STUDIES ON THE HIGH MOBILITY GROUP OF NON-HISTONE CHROMATIN PROTEINS

by

James Lawrence Fishback

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Approved by:

Gerald R Reeck

Major Professor

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INTRODUCTION

Histones and Chromatin Structure

Chromatin is the chromosomal material of interphase eukaryotic cells. It consists of DNA, two classes of protein (histone and non-histone), and a small amount of RNA (for reviews see 1-3). As an example, these components have mass ratios (setting DNA=1) of 1/0.79/1.19/0.06, respectively, in the chromatin obtained from Drosophila melanogaster embryos (4).

The histones have been well defined both physically and biologically. A summary of nomenclature and physical characteristics appear in Table 1. Histones are found in the chromatin of all eukaryotes and are highly conserved from an evolutionary standpoint (6,7). The estimated mutation rate of histone H4 is 0.06 per 100 amino acid residues per 100 million years, the lowest rate yet observed (8).

It has by now been well established that the histones are the major structural proteins of chromatin. The critical evidence began to accumulate around 1971 when Clark and Felsenfeld demonstrated that staphylococcal nuclease could digest only about 50% of the DNA in several types of chromatin (9). Hewish and Burgoyne in 1973 found that endogenous nucleases digested rat liver chromatin to 200 base pair fragments, as determined by gel electrophoresis (10). The data indicated that the DNA was being protected in some way by chromatin proteins (9).

In 1974, Olins and Olins examined individual chromatin fibers by electron microscopy (11). They noticed repeating structures, like

TABLE I

Class	Fraction	Lys/Arg	Total Residues	Molecular Weight
Very Lysine Rich	H1(I,f1,KAP)	22	215	21,500
Lysine Rich	H2A(IIb1,f2a2, ALG)	1.17	129	14,004
	H2B(IIb2,f2b,KSA)	2.5	125	13,774
Arginine Rich	H3(III,f3,ARE)	0.72	135	15,324
	H4(IV,f2a1,GRK)	0.79	102	11,282

All data for histones of calf thymus

Compiled from references 22 and 23

beads on a string, and termed the beads 'nu bodies'. This repeat unit is now termed the nucleosome. The authors speculated that this structure might be the result of histone - DNA complexes.

Kornberg was the first to put forth a possible model for the structure of chromatin (12). He and Thomas had found association products of histones in solution: an H3-H4 tetramer and an H2a-H2b oligimer (13,14). Kornberg reasoned that these four main histones in an octameric conformation, plus about 200 base pairs of DNA could form the nucleosome structure. A chromatin fiber could be the result of many such units joined together with DNA (12).

Reconstitution studies by Oudet et al. (15) showed that when equimolar amounts of the four histones were combined with naked DNA, structures were formed that closely resembled those seen by Olins and Olins (11) in their earlier study. This electron micrograph study was conclusive proof that the nucleosome was composed of histone-DNA complexes.

Noll's studies on rat liver nuclei with staphylococcal nuclease (16) confirmed the 200 base pair repeat unit found by Hewish and Burgoyne (10). In addition, Noll found that longer digestion could produce a stable particle of 140 base pairs. When other organisms were examined (see Table 2), the 200 base pair nucleosome repeat length was found to be variable, but the 140 base pair 'core particle' was found in all chromatins examined (17,18,19).

TABLE II

DNA Content of Nucleosome of Various Organisms

Cell Type	DNA Content of Nucleosome (Base Pairs)	Reference
Aspergillus	154	17
Yeast	165,163	24,25
Cells grown in culture		
HeLa	183,188	26,27
Hepatoma	188	27
Rat Liver	198,196	24,27,28
Chicken Erythrocyte	198,206,212	26,27,29
Sea Urchin Sperm	241	19
Moth	200	30

To summarize, the core histones exist as an octamer of two each of the four smaller histones. These are tightly complexed with approximately 140 base pairs of DNA, which constitutes the core particle. The remainder of the DNA in the repeating unit (nucleosome) is called the spacer region. Histone H1 is probably associated with this spacer region (20,21).

Non-histone Chromosomal Proteins

In addition to the histones, there is another class of proteins found in chromatin. The non-histone proteins are a highly heterogenous group, and rather ill-defined. As an operational definition, any protein found in isolated chromatin that is not one of the histones is termed a non-histone. It is possible that different chromatin isolation procedures could affect the non-histone population observed. In fact, Bhorjee and Pederson report contamination of chromatin by nuclear ribonuclearprotein particles, giving a false interpretation on the non-histone population (31). Other studies report similar findings (32,33).

Except for proteins with known enzymatic activities, the biological function of any given non-histone is unknown. Peterson and McKonkey resolved over 400 different non-histone peptides from HeLa cells using two dimensional electrophoresis (34), therefore, pinpointing the function of any one protein will be a difficult problem.

A good deal of recent research has centered on the fact that certain regions of the genome are selectively transcribed. As

indicated above, the histones have been assigned to primarily a structural role in chromatin, leaving the non-histones as candidates for specific gene regulation (35).

The most direct evidence that some non-histones function in a regulatory capacity comes from chromatin reconstitution studies. An example of such a study is that done by Tsai et al. on the chick oviduct system (36). This particular system is well suited to this type of work since the expression of the gene product (ovalbumin) is regulated by a hormone (estrogen) (37).

Tsai et al. isolated chromatin from the oviducts of chicks which had either received diethylstilbestrol stimulation for 14 days, or had been treated and then withdrawn from hormone administration. The non-histone chromatin proteins from each type were then extracted with 5M urea/ 50mM sodium phosphate (pH 7.5). The chromatin was then reconstituted by gradient dialysis against successively lower concentrations of NaCl. Results indicated that extractable non-histone proteins from stimulated chromatin were capable of activating the in vivo transcription of the ovalbumin gene in withdrawn chromatin. This was judged by use of complementary DNA (cDNA) that was prepared from isolated messenger RNA. This data seems to indicated that some protein or proteins in the non-histone fraction are capable of specific gene regulation (36).

The High Mobility Group Proteins

The high mobility group (HMG) chromosomal proteins were first detected by Johns as impurities in histone preparations (38). Later,

Walker and Johns demonstrated that this particular subset of the non-histone chromosomal proteins had some unique properties and was easily obtainable (39). Specifically, the HMG proteins can be extracted from chromatin with 0.35M NaCl, are soluble in 2% trichloroacetic acid (TCA), and are insoluble in 10% TCA. They derive their name from the fact that the original proteins isolated from calf thymus showed 'high mobility' on acid/urea polyacrylamide gels (39).

The amino acid composition of the HMG proteins is unusual (see Table 3). Approximately half of the amino acid residues of each protein is either acidic (Asp, Glu) or basic (Lys, Arg) residues. The entire sequences of HMG 14 and 17 have recently been determined (40,41). There has been a partial sequence of HMG 1 published which shows an unusual sequence of 41 consecutive Asp and Glu residues (42).

