Evidence for a Quantitative Tissue-Specific Distribution of the High Mobility Group Chromosomal Proteins†

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ABSTRACT: The quantitative tissue specificity of the high mobility group (HMG) chromosomal proteins was investigated. Perchloric acid (PCA) extracts of four different chicken tissues and erythrocytes contained three proteins which comigrated on $NaDodSO_4$–polyacrylamide gels with the HMG's 1, 2, and E from erythrocyte nuclei. These three HMG's from embryonic skeletal muscle and erythrocytes also comigrated on two-dimensional gels, employing an acid–urea system in the first dimension and an $NaDodSO_4$ system in the second. Interpretation of the two-dimensional gels suggested that the two low molecular weight proteins of this triplet arose from the HMG 2 band of the acid–urea gels. These have been designated HMG 2A and HMG 2B. Three proteins of similar molecular weights were also found in the PCA extracts of calf thymus. They were arranged in a similar but not identical pattern on two-dimensional gels. Thus, these three HMG's appear to be neither tissue nor species specific. In addition, the 2.0% PCA extracts of all chicken tissues examined contain a 38 000-dalton (38K) nuclear protein which coisolates with the HMG's. These four proteins are found in different relative amounts in each of the four chicken tissues and erythrocytes. They are found in the same relative amounts, however, in embryonic skeletal muscles from different chicken strains with widely different highly repetitive sequence content, suggesting that none of these individual proteins is selectively localized to constitutive heterochromatin. The quantitative tissue specificity of the HMG's and the 38K protein, however, suggests that they may participate in regulating cell-specific gene expression.

Recent studies suggest that the high mobility group (HMG) chromosomal proteins are involved in the structure of transcriptionally active chromatin (Levy-W. et al., 1977; Vidali et al., 1977; Weisbrod & Weintraub, 1979). HMG's are selectively released from nuclei during the preferential digestion of transcriptionally active genes by DNase I (Levy-W. et al., 1977, 1979; Levy-W. & Dixon, 1978; Levy-Wilson & Dixon, 1979; Vidali et al., 1977; Weisbrod & Weintraub, 1979; Weisbrod et al., 1980). Similarly, the extraction of HMG's from erythrocyte mononucleosomes eliminates the preferential DNase I sensitivity of globin genes, and this preferential sensitivity is restored by readdition of HMG's (Weisbrod & Weintraub, 1979). The HMG's thus appear to be responsible in part for those features of active chromatin structure which are reflected in DNase I sensitivity.

A question of some interest is whether the HMG's participate in determining tissue-specific gene expression or play a more common role in active chromatin structure in all tissues. The four major HMG subfractions (HMG 1, HMG 2, HMG 14, and HMG 17) have been identified in all bovine (Rabbani et al., 1978; Sterner et al., 1978), rodent (Levy-W. & Dixon, 1978), and avian (Rabbani et al., 1978; Sterner et al., 1978) tissues examined to date, although the quantitative distribution of the various HMG's has not been assessed. Of particular interest is the report by Sterner et al. (1978) of an HMG (HMG E) which appears to be specific to avian erythrocytes.

In the present study, we have pursued the question of tissue specificity by determining the quantitative distribution of HMG 1 and HMG 2 in various chicken tissues. Under conditions designed to prevent differential HMG recovery, a protein comigrating with HMG E was found in five chicken tissues and in calf thymus. An additional protein of molecular weight 38 000 (38K) was found to coisolate with the HMG's from various chicken tissues. Comparisons of the proportions of the HMG's, as well as the 38K protein, indicate a quantitative tissue-specific distribution of these chromosomal proteins.

Experimental Procedures

Tissues. Liver, brain, thymus, and erythrocytes were obtained from 6–8-week-old male White Leghorn chickens. The erythrocytes were collected by cardiac puncture and washed as described by Vidali et al. (1977). Embryonic chicken skeletal muscle was dissected from the legs of 18-day-old White Leghorn and Rhode Island Red embryos (SPAFAS, Norwich, CT). Calf thymus, obtained from freshly slaughtered animals at a local abattoir, was frozen in liquid nitrogen and transported to the laboratory. Chicken hepatocytes were prepared from 6–8-week-old males by the technique of Williams et al. (1978). Skeletal muscle cultures were initiated with myoblasts from 11-day-old chick embryo leg muscle. The cells were grown in vitro by employing the technique of Konigsberg (1972). The majority of the fibroblasts were removed by preplating for 40 min as described by Yaffee (1968). Five days after the initiation of the cultures, the myotubes were dislodged from the dishes with a rubber policeman in phosphate-buffered saline (PBS) and collected by centrifugation at 800*g* in a Sorval HL-8 rotor. Samples that were not to be extracted immediately were stored under liquid nitrogen.

Isolation of Nuclei. All steps were carried out at 0–4 °C. Nuclei were isolated from 18-day embryonic leg muscle by a modification and combination of the presoak muscle relaxation technique of Zak et al. (1972) and the filtration technique of Kuehl (1975). This procedure will be described in detail elsewhere (J. S. Gordon, R. Kaufman, and B. I. Rosenfeld, unpublished experiments). In brief, freshly dissected muscle was incubated at 4 °C for 2.5 h in three changes of a Contractile Apparatus Relaxing Solution (CARS) consisting of 250 mM sucrose, 5 mM EGTA, 5 mM sodium pyrophosphate (PP_i), 100 mM KCl, 5 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 50 mM $NaHSO_4$, and 1 mM phenylmethanesulfonyl fluoride (PMSF) in 25 mM Tris-HCl, pH

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7.5. The relaxed muscle was transferred to modified CARS, containing 0.1% Triton and 50 mM KCl, and homogenized. The bulk of the contractile apparatus was removed by sequential filtration through cheesecloth and Miracloth. Crude nuclei were pelleted at 1000*g* and resuspended in 2.2 M sucrose, 5 mM EGTA, 50 mM KCl, 5 mM $MgCl_2$, 1% bovine serum albumin (BSA, Sigma fraction V), 1 mM PMSF, and 25 mM Tris-HCl, pH 7.5. This was underlayed with a $^1/_4$ volume of the same solution without BSA and centrifuged at 80000*g* for 45 min. The purity and the characteristics of these nuclei will be described elsewhere (J. S. Gordon, R. Kaufman, and B. I. Rosenfeld, unpublished experiments). As noted below, the relaxing solution extracts most HMG's but does not extract the 38K protein.

