1983

Chromosomal distribution of Drosophila HMG-like proteins

James Andrew Bassuk
Iowa State University

Follow this and additional works at: https://lib.dr.iastate.edu/rtd

Part of the Zoology Commons

Recommended Citation
https://lib.dr.iastate.edu/rtd/8452

This Dissertation is brought to you for free and open access by the Iowa State University Capstones, Theses and Dissertations at Iowa State University Digital Repository. It has been accepted for inclusion in Retrospective Theses and Dissertations by an authorized administrator of Iowa State University Digital Repository. For more information, please contact digirep@iastate.edu.
INFORMATION TO USERS

This reproduction was made from a copy of a document sent to us for microfilming. While the most advanced technology has been used to photograph and reproduce this document, the quality of the reproduction is heavily dependent upon the quality of the material submitted.

The following explanation of techniques is provided to help clarify markings or notations which may appear on this reproduction.

1. The sign or "target" for pages apparently lacking from the document photographed is "Missing Page(s)". If it was possible to obtain the missing page(s) or section, they are spliced into the film along with adjacent pages. This may have necessitated cutting through an image and duplicating adjacent pages to assure complete continuity.

2. When an image on the film is obliterated with a round black mark, it is an indication of either blurred copy because of movement during exposure, duplicate copy, or copyrighted materials that should not have been filmed. For blurred pages, a good image of the page can be found in the adjacent frame. If copyrighted materials were deleted, a target note will appear listing the pages in the adjacent frame.

3. When a map, drawing or chart, etc., is part of the material being photographed, a definite method of "sectioning" the material has been followed. It is customary to begin filming at the upper left hand corner of a large sheet and to continue from left to right in equal sections with small overlaps. If necessary, sectioning is continued again—beginning below the first row and continuing on until complete.

4. For illustrations that cannot be satisfactorily reproduced by xerographic means, photographic prints can be purchased at additional cost and inserted into your xerographic copy. These prints are available upon request from the Dissertations Customer Services Department.

5. Some pages in any document may have indistinct print. In all cases the best available copy has been filmed.

University

Microfilms
International

300 N. Zeeb Road
Ann Arbor, MI 48106
Bassuk, James Andrew

CHROMOSOMAL DISTRIBUTION OF DROSOPHILA HMG-LIKE PROTEINS

Iowa State University Ph.D. 1983

University Microfilms International 300 N. Zeeb Road, Ann Arbor, MI 48106
PLEASE NOTE:

In all cases this material has been filmed in the best possible way from the available copy. Problems encountered with this document have been identified here with a check mark √.

1. Glossy photographs or pages √
2. Colored illustrations, paper or print
3. Photographs with dark background √
4. Illustrations are poor copy
5. Pages with black marks, not original copy
6. Print shows through as there is text on both sides of page
7. Indistinct, broken or small print on several pages √
8. Print exceeds margin requirements
9. Tightly bound copy with print lost in spine
10. Computer printout pages with indistinct print
11. Page(s) _______ lacking when material received, and not available from school or author.
12. Page(s) _______ seem to be missing in numbering only as text follows.
13. Two pages numbered _______. Text follows.
14. Curling and wrinkled pages
15. Other

University
Microfilms
International
Chromosomal distribution of
Drosophila HMG-like proteins

by

James Andrew Bassuk

A Dissertation Submitted to the
Graduate Faculty in Partial Fulfillment of the
Requirements for the Degree of
DOCTOR OF PHILOSOPHY

Major: Zoology

Approved: Members of the Committee:

Signature was redacted for privacy.

Charge of Major Work

Signature was redacted for privacy.

For the Major Department

Signature was redacted for privacy.

For the Graduate College

Iowa State University
Ames, Iowa

1983
TABLE OF CONTENTS

INDEX OF SYMBOLS AND ABBREVIATIONS iv

ABSTRACT 1

INTRODUCTION 2

LITERATURE SURVEY 4

Structure of the Nucleosome 4

Active Chromatin 6

Proteins Associated with Active Chromatin 7

Micrococcal Nuclease Digestion Patterns 10

Modification of Chromosomal Proteins 12

Phosphorylation 12
Acetylation 13
Methylation 15
ADP-ribosylation 16
Ubiquitination 17

Major Acid Soluble Proteins of Drosophila Chromatin 18

Histones 18
Protein D1 18
Protein D2 20
Protein A13 21
Active chromatin specific proteins 21

MATERIALS AND METHODS 24

Experimental Protocol 24

Embryos 24
Isolation of nuclei 24
Extraction of histones 24
Extraction of HMG-like proteins 25
Purification of A13 25
Preparation of anti-A13 26
Immunoprecipitation of A13 26
Immunofluorescence 27
Preparation of nucleosomes 27
INDEX OF SYMBOLS AND ABBREVIATIONS

