Isolation, Characterization, and Postsynthetic Modifications of *Tetrahymena* High Mobility Group Proteins†

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ABSTRACT: We have isolated four major high mobility group (HMG) proteins designated A, B, C, and D, together with ubiquitin from the ciliate protozoan *Tetrahymena*. These four HMG proteins are integral structural components of macronuclear nucleosomes. The proteins exhibit solubility properties, chromatographic behavior on carboxymethylcellulose, electrophoretic mobilities on various gel systems, and amino acid compositions similar to those of their mammalian counterparts. HMG-A is the largest, most acidic protein of the group and is phosphorylated in vivo at specific serine residues. HMG-B is both phosphorylated at serine residues and ADP ribosylated. HMG-C is not phosphorylated but is ADP ribosylated. HMG-D, the smallest, most basic protein of the group possesses an unusually high content of serine and threonine residues, and it is highly phosphorylated at both serine and threonine positions in the polypeptide chain.

The high mobility group (HMG) chromosomal proteins are a group of non-histone proteins that can be extracted from nuclei or chromatin with 0.35 M NaCl (Johns, 1982). HMG's can also be solubilized from whole cells with 5% perchloric acid (Goodwin et al., 1975) or 5% trichloroacetic acid (Watson et al., 1979). Four major HMG proteins have been isolated from mammalian cells: HMG-1, -2, -14, and -17 (Johns, 1982; Levy-Wilson & Dixon, 1978a). HMG-1 and -2 are larger and slightly more acidic than HMG-14 and -17 (Walker, 1982). The latter two proteins are fundamental structural components of transcriptionally active nucleosomes (Levy-Wilson et al., 1979; Egan & Levy-Wilson, 1981; Weisbrod & Weintraub, 1979). HMG's in mammalian cells are subject to various kinds of postsynthetic modifications, such as acetylation (Sterner et al., 1978, 1979), methylation (Boffa et al., 1979), phosphorylation (Levy-Wilson, 1981a; Hasuma et al., 1980), and glycosylation (Reeves et al., 1981). In trout testes, the two major HMG proteins, HMG-T (equivalent to mammalian HMG-1 and -2) and H6 (equivalent to mammalian HMG 17), are also ADP ribosylated (Wong et al., 1977; Levy-Wilson, 1981b). The precise functional significance of these modifications remains obscure.

Tetrahymena is a unicellular ciliate protozoan. Each cell possesses two distinct nuclei within the same cytoplasm. The macronucleus is transcriptionally active while the micronucleus is transcriptionally inert. Therefore, this organism is useful for correlating the role of HMG proteins with transcriptional activity. It is known that a major fraction of the macronuclear genome is transcribed (Stathopoulos et al., 1980); therefore, if the presence of HMG proteins is correlated with transcriptional activity, these proteins should be found in abundance in *Tetrahymena* macronuclei. To date, only two HMG proteins have been isolated from *Tetrahymena* and designated LG-1 and LG-2 (Hamana & Iwai, 1979). They are present in *Tetrahymena* macronuclei in half molar amounts compared to histone H1.

We present evidence that, in addition to these two HMG proteins already described (which we designate as C and B, respectively), there exist two other major (A and D) and several minor HMG-like proteins together with ubiquitin in macronuclei from *Tetrahymena* BVII or GL. We have asked whether these proteins are subject to secondary modifications, such as acetylation, phosphorylation, ADP ribosylation, glycosylation, and methylation.

Materials and Methods

Cell Culture and in Vivo Labeling. *Tetrahymena thermophila*, strain BVII, and *Tetrahymena pyriformis*, strain GL, were cultured axenically in 1% proteose peptone (Gorovsky et al., 1975). Cells were labeled for 20 h with either ^{3}H-labeled amino acids or [^{32}P]orthophosphate in media consisting solely of 10 mM Tris-HCl,[1] pH 8.0 (starved conditions). In this medium, incorporation of amino acids and phosphate into macromolecules is linear for several hours.

Isolation of Nuclei. Nuclei were obtained from *Tetrahymena* cells by a modification of the procedure of Zaug & Cech (1980). Exponentially growing cells were recovered by centrifugation for 20 min at 5000 rpm in a Sorvall GSA rotor. All subsequent steps were performed at 0 °C, and all solutions contained the protease inhibitor PMSF (phenylmethanesulfonyl fluoride) at a final concentration of 1 mM. The cell pellet was resuspended in a solution of TMS (0.25 M sucrose, 10 mM $MgCl_2$, 3 mM $CaCl_2$, 10 mM Tris-HCl, pH 7.5), at a density of 3×10^6 cells/mL. Nonidet P-40 (10% v/v) was added for a final concentration to 0.32%, and the cell suspension was agitated for about 1 h in a tissue homogenizer or until nuclei appeared under the microscope to be free of cellular debris. At that point, crystals of sucrose were added slowly (0.813 g/mL) and allowed to dissolve by continuing the homogenization for another 30–40 min on ice. Nuclei were then pelleted by centrifugation for 30 min at 10000 rpm in a Sorvall SS-34 or SA 600 rotor. The nuclear pellets were combined and washed 2 or 3 times more with TMS prior to counting and further use. This procedure, when applied to *Tetrahymena* GL cells, yields intact transcriptionally active macronuclei. With BVII cells, one obtains macronuclei in high yields (>80%) and variable amounts of the transcriptionally inactive micronuclei.

