper bracket). Amino acids in HMGs 1, 2, 14, and 17 that are identical to those in LG-1 have been given a dashed line; different amino acids have been indicated. Residue 34 of HMG 14 is an alanine, while the same residue in HMG 17 is a proline. Small bracket after position 7 in the LG-1 sequence indicates an insertion used to align residues 7–13 of LG-1 with residues 28–35 of HMG 14/17. (T) at position 43 indicates that only a tentative identification of this residue as threonine has been made. The first 30 residues of LG-1 have been sequenced twice. Shown in *B* is the primary sequence of three internal fragments of LG-2 (V8 1–3) generated by V8 protease. Arrow after position 5 of the upper sequence shown in *B* (V81) indicates the first residue of fragment V82, which completely overlaps with the remainder of V81. X in position 1 of V81 indicates that the amino acid at this position has not been identified. (K) below positions 6 and 7 of the lower sequence shown in *B* (V83) indicates that a significant amount of the PTH-derivative of lysine was also detected at these positions. Fragment V83 was sequenced twice. See text for further details.

1 and 2 (residues 102–112). The remainder of the partial sequence of LG-1 does not show significant homology to any other sequenced HMG protein.

Three attempts to obtain an amino-terminal sequence of LG-2 were unsuccessful, suggesting that the amino terminus of this polypeptide is blocked. To obtain an internal sequence from LG-2, preparative SDS gel strips were subjected to limited proteolytic digestion with V8 protease (see Materials and Methods). Three V8 peptides so obtained were excised, eluted, and subjected to automated microsequencing. The sequence obtained from these fragments (labeled V8 1–3) is shown in Fig. 4 *B*. Fragments V81 and V82 completely overlap except for five amino acids on the amino-terminal end of V81. A third V8 fragment, V83, produced the lower sequence shown in Fig. 4 *B*.

The first point to emerge upon a comparison between the partial amino acid sequences of LG-1 and LG-2 is that the sequenced regions of these two polypeptides do not share any regions of homology. Furthermore, unlike the amino-terminal half of LG-1, the two sequenced regions of LG-2 do not exhibit any significant homology to the other sequenced HMG proteins. Perhaps the most striking feature of the LG-2 sequence is the occurrence of six repeats of the dipeptide Ala-Pro broken up by a run of four consecutive aspartic acid residues (see the underlined residues in LG-2 V83). While the partial sequences of LG-1 and LG-2 shown in Fig. 4 are not complete enough to rule out a possible precursor–product relationship between LG-2 and LG-1, it is worth noting that neither LG-1 or LG-2 can be derived from histone H1 as discussed previously. The complete amino acid sequence of the *Tetrahymena* histone H1 has recently been determined (Wu et al., 1986), and it does not contain any of the partial sequences of LG-1 or LG-2.

Sequence comparisons between LG-1 and LG-2 and the HMG proteins from higher eukaryotes (see Walker, 1982) suggest that LG-1 and LG-2 are distinct from each other and are not strongly related to the higher eukaryotic HMG proteins. However, because only partial sequences have been obtained, the possibility that LG-1 is proteolytically derived from LG-2 as suggested by the similar V8 peptide maps (Fig. 2), or that LG-1 and LG-2 contain yet unsequenced regions of amino acid sequence homology to other HMG proteins cannot be ruled out.

Secondary Modification

Levy-Wilson et al. (1983) have previously studied several postsynthetic modifications affecting *Tetrahymena* HMG-like proteins. Their results suggested that LG-1 is ADP-ribosylated, that LG-2 is both ADP-ribosylated and phosphorylated, and that neither LG-1 or LG-2 is methylated or acetylated. As shown in Fig. 4 *B*, the amino acid sequence derived from the smallest V8 fragment of LG-2, V83, has two tentative residues (alanine, position 6, and proline, position 7) where significant amounts of lysine were also detected. The appearance of the PTH derivative of lysine at these positions, which was observed on two independent sequencing runs, may be due to a large amount of lysine lagging from cycle 5. However, earlier studies from our laboratory have shown that the PTH derivative of acetyl–lysine elutes at the position of alanine when applied to an HPLC system similar to that used in this study (see Chicoine et al., 1986, and Materials and Methods). Thus, the possibility that the alanine at position 6 of V83 is in fact a modified lysine residue (presumably acetylated) prompted us to reinvestigate some of the secondary modifications of both LG-1 and LG-2.