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>absorbance</td>
</tr>
<tr>
<td>ADP</td>
<td>adenosine diphosphate</td>
</tr>
<tr>
<td>Ag&lt;sup&gt;0&lt;/sup&gt;</td>
<td>silver, as a free element, oxidation number = 0</td>
</tr>
<tr>
<td>Ag&lt;sup&gt;+&lt;/sup&gt;</td>
<td>silver cation, oxidation number = 1</td>
</tr>
<tr>
<td>Ag&lt;sub&gt;2&lt;/sub&gt;O</td>
<td>silver oxide</td>
</tr>
<tr>
<td>Ag&lt;sub&gt;2&lt;/sub&gt;(NH&lt;sub&gt;3&lt;/sub&gt;)&lt;sup&gt;+&lt;/sup&gt;</td>
<td>diammine (ammoniacal) silver cation</td>
</tr>
<tr>
<td>A-T</td>
<td>adenine-thymine bases in DNA</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>A/U</td>
<td>acid/urea</td>
</tr>
<tr>
<td>bisacrylamide</td>
<td>N, N'-methylene-bis-acrylamide</td>
</tr>
<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>°C</td>
<td>degrees in Celsius</td>
</tr>
<tr>
<td>14&lt;sup&gt;C&lt;/sup&gt;</td>
<td>radioactive carbon isotope with 8 neutrons</td>
</tr>
<tr>
<td>CaCl&lt;sub&gt;2&lt;/sub&gt;</td>
<td>calcium chloride</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CC</td>
<td>chromocenter</td>
</tr>
<tr>
<td>cm</td>
<td>centimeter</td>
</tr>
<tr>
<td>D-76</td>
<td>Kodak developer type D-76</td>
</tr>
<tr>
<td>DMH</td>
<td>1,2-dimethylhydrazine, a carcinogen</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DNAase 1</td>
<td>deoxyribonuclease (EC 3.1.4.5)</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediamine tetraacetic acid</td>
</tr>
<tr>
<td>g</td>
<td>gram</td>
</tr>
<tr>
<td>G&lt;sub&gt;1&lt;/sub&gt;</td>
<td>cell cycle interval with no detectable DNA synthesis</td>
</tr>
</tbody>
</table>
cell cycle state, no DNA syn., prior to mitosis

G\textsubscript{2}

guanidine hydrochloride

GuCl

hour

h

state of histone H1 phosphorylation during interphase

H1\textsubscript{i}

state of histone H1 phosphorylation during metaphase

H1\textsubscript{m}

water

H\textsubscript{2}O

sulfuric acid

H\textsubscript{2}SO\textsubscript{4}

radioactive isotope of hydrogen, "tritium"

\textsuperscript{3}H

formaldehyde

HCHO

formic acid anion, forms the salt of formic acid

HCOO\textsuperscript{-}

hydrochloric acid

HCl

high mobility group

HMG

acetic acid

HOAc

heat shock

HS

immunoglobulin, class G

IgG

isoleucine

Ile

potassium hydroxide

KOH

leucine

Leu

common logarithm, base 10

log

molarity, units in moles liter\textsuperscript{-1}

M

milliamperes

mA

methionine

Met

milligram

mg

milliliter

ml

millimeter

mm
mM millimolar, units in millimoles liter\(^{-1}\)

\(M_r\) molecular weight

N amino

\(N\) normality, units in equivalents liter\(^{-1}\)