Labeling of Nuclei with [^{32}P]*NAD.* Purified nuclei derived from exponentially growing cells were resuspended in a solution containing 0.25 M sucrose, 10 mM Tris-HCl, pH 8.2, 60 mM KCl, 10 mM $MgCl_2$, and 4 mM NaF and incubated with 0.5

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[1] Abbreviations: Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; $NaDodSO_4$, sodium dodecyl sulfate; DTT, dithiothreitol; PCA, perchloric acid.

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mCi of [^{32}P]NAD$^+$ (10–50 Ci/mmol) for 45 min at 10 °C. Nonradioactive NAD$^+$ was then added at a final concentration of 1 mM for another hour (Levy-Wilson, 1981b).

HMG Isolation from Isolated Nuclei. HMG's were purified by extraction with 0.35 M NaCl from a minimum of 10^9 nuclei. The nuclear pellet, obtained as described above, was resuspended gently in a solution of 10 mM EDTA, pH 8.0. The suspension was homogenized on a Dounce homogenizer and maintained at 4 °C for 15–20 min to promote the formation of a chromatin gel. The lysed nuclei were recovered by centrifugation for 20–30 min at 10000 rpm in a Sorvall HB-4 rotor. The resulting chromatin suspension was homogenized in 10 mM Tris, pH 8.0, and NaCl was added to a final concentration of 0.35 M. The mixture (5–8 mL) was sheared in a Waring blender for 3 s at low speed and stirred for 45 min to 1 h at 0 °C, to promote solubilization of HMG's. The 0.35 M salt soluble fraction was recovered as a supernatant after centrifugation for 30 min at 10000 rpm in a Sorvall HB-4 rotor.

The residual nuclear pellet fraction was extracted again with 5–8 mL of the 0.35 M NaCl solution. The two supernatants were combined and trichloroacetic acid was added to a final concentration of 2%, to selectively precipitate the low mobility group proteins (LMG's). After 1 h in 2% Cl_3CCOOH, the contaminating LMG's were removed by centrifugation for 45 min at 10000 rpm in the Sorvall HB-4 rotor. The high mobility group proteins were recovered from the supernatant by precipitation with 22% Cl_3CCOOH for 16 h, at 4 °C. Excess Cl_3CCOOH was removed from the purified HMG's by repeated washing with 95% ethanol. The HMG's were lyophilized and stored at −20 °C. This procedure can be applied to either fresh or frozen nuclei. The average yield of HMG's is about 1 mg/(2 × 10^9 nuclei). HMG proteins were also isolated from whole cells by two other procedures, one based on their solubility in 3–5% perchloric acid (Goodwin et al., 1975) and the other based on the solubility of small HMG's in 5% Cl_3CCOOH (Watson et al., 1979).

Chromatography on Carboxymethylcellulose. [^{3}H]-Lysine-labeled HMG proteins (2 mg, 560000 cpm) were dissolved in 0.1 M LiCl, pH 8.0, and separated on a 1.5 cm × 3 cm column of carboxymethylcellulose (CM-52) equilibrated in 0.1 M LiCl, pH 9.0, as described previously (Levy-Wilson & Dixon, 1978a). The column was first washed with 25 mL of 0.1 M LiCl, pH 9.0, as described (Levy-Wilson & Dixon, 1978a). A linear gradient of 70 mL of 0.1–0.6 M LiCl was then applied at a flow rate of 12 mL/h. Fractions of 0.5 mL were collected and assayed. Peaks of labeled protein were pooled and analyzed.

Polyacrylamide Gel Electrophoresis of HMG Proteins. *Tetrahymena* HMG proteins were analyzed by polyacrylamide gel electrophoresis in various systems. Acid–urea gels containing or lacking Triton X-100 at a concentration of 0.22% were used as previously described (Egan & Levy-Wilson, 1981; Levy-Wilson, 1981a). Sodium dodecyl sulfate containing 10% acrylamide gels were also run according to the procedure of Laemmli (1970). Finally, two-dimensional gels, consisting of a first-dimension Triton-containing acid–urea gel (TAU) and a second-dimensional $NaDodSO_4$ gel, were used, as described by Allis et al. (1980), with some modifications. Conditions for staining of the gels and autoradiography of [^{32}P]orthophosphate-labeled samples were as previously described (Levy-Wilson, 1981a).

Amino Acid Analysis of Purified HMG's. Individual protein bands were sliced out of acid–urea or $NaDodSO_4$ gels and eluted for further analysis. Samples derived from acid–urea gels were eluted by incubation of the gel slices in 60% formic acid for three consecutive periods of 50 min, followed by centrifugation to recover the solubilized proteins and lyophilization. Hydrolysis of the proteins was at 110 °C for 20 h in 6 N HCl. Amino acid analysis was performed in a Durrum Model D-502 amino acid analyzer.