Initially, the HMG proteins from calf thymus were numbered 1 through 17. Recently, however, Goodwin and Johns have provided evidence that only HMG 1,2,14, and 17 occur *in vivo* (43). The other bands noticed on polyacrylamide gels are then presumably breakdown products from proteolysis. There is evidence that trout testis (44) and avian erythrocytes (45) may contain unique HMG proteins, and these are named HMG T and HMG E respectively. Goodwin and Johns have just published a paper in which they claim HMG E is actually another form of HMG 2, which they prefer to call HMG 2b (46). In addition, Alfagame *et al.* have isolated a protein from *Drosophila* that has an amino acid composition similar to the HMG proteins. It can also be extracted from chromatin under the same conditions as the HMG

TABLE III

Amino Acid Compositions of the High Mobility Group from Calf Thymus

	HMG 1[a]	HMG 2[a]	HMG 14[b]	HMG 17[c]
Asp	10.7	9.3	8.0	12.3
Thr	2.5	2.7	4.0	1.1
Ser	5.0	7.4	9.0	2.2
Glu	18.1	17.5	18.0	10.1
Pro	7.0	8.9	7.0	12.3
Gly	5.3	6.5	6.0	11.2
Ala	9.0	8.1	16.0	18.0
Cys	-	-	-	-
Val	1.9	2.3	4.0	2.2
Met	1.5	0.4	-	-
Ile	1.8	1.3	-	-
Leu	2.2	2.0	2.0	1.1
Tyr	2.9	2.0	-	-
Phe	3.6	3.0	-	-
His	1.7	2.0	-	-
Lys	21.3	19.4	21.0	24.7
Arg	3.9	4.7	5.0	4.5

All Values in Mole Percent

a. Reference 63.

b. Reference 41.

c. Reference 40.

proteins, but it has a much higher molecular weight (47).

Since the HMG proteins represent an easily obtainable subset of the non-histones, studies on their probable function has been intense. Goodwin and Johns seem to feel that the HMG proteins play mainly a structural role, considering they occur at about 10^6 molecules per nucleus (48), and are distributed through three eukaryotic kingdoms (49). Other investigators feel that the HMG proteins may have some role in transcription. Levy et al. (50) and Vidali et al. (51) reported that when chromatin is digested with DNase I (which should preferentially degrade transcribed sequences), HMG proteins are released. In several recent papers, Levy et al. (52,53) reported that micrococcal nuclease would preferentially release mononucleosomes enriched in both HMG proteins and transcribed sequences.

Another interesting line of work with HMG proteins has involved various types of labeling studies. Bustin showed that antibodies to HMG 1 reacted with chromatin (54). He extended this earlier work by labeling the antibodies to HMG 1 with a fluorescent dye. By using the fluorescence of the dye as a marker, micrographs showed that HMG 1 was not only associated with chromatin, but it was also present in the cytoplasm of whole cells (55). In a different type of study, Rechsteiner and Kuehl labeled HMG 1 with ^{125}I. When the labeled protein was injected into cells, it was rapidly transported from the cytoplasm to the nucleus, as visualized by autoradiography (56).

Javaherian et al. (57) reported that calf thymus HMG 1 and 2 can introduce negative supercoils into nicked PM2 DNA when the DNA

is ligated in the presence of either protein. The authors suggest that one possible explanation of this result would be if the proteins bound preferentially to single-stranded DNA. This possibility was recently supported when Bidney and Reeck isolated two proteins from cultured rat hepatoma cells that bound preferentially to single-stranded DNA (58). These two proteins appeared to be analogous to calf thymus HMG 1 and 2.

Blüthmann has reported the isolation from mouse myeloma cell of two DNA binding proteins (59). He also isolated another DNA binding non-histone from bovine lymphocyte (60). Filter binding studies showed that all of these proteins 'preferred' single stranded DNA. The protein isolated from lymphocyte is especially interesting since it was shown to contain 25.3% acidic and 18.3% basic amino acid residues. This is quite similar to the HMG proteins.

Blüthmann's isolation scheme differs significantly from that used by Bidney and Reeck. Bidney and Reeck used sequential DNA chromatography to select proteins that preferentially bind to single-stranded DNA. This method was first utilized by Herrick and Alberts to select for proteins with high affinity for single-stranded DNA (61). To apply the method, one seeks the proper solvent conditions so that proteins not binding to the initial double-stranded DNA column will remain bound tothe single-stranded DNA column. Blüthmann, however, used no such selection process. He first chromatographed his chromatin extracts on hydroxyapatite columns, then applied them to single-stranded DNA. In addition, Blüthmann's proteins were exposed

to 5M urea, which could alter their biological activity.

Single-stranded binding proteins are potentially important to DNA replication or transcription because they could destabilize the DNA double helix. Alberts and colleagues have extensively studied the helix destabilizing gene 32 protein of bacteriophage T4. Alberts and Frey (62) used single stranded DNA immobilized on a cellulose matrix to select for proteins that had a high affinity for single-stranded DNA. The result was the isolation of a protein which could dramatically reduce the T_m of poly dA-dT, as well as catalyze the renaturation of T4 single-stranded DNA.

In this thesis, single-stranded binding proteins from several sources will be examined in an attempt to discover whether or not HMG proteins have a general preference for single-stranded DNA. Sequential DNA chromatography is the method of choice, since it offeres the possibility of not only isolating individual proteins in reasonable purity, but the fact that the proteins prefer single-stranded DNA may give a clue to their possible biological functions.

MATERIALS AND METHODS

Cell Culture Conditions

HTC Cells (64). Hepatoma tissue culture cells (HTC cells) were grown in suspension culture in Swims 77 medium supplemented with: calf (5%) and fetal calf (5%) serums that had been held at 57° for 20 minutes / $NaHCO_3$ (0.5 g/l) / glucose (1.2g/l) / tricine (9g/l) / cystine (0.014g/l) / phenol red (10mg/l) / and, just before use, glutamine (0.29g/l). The pH of the medium was adjusted to 7.3 at 37° before filter sterilization (Millipore 0.22 micron pore size).

T.ni Cells (65). Trichoplusia ni (T.ni) cells derived from the ovary of the cabbage looper were grown in monolayer culture in Graces TC media (180 ml) supplemented with TC yeastolate (0.6 g) / lactalbumin hydrolysate (0.6 g) / Gentimycin (10mg) and 0.1M N,N-Bis(2 hydroxyethyl)-2 aminoethane sulfonic acid (BES) as recommended by Koval (66). The pH of the medium was adjusted to 6.2 with 1 N KOH before filter sterilization. 16 ml of fetal bovine serum was added just before use. Flasks had a growth area of 150 cm^2.

Determination of Protein, DNA, and RNA Concentrations

Protein concentrations were determined by a modification (67) of the method of Bradford (68) using Coomassie Brilliant Blue G-250 (Eastman). 200 mg of dye was dissolved in 50 ml of absolute ethanol. At this point the color of the solution should be a very bright blue. 100 ml of 85% phosphoric acid is then added, when causes a color change to brown. Distilled water is added to approximately 800 ml

total volume, and the solution is adjusted to pH 1 by addition of solid NaOH.

The pH is very hard to obtain since the dye seems to coat the electrode. It was sometimes easier to obtain the correct pH by just adjusting the color of the solution to dark green. When the 'proper' color was obtained, the mixture was diluted to 1 liter. A pH that is too low will give a brown colored solution, and a pH that is too high will give a blue solution.

A standard curve was generated with ovalbumin, using a stock solution to 1 mg/ml. The curve was linear from 0 to 30 micrograms, after which it became curvilinear. 0.2 ml of sample were added to 5 ml of dye reagent and gently shaken, the absorbance was read 2 minutes later at 595 nm.

DNA concentrations were determined spectrophotometrically at 260 nm in 1% sodium dodecyl sulfate (SDS), assuming $A_{260}^{0.1\%} = 21$. Alternatively, a diphenylamine assay was used (69).

RNA concentrations were determined using the modified orcinol assay of Almog and Shirey (70). 6% (w/v) orcinol (0.35 ml) in water was added to 5 ml concentrated HCl. A 1 ml sample was incubated with 4.0 ml of 85/15(v/v) H_2SO_4/H_2O for 24 hours at 40°. Then 0.1 ml of the above orcinol solution was added and the mixture was incubated with shaking at 100° for 30 minutes. Absorbance was read at 500 nm and a standard curve generated using 0-50 micrograms of yeast RNA per ml.