\(Na^+\) sodium cation, oxidation number = 1

NaCl sodium chloride

NaOH sodium hydroxide

\(N^G\) guanidino group of arginine

\(NH_3\) ammonia

NHC nonhistone chromosomal

nm nanometer

nmol nanomole

OD optical density

OH\(^-\) hydroxide anion

\(P\) phosphorous

\(^{32}P\) radioactive isotope of phosphorous, 7 neutrons

PAGE polyacrylamide gel electrophoresis

pBR322 a plasmid from *Escherichia coli*

PCA perchloric acid

pH negative log of the hydrogen ion concentration

Phe phenylalanine

PMSF phenylmethylsulfonyl fluoride

RNA ribonucleic acid

S sulfur

\(S\) sedimentation constant, Svedberg unit
S1  supernatant 1
S2  supernatant 2
$S_2O_8^{2-}$  persulfate anion
$^{35}S$ radioactive isotope of sulfur, 19 neutrons
SDS sodium dodecyl sulfate
SP serum protein
T transmittance
T/A/U Triton/acid/urea
TBE Tris-borate-EDTA buffer
TBS Tris-buffered-saline
TE Tris-EDTA buffer
TEMED N,N,N',N'-tetramethylethylenediamine
Tricine N-tris(hydroxymethyl)methyl glycine
Tris Tris(hydroxymethyl)aminomethane
T-T Tris-Tricine
tRNA transfer RNA
Tyr tyrosine
ug microgram
uH2A ubiquitinated H2A
uH2B ubiquitinated H2B
ul microliter
V volt
Val valine
$\times g$ times the force of gravity, 9.8 m s$^{-2}$
$1^o$, $2^o$, and $3^o$ mono-, di-, and tri-nucleosomes
Nuclei from *Drosophila melanogaster* embryos contain two major nonhistone chromosomal proteins which are extracted by 0.35 M NaCl and by 2% perchloric acid. A63 (M_r about 63,000) and A13 (M_r about 10,000) both contain high levels of basic and acidic amino acids, a property characteristic of high mobility group (HMG) proteins isolated from vertebrate tissues. Polytenic chromosomes were stained by immunofluorescent techniques with anti-A13 polyclonal antibodies. The staining pattern indicates that A13 does not specifically react with puffs, nor is the protein preferentially associated with heterochromatin or nucleoli.

There appears to be multiple binding sites for A13 throughout nuclei. A13 is rapidly released from nuclei by micrococcal nuclease. A13 cannot be completely released from nuclei by this nuclease, however, since even at very high digestion levels, the protein is also detected associated with nucleosomal fractions. At these high digestion levels, only histone H1-depleted mononucleosomes are solubilized in 5 mM EDTA. Some of these mononucleosomes also contain A13. The results indicate that A13 closely resembles the properties possessed by vertebrate HMGs 1 and 2.
INTRODUCTION

The wealth of information available on Drosophila makes it an important system for studies of chromatin structure and gene regulation. Foremost among the advantages is the fact that the genetics of Drosophila are better known than any other higher eucaryote. The ease of obtaining large numbers of well characterized mutants and its fairly rapid rate of growth with a fully described life cycle further complement the choice of Drosophila as an organism for study. Drosophila is also one of the few eucaryotic organisms in which polytene chromosomes can be readily observed. The giant chromosomes of Drosophila salivary glands have been of great importance in cytological studies of chromatin structure because they present a unique opportunity for studies of gene activation and transcription.

However, Drosophila DNA is unusual among higher eucaryotes in that, to date, there has been no report of 5'-methyl cytosine in the genomic DNA of the fruit fly. This characteristic differs from vertebrates, echinoderms, and higher plants in which the presence of hypermethylated DNA is correlated with gene inactivation in some systems (Razin and Riggs, 1980; Groudine et al., 1981; Naveh-Many and Cedar, 1981). This may indicate that the mechanisms of gene regulation in invertebrates (for Dipterans at least) are different from that of vertebrates.

Much effort has recently been focused on examining the presence, if any, of Drosophila analogues to vertebrate High Mobility Group (HMG) proteins. Vertebrate HMG proteins are a group of nonhistone chromosomal
(NHC) proteins two of which, HMGs 14 and 17, have been shown to be a component of active chromatin (Weisbrod and Weintraub, 1979, 1981). For *Drosophila melanogaster*, two major nuclear proteins, A63 and A13, have been found which have typical HMG chemical compositions and solubility characteristics (Bassuk, 1980). Neither protein co-electrophorese with known HMG proteins. In this dissertation and in previous studies (unpublished results from the laboratories of Dr. John E. Mayfield, Dept. of Zoology, Iowa State University, and Dr. Sarah C. R. Elgin, The Biological Laboratories, Harvard University), it has been shown that neither A63 nor A13 is located preferentially in polytene chromosome puffs. This result implies that neither protein plays a special role in the structure of active chromatin. In other studies, antisera have been obtained against calf thymus HMGs, and no cross-reactivity has been observed with *Drosophila* chromosomal proteins (McCoy et al., 1980; G. Howard, personal communication, The Biological Laboratories, Harvard University). Thus, at present it is uncertain as to whether or not the fruit fly possesses direct functional analogues of the vertebrate HMG proteins.

The long range goal of this research is to understand the various physical states of *Drosophila* chromatin and to determine how these states are related to the control of gene expression in this organism. This dissertation represents an attempt to characterize one *Drosophila* NHC protein, A13, and to compare its properties with vertebrate HMGs 1, 2, 14, and 17. The data presented also provide valuable general knowledge about the number of types and the basic properties of HMG-like proteins in this species.
LITERATURE SURVEY

Structure of the Nucleosome

All the DNA in the cell nucleus is believed to be packaged into a basic structure called the nucleosome. Each nucleosome contains a segment of DNA which wraps twice around the exterior of a core consisting of two each of the four core histones (histones H2A, H2B, H3, and H4). The average length of the DNA segment is about 200 base pairs (bp) and varies in length depending on species, tissue, and local environment within the cell nucleus (Lewin, 1980).