Protein bands derived from $NaDodSO_4$ gels were eluted in a solution of 0.05 M NH_4HCO_3,–0.1% $NaDodSO_4$ as described by Beemon & Hunter (1978). Hydrolysis was for 19 h at 115 °C in 6 N HCl. The amino acids were then converted into their *N*-heptafluorobutyrylisobutyl derivatives (Rhodes et al., 1981) and analyzed by both gas chromatography and mass spectroscopy.

Identification of Phosphorylated Residues. For each individual phosphorylated HMG protein, we determined the modified residues as follows. ^{32}P-Labeled protein bands were eluted from either acid–urea or $NaDodSO_4$ gels by the procedure of Beemon & Hunter (1978). The proteins were then partially hydrolyzed in 6 N HCl at 110 °C and subjected to two-dimensional thin-layer chromatography on cellulose plates as described by Sefton et al. (1980). Standards containing known amounts of known phospho amino acids were run in parallel with the samples, to allow for identification of the labeled residues.

Phosphatase Treatment of Phosphorylated HMG's. Aliquots (50–100 μg) containing some 5000–10000 cpm of phosphorylated HMG's were incubated in a total volume of 20 μL with calf intestinal alkaline phosphatase (2 units, Boehringer Mannheim) in a solution also containing $ZnSO_4$ (0.01 M, 3 μL), $MgCl_2$ (0.1 M, 3 μL), Tris-HCl (1 M, 3 μL), pH 8.0, and PMSF (0.025 M, 3 μL) (Levy-Wilson, 1981a). Alternatively, similar samples were incubated with rabbit muscle phosphoprotein phosphatase (Swarup et al., 1981) in a solution of 50 mM Tris-HCl, pH 7.5–1 mM DTT.

Snake Venom Phosphodiesterase Treatment of ADP-Ribosylated Proteins. Aliquots of ADP-ribosylated HMG's (~5000–10000 cpm) were incubated with snake venom phosphodiesterase for 2 h at 37 °C as previously described (Levy-Wilson, 1981b).

Micrococcal Nuclease Digestion of Tetrahymena Pyriformis (GL) Macronuclei. Intact macronuclei (100 A_{260}/mL) were fractionated into micrococcal nuclease sensitive and resistant fractions by the procedure of Levy-Wilson & Dixon (1978a). Digestion was performed in TMS buffer with micrococcal nuclease at a concentration of 0.4 unit/A_{260} of nuclei for 5–10 min at 37 °C. Separation of the various fractions was exactly as described by Egan & Levy-Wilson (1981).

Results

Tetrahymena Nuclei Have Four Major HMG Proteins and Ubiquitin. Nuclei were purified from *Tetrahymena thermophila* (BVII), and HMGs were isolated by extraction with 0.35 M NaCl, followed by selective Cl_3CCOOH precipitation. Figure 1 shows the electrophoretic profiles of these proteins on Triton-containing acid–urea gels (TAU). Four major proteins are visible in slot 2: A, B, U, and D. HMG-A comigrates on these gels with calf thymus HMG-1 and -2. HMG-B has a slightly faster mobility than HMG-1 and -2. U comigrates with calf thymus ubiquitin (slot 1) and copurifies with *Tetrahymena* HMGs by this procedure, as do their mammalian (Walker et al., 1978) and fish (Watson et al., 1978) counterparts. HMG-D comigrates with calf thymus HMG-17 (slot 3). There is an additional HMG protein, HMG-C, that cannot be seen in the particular salt extract illustrated in slot 2. This protein is only slightly salt soluble,

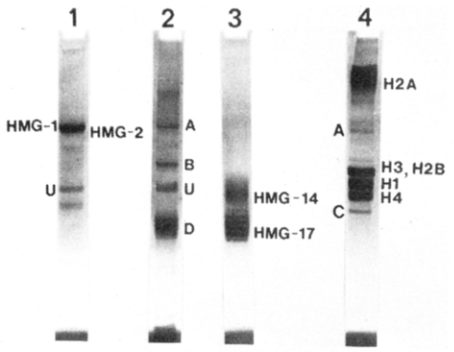

FIGURE 1: Triton–acid–urea (TAU) polyacrylamide gel electrophoresis of *Tetrahymena* HMG's. Slot 1 shows the mobilities of a standard sample of calf thymus HMG-1 and -2 and ubiquitin (U). Slot 2 shows HMG's from *Tetrahymena* BVII, extracted with salt. Slot 3 shows calf thymus HMG-14 and -17. Slot 4 represents 0.4 N sulfuric acid extract of the residual nuclear pellet after salt extraction of HMG's.