Nuclei from adult chicken liver and brain were prepared by a modification of the technique of Blobel & Potter (1966). The minced tissue was suspended in modified CARS containing 50 mM KCl but lacking $NaPP_i$ and EGTA. It was then homogenized sequentially in a Waring blender, operating at full speed for 30 s, followed by seven strokes of a motorized Potter-Elvejhem tissue grinder. The filtrate, obtained after passing the homogenate through two layers of cheesecloth, was centrifuged at 2250 rpm for 10 min in an HL-8 rotor. The sedimented nuclei were resuspended in 1.7 M sucrose in the above solution and underlayed with 2.2 M sucrose, also in the same solution, prior to centrifugation for 1 h at 25 000 rpm in a Beckman SW-27 rotor. The purified nuclei were resuspended in RSB (10 mM NaCl, 3 mM $MgCl_2$, and 1 mM PMSF in 10 mM Tris-HCl, pH 7.5) containing 0.1% Triton with a loose-fitting Dounce homogenizer and centrifuged at 2500 rpm for 10 min in an HL-8 rotor. The tissue was not exposed to detergent prior to sedimentation of the nuclei through dense sucrose to minimize erythrocyte disruption and, therefore, erythrocyte nuclear contamination. This was not necessary in the preparation of muscle nuclei as the vast majority of the erythrocytes were discarded during the presoaks. Adult erythrocyte nuclei were prepared by the technique of Weintraub & Groudine (1976) as described by Vidali et al. (1977).

HMG Extraction. The HMG's were extracted at 4 °C from whole tissues as described by Sanders & Johns (1974). Five to thirty grams of fresh tissue or rapidly thawed tissue was washed in 10 volumes of phosphate-buffered saline (PBS), drained, and minced with scissors. It was homogenized in a volume of 5 or 2% perchloric acid (PCA) equal to 1.5 times the tissue wet weight in a Sorval Omnimix set at half maximum speed for 1 min and then at full speed for 2 min. Assuming a tissue volume equal to its wet weight, the final PCA concentrations equal 3 and 1.2%, respectively. The pellet obtained after centrifugation at 38000*g* for 15 min in a SS-34 Sorval rotor was reextracted with the same volume and concentration of acid as above and combined with that of the first extraction. Assuming no fluid contribution by the pellet from the first extract, this resulted in final PCA concentration of 3.75 and 1.5%. Erythrocytes were extracted with twofold greater volumes of PCA adjusted to give the same final concentration to avoid trapping of the HMG in the heavy globin precipitates. The extracts were clarified by centrifugation at 20 000 rpm for 20 min in an SW-27 Beckman rotor. The resulting supernatant was mixed with 3.0 volumes of acetone and 0.2 volume of concentrated HCl. The precipitated H1's were then pelleted by centrifugation at 7000 rpm for 15 min in GS-3 rotor. Sanders & Johns (1974) used 3.5 volumes of acetone to precipitate the H1's. We found it necessary to use 3.0 volumes in order to prevent the partial precipitation of the 38 000-dalton protein which was identified in this study. The HMG's were recovered from the supernatant by the addition of another 2.0 volumes of acetone, followed by centrifugation as above. The H1 pellet was washed once with 5–10 mL of a 3.5:1 mixture of acetone–1 N HCl. The HMG- and H1-containing pellets were then washed 3 times with acetone, vacuum-dried, and resuspended in 62.5 mM Tris-HCl, 1 mM DTT, and 1 mM PMSF, pH 6.8.

When only small amounts of tissue were available, as in the case of hepatocytes and muscle cell cultures, recovery of the HMG's was ensured by not fractionating the PCA-soluble proteins. In this case, the PCA extract, which had been clarified by centrifugation in a Beckman SW-50.1 rotor at 40 000 rpm for 20 min, was mixed with 5 volumes of acetone and 0.2 volume of concentrated HCl to simultaneously precipitate both the H1 and HMG's.

The HMG's were prepared from nuclei at 4 °C by the techniques of Goodwin et al. (1973). After three to four washes in RSB, the nuclei were washed twice with 75 mM NaCl, 1 mM PMSF, and 25 mM EDTA, pH 7.5, and sedimented at 7000 rpm for 10 min in a Sorval HB-4 rotor. The combined supernatant, obtained after two resuspensions of the nuclei in 0.35 M NaCl (titrated to pH 7.0 with 0.1 N NaOH) and centrifugations as above, was clarified at 40 000 rpm for 1 h in a Beckman SW-50.1 rotor. The supernatant was brought to 2% Cl_3AcOH with the appropriate volume of 50% Cl_3AcOH. The acid-insoluble nonhistones were sedimented at 10 000 rpm for 10 min in an HB-4 rotor. The resulting supernatants were brought to 20% Cl_3AcOH and centrifuged as before. The pelleted proteins were sequentially washed with acetone–1 N HCl (6:1), 3 times with acetone, and once with ether, prior to vacuum drying and redissolving in 62.5 mM Tris-HCl, 1 mM DTT, and 1 mM PMSF, pH 6.8.

Electrophoretic Analysis. Proteins were resolved by Na-$DodSO_4$–polyacrylamide gel electrophoresis using the discontinuous buffer system of Laemmli (1970) with 17% polyacrylamide in the resolving gel. Electrophoresis was at 130 V for 7 h. The gels were fixed and stained in 0.25% Coomassie Blue, in 50% methanol–10% acetic acid, and destained in 50% methanol and 10% acetic acid.