Structurally, the nucleosome causes the DNA to be constrained into an ordered compact state instead of a disordered random coil. Biochemically, the twists and turns of the DNA in chromatin and its relative availability to various molecular agents is of paramount importance to genetic processes. Since the association between DNA and histones is presumably modulated by mechanisms which are central to cellular function, it is reasonable to suppose that research into the details of nucleosomal structure will play a central role in the understanding of gene regulation.

Micrococcal nuclease (from Staphylococcus aureus) digestion of chromatin reveals several levels of structural organization from which nucleoprotein particles can be isolated and studied; each of these are intermediates in the course of the hydrolytic reaction which progresses from the intact, high molecular weight DNA of the nucleus to a limit digest in which about 40% of the DNA is rendered acid soluble (that is, less than about 20 bp long). The major intermediates in the degradative process are as follows:
(1) a 40 S particle consisting of about eight nucleosomes (Hozier et al., 1977); (2) the nucleosome (Noll, 1974; Axel, 1975; Sollner-Webb and Felsenfeld, 1975; Shaw et al., 1976; Noll and Kornberg, 1977); (3) the chromatosome, a particle containing about 160 bp of DNA, one molecule of H1 (or H5 in birds), and an octamer of the four smaller histones (Varshavsky et al., 1976; Whitlock and Simpson, 1976; Bakayev et al., 1977; Todd and Garrard, 1979; Noll and Kornberg, 1977; Simpson, 1978a); and (4) the core particle, consisting of 146 bp of DNA and the four histone octamer (for reviews, see Kornberg, 1977; Felsenfeld, 1978; Igo-Kemenes et al., 1982; Wang, 1982). The 160 bp chromatosome and 146 bp core particle are likely to be structurally significant. During the course of digestion of the chromatosome to core particles, about one histone H1 per nucleosome is released (Simpson, 1978a), suggesting that H1 interacts with DNA segments that are hydrolyzed in this transition.

The dimensions of the 146 bp core particle corresponds to that of an oblate spheroid of 11 x 11 x 5.7 nm (Pardon et al., 1975, 1977; Hjelm et al., 1977; Suau et al., 1977; Finch et al., 1977; Finch and Klug, 1978) of which 7.3 x 7.3 x 4.0 nm is cylindrical protein (Bentley et al., 1981; Finch et al., 1981). The 146 bp DNA segment forms two annuli close to the top and bottom of the cylinder and corresponds to 1.75 turns of DNA superhelix (Felsenfeld, 1978; Finch et al., 1981).

There is general agreement that H1 is located at the region where DNA enters and exits the core particle (Noll and Kornberg, 1977; Simpson, 1978a; Thoma et al., 1979; Moyne et al., 1981). The central globular domain of H1 is able to close two full turns of DNA around the histone
octamer (Allan et al., 1980), and to cross-link to H2A (Boulikas et al., 1980) and H3 (Ring and Cole, 1979) by utilization of the cross-linking agent carbodiimide.

Interaction of H1 with the nucleosome core is further indicated by the finding that H1 depletion has a profound effect on the distribution of cuts within the core DNA which are generated late in the digestion of chromatin with micrococcal nuclease (Smerdon and Lieberman, 1981; Ishimi et al., 1981).

Active Chromatin

Evidence that active chromatin has a different structure from that of inactive chromatin comes from the utilization of relatively nonspecific endonucleases. Weintraub and Groudine (1976) have demonstrated that the chick erythrocyte globin gene is preferentially sensitive to digestion by DNAase I, but not to digestion by micrococcal nuclease. This resistance to micrococcal nuclease suggests that the globin gene is packaged into nucleosomes; whereas, its sensitivity to DNAase I indicates that globin nucleosomes are conformationally different from DNAase I resistant genes (such as the red cell ovalbumin gene) (Axel, 1975).

The sensitivity of actively transcribed genes to DNAase I reflects only a potential for a gene to be transcribed rather than transcription itself. For example, Physarum ribosomal DNA remains sensitive during mitosis when rRNA transcription is not detectable (Stalder et al., 1978), the mouse globin gene exhibits the same sensitivity in induced
(transcribed) as well as uninduced (nontranscribed) Friend erythroleukemia cells (Miller et al., 1979), and the ovalbumin gene remains susceptible in the hormone-withdrawn chick oviduct (Palmiter et al., 1977). The boundaries of these sensitive regions are very precise, for only sequences transcribed are sensitive; whereas, adjacent nontranscribed sequences are not (Flint and Weintraub, 1977).

Endonucleases have also proven helpful in examining the higher order structure of active chromatin. A very brief digestion with either micrococcal nuclease or DNAase 1 of Drosophila nuclei has shown preferential cleavage sites in a number of heat shock nuclei (Wu et al., 1979) which are best explained as features of higher order chromatin structure.

Proteins Associated with Active Chromatin

Since nuclease digestion experiments have indicated that the conformation of active nucleosomes is different from that of bulk nucleosomes, it is reasonable to suppose that the active structure is due to altered or modified histones and/or the presence of NHC proteins in the nucleosome.