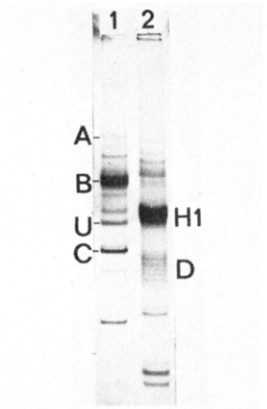

FIGURE 2: Solubilities of *Tetrahymena* HMG's in perchloric acid (PCA) and trichloroacetic acid. Electrophoresis, on a TAU gel, was as in Figure 1. Slot 1 shows HMG proteins soluble in 5% PCA; slot 2 shows HMG proteins soluble in 5% Cl_3CCOOH.

but it is readily solubilized together with the histones in 0.4 N H_2SO_4, as shown in slot 4. The electrophoretic behavior of HMG-C in TAU gels resembles that of calf thymus HMG-14 (slot 3). Therefore, we conclude that at least four major HMG proteins with electrophoretic mobilities similar to those of calf thymus HMG's exist in *Tetrahymena*. It should be noted that essentially the same pattern of HMG proteins is found in the macronucleus of *Tetrahymena pyriformis* (GL), a strain lacking micronuclei. This finding, together with our inability to date to find any HMG proteins in micronuclei prepared by the method of Gorovsky et al. (1975), makes it likely that in *Tetrahymena*, HMG's may be confined to the transcriptionally active macronuclei. In general, HMG's are noted for their solubility in dilute acid solutions. That *Tetrahymena* HMG's are not the exception to this rule is shown in Figure 2. When whole cells are subjected to fractionation with 5% PCA (slot 1) or 5% TCA (slot 2), HMG proteins can be recovered. Thus, HMG-B and -C are easily solubilized by the PCA procedure (slot 1), while HMG-D is extracted well with Cl_3CCOOH. A few other minor protein bands in addition to the four major HMG's are solubilized from whole cells by both procedures. Both these procedures have been recommended for tissues or cells with high contents of proteases (Goodwin et al., 1975).

The chromatographic behavior of *Tetrahymena* HMG's derived from nuclei of BVII cells on carboxymethylcellulose columns was also examined. Figure 3 displays the elution profile of a preparation of labeled HMG's on such a column. Various pools (1–6) were taken and the proteins from each pooled fraction identified on TAU gels. It is clear that HMG-A, seen on this gel as a triplet of bands, does not bind to the CM-52 column and elutes in the void volume. This behavior is identical with that of calf thymus HMG-1 and -2 in a similar column (Levy-Wilson & Dixon, 1978b). Considering both the mobilities of HMG-A on TAU gels and their behavior on CM-52 columns, we conclude that HMG-A is analogous to mammalian HMG-1 and -2. Nevertheless, the amino acid composition shown in Table I indicates that HMG-A is more acidic than either HMG-1 or -2 from calf thymus.

HMG-B and -C elute from the CM-52 column with ~0.35 M LiCl. HMG-D begins to elute at about 0.4 M LiCl, and it is totally eluted at 0.5 M LiCl. This protein, as demonstrated

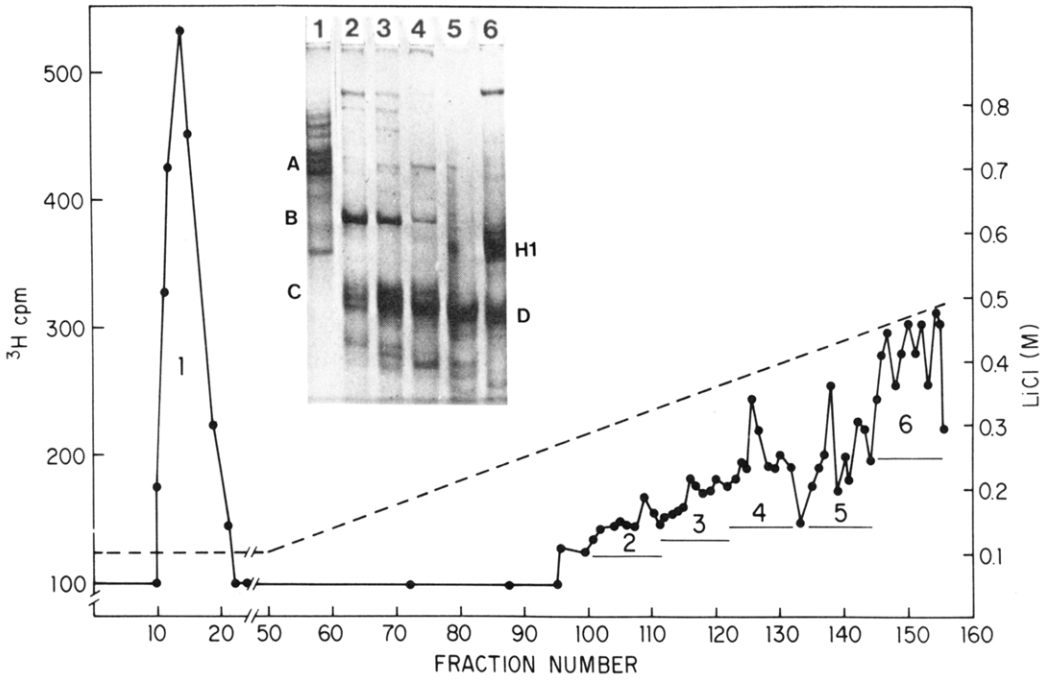

FIGURE 3: Carboxymethylcellulose chromatography of salt-extracted *Tetrahymena* HMG's: (—) ^{3}H cpm; (– –) LiCl concentration. The insert represents a TAU gel of aliquots taken from the various pools obtained from the columns.