Electrophoresis

Samples were prepared for electrophoresis by dialysis against the following buffer: 10% glycerol / 0.06M Trisphosphate (pH 6.7) / 1% β-mercaptoethanol / 1% SDS. The samples were placed in a boiling water bath for 15 seconds prior to electrophoresis. Samples were stored at -20°.

Alternatively, samples were concentrated for electrophoresis by precipitation with 20% TCA. 100% TCA was added to achieve the final concentration of 20%, then samples were allowed to set for approximately 1 hour at 4°. The samples were then centrifuged at 5000x g and the supernatant discarded. The pellet was washed twice with a small amount (5 ml) of acetone to remove remaining TCA, centrifuging at 5000x g after each wash. The pellet was then suspended in the desired amount of electrophoresis buffer (as above).

Polyacrylamide electrophoresis was conducted in the presence of SDS according to the procedure of Laemmli (71), except at half the bis concentration. Electrophoresis was carried out in slabs (0.2 x 14 x 14 cm) for 4 hours) at 25 ma and at 4°. Gels were strained overnight in 0.25% Coomassie Brilliant Blue R-250 (Sigma) / 50% ethanol / 10% acetic acid. The gels were destained in 25% ethanol / 10% acetic acid.

Densitometric scans were obtained with a spectrodensitometer (Schoeffel Instrument Corporation) equipped with an Omniscribe Recorder from Houston Instruments. The settings on the instrument were: gain, 10%; bias, negative; OD units, 1; time constant, 0.1 second. The

machine was set to the transmittance mode, and the wavelength used was 600 nm. It was usually necessary to switch the leads on the recorder to obtain a positive output. It is possible to adjust both the length and width of the scanning beam. It was best to set the width as narrow as possible to separate out distinct bands from the gel. The length of the scanning beam was different depending on the gel. If some spreading of the sample on the gel occurs, it was best to adjust the length to approximately the same value as the width. This meant that a small square of light could be seen, and this was placed so that the small square scanned down the middle of all the bands present on the gel. However, if no spreading of bands has occurred, I personally feel that the length of the scanning beam should be set so that the length of the beam is the same as the size of the bands on the gel.

Preparation of Double Stranded and Single Stranded DNA Columns

Sepharose Columns. 500 mg of calf thymus DNA (Sigma) was dissolved in 100 ml of 0.9% NaCl / 0.1% SDS. The DNA was purified by adding 100 ml of cold chloroform / isoamyl alcohol (25:1) and shaking vigorously. The emulsion was broken by centrifugation in galss centrifuge tubes for 10 minutes at 5000x g. The DNA in the supernatant was precipitated by the addition of 2 volumes of ice cold ethanol. The DNA was then collected on a glass rod and dissolved in 100ml of 0.9% NaCl/0.1% SDS and the extraction repeated an additional 2 times. The DNA was then collected on a glass rod and dissolved in 100ml of 0.9% NaCl/10mM Tris HCl (pH 7.5). The DNA

was sonicated by three 10 second bursts at a setting of 115 watts using a Branson Sonifier equipped with a microtip. The DNA was then dialyzed against an excess of 10 mM potassium phosphate (pH 8.5). Denatured (single stranded) DNA was obtained by placing a 50 ml portion of the above DNA in boiling water for 30 minutes, followed by rapid cooling in an ice bath.

Both the double and single stranded DNA were coupled to CNBr activated Sepharose 4B (Sigma) as described by Arndt-Jovin (72). Two 5 gram portions of activated Sepharose were treated for 30 minutes with 1mM HCl followed by collection of the beads on a sintered glass funnel. The treated Sepharose was then added to 50 ml of the above double stranded or single stranded DNA and coupling was allowed to proceed at 25° for 12 hours. The beads were then packed in a 1.3 x 13 cm glass column and washed with 10 mM Tris HCl (pH 7.5) followed by 2M NaCl/1mM Tris HCl (pH 7.5).

The double stranded DNA column was then equilibrated to 5% glycerol/10mM NaCl/50mM sodium acetate (pH 4.5)/1mM $ZnSO_4$. 10 units of S_1 nuclease (Calbiochem) was added to the column and slowly circulated for 24 hours. The column was then rinsed with 10mM Tris HCl (pH 7.5) and 2M NaCl/1 mM Tris HCl (pH 7.5). All columns were stored at 4° in 2M NaCl/1 mM Tris HCl (pH 7.5) when not in use.

Cellulose columns. DNA-cellulose was prepared according to the method of Herrick and Alberts (73). Salmon sperm DNA (Sigma) was purified as described above. The cellulose was first washed several times with boiling ethanol to remove contaminating pyridine, quickly

washed on a sintered glass funnel successively with 0.1M NaOH, 1 mM EDTA (pH 7.5), and 10 mM HCl solutions, and then washed with an excess of H_2O to neutrality. Following this step, the cellulose was lyophilized to dryness and stored.

To absorb DNA to cellulose, a solution of the salmon sperm DNA at 1-3 mg/ml/10 mM Tris HCl (pH 7.5)/1 mM EDTA is transferred to a glass beaker. Treated cellulose (as above) was added at approximately 1 gram of cellulose per 3 ml of solution. The lumpy mixture was spread on evaporating dishes to dry at room temperature. It was important to remove any remaining water by an overnight lyophilization. The dry powder is suspended in approximately 20 volumes of 10 mM Tris HCl (pH 7.5)/1 mM EDTA and left at 4° for a day.

After two washes to remove free DNA, the DNA cellulose was either stored frozen as a slurry in EDTA (pH 7.5)/0.15M NaCl or packed in columns and treated in a similar manner to the DNA Sepharose columns described above. Normally, about half of the original DNA is adsorbed to the cellulose. Double stranded DNA-cellulose was treated with S_1 nucleuse in an analogous manner to double-stranded DNA-Sepharose as above.

Nuclei and Chromatin Isolation

HTC Cells

Cells from suspension culture were harvested from growth medium by a 20 minute centrifugation at 5000x g. The cells were gently suspended in 0.05M Tris HCl (pH 7.5)/0.025M KCl/0.005M $MgCl_2$ (TKM) containing 0.25M sucrose (TKM/0.25M sucrose). The suspension was

briefly homogenized with a Potter-Elvehjem homogenizer, then collected by centrifugation at 5000x g for 10 minutes. This step was repeated to ensure the cells were completely free of contaminating growth medium.

Cells were lysed by suspension in TKM alone, containing no sucrose as an osmoticum. Using a tight fitting Potter-Elvehjem homogenizer, the suspension was homogenized extensively (50 strokes), cooling in ice every 5 strokes to avoid an excessive buildup of heat. In a typical preparation, cells from 4 liters of growth medium were suspended in 50 ml of TKM. The crude nuclei were pelleted by centrifugation at 5000x g for 10 minutes. This pellet was suspended in TKM/0.25M sucrose containing 1% Triton X-100, and homogenized for about 10 strokes. The nuclear pellet thus obtained was washed once with TKM/0.25M sucrose alone, then stored frozen at -20° in TKM/0.25M sucrose.

Chromatin was prepared from fresh or frozen nuclei by homogenizing the nuclear pellet in 10mM Tris HCl (pH 7.5). The chromatin was pelleted by centrifugation at 5000x g for 10 minutes. This was repeated twice to ensure full swelling of the chromatin.