The NHC protein fraction most often detected as being associated with active nucleosomes is that of the high mobility group (HMG). The four major calf thymus HMG proteins are HMGs 1, 2, 14 and 17. They have recently been extensively reviewed by Johns (1982). In Table 1 the properties of the HMG proteins from calf thymus are outlined and compared to those of the histones. The criteria normally used to identify HMGs are...
Table 1. Criteria used for identification of HMGs and histones

<table>
<thead>
<tr>
<th>Criterion</th>
<th>HMG proteins</th>
<th>Histones</th>
</tr>
</thead>
<tbody>
<tr>
<td>Extractability</td>
<td>Solubilized from chromatin by 0.35 M NaCl, and</td>
<td>Solubilized from chromatin by 0.2 M HCl, or</td>
</tr>
<tr>
<td></td>
<td>soluble in 2% TCA</td>
<td>0.4 N H₂SO₄, or by 3-4 M NaCl</td>
</tr>
<tr>
<td>Quantity</td>
<td>Ratio of total HMG to DNA = 0.03:1</td>
<td>Ratio of total histone to DNA = 1:1</td>
</tr>
<tr>
<td>Molecular Weight</td>
<td>Low molecular weight</td>
<td>Low molecular weight H1 = 21,000</td>
</tr>
<tr>
<td></td>
<td>HMGs 1 and 2 = 29,000</td>
<td>core histones = 11,500-15,300</td>
</tr>
<tr>
<td></td>
<td>HMG 14 = 10,700; HMG 17 = 9,200</td>
<td></td>
</tr>
<tr>
<td>Electrophoretic</td>
<td>High mobility in both acid-urea and SDS acrylamide</td>
<td>High mobility in both acid-urea and SDS</td>
</tr>
<tr>
<td>Mobility</td>
<td>gels, often running anomalously compared to H&lt;sub&gt;r&lt;/sub&gt;</td>
<td>acrylamide gels, often running anomalously</td>
</tr>
<tr>
<td></td>
<td></td>
<td>compared to H&lt;sub&gt;r&lt;/sub&gt;</td>
</tr>
<tr>
<td>Amino Acid</td>
<td>High in charged residues (25% basic and 20-30%</td>
<td>High in basic amino acids (20-40%)</td>
</tr>
<tr>
<td>Composition</td>
<td>acidic)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Low in hydrophobic residues</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>Mayes, 1982.
acid and salt extractability, electrophoretic mobility and amino acid composition. Unfortunately, these criteria are inadequate to define HMG proteins from lower eucaryotes; the HMG-like proteins from these organisms usually differ from calf thymus in one or more of these criteria. Without a functional assay, positive identification of the HMG proteins from lower eucaryotes can only be made when sequence homologies with higher eucaryotes are demonstrated. At present, very little sequence data is available for species other than calf and trout.

In trout testis, the HMG proteins have been sequenced and compared to the calf thymus HMGs. Trout testis HMG-T possesses 84% homology in the N-terminal end with HMGs 1 and 2 (Watson et al., 1977). The 69 residue protein H6 possesses 75% homology to the 89 residue HMG 17 (Watson et al., 1979). These HMG proteins have a high content of both basic and acidic amino acids, and sequence analyses have shown that the charge distributions in the molecules are polarized.

When HMGs 14 and 17 were eluted from chick erythrocyte chromatin with 0.35 M NaCl, the preferential DNAase 1 sensitivity of the globin gene was lost (Weisbrod and Weintraub, 1979). When 1 mole each of pure HMGs 14 and 17 were reconstituted onto 20 moles of HMG-depleted red cell nucleosomes (in the form of chromatin), the selective DNAase 1 sensitivity of the globin gene was restored. The ovalbumin gene used as a control showed no increased sensitivity either before or after reconstitution with HMGs 14 or 17. Affinity chromatography using HMGs 14 and 17 coupled to agarose, glass beads, or Glycophase (Pierce), resulted in a direct correlation between nucleosomes bound and DNAase 1 sensitive regions.
(Weisbrod and Weintraub, 1981). In contrast, chromosomal regions of intermediate DNAase I sensitivity which are adjacent to the highly sensitive regions did not bind to the columns.

The binding sites of HMGs 14 and 17 in active nucleosomes seem to be at each end of the nucleosome core and to cover or interact with inter-nucleosomal DNA. The evidence for this comes from the mapping of DNAase I cutting sites, thermal denaturation studies (Sandeen et al., 1980), and the characterization of subnucleosomal particles produced by extensive micrococcal nuclease digestion (Bakayev et al., 1977). Mardian et al. (1980) have shown that nucleosome cores can bind two HMG 14 molecules with high affinity. The same is true for HMG 17. HMG 14 and 17 do not bind independently of each other and most likely bind to the same two sites.