Table I: Amino Acid Compositions of *Tetrahymena* HMG's (mol %)

	BVII					calf thymus[a]				
residue	HMG-A	HMG-B	HMG-C	HMG-D	ubiquitin	HMG-1	HMG-2	HMG-14	HMG-17	ubiquitin
Asp	12.8	10.3	7.4	2.7	10.3	10.7	9.3	8.3	12.0	9.7
Thr	4.0	5.5	3.3	15.4	7.8	2.5	2.7	4.1	1.2	9.0
Ser	12.4	8.2	5.5	21.0	6.4	5.0	7.4	8.0	2.3	4.4
Glu	20.8	15.0	13.4	5.7	14.4	18.1	17.5	17.5	10.5	11.5
Pro	3.7	4.7	4.3	4.6	2.8	7.0	8.9	8.1	12.9	4.6
Gly	10.7	6.0	4.4	4.6	11.0	5.3	6.5	6.4	11.2	5.8
Ala	8.7	11.2	8.2	13.9	5.3	9.0	8.1	14.8	18.4	2.9
Cys										
Val	4.0	3.4	2.1	2.7	4.8	1.9	2.3	4.0	2.0	5.4
Met		0.7	0.9		0.7	1.5	0.4	0.1		1.2
Ile	2.3	3.3	2.1	0.6	6.9	1.8	1.3	0.3		9.0
Leu	4.0	5.7	3.2	1.5	9.9	2.2	2.0	2.0	1.0	12.4
Tyr	2.7	2.2	2.6	0.6	1.3	2.9	2.0	0.2		1.3
Phe	2.0	3.0	2.6	0.3	2.3	3.6	3.0	0.3		2.7
His		1.1	1.2	1.7	1.6	1.7	2.0	0.2		1.3
Lys	7.4	16.1	22.0	23.8	8.4	21.3	19.4	21.1	24.3	1.2
Arg	4.0	3.3	1.4	1.1	5.7	3.9	4.7	5.4	4.1	5.5

[a] Johns, 1982.

below, is highly modified, and its elution behavior from this column reflects this heterogeneity.

The amino acid compositions of these various HMG's have been determined. From the data in Table I, we conclude that, although similar to mammalian HMG's, these proteins have diverged considerably during evolution. In particular, the content of Lys + Arg increases from A to D and the content of acidic residues (Asp + Glu) decreases markedly from A to D, making A the most acidic HMG and D the most basic one. Despite minor differences, HMG-B and -C resemble mammalian HMG's more than do HMG-A and -D. *Tetrahymena* ubiquitin is very similar to calf thymus (Walker et al., 1978) and trout testis (Watson et al., 1978) ubiquitin. The HMG-D protein has the most interesting characteristics. It is very small (M_r <7000) as judged by its rapid mobility on $NaDodSO_4$ gels (not shown). Although on TAU gels it comigrates with HMG-17 from calf thymus, its amino acid composition differs (Table I) in that HMG-D has a much lower content of acidic residues and a much higher level of serine and threonine. Furthermore, HMG-D electrophoreses on acid–urea gels as a series of at least six bands, suggesting a high degree of modification (Figure 1).

Localization of Tetrahymena HMG's in Chromatin Subunits. Having isolated and characterized these HMG proteins, we wished to determine whether these proteins were bound to the nucleosome core or whether they were associated with micrococcal nuclease sensitive "linker" regions. This was accomplished by incubating intact macronuclei (from GL cells) with low levels of micrococcal nuclease by the procedure of Levy-Wilson & Dixon (1978a). This procedure generated four fractions: S_1, MN_1, MN_2, and pellet, in order of their sensitivity to micrococcal nuclease. S_1 consists of structurally incomplete nucleosome cores and linker regions. MN_1 consists largely of mononucleosome core particles that are soluble in 0.1 M NaCl. MN_2 comprises mono- and dinucleosomes that are insoluble in 0.1 M NaCl. The pellet (P) contains mostly tetra- and pentanucleosomes and larger sized material.

When acid-soluble proteins derived from these fractions were examined (Figure 4), it was found that all four HMG's, A, B, C, and D, are associated with nucleosomes. The MN_1 core particles contain these proteins in high amounts. Furthermore, A, B, and C may also be localized in the linker regions as judged by their presence in S_1. These data prove that *Tetrahymena* HMG's are important structural components of the nucleosome present in macronuclei at stoichiometries comparable to those of the histones.

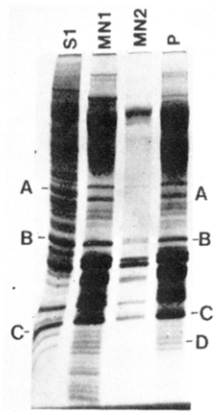

FIGURE 4: TAU gel of the 0.4 N H_2SO_4 extract from fractionated macronuclear chromatin from *Tetrahymena*.

Phosphorylation of Tetrahymena HMG Proteins. The striking multiband pattern observed in all preparations of *Tetrahymena* HMG-D, similar to mammalian HMG-17, prompted us to ask whether this series of bands could be the result of phosphorylation. Mammalian HMG-17 is known to be phosphorylated and ADP ribosylated. *Tetrahymena* cells were labeled in vivo with [^{32}P]orthophosphate, followed by purification of nuclei and isolation of HMG proteins as described under Materials and Methods. Figure 5A shows a stained gel of phosphorylated HMG's; the corresponding autoradiogram is shown in Figure 5B. The data clearly show that *Tetrahymena* HMG-D (slot 1) is highly phosphorylated in vivo. Furthermore, HMG-A and -B and ubiquitin are also phosphorylated to a lower extent than HMG-D. HMG-C does not appear to be phosphorylated in *Tetrahymena* (not shown).