The staining intensity of each band was determined by densitometry of a Polaroid transparancy of the gel using a Joyce-Loebl microdensitometer. The area under each peak was determined with a sonic digitizer (Science Accessory's Model NT501). When the tracings of two peaks partially overlapped, the half-area of the more defined peak was obtained by dropping a perpendicular from the maximum to the base line. This was then multipled by 2 and subtracted from the total area covered by both peaks to determine the area under each peak. In all cases, different concentrations of the same sample were run on each gel to ensure that the binding of Coomassie to each band was linearly related to protein concentration and that the gels were photographed at exposures in the linear response range of the film.

A modification of the two-dimensional polyacrylamide gel electrophoresis technique devised by Savic & Poccia (1978) employing acid–urea gel electrophoresis in the first dimension and an $NaDodSO_4$ system in the second was employed. Duplicate samples were prepared and electrophoresed at 200 V for 7 h on acid–urea gels, as described by Panyim & Chalkley (1969). One gel of each pair was fixed and stained as above, while the other was equilibrated in 2% $NaDodSO_4$, 80 mM Tris-HCl, pH 6.8, 10% glycerol, 100 mM DDT, and 0.2% Bromphenol Blue prior to electrophoresis in the second dimension. The second dimension 20% polyacrylamide resolving

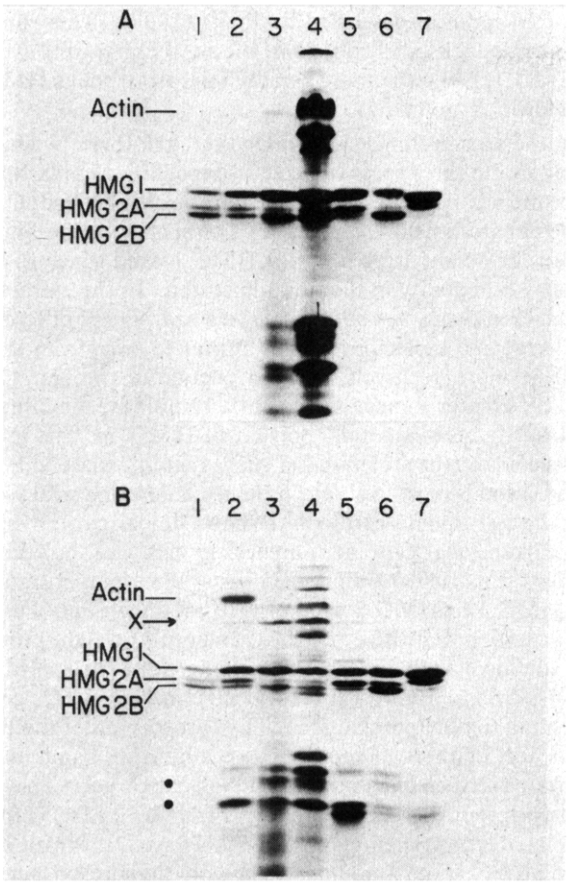

FIGURE 1: Discontinuous $NaDodSO_4$–polyacrylamide gel electrophoresis of the HMG fraction of the 5% (A) and 2% (B) PCA extracts of various chicken tissues. 18-day embryonic skeletal muscle (2) and adult liver (3), brain (4), thymus (5), and erythrocytes (6), as well as calf thymus (7), were extracted as described under Experimental Procedures. The HMG fraction obtained by acetone–HCl fractionation, as described under Experimental Procedures, was resolved on 17% acrylamide gels. Lane 1 displays HMG's extracted from purified erythrocyte nuclei by 0.35 M salt extraction (as described under Experimental Procedures). The reason for adapting the nomenclature of HMG 2A and HMG 2B for proteins previously termed HMG 2 and HMG "E", respectively (Sterner et al., 1978), is explained in the text. The arrow delineates a 38 000-dalton peptide coisolating with the HMG's. The closed circles mark the anticipated mobilities for HMG's 14 and 17.

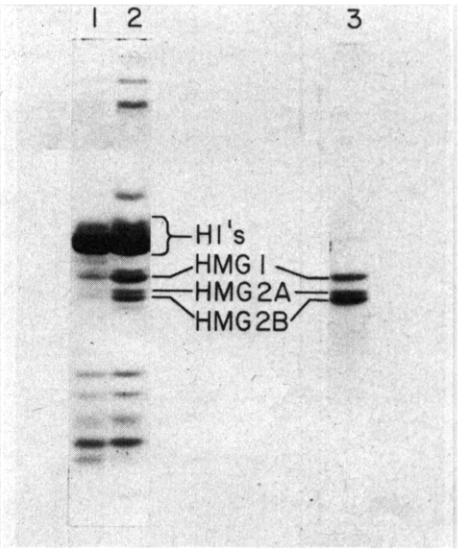

FIGURE 2: Discontinuous $NaDodSO_4$–polyacrylamide gel electrophoresis of the 2% PCA extracts of erythrocyte free hepatocytes (1) and primary embryonic skeletal muscle (2) tissue cultures. The chicken hepatocytes and 5-day embryonic muscle cultures were prepared and extracted as described under Experimental Procedures. The proteins obtained by precipitation with 5 volumes of acetone and 0.2 volume of HCl were resolved on 17% polyacrylamide gels (as described in the legend to Figure 1). The HMG's from erythrocyte nuclei (3) isolated as described in the legend of Figure 1 were resolved on the same slab gel.

$NaDodSO_4$ gel, 1.35 mm thick, 8.0 cm long, and 25.5 cm wide, was prepared in an apparatus similar in design to that described by O'Farrell (1975). The extended width allows simultaneous electrophoresis of two or more different first-dimension gels. The stacking gel was 3.5 cm long. The first-dimension gels were embedded in 0.25% agarose in the equilibration buffer at a height of 0.5 cm above the stacking gel. The gels were subjected to 130 V for 20 h and then stained and destained as above.

Miscellaneous. Protein was measured by the technique of Lowry et al. (1951). All solutions were made with deionized water by using analytical-grade reagents. The actin, troponin T, and tropomyosin complex isolated from 18-day chicken embryo muscle (Roy et al., 1976) was a generous gift of Dr. Satyapriya Sarkar.

