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Major High Mobility Group like Proteins of *Drosophila melanogaster* Embryonic Nuclei†

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ABSTRACT: Nuclei from *Drosophila melanogaster* embryos contain three major proteins which are extracted by 0.35 M NaCl and by 2% perchloric acid. One of these is histone H1, and we refer to the other two as A63 and A13 in accordance with their molecular weights determined by electrophoresis on sodium dodecyl sulfate ($NaDodSO_4$)–polyacrylamide gels (63 000 and 13 000, respectively). The molecular weight of A13, based on its amino acid composition, is approximately 10 000. The amino acid analyses of A63 and A13 show that both of these proteins have high proportions of acidic and basic amino acid residues, a property characteristic of the high mobility group proteins isolated from vertebrate tissues. While A13 comigrates with histone H2A on $NaDodSO_4$–polyacrylamide gels and with H2B on acid/urea gels, it can be readily resolved from the histones by Triton/acid/urea–$NaDodSO_4$ two-dimensional electrophoresis.

Four major acid-soluble nonhistone chromosomal proteins named high mobility group 1 (HMG-1),[1] HMG-2, HMG-14, and HMG-17 have been identified in both mammals (Sanders & Johns, 1974; Rabbani et al., 1978a) and birds (Rabbani et al., 1978b; Sterner et al., 1978). In addition, HMG-like proteins have been reported in trout (Watson et al., 1977; Marushige & Dixon, 1971; Wigle & Dixon, 1971), flies (Franco et al., 1977), yeast (Weber & Isenberg, 1980), and plants (Spiker et al., 1978).

While the precise function of the HMG proteins is not known, there are many indications that they function by participating in the basic nucleosome structure. Thus, HMG-14 and HMG-17 as well as trout H6 have been shown to be associated with the nucleosomes of transcriptionally competent chromatin (Levy-W. et al., 1979; Weisbrod & Weintraub, 1979; Weisbrod et al., 1980). HMG-14 and HMG-17 have been shown to confer DNase I sensitivity on the chick erythrocyte globin chromosomal domain (Weisbrod & Weintraub, 1979; Weisbrod et al., 1980) and to bind to "active" nucleosomes (Weisbrod & Weintraub, 1981). They also appear to partially inhibit histone deacetylases in vitro (Candido et al., 1980).

In the present paper, we report a characterization of proteins eluted from *Drosophila* embryonic nuclei by standard procedures known to extract HMG proteins. We find that *Drosophila* contains two major proteins having typical HMG chemical compositions and solubility characteristics. However, when compared to calf thymus and trout testis HMG proteins, these *Drosophila* proteins differ in both molecular weight and amino acid composition. Whether or not *Drosophila* contains analogues to HMG-1, HMG-2, HMG-14, and HMG-17 remains unclear, and the answer to this question must await a definition of these proteins based on functional characteristics as well as physical characteristics.

Experimental Procedures

Embryos. *Drosophila melanogaster* Oregon R embryos were collected 6–18 h after fertilization, washed, and stored at −70 °C until needed. All samples were handled at 0–4 °C, unless otherwise noted, and PMSF (0.2 mM) was present in all solutions to inhibit proteolysis.

Preparation of Nuclei. Nuclei were prepared by the method of Hewish & Burgoyne (1973) as modified by Mayfield et al. (1978). Purified nuclei were used immediately for the extraction of proteins.

Extraction of HMG-like Proteins. HMG-like proteins were extracted from homogenized nuclei (DNA at 10–15 mg mL^{-1}) by the addition of 70% PCA to a final concentration of 2%. After the suspension was gently stirred on ice for 30 min, it was centrifuged for 30 min at 15000*g*. The clear supernatant was neutralized to pH 7 with 50% KOH.

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[1] Abbreviations: PCA, perchloric acid; Cl_3CCOOH, trichloroacetic acid; PMSF, phenylmethanesulfonyl fluoride; $NaDodSO_4$, sodium dodecyl sulfate; HMG, high mobility group.

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Extraction of Histones. Histones were obtained from homogenized nuclei by the addition of an equal volume of cold 0.8 N H_2SO_4. After the mixture was stirred on ice for 30 min, it was centrifuged for 30 min at 15000*g*, and the pellet was reextracted with cold 0.4 N H_2SO_4. Four volumes of ethanol was added to the combined supernatants, and the histones were allowed to precipitate overnight at −20 °C and collected by centrifugation for 15 min at 15000*g*. The histone pellet was washed twice with ethanol and dried under vacuum.