As shown in Fig. 5 (*A* and *B*), LG-1 and LG-2 are not labeled postsynthetically with [^{3}H]sodium acetate under conditions where ^{3}H-acetate is efficiently incorporated into all of the core histones (see Materials and Methods). LG-1 and LG-2 are also not acetylated when cells are labeled in the absence of cyclohexamide in order to assay synthesis-dependent acetylation (Allis et al., 1985). These results confirm the previous results of Levy-Wilson et al. (1983) and strongly suggest that the presence of both alanine and lysine at cycle 6 of LG-2 fragment V83 is not due to the presence of acetyl–lysine. Similarly, it is clear that the PTH derivative of methyl–lysine does not co-migrate with alanine under our HPLC column conditions. Thus, it is unlikely that residue 6 of V83 represents methyl–lysine. The basis for the heterogeneity of LG-2 at this position is not certain.

Fig. 5 (*C* and *D*) also shows that both LG-1 and LG-2 are not phosphorylated when vegetative cells are labeled with ^{32}P-orthophosphate under conditions that strongly label histones H1 and H2A. The absence of ^{32}P-phosphate on LG-2 contradicts the previous results of Levy-Wilson et al. (1983). This contradiction is most likely explained by the failure of these authors to use a two-dimensional gel system to separate LG-2 from minor phosphorylated polypeptides that co-migrate in this region of acid-urea gels.