Results

Three High Molecular Weight HMG's Common to All Chicken Tissues. The HMG fraction was obtained from 5% (Figure 1A) and 2% (Figure 1B) PCA extracts of whole tissues. This approach was selected so as to minimize proteolytic degradation while maximizing recovery. Goodwin et al. (1978) have shown that this procedure dramatically reduces proteolytic degradation. It also ensures complete recovery by avoiding the loss of some HMG to the cytosol after nuclear isolation (Bustin & Niehart, 1979; J. S. Gordon, J. Bruno, and J. I. Lucas, unpublished experiments).

The HMG fraction from 18-day embryonic skeletal muscle (lane 2), liver (lane 3), brain (lane 4), thymus (lane 5), and erythrocytes (lane 6) is resolved by $NaDodSO_4$–polyacrylamide electrophoresis (Figure 1). All five extracts contain three proteins comigrating with the HMG's isolated from the 0.35 M NaCl wash of erythrocyte nuclei (Figure 1, lane 1). These proteins have been identified by Sterner et al. (1978) in order of increasing mobility as HMG's 1, 2, and E. They have been denoted HMG's 1, 2A, and 2B, respectively, in Figure 1 for reasons to be developed further in this study. Thus, in contrast to their (Sterner et al., 1978) report, we find that the lowest molecular weight member of this triplet is not limited to chick erythrocytes. In addition, a protein with a similar mobility is also found in small quantities in calf thymus extracts (Figure 1, lane 7).

The presence of the fastest migrating member of the triplet in all tissues does not result from contamination by erythrocytes, since in some tissues, such as muscle, it is found in greater amounts than a protein comigrating with the erythrocyte-specific histone 5 (data not shown). Nor can erythrocyte contamination explain its extraction from calf thymus. More convincingly, the protein is present in proportions similar to that found in whole tissues, in extracts of fully differentiated 5-day-old muscle culture (Figure 2, lane 2), from which the erythrocytes were removed by frequent media changes, and in preparations of purified hepatocytes (Figure 2, lane 1) which can be shown to be 99% free of erythrocytes (Williams et al., 1978).

The low molecular weight member of this triplet does not correspond to any of the other HMG's previously identified on low pH gel systems. It is too large to be either HMG 14 or 17. It is unlikely to be the peptide called HMG 3 (Goodwin & Johns, 1973) since this species has been shown to result from

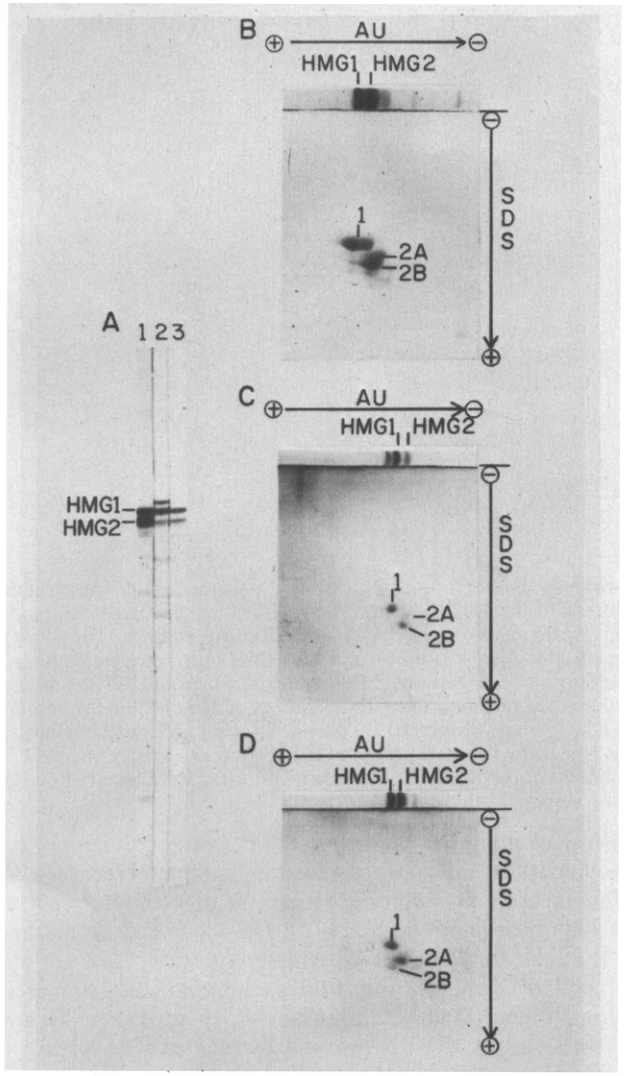

FIGURE 3: Two-dimensional polyacrylamide gel electrophoresis of the proteins in the HMG fraction of the 2% PCA extracts of various tissues. The HMG fractions from chicken muscle (1), erythrocytes (2), and calf thymus (3) analyzed in Figure 1 were subjected to acid–urea–polyacrylamide gel electrophoresis as described under Experimental Procedures. These first-dimension gels containing the proteins from muscle (B), erythrocytes (C), and calf thymus (D) were then equilibrated and subjected to $NaDodSO_4$ (SDS in the figure) gel electrophoresis, as described under Experimental Procedures. Parts C and D reproduce two parts of the same second-dimension gel, to which the first-dimension gels were applied.

an H1 degradation which is prevented by the extraction technique employed here (Goodwin et al., 1978). The data from Sterner et al. (1978) suggest that HMG E is only partially resolved from HMG 2 on low pH gels and carboxymethyl-Sephadex chromatography. Thus, if the low molecular member of the triplet corresponded to HMG E, it would be expected to comigrate with HMG 2 on acid–urea gels. This was tested by resolving the HMG's by two-dimensional gel electrophoresis (Savić & Poccia, 1978). The HMG's were resolved in the first dimension by an acid–urea gel system, followed by an $NaDodSO_4$ system in the second dimension. On the first-dimension acid–urea gels, the HMG's from all three sources contained, in common, two major species with a similar migration (Figure 3A). This pair of proteins also comigrated when extracts from each tissue were separately electrophoresed in neighboring lanes of an acid–urea slab gel or when extracts from two tissues were combined and electrophoresed on the same cylindrical gel (data not shown). The same pattern (data not show) was obtained in acid gels without urea, prepared as described by Johns (1967). Thus, on the basis of previous identifications, the slowly migrating species is HMG 1, while the more rapidly migrating one is HMG 2 (Goodwin & Johns, 1973).