That the ^{32}P labeling indeed corresponds to phosphate groups covalently bound to the HMG proteins was validated by incubating an aliquot of these proteins with calf intestinal alkaline phosphatase. This enzyme has been used previously to remove phosphate groups covalently bound to mammalian HMG proteins (Levy-Wilson, 1981a). In slot 2, Figure 5B, we observe that alkaline phosphatase does indeed remove most of the ^{32}P label associated with HMG-A and -B ubiquitin; however, it only removed a small fraction of the label in HMG-D. It has been recently shown that calf intestinal alkaline phosphatase dephosphorylates phosphoserine residues in histones at a rather low rate (Swarup et al., 1981).

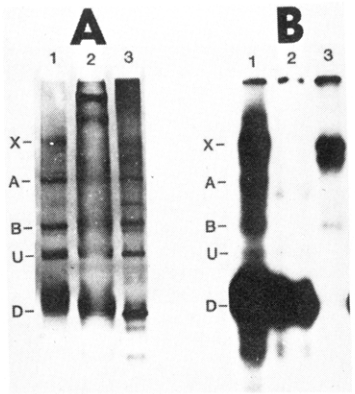

FIGURE 5: Phosphorylation of *Tetrahymena* HMG's. Part A illustrates the stained portion of a TAU gel of phosphorylated HMG's. Part B corresponds to the autoradiogram of the gel of part A. Slot 1 is a sample of phosphorylated HMG's. Slot 2 depicts the action of alkaline phosphatase on a sample identical with that displayed in slot 1, and slot 3 shows the action of rabbit muscle phosphoprotein phosphatase upon the same sample.

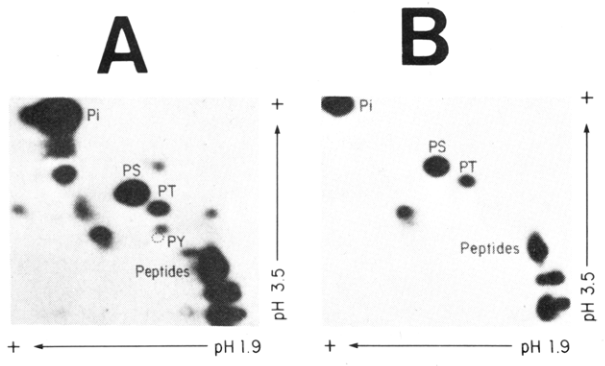

FIGURE 6: Two-dimensional thin-layer chromatography of an acid hydrolysate of *Tetrahymena* HMG's. P_i, inorganic phosphate; PS, phosphoserine; PT, phosphothreonine; PY, phosphotyrosine. Panel A corresponds to the whole HMG fraction; panel B corresponds to HMG-D.

Therefore, to remove quantitatively the phosphate labeling of HMG-D, we used rabbit muscle phosphoprotein phosphatase, an enzyme that preferentially dephosphorylates phosphoserine residues (Swarup et al., 1981).

Our results are shown in Figure 5, slots 3. The stained multibanding pattern of HMG-D, Figure 5A, is reduced to only one band of high electrophoretic mobility, corresponding to the parental, unmodified species, after treatment of the sample with phosphoprotein phosphatase (Figure 5B).

To determine unequivocally the chemical nature of the phosphorylated residues, we performed a careful two-dimensional thin-layer chromatographic analysis of partial hydrolysates of each one of the phosphorylated *Tetrahymena* HMG's (Figure 6). When the total HMG fraction, prepared by salt extraction of *Tetrahymena* nuclei, was analyzed in this fashion, we found serine and threonine to be the phosphorylated residues. No phosphotyrosine was present (Figure 6A). HMG-D was phosphorylated at both serine and threonine residues, with an approximate ratio of phosphoserine (PS) to phosphothreonine (PT) of 5:1. HMG-A and -B and ubiquitin were all phosphorylated mainly at phosphoserine residues.

ADP Ribosylation of Tetrahymena HMG Proteins. Having established that *Tetrahymena* HMG's and ubiquitin are phosphoproteins, we asked whether this same group of proteins is subject to another secondary modification, ADP ribosylation, known to occur in mammalian (Reeves et al., 1981) and trout HMG's (Levy-Wilson, 1981b). To this end, *Tetrahymena*

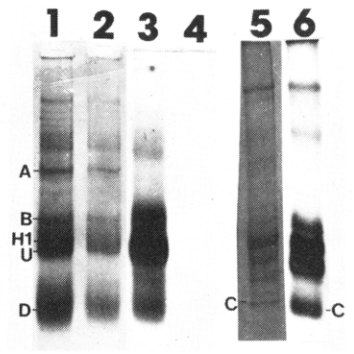

FIGURE 7: ADP ribosylation of *Tetrahymena* HMG's. Slot 1 is a sample of ADP-ribosylated HMG's. Slot 3 is the corresponding autoradiogram. Slot 2 is the stained gel of the same sample in slot 1 after alkali treatment to remove the ADP-R label. Slot 4 shows the autoradiogram of the sample after alkali treatment. Slot 5 shows a stained TAU gel of the acid-soluble proteins remaining after salt extraction of the HMG's. Slot 6 is the corresponding autoradiogram.