Micrococcal Nuclease Digestion Patterns

It appears that there are at least two sub-populations of HMGs 1 and 2 in rabbit thymus, one of which is fairly tightly bound to the nucleosome, and another more loosely bound fraction which is released from chromatin by very brief digestion with micrococcal nuclease digestion (Mathew et al., 1979). These differences in binding may be related to their known microheterogeneity, as demonstrated on isoelectric focusing gels (Goodwin et al., 1976).

Limited digestion of trout-testis nuclei with micrococcal nuclease followed by a low-speed centrifugation led to the release into the
supernatant solution (S1) of significant amounts of HMG-T and ubiquitin but no core histones or H6 (Levy-Wilson et al., 1979). Since it is known that micrococcal nuclease attacks the linker regions in chromatin preferentially, this result was interpreted as indicating that HMG-T and ubiquitin were located in a particularly micrococcal nuclease-sensitive subset of linker regions. The location of HMG-T in linker regions received support from the observation that a specific antibody to trout HMG-T, prepared in goats, when tagged with a fluorescent label, could be shown to bind only to nucleosome oligomers containing intact linker regions and derived by limited micrococcal nuclease digestion (Peters et al., 1979). The fluorescent anti-HMG-T did not bind to these core nucleosomes.

More detailed studies of the distribution of HMG-T have indicated, however, that only a portion of the total HMG-T is rapidly released by limited micrococcal nuclease digestion (Kuehl et al., 1979) and that a distinct population of HMG-T molecules is resistant to micrococcal nuclease digestion and even to extraction with 0.6 M NaCl.

Thus, the sequence related proteins, HMGs 1 and 2 from calf thymus, and HMG-T from trout testis, share micrococcal nuclease digestion patterns and appear to be localized in chromatin in a similar manner. HMGs 1, 2, and T do not appear to be preferentially bound to active chromatin and their cellular function is presently unknown.
Modification of Chromosomal Proteins

Postsynthetic histone modification by phosphorylation, acetylation, methylation, poly-ADP ribosylation and ubiquitination is a well characterized feature of chromatin (for reviews, see Allfrey, 1977; Igo-Kemenes et al., 1982). These modifications have been correlated to transcriptional activity.

Phosphorylation

The modification of chromosomal proteins by phosphorylation is a complex phenomenon involving all of the histones and large numbers of nonhistone proteins, including subunits of RNA polymerase (Bell et al., 1977).

Phosphorylation of histones occurs during S phase, mitosis, and as a response to hormonal stimulation. In each case, different and highly specific serines and threonines are phosphorylated (Isenberg, 1979). Unique phosphorylations of H4 histidines and H1 lysines have been found and, in contrast to serine and threonine phosphates, are alkali-stable and hot-acid-labile (Chen et al., 1974; Smith et al., 1974).

Different levels of chromatin organization may be correlated with different types of phosphorylation. One type of H1 phosphorylation, designated as H1\(_1\) (occurring during interphase, containing 1-3 phosphoserines, and representing a fraction of H1 molecules), may be correlated with a reorganization at the molecular level (Hohman et al., 1976; Gurley et al., 1978a). A second type of histone H1 phosphorylation, termed
$H_{1m}$ (occurring during mitosis, containing 3-6 phospho-serines and -threonines, and representing almost all $H_1$ and $H_{1j}$ molecules), and $H_3$ phosphorylation may be correlated with condensation to chromosomes (Hohman et al., 1976; Gurley et al., 1978a). $H_2A$ phosphorylation has been linked to heterochromatin condensation (Gurley et al., 1978b) and increases after hormonal stimulation (Prentice et al., 1978). These observations are only correlative and any cause and effect relationships are yet to be established.

A most elegant set of experiments concerning HMG phosphorylation have utilized the thiophosphate analogue of ATP, adenosine-5'-O-(3-thiophosphate) (ATP-$\gamma$S) as a thiophosphoryl group donor in kinase-mediated transfer reactions. The labelling of the serine residues of $H_1$, $H_2B$ and HMG 1 by ATP-$\gamma$S$^{35}$S has been observed using a purified cAMP-dependent protein kinases (Sun et al., 1980). The incorporation of $^{32}$P-phosphate into HMGs 14 and 17 has been detected in Ehrlich ascites and L1210 cells (Saffer and Glazer, 1980).

**Acetylation**

All four core histones (except Tetrahymena H4, Glover and Gorovsky, 1979) undergo cytoplasmic amino-terminal acetylation of serines (Liew et al., 1970). The acetylation of residues internal to the polypeptide chain occurs on lysines in the N-terminal third of all four core histone molecules (Dixon et al., 1975).