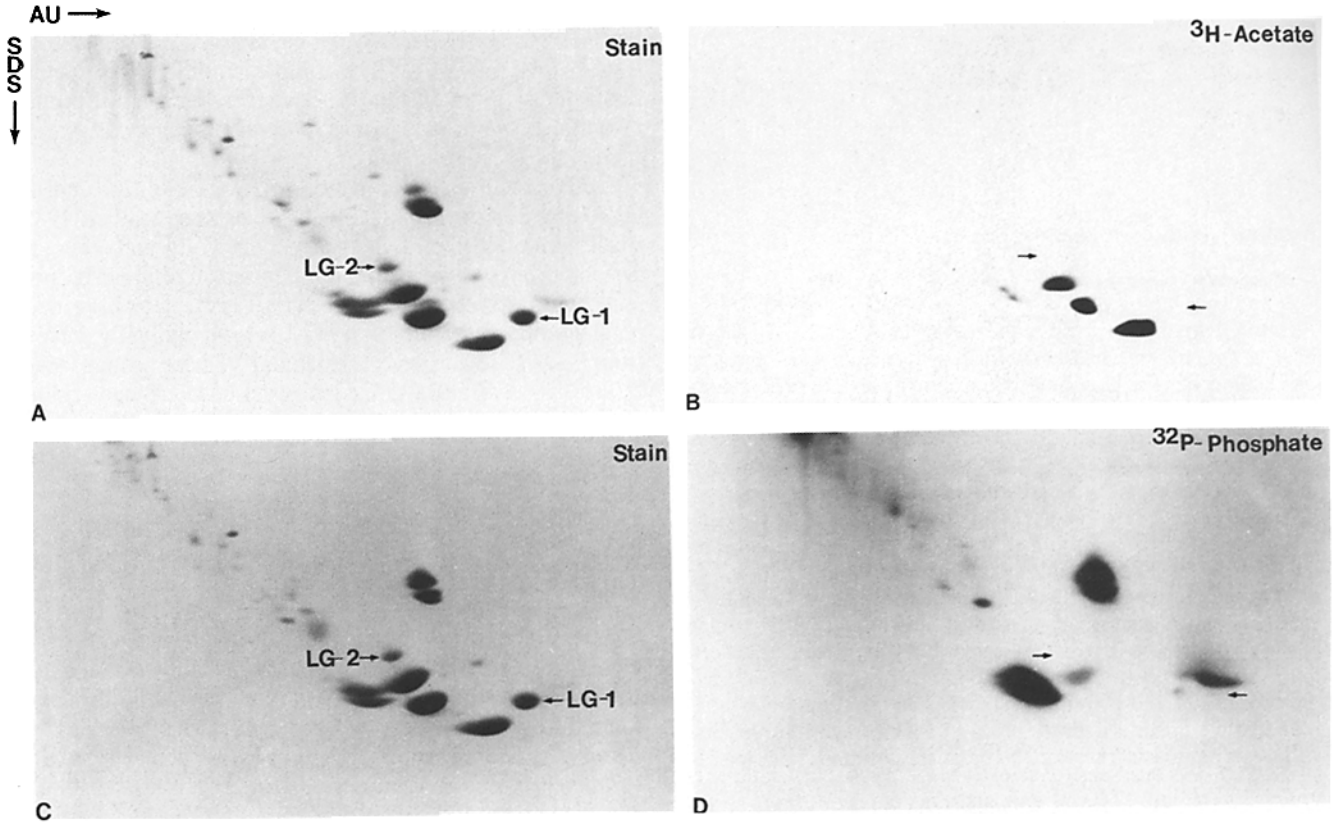

Figure 5. Patterns of acetylation and phosphorylation associated with macronuclear total acid-soluble protein. Growing *Tetrahymena* were labeled with either [^{3}H]sodium acetate (*A* and *B*) or ^{32}P-orthophosphate (*C* and *D*) (see Materials and Methods) before macronuclei were isolated. Total acid-soluble proteins were subjected to electrophoresis on two-dimensional gels (acid–urea by SDS) and analyzed by staining (*A* and *C*) or fluorography (*B* and *D*). Arrows in *B* and *D* indicate the positions of LG-1 and LG-2. A highly phosphorylated band that migrates directly above LG-1 in this gel system has consistently been observed.

Immunological Comparisons

To further address the question of possible relationships between LG-1, LG-2, and other HMG-proteins, SDS gel-purified LG-1 and LG-2 were used to immunize rabbits, and polyclonal antibodies raised to both of these proteins were obtained. As can be seen from the immunoblot in Fig. 6, antibodies against either LG-1 or LG-2 recognize only the original antigen when tested against total macronuclear protein (lanes *6* and *10*). This result, along with the absence of sequence homology, strongly suggests that a precursor–product relationship does not exist between LG-1 and LG-2. Furthermore, these data suggest that LG-1 and LG-2 do not arise from a larger yet unidentified precursor in macronuclei. It must be pointed out, however, that the polyclonal antibodies specific to each protein may be recognizing a small number of unshared epitopes. When the antibodies specific for either LG-1 or LG-2 are tested against HMG proteins isolated from calf thymus or chicken erythrocytes under conditions where strong reactivity to the original *Tetrahymena* antigen is observed, cross-reactivity to the calf or chicken HMG proteins does not occur (Fig. 6, lanes *7*, *8*, *11*, and *12*). Similarly, LG-1 and LG-2 antibodies fail to cross react with yeast HMG-like proteins (data not shown). Antiserum against LG-1 occasionally exhibits very weak cross-reactivity to both the *Tetrahymena* and calf thymus H1 molecules, whereas polyclonal antibodies against the *Tetrahymena* H1 never exhibit any cross-reactivity to either LG-1 or LG-2 (data not shown). These immunological results along with the sequence comparisons (Fig. 4) suggest that the *Tetrahymena* HMG proteins, LG-1 and LG-2, are significantly different from each other and from the higher eukaryotic HMG proteins.

Synthesis of LG-1 and LG-2 during Conjugation

The immunological and amino acid sequence data suggest that LG-1 and LG-2 are two distinct proteins. To gain more insight into possible functions of these proteins, the synthesis of LG-1 and LG-2 during the sexual phase of the *Tetrahymena* life cycle, conjugation, was investigated. Conjugation is an ordered developmental process during which cells of opposite mating type pair, undergo meiosis, and genetic exchange that results in the formation of new somatic (macro-) and germinal (micro-) nuclei (Ray, 1956). To determine when during conjugation LG-1 and LG-2 are synthesized, mating cells were pulsed for 1 h with [^{3}H]lysine at various times throughout conjugation. LG-1 and LG-2 were then isolated by extracting whole cells directly in 5% perchloric acid (see Materials and Methods). Direct extraction of whole cells or tissue in perchloric acid is a procedure developed by Johns and co-workers (see Nicolas and Goodwin, 1982) to isolate HMG proteins with a minimal amount of proteolysis. This method was used to reduce the possibility that any