Chromatography of HMG-like Proteins. The neutralized 2% PCA soluble protein sample was made 6 M in urea and 50 mM in Tris–acetate, pH 7.8, and applied to a 1.5 × 16 cm Bio-Rex 70 column (Na^+ form, 50–100 mesh, Bio-Rad) previously equilibrated with 6 M urea/50 mM Tris–acetate buffer, pH 7.8. The sample was eluted with a linear, 0–10%, gradient of guanidine hydrochloride at a flow rate of 50 mL h^{-1}, and 2.0-mL fractions were collected at room temperature. Aliquots (0.1 mL) were withdrawn from selected fractions for electrophoresis.

Desalting on Sephadex G-25. Protein-containing fractions were pooled and applied in 5-mL batches to a 1.5 × 16 cm Sephadex G-25 (medium grade) gel filtration column and desalted by elution with 0.01 N acetic acid. Pooled fractions were lyophilized and analyzed by electrophoresis. The total protein content was determined by the method of Lowry et al. (1951) with bovine serum albumin as a standard.

Extinction Coefficients. The ultraviolet spectra of A13 in water and of A63 in 0.01 N acetic acid were used to calculate extinction coefficients for these proteins based on the Lowry determinations. After corrections for Rayleigh scattering were made, the extinction coefficient for A13 at 280 nm is 1.03 mL mg^{-1} cm^{-1} and at 230 nm is 3.35 mL mg^{-1} cm^{-1}. These values agree closely with those calculated from the amino acid analyses (ϵ_{280} = 1.04 mL mg^{-1} cm^{-1} and ϵ_{230} = 3.4 mL mg^{-1} cm^{-1}). The extinction coefficients of A63 were determined as the following: ϵ_{280} = 0.32 mL mg^{-1} cm^{-1} and ϵ_{230} = 3.75 mL mg^{-1} cm^{-1} based on the Lowry protein determination with bovine γ-globulin as a standard and ϵ_{280} = 0.34 mL mg^{-1} cm^{-1} and ϵ_{230} = 4.00 mL mg^{-1} cm^{-1} based on the Bio-Rad Bradford protein determination also with bovine γ-globulin as the standard.

Gel Electrophoresis. For two-dimensional electrophoresis, acid/urea–polyacrylamide tube gels containing 12% acrylamide, 0.9 N acetic acid, 6 M urea, and 0.7% Triton DF-16 were used for the first dimension, while the second dimension was performed by using $NaDodSO_4$–polyacrylamide slab gels (20 cm). Electrophoresis was performed exactly as described by Allis et al. (1979). For the estimation of molecular weights, $NaDodSO_4$–polyacrylamide (11.5%) slab gels were employed, utilizing bovine serum albumin, human IgG heavy chain, human IgG light chain, and cytochrome *c* as markers.

Amino Acid Analyses. Purified A13 was hydrolyzed in triplicate for 24, 48, and 72 h in 6 N HCl and analyzed on a Durram D400 amino acid analyzer by using the sodium citrate buffer system. Cysteine was determined as cysteic acid after performate oxidation. Triplicate samples of A63 were hydrolyzed for 24, 48, and 72 h in 6 N HCl and analyzed on a Beckman 121M amino acid analyzer.

Results

Extraction and Chromatography of HMG-like Proteins. Homogenized nuclei were extracted by various reagents which have commonly been used to extract histones or lysine-rich nonhistone proteins from the nuclei of many species. These included several concentrations of either Cl_3CCOOH or PCA, 0.35 M NaCl followed by 2% Cl_3CCOOH or 2% PCA, and 0.4 N H_2SO_4. The extracted proteins were then analyzed by $NaDodSO_4$–polyacrylamide gel electrophoresis. From these studies, it is clear that there are three major nuclear proteins which are consistently extracted by low concentrations of both Cl_3CCOOH and PCA. All three of these proteins are also extracted by 0.35 M NaCl and therefore satisfy the solubility requirements established by Goodwin et al. (1973) as defining HMG proteins from mammalian tissues. The most prominent of the three proteins is histone H1; we have labeled the others A63 and A13 because of their acid solubility and in accordance with their apparent molecular weights by $NaDodSO_4$–polyacrylamide gel electrophoresis (63 000 and 13 000, respectively). These proteins appear to be present at roughly 5% and 20% of the amount of histone H1, respectively.