Numerous correlations have been made to link acetylation with gene activity and Yamamato and Alberts (1976) have proposed that histone
acetylation-deacetylation can provide the flexibility needed to increase accessibility to RNA polymerase. However, when sodium butyrate is used to inhibit histone deacetylases (Candido et al., 1978; Sealy and Chalkley, 1978), and hence maintain histone hyperacetylation, subsequent analysis of core particles following DNAase 1 digestion has yielded conflicting results. Simpson (1978b) reported no increased DNAase 1 sensitivity; whereas, Vidali et al. (1978) did observe a significant increase. In addition, in vitro studies of acetylated SV40 nucleosomal complexes does not affect the kinetics of transcription by calf thymus RNA Polymerase I or II (Mathis et al., 1978). So although there are conflicting reports on a correlation between gene activity and histone acetylation, the exact role of acetylation, if any, is not known.

The acetylation of lysine residues in all four major HMG classes has been observed. When calf-thymus homogenates were incubated with $^3$H-acetate and HMGs 1 and 2 were isolated and analyzed, the presence of radioactive ε-N-acetyl lysine was established. These acetylated lysines of HMG 1 were mapped to lysine residues at positions 2 and 11, representing 50% of the total lysine present in the NH$_2$-terminal region of the HMG molecule (Sterner et al., 1979; 1981).

Purified HMGs 14 and/or 17 will partially inhibit endogenous mouse cell histone deacetylase enzymes when added to an in vitro assay mixture (Reeves and Candido, 1980). The ability to suppress histone deacetylation may represent an important aspect of function of these HMG molecules, and may be coupled to their preferential disposition on nucleosomes enriched in acetylated histones and transcribed sequences.
Methylion

Methylation of histones has been observed from a variety of sources for lysines 9 and 27 of H3 (Hooper et al., 1973) and lysine 20 of H4 (DeLange et al., 1969). Chick erythrocyte H1 and H5 may have methylated histidines (Gershey et al., 1969). The lysines of H3 may also possess more than one methyl group (Hooper et al., 1973) and are adjacent to the sites of H3 phosphorylation (Dixon et al., 1975).

Histone methylation occurs late in S phase or in G2 (Borun et al., 1978; Thomas et al., 1975), after DNA synthesis (Tidwell et al., 1968), and appears to be irreversible (Thomas et al., 1975).

In striking contrast, methylation of HMGs involves arginine residues rather than specific lysine residues (Boffa et al., 1979). When calf thymus HMGs 1 and 2 were purified and analyzed, it was found that 8-9% of the total arginine in HMG 2 is present as N^G, N^G-dimethyl arginine and that 3-4% of HMG 1 arginines were in the asymmetric dimethylated form (Boffa et al., 1979). The positions of the modified arginine in the primary structure of the proteins is not known.

Interestingly, when cells are treated with alkylating carcinogens such as 1,2-dimethylhydrazine (DMH), methyl groups derived from (methyl-^3H) DMH are transferred to both lysine and arginine residues in both histones and HMGs (Allfrey, 1977). Since these amino acids participate in electrostatic interactions with DNA, it is possible that the direct actions of carcinogens result in aberrant, irreversible alterations in the structure and function of chromatin. Moreover, this type of chromosomal damage appears to be most severe in the more accessible, HMG-enriched regions of
chromatin, as judged by the finding that the methylation of the HMG proteins by $^3$H-DMH is three times greater than that of the histones in the carcinogen-treated cells (Boffa et al., 1981).

**ADP-ribosylation**

The enzyme poly(ADP)-ribose synthetase catalyzes the transfer of ADP-ribose units to acceptor sites on a variety of nuclear proteins (for reviews, see Hilz and Stone, 1976; Hayaishi and Ueda, 1977). Following transfer of one ADP-ribose to a nuclear protein, successive transfer of other residues of ADP-ribose to the initial one is achieved, generating oligomers and polymers of ADP-ribose (Miwa et al., 1979).

Evidence has been obtained for the natural occurrence of poly(ADP)-ribosylated histones in rat liver (Veda et al., 1975; Smith and Stocken, 1973). Labelling studies with isolated nuclei indicate that many non-histone proteins are ADP-ribosylated including members of the HMG group (Wong et al., 1977; Giri et al., 1978; Levy-Wilson, 1981). Isolated trout testis nuclei modify protein H6 to an average (ADP)-ribose polymeric chain length of 4.5 units (Wong et al., 1977).

When mouse mammary carcinoma cells are labelled with $^3$H-adenosine, the four HMG proteins, 1, 2, 14, and 17, as well as H1, are ADP-ribosylated (Tanuma and Johnson, 1983). Proof that label on the proteins was ADP-ribose was provided by chromatographic identification of ADP-ribose following alkaline hydrolysis and also by the observation that the inhibitor of (ADP-ribose) synthetase, 3-aminobenzamide, prevented incorporation of the labelled adenosine. These observations are of extreme interest because of
the association of these HMG proteins with actively transcribed genes. Hence, ADP-ribosylation could serve in some capacity to regulate gene expression.