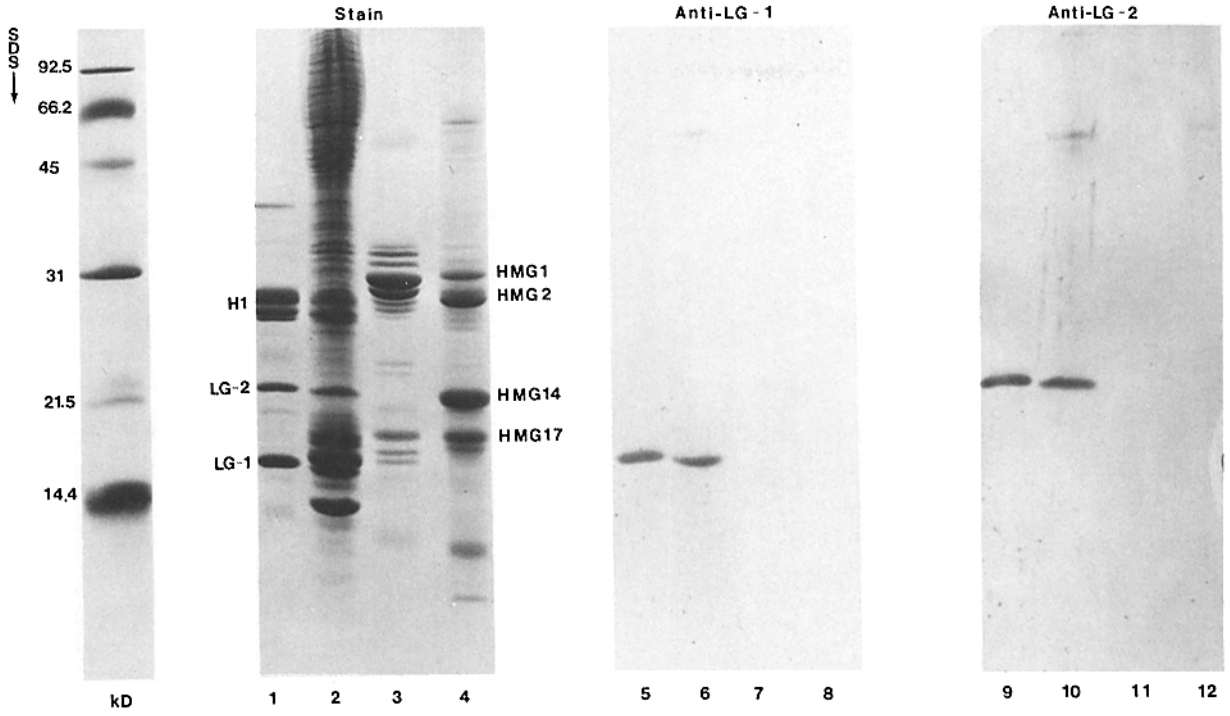

Figure 6. Immunoblotting of total macronuclear protein and HMG 1, 2, 14, and 17. Macronuclear 5% PCA-soluble protein (lanes *1*, *5*, and *9*), SDS-dissolved macronuclei (lanes *2*, *6*, and *10*), calf thymus HMGs 1, 2, 14, and 17 (lanes *3*, *7*, and *11*), and chicken erythrocyte HMGs 1, 2, 14, and 17 (lanes *4*, *8*, and *12*) were subjected to electrophoresis in 15% SDS gels, blotted to nitrocellulose, and probed with polyclonal antibodies raised against either LG-1 or LG-2 (see Materials and Methods). Lanes *1–4*, parallel Coomassie-stained gel. Lanes *5–8*, a blot with anti-LG-1 (1/200). Lanes *9–12*, a blot with anti-LG-2 (1:200). All samples shown were subjected to electrophoresis in the same polyacrylamide gel; spaces between some of the lanes were included for clarity. Molecular mass markers expressed in kilodaltons.

differences observed in the levels of LG-1 or LG-2 throughout conjugation were the result of differential proteolysis.

As can be seen in Fig. 7, although the stainable amount of LG-1 and LG-2 remains approximately constant throughout conjugation (Fig. 7 *A*), these proteins are synthesized maximally at different times (Fig. 7 *B*). Synthesis of LG-1 begins to increase from a basal level seen in starved and young conjugating cells (Fig. 7 *B*, lanes *1–7*) at 6–7 h (Fig. 7 *B*, lane *8*) and continues to increase gradually thereafter (cf. *7*, *9*, *11*, and *13* h, Fig. 7 *B*, lanes *8–11*). This time period (7–13 h) corresponds precisely to the developmental period when new developing macronuclei (anlagen) differentiate from micronuclei and begin to acquire newly synthesized macronuclear-specific chromatin-associated proteins (Allis and Wiggins, 1984; Wenkert and Allis, 1984; Chicoine et al., 1984).