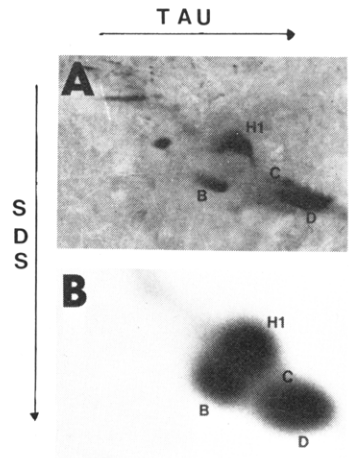

FIGURE 8: Two-dimensional polyacrylamide gel electrophoresis of ADP-ribosylated *Tetrahymena* HMG's. Panel A shows the stained gel. Panel B shows the corresponding autoradiogram.

nuclei were incubated wiith [^{32}P]NAD$^+$, followed by purification of the HMG fraction and gel electrophoresis as indicated under Materials and Methods. The results are shown in Figure 7; slot 1 shows the pattern of stained HMG proteins after ADP ribosylation of nuclei. Slot 3 shows the autoradiographic pattern of [^{32}P]ADP-ribosylated HMG's. Three major labeled bands can be seen, comigrating with HMG-B, ubiquitin, ahd HMG-D, respectively. That the labeling corresponds to ADP-R groups was shown by the alkali lability of the ^{32}P label. In slot 4, we show the effect of treatment of the ADP-ribosylated sample of slot 2 with 0.1 N NaOH. All the labeling is abolished by such a treatment. Essentially the same result was obtained by action of snake venom phosphodiesterase upon the labeled HMG sample, thus confirming that these proteins are ADP ribosylated.

When an acid extract of the residual nuclear pellet was analyzed in a similar fashion (slots 5 and 6), it appeared that HMG-C was also ADP ribosylated. Because this preparation of ADP-ribosylated HMG's contained some histone H1, which on TAU gels runs slightly ahead of ubiquitin, and H1 is known to be a major acceptor for ADP-R groups in mammalian cells, we analyzed the distribution of ADP ribose groups of the HMG fraction in two-dimensional gels. The results, shown in Figure 8, confirmed the data concerning labeling of HMG-B, -C, and -D and further demonstrated that H1 is also ADP ribosylated. Because ubiquitin ran off the bottom of the gel, it still remains to be proven whether it is ADP ribosylated.

In addition to the above-mentioned studies, we asked whether *Tetrahymena* HMG's could be acetylated or methylated. Cells were labeled with [^{3}H]acetate and HMG's purified and analyzed by gel electrophoresis as described above. In three independent sets of labeling experiments, we were unable to detect significant levels of acetylation, under conditions that gave consistently high levels of acetylation of the *Tetrahymena* arginine-rich histones H3 and H4, isolated from the same labeled nuclei. Therefore, we conclude that *Tetrahymena* HMG proteins are not acetylated at levels detectable by our procedures.

We then asked whether *Tetrahymena* HMG's could be methylated, when isolated nuclei are incubated with *S*-adenosyl[^{3}H]methylmethionine. The data obtained showed no incorporation of labeled methyl groups into HMG's, under conditions in which histone H2A and the non-histone protein A24 were methylated in the same nuclear preparation. Therefore, we conclude that *Tetrahymena* HMG's are not methylated, as assayed by our procedure.

Discussion

We have isolated a group of non-histone proteins from *Tetrahymena*, with properties similar to those of mammalian high mobility group proteins. Four major HMG proteins designated A, B, C, and D together with ubiquitin and several minor HMG-like proteins can be isolated from *Tetrahymena* cells or isolated nuclei. HMG-A has chromatographic properties in carboxymethylcellulose columns and electrophoretic mobility on acid–urea gels resembling those of mammalian HMG-1 and -2. Its overall amino acid content is also similar to that of HMG-1 and -2. However, HMG-A has a much lower content of lysine and a much higher content of serine residues than these mammalian proteins. Indeed, HMG-A is much more acidic than most mammalian HMG's. Our studies on the in vivo incorporation of labeled phosphate indicate that HMG-A is phosphorylated at specific serine residues.