In the second-dimension $NaDodSO_4$ gel, these two bands resolved into three spots with relative mobilities similar to the three main bands observed by one-dimensional $NaDodSO_4$ gel electrophoresis (Figure 3B–D). The spot with the highest molecular weight arose from the HMG 1 band which showed the slower mobility in the first dimension. In the extracts of the chicken tissue, two additional spots with similar differences in charge and molecular weight appear to arise from HMG 2. The more positively charged peptide of the pair has a slightly greater molecular weight. Its relative mobility on $NaDodSO_4$ gels is similar to that of HMG 2. The lowest molecular weight protein has a charge intermediate to HMG 1 and 2 and is most likely the protein comigrating with HMG E. The calf thymus extract also shows similar pattern on the two-dimensional gels. It is unclear in this case, however, if the lowest molecular weight member of the triplet arises from the HMG 1 or HMG 2 band of the first dimension. Further confirmation that these two lower molecular weight proteins are identical with the peptides comigrating with HMG 2 (HMG 2A) and HMG E (HMG 2B) on $NaDodSO_4$ gels is the similarity in approximate relative proportions of these two spots and of the two rapidly more migrating bands of the triplet, as seen on one-dimensional $NaDodSO_4$ gels. Thus, the spot with the $NaDodSO_4$ mobility of HMG 2 (2A) is found in relatively low amounts in the erythrocyte preparation but in relatively large amounts in the calf thymus extract. In muscle extracts, both spots are found nearly in equal amounts.

From this two-dimensional electrophoresis study, it appears that a protein very similar if not identical with HMG E can be extracted from all the chicken tissues and calf thymus. The most reasonable explanation is that HMG 2 in chicken consists of two different peptides with slightly different molecular weights and charges. Tentatively, the larger of the two will be termed HMG 2A and the smaller which was previously termed HMG E (Sterner et al., 1978) will be termed HMG 2B. While it is unclear if the lowest molecular weight peptide from calf thymus is identical with the peptide from chickens, it is similar enough in size and charge to be also designated HMG 2B.

Other minor spots besides the HMG's can also be resolved in the second dimension (Figure 3B–D). For instance, an unidentified erythrocyte peptide with a molecular weight greater than the HMG's is seen to migrate on $NaDodSO_4$ gels similarly to an H1 (Figure 3C). It does not, however, migrate faster than the HMG's on acid gels (Figure 3A,C) as would be expected for H1's (Goodwin & Johns, 1973). Also, as seen in Figure 3B in which the muscle HMG's are overloaded, there are a number of minor spots which could be HMG varients.

A 38 000-Dalton HMG-like Protein in Chicken Tissues. With the exception of brain, the 5.0% PCA extracts contain very few proteins other than the three HMG's. The 2.0% extracts, however, contain substantially more proteins. For example, two lower molecular weight proteins (marked by closed circles in Figure 1B) found in all the samples including calf thymus have mobilities on these gels expected for HMG's 14 and 17. They were not further characterized in this study. Attention was focused on the 38K protein, labeled X in Figure 1B, which migrates somewhat ahead of actin and shares many characteristics with the lower molecular weight HMG's. As seen in Figure 1B, it is easily detectable to chicken muscle, liver, and thymus and barely detectable in erythrocytes. It

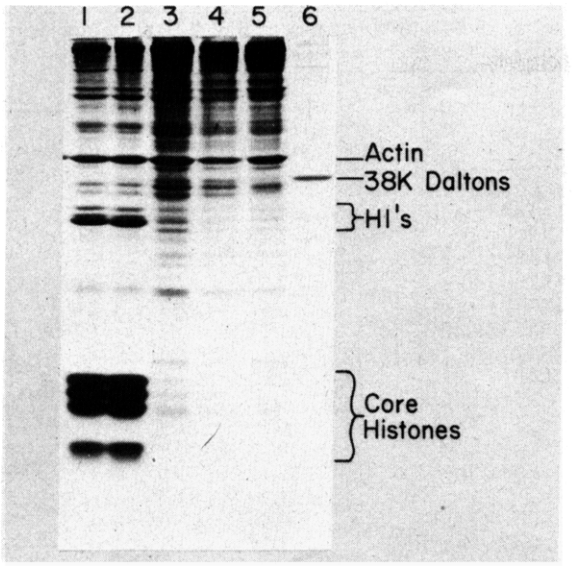

FIGURE 4: Electrophoretic analysis of the protein populations of the various fractions obtained during the isolation of the 38 000 molecular weight acid-soluble NHC protein from skeletal muscle nuclei. The acid-soluble nonhistones were prepared as described under Experimental Procedures from nuclei isolated from 18-day embryonic skeletal muscle. The distribution of the proteins in unextracted nuclei (1), nuclei extracted with 0.075 M NaCl (2), the 0.35 M NaCl extract of the nuclei (3), the 0.35 M NaCl extract after clarification by centrifugation at 40 000 rpm for 1 h in an SW-50.1 rotor (4), and the 2.0% Cl_3AcOH insoluble (5) and soluble fraction (6) was compared after electrophoresis on 15% discontinuous $NaDodSO_4$–polyacrylamide gels at 130 V for 4 h.