Unlike LG-1, LG-2 synthesis is induced as early as 1 h after cells of opposite mating type have been mixed and typically reaches a maximum at 3 h (Fig. 7 *B*, lanes *5* and *6*). After this early period, LG-2 synthesis decreases between 4 and 7 h (Fig. 7 *B*, lanes *7* and *8*), and then gradually increases again during the period of anlagen differentiation (7–14 h, Fig. 7 *B*, lanes *8–11*) in a manner similar to that seen with LG-1. Thus, while LG-1 and LG-2 appear to be coordinately synthesized during the development of new macronuclei, a specific induction of LG-2 synthesis takes place during the early stages of conjugation (2–4 h). Furthermore, similar results are obtained when preparations of macronuclei (or anlagen) prepared from mating cells (labeled as in Fig. 7) are subjected to analysis similar to that shown in Fig. 7 (data not shown). This result suggests that LG-1 and LG-2 are synthesized and deposited into macronuclei or anlagen during these intervals of conjugation.

Maximal synthesis of LG-2 at 3 h takes place during meiotic prophase at a time when a burst of both conjugation-specific protein and RNA synthesis is known to occur (Martindale et al., 1985). Interestingly, this early synthesis coincides with a marked decrease in the electrophoretic mobility of histone H1 (see *arrows*, Fig. 7 *A*, lane *5*, and Fig. 7 *C*, lane *3*), which is observable at 1 h and continues until 7 h (see Fig. 7 *A*, lane *8*, and Fig. 7 *C*, lane *7*). This shift in the electrophoretic mobility of H1 has previously been shown to be a result of hyperphosphorylation (Glover et al., 1981). Equally striking is the finding that at 7 h, a period that closely coincides with the initiation of anlagen differentiation, a significant amount of H1 is dephosphorylated (see *arrows*, Fig. 7 *A*, lane *8*, and Fig. 7 *C*, lane *7*). These shifts in the electrophoretic mobility of H1 are extremely reproducible and particularly well resolved when perchloric acid-soluble proteins are resolved in long acid-urea gels (see Fig. 7 *C*). The significance of these changes in the phosphorylation levels of H1 and their relationship, if any, to the early induction of LG-2 synthesis is presently not clear (see Discussion).

Discussion

Two proteins, LG-1 and LG-2, isolated from macronuclei of *Tetrahymena thermophila* have previously been classified as