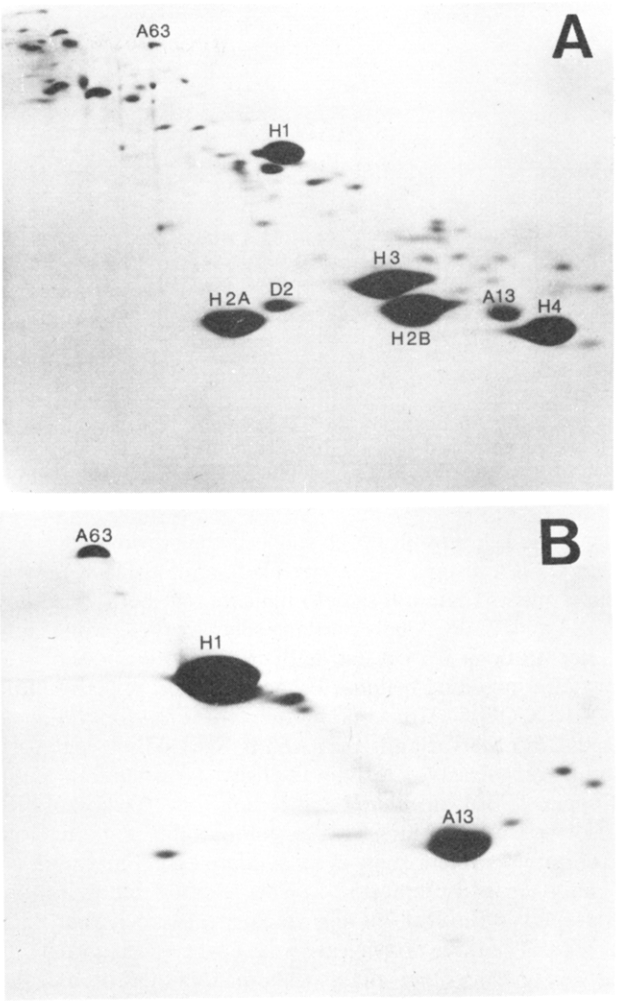

FIGURE 1: Two-dimensional electrophoretic polyacrylamide gels of the acid-soluble proteins extracted from nuclei. (A) Proteins solubilized by 0.4 N H_2SO_4. (B) Proteins solubilized by 2% PCA. Samples were first run in 12% polyacrylamide tube gels containing 6 M urea, 0.9 N acetic acid, and 0.7% Triton DF-16 with the cathode to the right. The proteins were then separated in a second dimension by using 22% polyacrylamide slab gels containing $NaDodSO_4$ with the anode at the bottom.

Figure 1 shows these proteins resolved by two-dimensional gel electrophoresis. Figure 1A demonstrates that the histones are the dominant proteins extracted by 0.4 N H_2SO_4, a fact that has been known for many years. A63, A13, a small histonelike protein which we have tentatively identified as D2 (Palmer et al., 1980), and a large number of other nonhistone proteins are also extracted from *Drosophila* nuclei by this reagent. Figure 1B demonstrates that 2% PCA is a far more selective solvent. Both A63 and A13 are almost completely soluble. The core histones are not soluble in 2% PCA, and

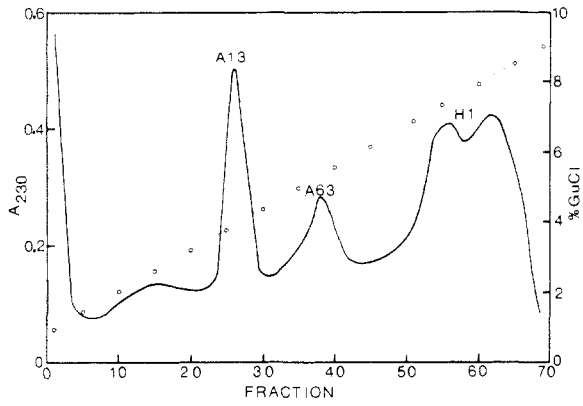

FIGURE 2: Chromatography of 2% PCA soluble proteins on a Bio-Rex 70 column developed with a 0–10% guanidine hydrochloride gradient in 6 M urea and 50 mM Tris–acetate, pH 7.8. (—) Absorbance at 230 nm; (O- - -O) concentration of guanidine hydrochloride.

neither is D2, a result which supports the previous conclusion that D2 is a histone H2 variant (Palmer et al., 1980). Other experiments (data not shown) indicate that both 2% Cl_3CCOOH and 5% PCA only partially solubilize A13 while higher concentrations of PCA extract increasing quantities of other proteins including histones H2A and H2B. A 10% solution of Cl_3CCOOH extracts no proteins from *Drosophila* nuclei. A useful observation is that A63 on $NaDodSO_4$–polyacrylamide gels displays a distinct reddish color when stained with Coomassie Brilliant Blue R and destained in 10% glacial acetic acid for 1 week or longer. This color readily distinguishes it from other protein spots even in complex gel mixtures.