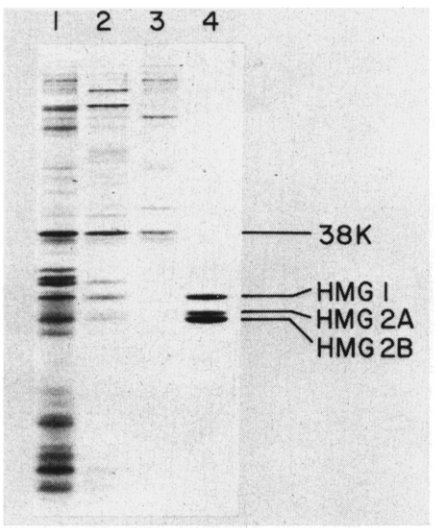

FIGURE 5: Electrophoretic comparison of the Cl_3AcOH-soluble nonhistones from nuclei of various chicken tissues. The protein distribution of the Cl_3AcOH-soluble chicken nonhistone proteins was prepared from the 0.35 M NaCl extract of nuclei of adult brain (1), liver (2), 18-day embryo skeletal muscle (3), and adult erythrocytes (4) as described in the legend of Figure 4, with the exception that the 0.35 M NaCl extract was fractionated with 1.5% Cl_3AcOH. The proteins were resolved as in Figure 4.

is not detectable in the calf thymus extract even when the gels are overloaded. It has not been resolved in whole brain extracts because a cytoplasmic protein of similar molecular weight is also soluble in 2.0% PCA.

The 38K protein is nuclear as it can be isolated from purified liver, brain, and muscle nuclei (Figures 4 and 5) by the same procedures originally used to identify the HMG's (Goodwin & Johns, 1973). A typical isolation from the nuclei of relaxed skeletal muscle is detailed in Figure 4. It is seen that the 38K protein is detected in the nonhistone proteins extracted from

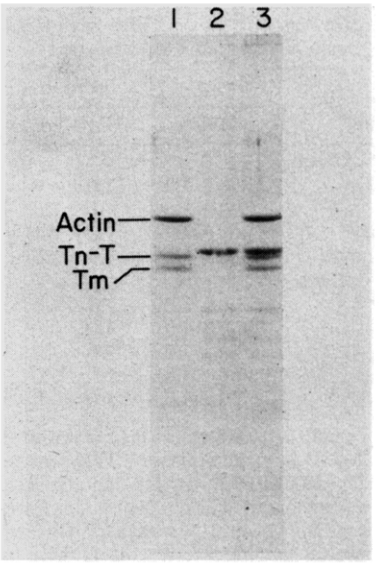

FIGURE 6: Electrophoretic comparison of the 38 000-dalton acid-soluble nonhistone chromosomal protein with skeletal muscle contractile proteins. The electrophoretic mobility of 18-day embryo leg skeletal muscle actin, troponin T, and tropomoysin (1) is compared with that of the 38 000-dalton protein (2) isolated as in Figure 4 by combining the preparations (3) and electrophoresing as in Figure 4.

these nuclei with 0.35 M NaCl (Figure 4, lane 3). It then partitions to the HMG fraction after bringing the extract to 1.5% Cl_3AcOH (Figure 4, lane 6). A protein with a similar mobility, as well as HMG's 1, 2A, and 2B, can be detected in similarly prepared HMG fractions from purified chicken liver and brain nuclei (Figure 5). Note that the HMG's are not detected in the acid-soluble nonhistone fraction from muscle because they were extracted by the relaxing solution during preparation of the nuclei. Preparation of nuclei at lower ionic strengths, however, results in the recovery of the HMG's (J. S. Gordon, J. Bruno, and J. J. Lucas, unpublished experiments).

The presence of the 38K protein in skeletal muscle raises the possibility that it is a cytoplasmic contaminant arising from the contractile apparatus. A likely candidate would be troponin T which has a similar molecular weight (Roy et al., 1976) and is also soluble in dilute acid (Hartshorne & Mueller, 1969). This possibility was eliminated by demonstrating that the 38K protein isolated with the HMG's from 18-day embryonic skeletal muscle nuclei does not comigrate with any of the proteins in the actin, troponin, and tropomyosin complex, even when both samples are electrophoresed in the same lane (Figure 6, lane 3). On the basis of its mobility in $NaDodSO_4$–polyacrylamide gels, the molecule weight of this protein is 38 000.

Quantitative Tissue-Specific Distribution of the PCA-Extractable Proteins. Observations of Figure 1 suggest that the relative proportions of each of the three HMG's and the 38K protein vary from tissue to tissue. This was verified by quantitating the percent of the total stain in each of the three HMG bands. The results are summarized in Table I. The relative amounts of HMG's 1, 2A, and 2B extracted from muscle, liver, thymus, and erythrocytes are independent of the PCA concentration. Whether this also holds for brain cannot be determined because of the inability in most cases to resolve HMG 2B in the 2.0% PCA extract from a cytoplasmic (compare with Figure 5, lane 1) protein with a slightly lower molecular weight (Figure 1B, lane 4).

It is readily apparent that the relative amounts of the three HMG's from each tissue are different. The differences be-

Table I: Relative Amounts of the Three High Molecular Weight HMG's Extracted from Various Chick Tissues[a]

tissue	rel amounts HMG 1	HMG 2A	HMG 2B
2% PCA Extract			
muscle	52.0 ± 4.0	27.0 ± 4.0	21.0 ± 5.0
liver	71.0 ± 3.0	5.0 ± 1.0	25.0 ± 2.0
thymus	53.0 ± 4.0	39.0 ± 2.0	8.0 ± 2.0
erythrocyte	45.0 ± 6.0	18.0 ± 3.0	37.0 ± 7.0
5% PCA extract			
muscle	53.0 ± 3.0	27.0 ± 5.0	20.0 ± 5.0
liver	76.0 ± 4.0	4.0 ± 1.0	20.0 ± 4.0
brain	67.0 ± 3.0	9.0 ± 2.0	24.0 ± 3.0
thymus	54.0 ± 4.0	39.0 ± 6.0	7.0 ± 3.0
erythrocyte	42.0 ± 3.0	15.0 ± 6.0	43.0 ± 7.0

[a] The HMG fraction from various tissues was prepared and subjected to $NaDodSO_4$-polyacrylamide gel electrophoresis as described in the legend of Figure 1. The staining intensity of the HMG bands was quantitated as detailed under Experimental Procedures. The staining intensity of each HMG is expressed as the percent of the total staining intensity of all three bands. The values represent the average for a minimum of 3 (erythrocytes) to a maximum of 11 (muscle) separate preparations plus or minus the average error.

tween tissues exceed the differences observed when the same tissue is extracted at different PCA concentrations. Thus, liver and brain, which show the closest similarity, have a twofold difference in the relative amounts of HMG 2A. This difference is substantially greater than the variability in the relative amounts of HMG 2A obtained when either tissue is extracted with different PCA concentrations. The quantity of the 38K protein relative to the HMG's also show tissue variability. It makes up 10% of the four proteins in muscle and liver extracts, approximately half as much in thymus, while it is detectable but not measurable in erythrocytes.