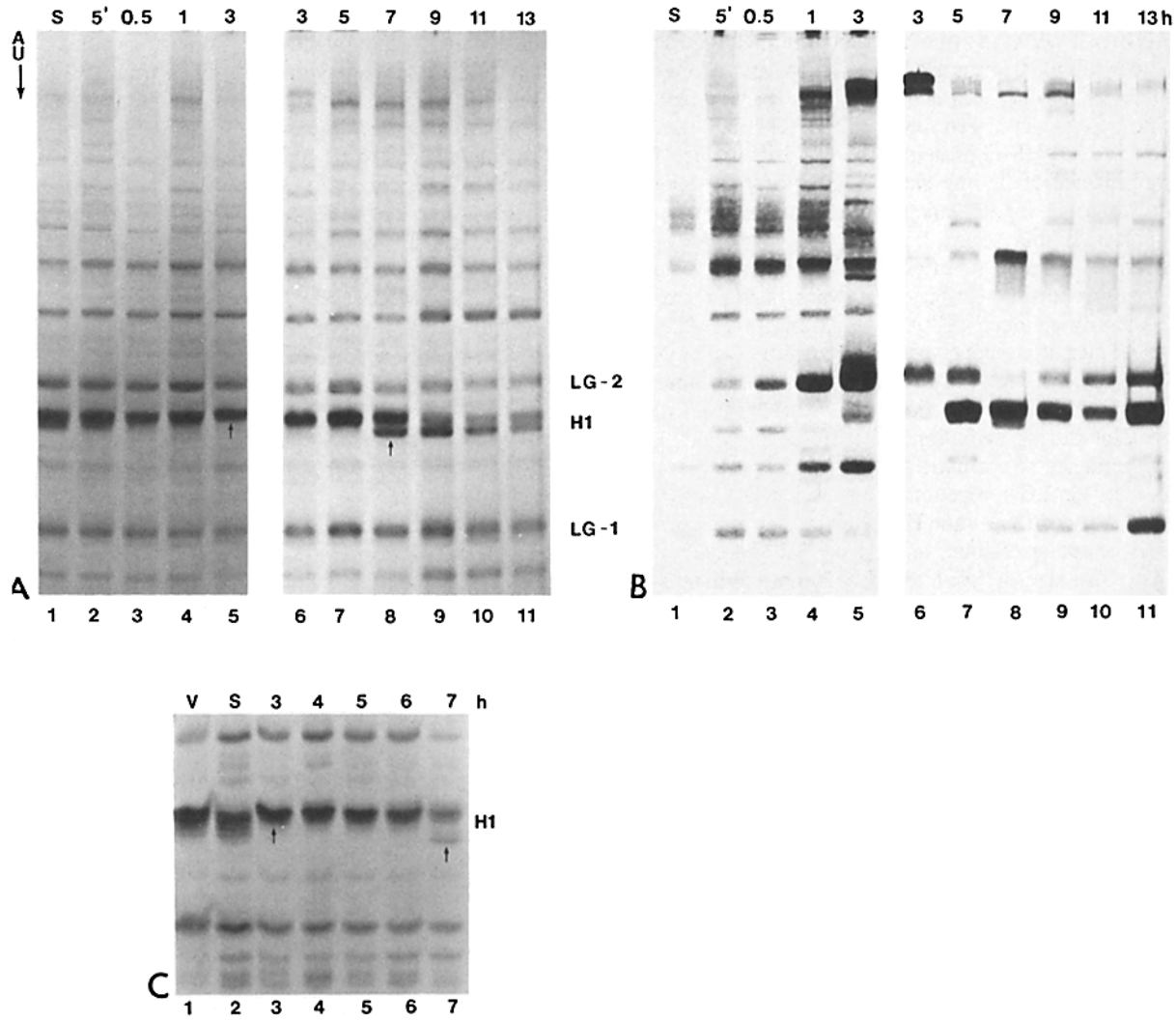

Figure 7. Synthesis of LG-1 and LG-2 during conjugation. Mating cells were pulsed for 1 h with [^{3}H]lysine (1 μCi/ml) at various times throughout conjugation; total cell PCA-soluble protein was isolated (see Materials and Methods) and resolved on 15% acid-urea gels. (*A*) Stained gels; (*B*) fluorograph of the same gels shown in *A*. Lanes *1–5* (*A* and *B*) correspond to a single experiment in which the percentage of paired cells was 98%. Lanes *6–11* (*A* and *B*) correspond to a second experiment in which the percent pairing was 92%. The two independent experiments overlap at the 3-h time point (see lanes *5* and *6*). Lane *1*, starved cells were labeled for 1 h; lane *2*, 5 min. Starved cells were labeled for 55 min before mixing cells of opposite mating types. Conjugation was then allowed to proceed for 5 min; lane *3*, 30 min. Starved cells were labeled for 30 min before mixing opposite mating types. Conjugation was then allowed to proceed for 30 min; lane *4*, cells labeled from 0–1 h of conjugation; lanes *5* and *6*, 2–3 h; lane *7*, 4–5 h; lane *8*, 6–7 h; lane *9*, 8–9 h; lane *10*, 10–11 h; lane *11*, 12–13 h. (*C*) Close up of the histone H1 region of a Coomassie-stained long acid-urea gel used to resolve changes in the level of H1 phosphorylation. Lane *1*, vegetative cells labeled for 1 h; lane *2*, starved cells labeled for 1 h; lane *3*, cells labeled from 2–3 h of conjugation; lane *4*, 3–4 h; lane *5*, 4–5 h; lane *6*, 5–6 h; lane *7*, 6–7 h. Arrows in *A* and *C* denote the position of hyperphosphorylated H1 at 3 h (lanes *A 5* and *C 3*) and the position of dephosphorylated H1 at 7 h (lanes *A 8* and *C 7*). The data for *C* were obtained from an independent experiment in which the percent pairing was 85%.

being members of the high mobility group family of chromosomal proteins (Hamana and Iwai, 1979; Levy-Wilson et al., 1983). The copurification of LG-1 and LG-2 with *Tetrahymena* histone H1 and the low yield of LG-1 and LG-2 in nuclei prepared by the method of Gorovsky et al. (1975), however, suggested the possibility that LG-1 and LG-2 could be proteolytic breakdown products of H1. In this study partial amino acid sequences of both LG-1 and LG-2 have been determined, establishing that the above hypothesis cannot be the case.