Rat Liver

Nuclei were isolated by a slight modification (74) of the method of Blobel and Potter (75). 15 grams of frozen rat liver (Pel Freeze) were minced in a 30 ml volume of TKM/0.25 M sucrose. This was homogenized with a Potter Elvehjem homogenizer for approximately 15 strokes. The homogenate was then filtered through two layers of cheese

cloth. Three volumes of TKM/2.1M sucrose were then added, along with 10 grams of solid sucrose. The additional sucrose was dissolved by shaking, and the solution was then layered on 10 ml pads of TKM/2.1M sucrose in 40ml nitrocellulose centrifuge tubes. These were centrifuged on a Spinco SW-27 rotor at 125,000 x g for 1 hour at 4°. The top layer of cytoplasm was carefully removed by suction and the sides of the tubes washed with TKM buffer. The TKM/2.1M sucrose pad was then removed and the nuclear pellet suspended in TKM/0.25M sucrose. The suspension was homogenized briefly, and the nuclei collected by centrifugation at 5000x g for 10 minutes. This pellet was then suspended in TKM/0.25M sucrose containing 1% Triton X-100. The suspension was homogenized with about 10 strokes, and the nuclei collected by centrifugation at 5000x g for 10 minutes. The nuclei were washed once in TKM/0.25M sucrose alone, then stored frozen in TKM/0.25M sucrose at -20°.

Chromatin was prepared from fresh or frozen nuclei by homogenizing the nuclear pellet in 10 mM Tris HCl (pH 7.5). This step was repeated 2-3 times to ensure that the chromatin had swelled fully.

Rat liver cytoplasm was obtained by homogenizing 15 grams of frozen rat liver in 30 ml of TKM/0.25M sucrose containing 1% Triton X-100. The cytoplasm fraction was then separated from nuclei and other contaminants by centrifugation at 10,000x g for 10 minutes.

Calf Thymus

Approximately 30 grams of frozen calf thymus (Pel Freeze) was minced and placed in a Potter-Elvehjem homogenizer along with 200 ml

of 0.25M sucrose/5mM $CaCl_2$/50mM Tris HCl (pH 7.5). (Buffer A) containing 1% Triton X-100 and 1mM phenyl methane sulfonylfluoride (PMSF-Sigma). After approximately 20 strokes, the homogenate was centrifuged at 5000x g for 10 minutes. The supernatant was saved as the cytoplasm fraction, and the pellet suspended in 200 ml of buffer A containing 1% Triton X-100. The suspension was homogenized briefly, and the resulting homogenate was filtered through two layers of cheese cloth to remove fat and connective tissue. The filtrate was centrifuged at 5000x g for 10 minutes. The resulting nuclear pellet was washed once in buffer A alone.

To obtain chromatin, the nuclear pellet was washed successively with 10mM Tris HCl, 5mM Tris HCl, and 1mM Tris HCl, all at pH 7.5. The final chromatin pellet was rather tenuous, and care had to be taken in pouring off the supernatant so as not to lose any of the chromatin. Typically, some 50 ml of chromatin at 2mg/ml were obtained by this procedure.

T.ni Cells

T.ni cells were harvested from growth medium by centrifugation at 500 x g for 20 minutes. The pellet was suspended in buffer A, homogenized, and collected by centrifugation at 5000x g for 10 minutes. This step was repeated an additional time to ensure any contaminating growth medium had been removed, To lyse the cells, they were suspended in buffer A containing 1% Triton X-100. The suspension was homogenized for about 15 strokes, and the nuclei collected by centrifugation at 5000x g for 10 minutes. The nuclei thus obtained

were washed once in buffer A alone, then stored frozen at -20° in buffer A.

Chromatin was prepared from fresh or frozen nuclei by homogenizing the nuclear pellet in 1mM Tris HCl/1mM EDTA (pH 7.5). The homogenate was allowed to set on ice for 10 minutes before the chromatin pellet was collected by centrifugation at 5000x g for 10 minutes. This was repeated twice to ensure full swelling.

Amino Acid Analysis

Protein samples that had been dialyzed against deionized H_2O were hydrolyzed in 6N HCl at 110° for 24 hours. Analyses were performed on a Beckman 121 Amino Acid Analyzer equipped with a Columbia Scientific integrator (Supergrator-3).

Conductivity Measurements

NaCl concentrations were determined with a Model 31 Conductivity Bridge from the Yellow Springs Instrument Company. A standard curve was prepared, and temperature of both samples and standards was 4°.

Sucrose Gradient Centrifugation

Solid scurose and NaCl were added to 2ml of insect chromatin to give 10% sucrose (w/v) and 0.75M NaCl. The samples were then layered over 9.5 ml sucrose gradients, which contained 0.75M NaCl. The gradients were from 10 to 40% sucrose, and were constructed in cellulose nitrate tubes (9/16 x 3 1/2 in.) over 0.5ml of 60% sucrose/ 0.75M NaCl. Samples were subjected to centrifugation in a Spinco SW-41 rotor at 240,000x g for 6 hours. Gradients were collected in 0.7ml

fractions by pumping 80% sucrose/2M NaCl into the bottom of the tubes. SDS dissociation studies were done in an analogous manner, with 2.5% SDS instead of 0.75M NaCl (76).

RESULTS AND DISCUSSION

Calf Thymus HMG Proteins

The HMG proteins were first discovered in calf thymus tissue by Johns (38). Further study by Goodwin and Johns (43) indicated that there were four main HMG proteins found *in vivo*, namely HMG 1,2,14, and 17. Goodwin and Johns also noted that there were high levels of proteolytic enzymes in calf thymus, and that they were not fully inhibited by PMSF (43). Therefore, in the following chromatography experiments, all manipulations were carried out at 4° and completed as rapidly as possible (within 48 hours). In addition, PMSF was added at several steps in the isolation procedure.

Nuclei and chromatin were obtained as outlined in Materials and Methods. The chromatin was extracted by adding solid NaCl to a final concentration of 0.35M. The salt was added slowly as the chromatin was being stirred with a magnetic stir bar. After all the NaCl had been added, the mixture was allowed to stir in the cold for an additional 30 minutes. It proved prudent to add 1M PMSF at this point to adjust the chromatin extract to a final concentration of 1mM PMSF.

The extracted protein was separated from DNA and any remaining bound protein by centrifugation at 25,000x g for 30 minutes. The supernatant was diluted with 1mM Tris HCl (pH 7.5) to a final concentration of 0.2M NaCl. Normally, this caused some protein to precipitate, and this was removed by a further centrifugation at 10,000x g for 10 minutes.

The A_{220} of the supernatant was measured, and from 50-100 A_{220} units of protein were then loaded onto a double stranded DNA cellulose column equilibrated to 0.2M NaCl/1mM Tris HCl (pH 7.5). DNA cellulose and DNA Sepharose behaved equivalently in all experiments, however DNA cellulose was used routinely since double stranded DNA is coupled rather inefficiently to CNBr activated Sepharose (72). After the column was loaded, it was washed with 0.2M NaCl/1mM Tris HCl (pH 7.5) until the A_{220} of the fractions reached a baseline level. The protein which was washed off the column by this 0.2M NaCl wash is hereafter called the double-stranded breakthrough or runthrough. Any protein bound to the double-stranded column was eluted with 2M NaCl/1mM Tris HCl (pH 7.5). At this ionic strength (0,2 M NaCl), it was determined by Coomassie Blue binding assay that approximately 25% of the total protein bound to the double-stranded DNA column. Figure 1 shows a profile from double-stranded DNA cellulose, showing the fractions pooled as the double-stranded DNA cellulose, showing the fractions pooled as the double-stranded runthrough.

The double-stranded runthrough was next loaded onto a single-stranded DNA Sepharose column equilibrated to 0.2M NaCl/1mM Tris HCl (pH 7.5). The column was washed with 0.2M NaCl/1mM Tris HCl (pH 7.5) until the A_{220} of the eluting fractions reached a baseline level. Then a 0.2M to 0,8M linear NaCl gradient was started (See Figure 2).

Samples for SDS gels were obtained for each step in the procedure. Fractions from the beginning, middle, and end of the protein peak

FIGURE 1: DOUBLE STRANDED DNA CELLULOSE CHROMATOGRAPHY OF CALF THYMUS CHROMATIN PROTEINS.