HMG-B has previously been described by Hamana & Iwai (1979) as a *Tetrahymena* HMG protein. This protein resembles mammalian HMG-1 and -2 in overall amino acid composition; it has a slightly faster electrophoretic mobility on acid–urea gels than do the mammalian proteins, but its chromatographic properties on CM-52 resemble those of HMG-14. HMG-B, like HMG-A, is phosphorylated in vivo at specific serine residues and, in contrast to A, B is also a substrate for the enzyme poly-ADPR-polymerase. HMG-C corresponds to the second HMG protein previously described by Hamana & Iwai (1979). C has electrophoretic mobility in TAU gels midway between that of mammalian HMG-14 and HMG-17. Its chromatographic behavior on CM-52 columns and amino acid composition also resemble those of the two small mammalian HMG's. Compared to B, C has a much lower solubility in 0.35 M NaCl, but it is readily soluble in either dilute sulfuric or perchloric acid. HMG-C does not appear to be phosphorylated in *Tetrahymena* cells, and indeed, it exhibits the lowest content of serine residues (5 mol % compared to 12 for HMG-A, 8 for HMG-B, and 21 for HMG-D). Nevertheless, HMG-C incorporates ADP ribose groups when isolated nuclei are labeled with [^{32}P]-NAD^+.

The fourth major *Tetrahymena* HMG protein, HMG-D, is by far the most interesting new protein of the group. It is a small and extremely basic protein, with an electrophoretic mobility on acid–urea gels equivalent to that of mammalian HMG-17. This protein is remarkable in its very high content of serine and threonine residues (36 mol %), much higher than any other major chromosomal protein so far described. Perhaps because of its unusual composition, HMG-D appears to be a preferred substrate for nuclear protein kinases, and thus, it is found in vivo in a highly phosphorylated form. The ratio of phosphoserine to phosphothreonine is approximately 5:1. The various phosphorylated forms have slightly different electrophoretic mobilities on TAU gels and slightly different elution profiles from CM-52 columns. In addition to being highly phosphorylated, HMG-D is also ADP ribosylated.

In addition to these four major HMG proteins, ubiquitin is also extracted by the same procedures. *Tetrahymena* ubiquitin shares all of the properties of higher eucaryote ubiquitins. In *Tetrahymena*, ubiquitin is phosphorylated at specific serine residues and perhaps also ADP ribosylated.

Our data show that the HMG proteins are integral components of the nucleosome core and occur in a rougly half molar amount compared to the histones. *Tetrahymena* HMG's are widely distributed along the various macronuclear chromatin domains and are not confined to the most nuclease-sensitive regions. This may be attributable to the fact that a major proportion of the chromatin in *Tetrahymena* macronuclei is transcriptionally active (Stathopoulos et al., 1980).

The functional significance of the high degree of secondary modifications that affect this group of proteins is yet known. However, one possibility is that these various modifications play a role in altering the dynamics of gene expression. Thus, some sets of genes may be turned on or off when the domain in which they reside is subject to such HMG modifications. Both phosphorylation and ADP ribosylation have the net effect of reducing the overall positive charge of proteins. If this occurs in the DNA-binding domain or the HMG's, it may weaken the interactions with DNA, and thus allow enzymes such as RNA or DNA polymerase to act upon the DNA template to either transcribe or replicate DNA. It has been shown that mammalian HMG-1 and -2 can indeed unwind DNA (Javaherian et al., 1978). In the case of HMG phosphorylation, ^{32}P incorporation into HMG-17 of cultured nerve cells is stimulated by the addition of nerve growth factor, suggesting a positive correlation with transcription (Halegoua & Patrick, 1979). Furthermore, both HMG-14 and -17 are hyperphosphorylated in transcriptionally active nucleosome cores from butyrate-treated HeLa cells (Levy-Wilson, 1981a). The precise understanding of the role of HMG modification will have to await the results of work on the reconstitution of a specific functional gene from purified DNA and chromosomal proteins, in the unmodified and modified states. Nevertheless, the mere finding of these modifications in HMG proteins from various organisms may imply structural and functional importance.

Recently, it has been shown that calf thymus HMG's are glycoproteins. We wished to ascertain whether fucose or mannose residues occur in *Tetrahymena* HMG's. We asked whether two lectins, gorse seed lectin, specific for fucosyl residues, and concanavalin A, exhibiting high affinity for mannosyl residues, would bind to *Tetrahymena* HMG's separated on TAU gels. These preliminary results (data not shown) indicate that HMG-D is indeed a glycoprotein, as judged by binding to these two lectins. The specificity of the binding was demonstrated by using the cognate sugars in competition assays. Binding of gorse seed lectin to HMG-D was competed for by an excess of fucose. Similarly, concanavalin A binding was competed for by mannose and glucose.

It has recently been shown that fucosyl residues are found covalently attached to serine and threonine residues in low

molecular weight proteins from rat tissues (Klinger et al., 1981). Interestingly enough, the HMG protein exhibiting the highest extent of reaction with gorse seed lectin is HMG-D.

The methods used to demonstrate glycosylation of HMG's have recently been used to demonstrate glycosylation of histones and have been validated by experiments involving incorporation of radioactive sugars into these proteins (Levy-Wilson, 1983).

The presence of sugar residues in HMG's raises the possibility that these proteins may be associated with the nuclear membrane. In particular this is a very attractive possibility for HMG-D. The high levels of phosphorylation and ADP ribosylation of this protein compared to those of the other HMG's may imply that the membrane location facilitates access to kinases and ADP-ribosylating enzymes. Perhaps, some of the transcriptionally active DNA sequences are localized close to the nuclear membrane, from which they deliver the RNA transcripts to their final cytoplasmic destination.

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Registry No. Ubiquitin, 60267-61-0.

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