The question remains as to whether either LG-1 or LG-2 are in fact HMG proteins. In this report we have used a relatively new procedure, elutive intercalation, to ask whether LG-1 and LG-2 behave similarly to HMGs 14 and 17 from higher organisms (Schröter et al., 1985). Indeed, both LG-1 and LG-2 are specifically released from macronuclei by this procedure which suggests that these polypeptides may share some property (properties) with the smaller pair of HMG proteins (14 and 17). Considering their solubility in 5% PCA (and TCA), their high mobility in polyacrylamide gels, their amino acid compositions, and their extractability from chromatin by 0.35 M NaCl and now elutive intercalation (Hamana and Iwai, 1979; Levy-Wilson et al., 1983; this report), we feel that it is reasonable to consider these proteins as being

HMG-like. However, when the partial amino acid sequences of LG-1 and LG-2 are compared with the complete amino acid sequences of calf thymus HMGs 1, 2, 14, and 17 (see Walker, 1982, for amino acid sequences), only two small regions of homology have been identified in LG-1 that exist in these well-studied HMG proteins (see brackets in Fig. 4 *A*). The significance of finding short regions of homology from both classes of higher eukaryotic HMG proteins in LG-1 is not clear. In this regard, however, it is interesting to note that a *Tetrahymena* H2A variant, hv1, has recently been shown to have properties exhibited by two separate mammalian H2A family members (H2A.X and H2A.Z, see Allis et al., 1986). Thus, it is conceivable that primitive eukaryotes like *Tetrahymena* contain proteins (like LG-1) whose structural and functional properties have been imparted to multiple proteins during evolution.

The high frequency of aromatic and certain apolar amino acids (V, L, and I) in LG-1 appears to rule out a strong structural similarity between LG-1 and HMG 14 or 17 (which contain few of these apolar residues and are devoid of aromatic amino acids). The size of LG-1 and LG-2, approximately half the molecular weight of HMG 1 or 2, and the selective extractability of these proteins by elutive intercalation also suggests that these proteins are not strongly related to the larger class of HMG proteins. These conclusions are reinforced by the failure of polyclonal antibodies specific to either LG-1 or LG-2 to cross react with HMG 1, 2, 14, or 17 isolated from calf thymus or chicken erythrocyte. The absence of immunological cross-reactivity and amino acid sequence homology is not surprising in light of recent results of Vanderbilt and Anderson (1985), who failed to observe cross-reactivity when *Drosophila* and yeast extracts were tested with a series of monoclonal antibodies against hen erythrocyte HMGs 1 and 2, and Landsman et al. (1986), who failed to observe any cross-hybridization when Southern blots of *Drosophila* and yeast DNA were probed with a clone to the human HMG 17 gene. In view of the paucity of amino acid homology and immunocross-reactivity between LG-1 and LG-2 and other sequenced HMG proteins, we feel that it is best to consider these proteins as being only "HMG-like".

The possibility that LG-1 and LG-2, like the members of each pair of higher eukaryotic HMG proteins, are strongly related to each other appears to be ruled out by the absence of amino acid sequence homology and immunological cross-reactivity between these two proteins. However, because only partial amino acid sequences have been obtained, the possibility that LG-1 and LG-2 share yet undetermined homologies in primary structure cannot be completely eliminated until the complete amino acid sequence of these proteins is known.

The temporal order of LG-1 and LG-2 synthesis during the sexual phase of the *Tetrahymena* life cycle, conjugation, also supports the hypothesis that LG-1 and LG-2 are two distinct proteins. Both LG-1 and LG-2 are synthesized relatively late in conjugation (7–13 h) at a time when developing new macronuclei are beginning initial rounds of endoreplication (2–8C, Allis and Dennison, 1982) and therefore require newly synthesized chromosomal proteins. Not surprisingly, other macronuclear-specific histones are also synthesized during this period (Allis and Wiggins, 1984; Wenkert and Allis, 1984; Chicoine et al., 1984). However, a specific peak of LG-2 synthesis also occurs early in conjugation (3 h) at a time when only basal synthesis of LG-1 is detected. The finding that LG-2 is synthesized independently of LG-1 during this early period of conjugation suggests that these proteins may have different functions.