50 A_{220} units of the 0.35M extract were diluted to 0.2M NaCl/1mM Tris HCl (pH 7.5) and applied to double-stranded DNA cellulose equilibrated to the same conditions. Bound material was eluted by the application of 2.0M NaCl at the indicated fraction. The flow rate was 50ml/hour and the fraction volume was 7ml. The bar indicates fractions pooled for further analysis.

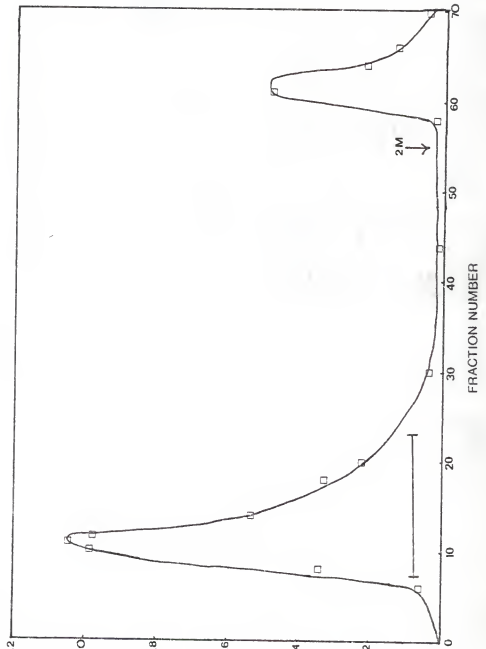
1.2
1.0
0.8
0.6
0.4
0.2
A_{220}
0
10
20
30
40
50
60
70
FRACTION NUMBER
2M

FIGURE 2: SINGLE STRANDED DNA SEPHAROSE CHROMATOGRAPHY OF CALF THYMUS CHROMATIN PROTEINS.

The protein not binding to double stranded DNA cellulose was pooled and applied to single-stranded DNA Sepharose equilibrated to 0.2M NaCl/ 1mM Tris HCl (pH 7.5). The column was washed with the same buffer and the bound protein eluted by the application of a 150ml 0.2 - 0.8M NaCl linear gradient. The flow rate was 20ml/hour and the fraction volume was 5ml. The A_{220} readings more accurately depict the elution profile, while the protein readings as determined by Coomassie Blue binding assay presumably more accurately represent the actual protein content in the runthrough and the peak eluted by the salt gradient (see text for discussion).

A220 (—●—)
0.50
0.25
0.8
0.6
0.4
0.2
PROTEIN (µg/tube)
200
150
100
50
10
20
30
40
50
60
FRACTION NUMBER

eluted by the salt gradient were also obtained. The gel samples were prepared by precipitation with 20% TCA as outlined in methods. In addition, a preparation of calf thymus HMG proteins was run as a standard (39). The results of the gel appear in Figure 3. It is interesting to note that some of the lower molecular weight HMG proteins appear bound to double-stranded DNA, while HMG 1 and 2 appear in the breakthrough. One of these low molecular weight proteins, HMG 3, has been shown by Goodwin and Johns to be the result of proteolysis of HMG 1 (43). This is exceedingly curious since HMG 1 itself does not show any indication of binding to double-stranded DNA. In the samples obtained across the salt gradient on single-stranded DNA, HMG 1 can clearly be seen to elute before HMG 2.

NaCl concentrations across the salt gradient were checked by conductivity readings, as outlined in methods. From such measurements it was possible to determine that HMG 1 began to elute from single-stranded DNA at a salt concentration of 0.3M. HMG2 eluted at a slightly higher concentration.

In the course of running SDS gels of column profiles, it was noted that the single-stranded DNA breakthrough had to be concentrated 5 to 10 times more than other samples in order to be seen clearly on the gels. When the Coomassie Blue binding assay was used as a check on A_{220} readings, it was found that the breakthrough had a lower protein content than one would expect from examining the absorbance readings. When the breakthrough was concentrated by lyophilization, it was found that the A_{260} of the resuspended material was greater

FIGURE 3: SODIUM DODECYL SULFATE SLAB GEL ELECTROPHORESIS OF CALF THYMUS CHROMATIN PROTEINS.

Samples from sequential DNA chromatography were prepared for electrophoresis as described in methods. The samples were immersed in boiling water for 15 seconds just prior to use. Track 1: Proteins dissociated from calf thymus chromatin with 0.35M NaCl. Track 2: Material retained by double-stranded DNA column and subsequently eluted with 2M NaCl. Track 3: Material that failed to bind to double-stranded DNA equilibrated to 0.2M NaCl. This material was subsequently loaded on single stranded DNA Sepharose. Track 4: Material that failed to bind to single stranded DNA. Track 5-7: Material eluted from the single-stranded DNA column by NaCl gradient in fractions 36, 39 and 42 (see Figure 2). Track 8: A preparation for calf thymus HMG proteins.


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than the A_{280}. This could be due to contamination of the protein preparation by endogenous nucleic acids, or it could be due to leaching of DNA from the double-stranded DNA cellulose column, since the DNA is not covalently attached to the cellulose.

The proteins eluted from single-stranded DNA were subjected to amino acid analysis. From Table IV, it is clear that the composition agrees closely with those of HMG 1 and 2.

As mentioned above, it is possible for DNA to leach off of the double-stranded DNA cellulose column. Loss of DNA will lead to reduced binding capacity, and the amount of protein applied to the column must then be reduced appropriately. Figure 4 shows the profile when the double-stranded DNA column had been somewhat overloaded. One can see in the runthrough from the double-stranded DNA column proteins of the same electrophoretic mobility as those observed bound to the column. Some of these same proteins also appear bound to single-stranded DNA. This particular gel represents perhaps the best method of determining the loading capacity of a column. It is preferred over other methods since it can be used even for an extract from a different type of tissue. The procedure would be to deliberately underload the double-stranded column with a small amount of protein from an extract. The resulting gel is carefully observed to determine which proteins bind to double-stranded DNA and which appear in the breakthrough. When the capacity of the column is exceeded in future experiments, it will be known because protein bands will appear in the double-stranded runthrough that were not there when the column

TABLE IV

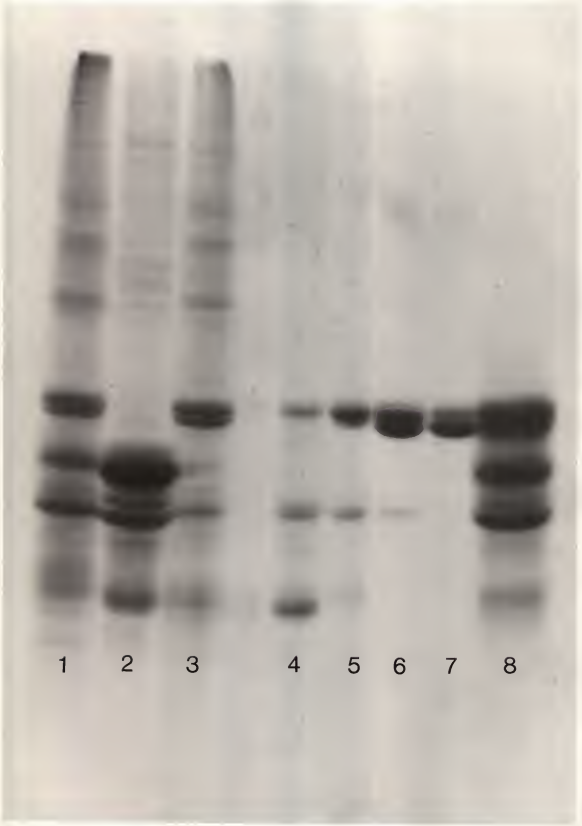
Amino Acid Compositions of Single-Stranded DNA Binding Proteins and of HMG 1 and HMG 2 (values in mole percent)

	Single-stranded DNA Binding Proteins	HMG 1 & HMG 2[a]
Lys	17.5	20.4
His	2.2	1.8
Arg	4.3	4.3
Asp	9.8	10.0
Thr	2,8	2.6
Ser	6.9	6.2
Glu	17.1	17.8
Pro	7.4	8.0
Gly	8.4	5.9
Ala	8,9	8.6
Cys	-	-
Val	4.2	2.1
Met	1.4	1.0
Ile	2.7	1.6
Leu	3.3	2.1
Tyr	1.3	2.4
Phe	3.8	3.3

[a]Unweighted averages of the compositions reported by Rabbani et al. (63)

FIGURE 4: SODIUM DODECYL SULFATE SLAB GEL ELECTROPHORESIS OF CALF THYMUS CHROMATIN PROTEINS.