The tissue-specific proportions of the three HMG's are not the result of differential extraction due to proteolysis or other unknown causes. Supporting evidence comes from a number of observations. Extractions at low pH should inactivate most if not all proteases. The inclusion of pepsatin, an inhibitor of acid proteases (Umezawa et al., 1970), also does not affect the relative proportion of thymus HMG's (data not shown). Differential extraction from each tissue is made unlikely by the recovery of the same proportions of each HMG upon extraction with 2 and 5% PCA. Differential recovery was tested directly by extracting a mixture of chicken erythrocytes and thymus as detailed in Table II. In these experiments, HMG's were extracted from erythrocytes, thymus, and a combination of both tissues in the same proportions with 2.0% PCA. Approximately the same amount of Lowry-determined protein (Lowry et al., 1951) from each extract was then run in adjacent wells of the same $NaDodSO_4$ slab gel. As seen in Figure 7, the combined extract contains proportionally more HMG 2B than thymus alone and more HMG 2A than erythrocytes alone, as would be expected if the HMG proportions were additive. Complete additivity was confirmed by quantitating the staining intensity of each band. The results of two such experiments are detailed in Table II. It is difficult to control the exact amount of HMG's loaded on the gel because of the presence in each 2.0% PCA extract of differing amounts of extraneous proteins which contribute to the Lowry determination. This requires the following analysis, which is valid as long as the staining intensity of each HMG band is linearly related to the amount of protein it contains. The ratio of the sums of staining intensity of the total HMG triplet in the erythrocyte and thymus lanes to the total triplet staining intensity for combined extract was compared to the ratios of

Table II: Quantitative Recovery of HMG's from a Combined Thymus and Erythrocyte Extract[a]

	area HMG 1	HMG 2A	HMG 2B	TA
expt 1				
(1) Th[b]	8.71	7.85	2.39	18.97
(2) Er[c]	3.88	2.00	4.00	9.87
(3) sum (Th + Er)	12.59	9.87	6.39	28.84
(4) C[d]	7.57	6.90	3.20	17.67
(5) (Th + Er)/C	1.66	1.43	2.00	1.63
(6) $[(Th + Er)/C]/[(TA_{Th} + TA_{Er})/TA_C]$	1.01	0.87	1.20	
expt 2				
(1) Th	10.46	5.29	1.61	17.36
(2) Er	8.70	2.19	12.06	22.95
(3) sum (Th + Er)	19.16	7.48	13.67	40.31
(4) C	21.29	9.42	21.00	51.71
(5) (Th + Er)/C	0.90	0.79	0.65	0.77
(6) $[(Th + Er)/C]/[(TA_{Th} + TA_{Er})/TA_C]$	1.17	1.03	0.85	

[a] The areas (cm^2) under the peaks from densimetric tracings of the electrophoretograms of HMG fractions generated as described in the legend to Figure 6 were calculated as described under Experimental Procedures. The total area (TA) is determined from a tracing encompassing all three HMG peaks. The tracings for experiment 2 are presented in Figure 6. [b] Th = thymus. [c] Er = erythrocyte. [d] C = combination of thymus and erythrocyte.

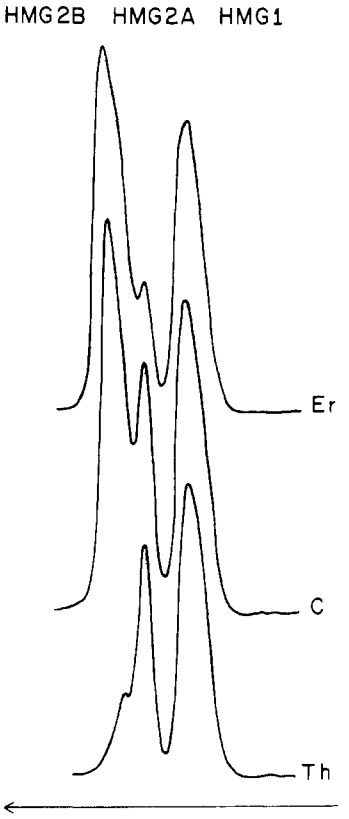

FIGURE 7: Quantitative recovery of HMG's on PCA extraction of whole tissues. The HMG fractions were prepared, as described in the legend of Figure 1, from chicken erythrocytes (Er) and thymus (Th) separately and a mixture (C) containing the same amounts of each tissue used in the individual extracts. The densitometric tracings of Polaroid transparancies of the 17% $NaDodSO_4$-polyacrylamide gel analysis of each fraction were obtained as described under Experimental Procedures.

the sum of each individual band in the erythrocyte and thymus lanes to each band in the combination. Equal ratios confirm

Table III: PCA-Soluble Proteins in Muscles of Chick Strains with Genomes Varying in Highly Redundant DNA Concentrations[a]

strain	rel amounts			
	38K peptide	HMG 1	HMG 2A	HMG 2B
White Leghorn	9.0 ± 2.0	49.0 ± 5.0	24.0 ± 5.0	18.0 ± 5.0
Rhode Island Red	7.0 ± 3.0	45.0 ± 2.0	27.0 ± 2.0	21.0 ± 2.0

[a] The relative amounts of the various HMG's are determined and expressed as detailed in the legend for Table I. The values represent the average for three separate experiments in which muscles from embryos from both strains were simultaneously extracted, plus or minus the average error.

additivity. As seen in Table II, the value for each HMG was within at least 20% of 1.0 for each individual experiment. If the ratios for both experiments are averaged, they fall within 5% of the value of 1. This is within the error of the staining intensity measurements. Thus, selective loss of any of the HMG's during isolation cannot account for the substantial difference in the relative amounts of HMG's 2A and 2B found in these tissues.