The finding that LG-2 is synthesized early in conjugation (3 h, which corresponds to meiotic prophase) is an unexpected result. One would not necessarily expect "parental" macronuclei, which do not divide and will ultimately be degraded later in conjugation (see Wenkert and Allis, 1984), to require newly synthesized chromosomal proteins. We point out, however, that macronuclei are quite active in synthesis of both meiotic-specific RNA (Martindale et al., 1985) and histone (Allis and Wiggins, 1984) to support the active divisions made by micronuclei during this interval of conjugation. In fact, it is a formal possibility that the LG-2 synthesized during this early period of conjugation is associated with micronuclei. We feel, however, that this is unlikely since a considerable amount of the LG-2 synthesized during this early period of conjugation is associated with nuclear preparations that are greatly enriched in macronuclei. Furthermore, a considerable amount of the macronuclear-specific histone H1 is also synthesized during prezygotic stages of conjugation (see Fig. 7 *B*, lane *7*), even though these macronuclei do not divide. Interestingly, the early induction of LG-2 synthesis always precedes that of H1 (see Fig. 7 *B*).

The prezygotic synthesis of LG-2 has also been shown to coincide with another conjugation-induced event, the hyperphosphorylation of histone H1. This hyperphosphorylation is apparent as early as 1 h into the conjugation process and continues until 7 h when an abrupt dephosphorylation is observed. Earlier studies have determined two other conditions during which the *Tetrahymena* H1 is hyperphosphorylated: (*a*) an increase in phosphorylation is seen when vegetatively growing cells are compared with starved cells (Allis and Gorovsky, 1981; and see Fig. 7 *C*, lanes *1* and *2*); and (*b*) a hyperphosphorylation occurs when starved cells are subjected to stress such as heat shock (Glover et al., 1981). To further demonstrate that the synthesis of LG-2 is not simply a stress-induced phenomenon and is indeed conjugation specific, starved cells have been placed on a fast shaker under conditions that block costimulation, a prerequisite for conjugation (Bruns and Brussard, 1974). Cells treated in this manner and labeled with [^{3}H]lysine between 2 and 3 h do not hyperphosphorylate H1 or synthesize LG-2 (data not shown). Also, when starved cells are heat shocked under conditions that induced hyperphosphorylation of H1 (Glover et al., 1981) synthesis of LG-2 does not take place (data not shown). Thus, the induction of LG-2 synthesis and the hyperphosphorylation of H1 that occur in prezygotic conjugating cells is not apparently a result of stress and can be dissociated from stress-induced H1 hyperphosphorylation.

H1 is known to be an important mediator of higher order chromatin structure (Thoma and Koller, 1977; Thoma et al., 1979), and it has been suggested that the stress-induced phosphorylation of H1 in *Tetrahymena* may result in changes in chromatin structure that play a role in either the induction of stress protein RNA synthesis or in the general depression of RNA synthesis that usually accompanies the stress response (Glover et al., 1981). Interestingly, the macronucleus of an early conjugant may be undergoing a series of events similar to that of the macronucleus of a heat-shocked cell.

Both nuclei are activating a set of novel genes (stress proteins or meiosis-specific proteins, respectively; see Martindale et al., 1985), and the macronucleus of an early conjugant, which will be degraded later in conjugation, may also be shutting down a large part of its genome. The possibility that the synthesis of LG-2 and the hyperphosphorylation of H1 are somehow linked to either of these events is under examination.

Besides questions of relatedness between LG-1 and LG-2 and other HMG proteins, the identification of the functions of these two proteins remains an important issue. Both LG-1 and LG-2 are associated with sucrose gradient-pure mononucleosome core particles (data not shown; see Levy-Wilson et al., 1983), which suggests that both proteins are integral components of macronuclear chromatin. The prezygotic induction of LG-2 synthesis suggests the possibility that this protein may be involved in conjugation-specific events that take place in "parental" macronuclei. On the other hand, the gradual increase in the synthesis of LG-1 and LG-2 that takes place during the development of new macronuclei suggests that both LG-1 and LG-2 are required for the formation of transcriptionally active chromatin.

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