Samples from sequential DNA chromatography were prepared for electrophoresis as described in methods. The samples were immersed in boiling water for 15 seconds just prior to use. Track 1: 0.35M NaCl extract of calf thymus chromatin. Track 2: Protein bound to double stranded DNA and subsequently eluted with 2M NaCl. Track 3: Material that did not bind to double-stranded DNA at 0.2M NaCl. This material was pooled and applied to single-stranded DNA Sepharose. Track 4-7: Material from fractions taken across the peak of material eluted by the NaCl gradient. Track 8: A preparation of calf thymus HMG proteins.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was run at well below its capacity. Of course, this will have to be done every time a new extract is tried on a column, and also every time a new column is made since the cellulose will adsorb different amounts of DNA each time.

Experiments on calf thymus cytoplasm were also conducted using sequential DNA chromatography. The supernatant from the first centrifugation of the homogenized tissue was designated as the cytoplasm fraction as outlined in methods. This supernatant was dialyzed overnight against a large excess of 0.2M NaCl/1mM Tris HCl (pH 7.5). The dialysate was then centrifuged at 10,000x g to remove mitochondria and any precipitated protein.

The sample was then loaded on double-stranded DNA cellulose equilibrated to 0.2M NaCl/1mM Tris HCl (pH 7.5). The column was washed to baseline with 0.2M NaCl/1mM Tris HCl (ph 7.5). The double-stranded runthrough (which can be easily seen because of its red color) was pooled and applied to single-stranded DNA equilibrated to the same ionic strength. The single-stranded binding proteins were eluted with stepwise washes of 0.5M NaCl and 2M NaCl, both containing 1mM Tris HCl (pH 7.5). The proteins eluted from single stranded DNA were visualized on SDS gels, and this is shown in Figure 5. No prominent protein could be seen at the same electrophoretic mobility as HMG 1. This may not be unusual in view of the high levels of proteolytic enzymes in this tissue.

Rat Liver HMG Proteins

Nuclei and cytoplasm fractions were obtained as outlined in

FIGURE 5: SODIUM DODECYL SULFATE SLAB GEL ELECTROPHORESIS OF CALF THYMUS PROTEINS.

Samples from sequential DNA chromatography of calf thymus cytoplasm were prepared for electrophoresis as described in methods. Track 1: Material that did not bind to single-stranded DNA Sepharose. This material had previously been chromatographed on double-stranded DNA cellulose equilibrated to 0.2M NaCl/1mM Tris HCl (pH 7.5). Track 2: Material eluted from single-stranded DNA by 0.5M NaCl. Track 3: Material eluted from single-stranded DNA by 2M NaCl. Track 4: A preparation of calf thymus HMG proteins.


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methods. Chromatin was swelled with successive washes of 10mM Tris HCl (pH 7.5). When 1mM Tris HCl was used in an attempt to swell the chromatin further, it became transparent, tenuous, and difficult to handle. Morphologically, it was not at all like chromatin obtained from HTC cells or calf thymus that had been swelled in 1mM Tris HCl. Therefore, 10mM Tris HCl was used to swell chromatin in both rat and HTC cells, as outlined in methods.

The chromatin was extracted by adding solid NaCl to a final concentration of 0.35M. The salt was added slowly, and the mixture was allowed to stir in the cold for 30 minutes. Following this, the mixture was centrifuged at 25,000x g to remove DNA and any protein remaining bound to it.

The protein extract was then diluted to a final concentration of 0.2M NaCl with 1mM Tris HCl (pH 7.5). Often, a precipitate formed, and this was removed by centrifugation at 10,000x g for 10 minutes. The extract was then loaded on double-stranded DNA cellulose equilibrated to 0.2M NaCl/1mM Tris HCl (pH 7.5). Fractions of 7ml were collected, and the breakthrough from double-stranded DNA was pooled and loaded onto single-stranded DNA Sepharose equilibrated to the same ionic strength. Proteins bound to double-stranded DNA were eluted with a 2M NaCl wash.

The proteins bound to single stranded DNA were eluted by stepwise washes of 0.3M, 0.5M, and 2M NaCl/1mM Tris HCl (pH 7.5). Samples for SDS gels were prepared by precipitation with 20% TCA as described in methods. Figure 6 shows the results of a gel following chromatography

FIGURE 6: SODIUM DODECYL SULFATE SLAB GEL ELECTROPHORESIS OF RAT LIVER PROTEINS.

Samples of rate liver chromatin proteins and rat liver cytoplasm were prepared for electrophoresis as described in methods. Track 1: Material from a 0.35M extract of rat liver chromatin that did not bind to double-stranded DNA. Track 2: Material that bound to double-stranded DNA cellulose that was subsequently eluted with 2M NaCl. Track 3: Material that did not bind to single-stranded DNA Sepharose. Track 4: Material eluted from single-stranded DNA with 0.3M NaCl. Track 5: Material eluted from single-stranded DNA with 0.5M NaCl. Track 6: Material from rate cytoplasm that was eluted from single-stranded DNA with 0.3M NaCl. This material had previously been chromatographed on double-stranded DNA cellulose. This sample is 1/10 as concentrated as the material appearing in Track 4.


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of the chromatin extract. The histones should not be extractable with 0.35M NaCl (76), however, they appeared in every extract done with rat chromatin. One possible explanation of this result is that DNA containing bound histones was not completely removed by the 25,000x g centrifugation step. Checks on this indicated that 90% of the A_{260} units were removed by this step.

An attempt was made to roughly quantitate the amount of HMG 1 present in rat liver nuclei versus rat liver cytoplasm. This was done by preparing nuclei from 15 grams of frozen rat liver, and preparing cytoplasm from an equivalent amount of tissue. Both of these methods are outlined in the materials and methods section. Protein from a 0.35M NaCl extract of chromatin was subsequently run over the double and single stranded columns, and the cytoplasm was also run over the same columns a day later after they had been washed and re-equilibrated to 0.2M NaCl. The proteins bound to single-stranded DNA in each case were eluted by successive washes of 0.3M, 0.5M, and 2M NaCl/1mM Tris HCl (pH 7.5).

Much more protein eluted at 0.3M NaCl from rat cytoplasm than from rat chromatin as judged by A_{220} readings. This protein was contained in a correspondingly larger volume of eluate. Samples were concentrated for electrophoresis by precipitation with 20% TCA as outlined in methods. At the far right of the gel in Figure 6, the sample from rat cytoplasm was concentrated from 1/100 of the total volume of the eluate. The sample in track 4, however, was concentrated from 1/10 of the total volume of the 0.3M eluate. I

estimate track 6 has about half as much HMG 1 as track 4, therefore, HMG 1 is present in cytoplasm of rat liver at roughly 5 times as great an amount as in chromatin.