PCA nuclear extracts (2%) were fractionated by chromatography on Bio-Rex 70. Figure 2 represents a typical elution profile. The three proteins are well separated from each other as well as from other minor contaminants by this procedure.

Amino Acid Compositions of A63 and A13. Fractions corresponding to A63 and A13 were collected, the urea and salt were removed by chromatographic desalting, and the amino acid composition of the proteins was determined as described under Experimental Procedures. The purity of A63 was assessed by $NaDodSO_4$–polyacrylamide gel electrophoresis and judged to be greater than 90%. A13 was considered to be free from contaminants as analyzed by two-dimensional Triton/acid/urea–$NaDodSO_4$–polyacrylamide gel electrophoresis. Table I presents the amino acid compositions of these proteins.

Both A13 and A63 are lysine rich, but they also contain large amounts of the acidic amino acids. Clearly, A13 is not a histone, since its amino acid composition is substantially different from that of four core histones, histone H1, or any reported *Drosophila* H1 cyanogen bromide cleavage product (Alfageme et al., 1974).

The probable molecular weight of A13, calculated from its amino acid composition, is 10000. This is consistent with the observation we and others have made that HMG-type proteins frequently run more slowly in $NaDodSO_4$–polyacrylamide gels than predicted by their true molecular weight.

Table I also lists for comparison the amino acid compositions of the *Drosophila melanogaster* chromosomal proteins D1 (Alfageme et al., 1980) and D2 (Palmer et al., 1980), and the HMG-like protein CMC-1 from the fruit fly *Ceratitis capitata* (Franco et al., 1977).

The UV spectrum of A13 (data not shown) indicates the presence of tryptophan. Assuming a molar extinction coefficient at 280 nm for tryptophan of 5560 L mol^{-1} cm^{-1} and for tyrosine of 1200 L mol^{-1} cm^{-1} (Sober, 1970), and assuming the presence of one tyrosine per A13 molecule based on the amino acid analysis and a protein molecular weight of 10000, we then determined that one tryptophan would give a calculated extinction coefficient of 0.676 mL mg^{-1} cm^{-1} while two tryptophan residues would give 1.23 mL mg^{-1} cm^{-1}. On this basis, one may estimate that A13 contains two tryptophan residues.

Table I: Amino Acid Analysis (mol %) of A13, A63, D1, D2, and CMC-1

	A13	A63	D1[e]	D2[f]	CMC-1[g]
Asx	9.7	10.7	16.0	5.7	17.2
Thr	3.8[a]	3.4[a]	3.1	5.0	4.8
Ser	11.2[a]	11.1[a]	10.6	6.6	7.7
Glx	13.3	8.9	10.7	10.0	10.2
Pro	3.9	8.7	8.0	2.7	7.2
Gly	13.6	13.3	13.2	11.1	11.4
Ala	10.6	11.3	9.6	13.9	8.3
Val	4.6	4.8	4.8	5.9	0.0
Cys	trace	ND[d]	0.2	ND[d]	2.3
Met	1.2	0.3	0.2	0.0	0.4
Ile	2.2	1.5	1.7	6.8	1.8
Leu	3.7	2.0	1.5	10.0	1.5
Tyr	1.6	1.2	0.7	1.6	0.8
Phe	1.3	trace	<0.1	0.8	trace
Lys	10.4	13.9	11.5	9.5	17.6
His	1.1	1.5	1.3	3.6	0.6
Arg	4.3	7.6	7.4	6.8	8.2
Trp	3.7[b]	0.5[b]	<0.1	ND[d]	ND[d]
Lys/Arg	2.4	1.8	1.6	1.4	2.1
basic/acidic	0.7	1.2	0.8	1.3	0.9
hydrophobic[c]	14.6	9.8	8.9	25.1	4.5
basic + acidic	38.8	41.1	45.6	32.0	53.2

[a] Extrapolated to zero hydrolysis time. [b] Estimated spectrophotometrically. [c] Val, Met, Ile, Leu, Tyr, and Phe. [d] ND = not determined. [e] Alfageme et al. (1980). [f] Palmer et al. (1980). [g] Franco et al. (1977).