Since the HMG's are present in small quantity relative to histones, the HMG's may be involved in the structure of specific chromatin fractions. We asked about a preferential association of HMG's with constitutive heterochromatin by comparing their proportions in skeletal muscle from White Leghorn and Rhode Island Red embryos. While these strains differ markedly in their content of highly repetitive DNA (Colbert et al., 1976), the data of Table III show no significant differences in the relative proportions of the HMG's and the 38K protein.

Discussion

This investigation has shown that three high molecular weight HMG's previously isolated from avian erythrocytes (Vidali et al., 1977; Sterner et al., 1978) are also found in four other chicken tissues. In addition, a 38 000-dalton protein with characteristics similar to those of the HMG's has been discovered. Most importantly, it is evident that the relative amounts of these four proteins are tissue specific.

In a previous study (Sterner et al., 1978), the two higher molecular weight species of the chicken erythrocyte HMG triplet have been shown by gel electrophoresis, ion-exchange chromatography, and partial sequence analysis to correspond to calf thymus HMG's 1 and 2. The lower molecular weight (HMG E) member of the triplet, which was reported to be erythrocyte specific, was shown to be distinct from HMG's 1 and 2 by primary sequence (Sterner et al., 1978) and immunological analysis (Romani et al., 1979). In the present study, a protein corresponding to HMG E was found in at least four other chicken tissues and, possibly, calf thymus. During the preparation of this manuscript, a report (Mathew et al., 1979) appeared showing that HMG 2 from both chicken thymus and erythrocytes could be separated by chromatography into two subspecies of differing molecular weight and primary sequence. These two proteins appear to be identical with the two smaller members of the HMG triplet reported here on the basis of similar molecular weights and charge properties and their relative amounts in chicken thymus and erythrocytes. Thus, it is likely, though not proven, that the HMG 2B species we have identified in various tissues corresponds to HMG E and the HMG 2a species reported by Mathew et al. (1979). This conclusion is supported by the observation that the high molecular weight HMG's isolated from muscle and erythrocytes also have similar mobilities on two-dimensional gel electrophoresis. The two-dimensional analysis of the calf thymus HMG's suggests that the low molecular weight member of the triplet is very similar but definitely not identical with the comparable protein from chicken. Final verification that the lowest molecular weight member of the triplet is identical in all chicken tissues will require sequence analysis.

In this investigation we have also identified a protein with properties similar to the HMG's but having a molecular weight exceeding that previously reported for the HMG's. Like the HMG's, the 38K protein is extracted from nuclei at 0.35 M NaCl (Goodwin et al., 1973), is soluble at low acid concentrations (Goodwin et al., 1973), and partitions with the HMG's in acetone–HCl (Sanders & Johns, 1974). In addition, it can be separated from the H1's with the other HMG's by elution from carboxymethyl-Sephadex columns with 0.6 M NaCl (D. Levy, M. Thoguluva, and J. S. Gordon, unpublished data). It does, however, show subtle differences on comparison with HMG's 1, 2A, and 2B. The 38K protein appears to be more tightly bound to nuclei since it is retained within skeletal muscle nuclei under conditions which extract the other HMG's (Figure 4). It is also soluble at slightly lower PCA concentrations. Although these subtle differences should not rule against its tentative identification as an HMG, its confirmation as an HMG will require sequence analysis to determine if it contains high amounts of acidic and basic residues distributed in a manner leading to the charge asymmetry characteristic of the HMG's (Walker et al., 1976a,b, 1977, 1978, 1979).

This study has demonstrated that HMG's 1, 2A, and 2B and the 38K protein are found in all the chicken tissues studied but in different proportions. These tissue-specific quantitative differences do not appear to result from differential recovery because when two tissues are extracted in combination, the original proportions of each HMG are recovered. Thus, the HMG's are similar to the H1's (Bustin & Cole, 1968; Kinkade, 1969; Panyim et al., 1971) in showing a quantitative tissue specific heterogeneity.

The finding of nearly identical proportions of HMG 1, HMG 2A, HMG 2B, and the 38K protein in skeletal muscles from White Leghorn and Rhode Island Red embryos suggests that none of these proteins is preferentially localized to constitutive heterochromatin. Since the White Leghorn genome contains 1–2% highly repetitive sequence while the Rhode Island Red genome contains over 30% highly repetitive sequence (Colbert et al., 1976), it is likely that a preferential localization of any one of these chromatin proteins would have been detected.

The tissue-specific distribution of the high molecular weight HMG's demonstrates that changes in chromatin structure occur during cell differentiation. Since the HMG's are associated with active chromatin regions (Vidali et al., 1977; Levy-W. et al., 1979; Weisbrod & Weintraub, 1979), the tissue-specific HMG pattern may underly the distribution of particular chromatin structures required for cell-specific gene expression. Little is known, however, about the localization and function of HMG's 1 and 2 in chromatin. Evidence from nuclease digestion experiments suggest that these HMG's may be localized on nucleosomal linker regions (Levy-W. et al., 1977, 1979). This, taken with the interaction of the high molecular weight HMG's and H1 in solution (Smerdon & Isenberg, 1976; Yu & Spring, 1977), suggests that these two sets of proteins may interact in regulating different supranucleosomal structures associated with the expression of different gene sets. If this is the case, these structures must be organized by a third class of chromosomal components with sufficient heterogeneity to recognize specific DNA sequences. The

limited HMG heterogeneity is insufficient for gene-specific sequence recognition but could suffice for interactions with common domains in specific regulatory proteins. Alternatively, the limited HMG heterogeneity could suffice for direct interaction with a limited set of moderately redundant DNA sequences. Analyses of HMG interactions during specific developmental transitions should prove particularly useful in testing these possibilities.

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