Quantitation of HMG Proteins in Rat Liver vs. HTC Cells

Bidney and Reeck (58) have reported that hepatoma tissue culture cell chromatin was enriched in two non-histone chromatin proteins (NH1 and NH2) compared to adult rat livers. I have made an attempt to quantitate the enrichment of these proteins in HTC cells. Since NH1 and NH2 are apparently HMG proteins (58), selective TCA precipitation as described by Goodwin and Johns (39) would be useful to eliminate low mobility group proteins, and would, therefore, facilitate the use of a densitometer for quantitation.

Chromatin from HTC and normal rat liver was prepared simultaneously as described in methods. The two chromatin preparations were then adjusted to the same A_{260} with 10mM Tris HCl (pH 7.5). The chromatin was then extracted with 0.35M NaCl, as described, and the extracted protein separated from DNA and remaining bound protein by centrifugation at 25,000x g for 30 minutes. This centrifugation typically pelleted 85-90% of the A_{260} units. 4.9 ml of the extract from each type of chromatin was then adjusted to 2% TCA by the addition of 0.1 ml of 100% TCA. After sitting in the cold for 1 hour, the solutions were centrifuged at 5000x g for 10 minutes to remove precipitated protein (the low mobility group).

The supernatant was then adjusted to 20% TCA to precipitate the HMG proteins. The precipitate was collected by centrifugation

as above, washed twice with a small amount of acetone, and dissolved in equivalent amounts of electrophoresis buffer, as described in methods.

These samples from equivalent amounts of chromatin were then loaded on SDS gels, along with Bovine Serum Albumin (BSA-Sigma) standards of 0.5, 1.0, 2.5, and 5 micrograms. The resulting gel was then scanned with a spectrodensitometer as outlined in methods. The instrument gave a roughly linear response to increasing concentrations of BSA; a standard curve is shown in Figure 7. The relative peak area was obtained by tracing the peaks from the recorder, cutting them out, and weighing them. The area is then relative to the first standard of 0.5 micrograms.

When this was done for the peaks corresponding to HMG 1 and 2, an enrichment of 5 to 8 times was obtained for HTC. The gel in Figure 8 gave an enrichment of 8.25 times. The recorder trace from the scan of this particular gel follows in Figure 9. Various amounts of sample were run on this gel, and with larger amounts some spreading is noted. This made accurate scanning difficult, as alluded to in methods. Accurate quantitation will have to await development of a radioimmunoassay for these proteins.

Insect Single Stranded DNA Binding Proteins

Ovarian cells from the cabbage looper, Trichoplusia ni, were grown in monolayer cultures as outlined in methods. Approximately 1×10^9 cells could be obtained after two weeks of culture. If the cells were transferred from a seed flask as they reached confluency,

FIGURE 7: STANDARD CURVE FOR DENSITOMETRIC SCANNING.

Bovine Serum Albumin was loaded on SDS slab gels in amounts of 0.5, 1.0, 2.5, and 5 micrograms. Scanning was performed as outlined in methods. Peaks were traced, cut out and weighed. Area is relative to the first standard of 0.5 micrograms.

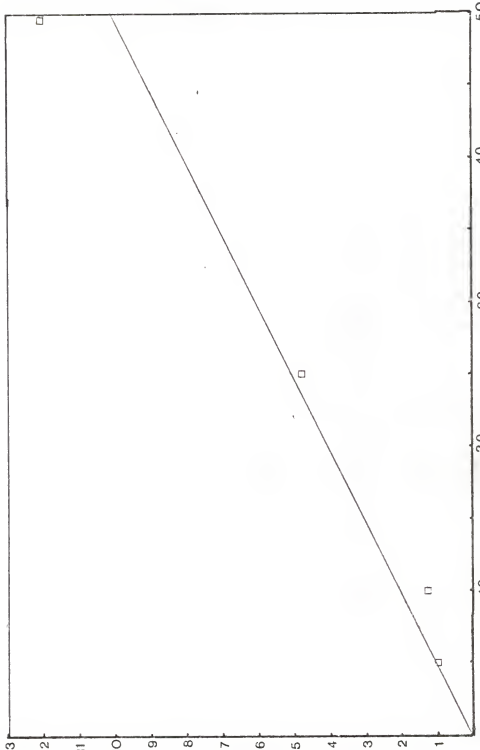
RELATIVE PEAK AREA
13
12
11
10
9
8
7
6
5
4
3
2
1
0
1.0
2.0
3.0
4.0
5.0
BSA (micrograms)

FIGURE 8: SODIUM DODECYL SULFATE SLAB GEL ELECTROPHORESIS.

Samples were prepared as described in the text. Track 1: 5 microliters of rat liver sample. Track 2: 5 microliters of HTC sample. Track 3: Same as Track 2. Track 4: 15 microliters of same rate sample. Track 5: 15 microliters of same HTC sample.


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FIGURE 9: RECORDER OUTPUT FROM DENSITOMETRIC SCAN.

This represents the relevant portions of the scan of the gel seen in Figure 8. Both were scanned at an attenuation of 8 as described in methods. The HTC peak was about 8.25X the area of the rat peak.

HTC
RAT

approximately 60 monolayer flasks were needed during the two week growth period. In the original report by Hink (65), it was claimed that T.ni could be grown in suspension culture. In our hands, this has not been possible. Using the monolayer technique, about 6ml of chromatin at 1.5 mg/ml can be obtained every two weeks.

DNA, RNA, and protein levels were measured in nuclei and chromatin, as described in methods. The results are presented in Table V, along with the results of Elgin and Hood for Drosophila embryos (4).

Essentially all of the protein bound to the DNA in chromatin can be dissociated by the detergent, SDS. In Figure 10, track 1 shows the proteins dissociated from T.ni chromatin by SDS. This sample was obtained by sedimenting the chromatin through a 10-40% sucrose gradient, and dialyzing the protein peak for use in selectrophoresis, as described in Materials and Methods. Track 2 shows proteins dissociated with 0.75M NaCl, obtained in an analogous manner to the SDS dissociated proteins just described. 0.75M NaCl removes histone H1 as expected (76). Extraction of chromatin with sulfuric acid yielded the histone proteins seen in track 3.

In light of the results of Alfagame et al. (47) with Drosophila 'HMG' proteins, it seemed useful to examine TCA solubility properties and behavior on double and single-stranded DNA of chromatin proteins from the T.ni cell line. Alfagame's result would seem to require a nomenclature change, since a protein with a molecular weight higher than that of HMG 1 from calf thymus could not realistically be included in the 'high mobility group'.

TABLE V

	T.ni Nuclei (values in picograms/nucleus)		
Sample A	11.06	8.75	59.25
Sample B	8.86	3.18	54.3
Sample C	9.79	4.63	60.13
	9.90	5.52	56.24
	T.ni Nuclei Mass Ratios (DNA = 1)		
Sample A	1	0.79	6.36
Sample B	1	0.36	6.13
Sample C	1	0.47	6.14
	1	0.54	5.88
	Drosophila melanogaster chromatin (DNA=1) (Ref. 4)		
	1	0.06	1.98
	T.ni Chromatin Mass Ratio (DNA=1)		
Sample A	1	0.27	2.06
Sample B	1	0.30	2.89
Sample C	1	0.30	3.49
	1	0.29	2.81

FIGURE 10: SODIUM DODECYL SULFATE SLAB GEL ELECTROPHORESIS OF T.ni CHROMATIN PROTEINS.

Samples for electrophoresis were prepared as described in methods. Track 1: Protein peak from 10-40% sucrose gradient containing 2.5% SDS, as described in methods. Track 2: Protein peak from 10-40% sucrose gradient containing 0.75M NaCl, as described in methods. Track 3: Material from insect chromatin that was soluble in 0.4N H_2SO_4. Track 4: Material dissociated from insect chromatin by 0.35M NaCl. Track 5: Material from 0.35M NaCl extract that was soluble in 2% TCA. Track 6: Material that bound to single-stranded DNA that was subsequently eluted with 2M NaCl. Track 7: A preparation of calf thymus HMG proteins.