Spectral analysis of A63 also suggests the presence of a small amount of tryptophan. The absorbance values for 294 and 280 nm, at pH 12, yield a ratio of Tyr/Trp = 2.4 when analyzed by the method of Beaven & Holiday (1952). On this basis, we estimate the presence of about 0.5 mol % of tryptophan. This value would yield a calculated extinction coefficient of 0.4 mL mg^{-1} cm^{-1}, in reasonable agreement with the measured value of 0.33 mL mg^{-1} cm^{-1} (see Experimental Procedures).

Discussion

Chromatin studies over the past few years using chick nuclei have shown that proteins HMG-14 and HMG-17 bind to a subset of nucleosomes and confer on these nucleosomes particular sensitivity to digestion by DNase I. Furthermore, this same subset of DNase I sensitive nucleosomes can be shown to contain DNA sequences which are normally expressed in that tissue, while many sequences which are not expressed are absent [e.g., see Weintraub & Groudine (1976) and Garel & Axel (1976)]. The evidence presented by Weintraub and his colleagues makes it difficult to escape the conclusion that these proteins play a major role in either the maintenance or the regulation of gene expression in chick red blood cell nuclei. Because of the extensive use of *Drosophila melanogaster* as an experimental organism for the study of eukaryotic gene expression, it is of major importance to ascertain whether or not this organism has nuclear proteins analogous to the vertebrate HMG proteins. The present study clearly shows that there are two and only two major nuclear proteins to be found in *Drosophila* embryonic nuclei with physical properties similar to those of HMG proteins found in higher organisms.

A63 is unusual because it is over twice the size of HMG-1 and HMG-2, the larger of the mammalian HMG proteins. A

protein of similar size and solubility properties, termed D1, has been previously described (Alfageme et al., 1980). Despite the still unresolved differences in amino acid composition (see Table I), we believe that A63 and D1 are in fact the same protein. The amino acid composition of this protein is rather typical of HMG proteins, with the basic and acidic amino acids each constituting about 20% of the total. The high proportions of proline and glycine may indicate an extended conformation. The protein CMC-1 from *Ceratitis capitata*, although smaller and somewhat different in overall composition (see Table I), shares these properties and may well be the functional analogue of A63/D1 for this species.

A63 is known to be associated with polynucleosomes following digestion with either micrococcal nuclease or DNase I (Bassuk & Mayfield, 1980, 1981). Mayfield et al. (1978) reported the preparation of an antiserum to a small group of *Drosophila* nuclear proteins, referred to as "band 2", which were released from nuclei by brief digestion with DNase I. When used to stain salivary gland polytene chromosomes, this serum clearly reacted with puffs and with a limited set of developmentally active nonpuffed bands (loci). The principal protein present in band 2 was A63. Since the band 2 antigen is known to be extracted from polytene chromosomes by acid (Mayfield et al., 1978), it was reasonable to suppose that A63 plays some role in gene activity. However, a rabbit antiserum has been prepared against purified A63 and used to stain *Drosophila* polytene chromosomes (data not shown). This serum does not specifically stain puffs and in fact indicates that A63 is generally distributed over the entire chromosomes. Antisera to D1 have also been made and used to stain polytene chromosomes (Alfageme et al., 1980). These studies show that D1 is also distributed widely over the chromosomes and under carefully controlled cytological conditions is preferentially located in chromosomal regions containing (A + T)-rich DNA.

A13, though clearly a protein of the HMG type, is somewhat less basic than most recognized HMG proteins (Table I). The only other reported *Drosophila* protein with which it might be confused is D2 (Palmer et al., 1980). D2, however, more closely resembles the histones and is clearly different from A13 in amino acid composition and electrophoretic mobility (see Table I and Figure 1A). At present, nothing is known about the chromosomal distribution of either A13 or D2.

Inspection of Figure 1B reveals that at the level of 1–2% of histone H1 there are a large number of nuclear proteins which are extracted by 2% PCA. The recovery of these proteins in the extract is quite variable, but the position of the spots on the two-dimensional gel is reproducible. These proteins include the core histones, which have a very limited solubility in this reagent, as well as presumed degradation products of histone H1. It is likely that additional minor HMG-like proteins exist in this collection, but no attempt has been made in the present study to isolate and characterize them.

Whether or not either A13 or A63 plays a role in gene expression is at present unknown. However, the present study defines the field of promising HMG-like proteins in *Drosophila*. It is likely that if functional analogues of vertebrate HMG-1, HMG-2, HMG-14, and HMG-17 are to be found in *Drosophila* nuclei, A63, A13, and possibly D2 will be the proteins involved.

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