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Chromatin was prepared as outlined in methods, and it was extracted with 0.35M NaCl as described. Following centrifugation at 25,000x g for 30 minutes, the extract was diluted to 0.2M NaCl with 1mM Tris HCl/1mM EDTA (pH 7.5). The extract was then applied to double-stranded DNA cellulose equilibrated to 0.2M NaCl/1mM Tris HCl (pH 7.5). The breakthrough from double-stranded DNA was pooled and applied to single-stranded DNA Sepharose equilibrated to the same ionic strength. Proteins bound to single-stranded DNA were eluted with 2M NaCl/1mM Tris HCl (pH 7.5).

Part of the 0.35M NaCl extract was adjusted to 2% TCA and allowed to set in the cold for 30 minutes. Precipitated protein was then removed by centrifugation at 5000x g for 10 minutes. The supernatant was then adjusted to 20% TCA to precipitate any 'HMG' proteins. The results of this experiment appear in Figure 10, along with the SDS and 0.75M NaCl studies. The results here are disappointing, since it appears that almost no proteins were removed by precipitation with 2% TCA. A single stranded DNA binding protein from Tni also appears in this gel, and it is of high molecular weight. Although a complete set of standards was not run, this protein has an electrophoretic mobility similar to that of BSA, molecular weight 68,000.

A complete gel of the DNA chromatography experiment described above appears in Figure 11. The same protein is observed bound to single-stranded DNA in this experiment. For comparison, track 6 shows a preparation of calf thymus HMG proteins.

FIGURE 11: SODIUM DODECYL SULFATE SLAB GEL ELECTROPHORESIS T.ni CHROMATIN PROTEINS.

Samples from sequential DNA chromatography of proteins dissociated from T.ni cell chromatin were prepared as described in methods. Track 1: Proteins dissociated by 0.35M NaCl. Track 2: Material that did not bind to double-stranded DNA cellulose equilibrated to 0.2M NaCl/1mM Tris HCl (pH 7.5). Track 3: Material bound to double-stranded DNA cellulose that was subsequently eluted with 2M NaCl. Track 4: Material that did not bind to single-stranded DNA Sepharose. Track 5: Material that bound to single-stranded DNA. Track 6: A preparation of calf thymus HMG proteins.

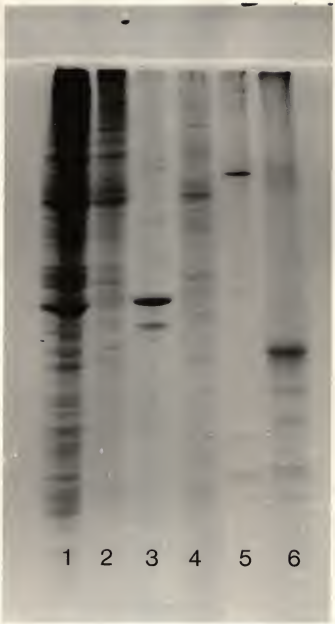
1 2 3 4 5 6

Figure 12 shows a gel in which the double stranded DNA column has apparently been overloaded. Several proteins now appear on the single-stranded DNA column, some with mobilities similar to the HMG proteins from calf thymus. Toward the bottom of the gel, both in the 0.35M extract, and in the proteins bound to double-stranded DNA, the low molecular weight histones can be seen. This often occurred in extracts of insect chromatin.

FIGURE 12: SODIUM DODECYL SULFATE SLAB GEL ELECTROPHORESIS OF T.ni CHROMATIN PROTEINS.

Samples from sequential DNA chromatography of proteins dissociated from T.ni cell chromatin were prepared as described in methods. Track 1: Proteins dissociated by 0.35M NaCl. Track 2: Material that did not bind to double-stranded DNA cellulose equilibrated to 0.2M NaCl/1mM Tris HCl (pH 7.5). Track 3: Material that bound to double-stranded DNA cellulose and was subsequently eluted with 2M NaCl. Track 4: Material that did not bind to single-stranded DNA Sepharose and was subsequently eluted with 2M NaCl.


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CONCLUSIONS

The fact that calf thymus, rat and HTC cell HMG proteins prefer single stranded DNA is interesting because Herrick and Alberts (61) have postulated that any protein showing preferential affinity for single-stranded DNA will act as a helix destabilizing protein, if the appropriate kinetic pathways exist. Such proteins would be of importance in replication, since strand separation is required for duplication, yet single stranded DNA shows a tendency to form intrachain hydrogen bonds at 37°, thereby destroying the base sequence. Alberts and Frey (62) have shown that for T4 phage, the gene 32 protein binds to single-stranded DNA in stoichiometric amounts, thus serving two functions in the replication process. First, the DNA double helix is 'melted', allowing polymerases to read off the relevant base sequence. Second, the single-stranded DNA is stabilized, preventing intrachain hydrogen bonding, and perhaps also holding the single-stranded chain in the proper orientation for the polymerases to act.

The enrichment of HMG 1 and 2 found in HTC cells is consistent with the HMG proteins being involved in replication events, but this is far from having been proven. As noted above, gene 32 protein was involved in stoichiometric, not catalytic amounts.

The results of Javaherian et al. (57) with HMG 1 and 2 also reinforce the conclusions drawn here. Their results indicate that these two proteins can either unwind the double helix, or induce a supercoiling of the DNA. They go on to state that one possible explanation

of these results would be if the proteins show a preferential affinity for single-stranded DNA.

The results with cytoplasm reinforce the findings of Bustin (55), that there is cross-reactivity with cytoplasmic components by antibodies elicited to HMG 1 from calf thymus. However, the results with calf thymus are puzzling. Since the cytoplasm was dialyzed overnight, proteolytic enzymes could conceivably have degraded HMG 1 so that it could not be seen in the extract.

From the results of the studies done on the T.ni cell line, one thing is immediately obvious. That is, there appear to be no analogs to HMG 1 and 2 as they appear in calf thymus, rat liver, and HTC cells. There is, however, a much higher molecular weight protein of T.ni that displays selective affinity for single-stranded DNA (47). How such high molecular weight proteins in insects are related to the HMG proteins is unclear. Elgin and Hood (4) reported a high molecular weight protein in Drosophila embryos that disappeared as the insect matured. It would be interesting to examine this protein to see if it had preferential affinity for single-stranded DNA.

If such proteins in insects are found to be somehow related to the HMG proteins from calf thymus, then the entire group of proteins will have to be redefined. As it is, the HMG group represents a strictly arbitrary classification of proteins on the basis of their solubility in TCA. Perhaps all of the proteins would fit better under the label of single-stranded binding proteins, as I was careful to call the proteins isolated from T.ni.

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STUDIES ON THE HIGH MOBILITY GROUP OF NON-HISTONE CHROMATIN PROTEINS

by

James Lawrence Fishback

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Graduate Biochemistry Group

Kansas State University
Manhattan, Kansas

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Non-histone chromatin proteins from calf thymus, rat liver, hepatoma tissue culture cells, and Trichoplusia ni, an insect cell line, were dissociated from chromatin with 0.35M NaCl. The chromatin extracts were studied using the technique of sequential DNA chromatography, Results indicate that two high mobility group proteins (HMG 1 and HMG 2) show preferential affinity for single stranded DNA. Furthermore, HMG 1 and HMG 2 appear to be tumor enriched in hepatoma tissue culture cells. Trichoplusia ni cells do not appear to have analogs of HMG 1 and HMG 2, however, a high molecular weight single-stranded DNA binding protein was observed. The possible function of single-stranded DNA binding protein is discussed.