**Ubiquitination**

Protein A24 (molecular weight approximately 27,000 in rat liver) was first identified as a branched protein composed of H2A joined by an isopeptide linkage to the 74 residue NHC protein "ubiquitin" (Goldknopf and Busch, 1977; Mawtinson et al., 1979).

Recently, it has been shown that H2B may undergo an isopeptide linkage with ubiquitin (West and Bonner, 1980a, Wu et al., 1981). Approximately 10% of the H2A and 1.5% of H2B are in the form of ubiquitin in mouse L1210 leukemia cells (West and Bonner, 1980a,b). The conjugated moieties of H2A and H2B were found to be in rapid equilibrium with the pool of free ubiquitin, both in dividing and nondividing cells (Wu et al., 1981). The synthesis of ubiquitin and the formation of ubiquitinated histones are not linked to DNA synthesis, nor are the conjugates present in isolated metaphase chromosomes (Wu et al., 1981), suggesting some involvement in the restructuring of chromatin in the G1 and G2 phases of the cell cycle. If modification applies to nuclear proteins with more rapid rates of synthesis and turnover in nondividing cells (such as HMGs), the coupling to ubiquitin could represent a normal pathway of protein degradation.
Major Acid Soluble Nuclear Proteins of Drosophila Chromatin

Histones

Drosophila melanogaster possesses the normal complement of all five histones, H1, H2A, H2B, H3, and H4. When compared to sea urchins, birds, and mammals, D. melanogaster H1 is slightly larger, less basic, and contains increased amounts of serine, aspartate, valine, and isoleucine (Rodriguez-Alfageme et al., 1974). Its H2A contains one methionine, whereas H2A from calf, trout, and sea urchin do not (Rodriguez-Alfageme et al., 1974). D. melanogaster H2B has been sequenced (Elgin et al., 1979) and the N-terminal portion of the molecule (residues 1-26) displays little conservation of sequence when compared to H2B of calf, trout, and Patella; whereas, the remainder of the protein is highly conserved.

Protein D1

The first report of the basic Drosophila embryonic nuclear protein, termed D1, was published by Rodriguez-Alfageme et al. (1974). This protein was detected on polyacrylamide gels from a 20% TCA precipitate of a 0.25 M HCl extract of proteins solubilized by 2 M NaCl. D1 is rich in both acidic and basic amino acids (24% and 21%, respectively). It shares many properties with calf thymus HMGs, notably, solubility in 5% PCA, extractability from chromatin by 0.35 M NaCl, and a high content of basic and acidic amino acids. However, it differs from those protein of the HMGs thus far isolated in having a higher molecular weight and in having
aspartic acid rather than glutamic acid as its most abundant amino acid.

Based on its amino acid composition, acid and salt extractability, electrophoretic mobility, and its location in polynucleosomes, D1 is the same protein as the previously described D. melanogaster NHC protein A63 (Bassuk, 1980; Bassuk and Mayfield, 1980; Bassuk and Mayfield, 1982).

The amount of D1 in embryonic nuclei is 2-5% that of histone H1 on a molar basis, and the amount in salivary gland chromatin, while not yet accurately determined, is not much more than that.

An interesting staining characteristic of D1 is that it stains green with Amido Black and stains red with Coomassie Brilliant Blue R (Bassuk and Mayfield, 1982). D1 has been further characterized by immuno-fluorescence localization in polytene chromosomes of D. melanogaster (Rodriguez-Alfageme et al., 1976). This study noted a correlation between the chromosomal sites that fluoresce most intensely with anti-D1 antibodies and the sites known to fluoresce most intensely with quinacrine (Vosa, 1970; Adkisson et al., 1971; Ellison and Barr, 1972; Mayfield and Ellison, 1975; Hochman, 1976). A more carefully controlled follow-up investigation revealed that, under conditions that minimize nonspecific antibody interactions, D1 is widely distributed throughout the cytoplasm and nucleus, present in many chromomeres, but most abundant in chromosomal sites that contain the A-T rich satellite DNA of buoyant density 1.672 g ml⁻¹ (Rodriguez-Alfageme et al., 1980). The authors conclude that the observed distribution, taken together with available evidence about the nucleotide sequences present in this satellite (Brutlag and Peacock, 1975; Endow et al., 1975), suggests that D1 preferentially binds to chromatin
containing sequences AATAT and/or AATATAT.

Most recently, Levinger and Varshavsky (1982) have utilized two-dimensional mapping of nucleosomes to compare the structure of mononucleosomes from different regions of the \textit{D. melanogaster} genome. They reported the finding that D1 associates with 1.688 satellite DNA containing mononucleosomes. This satellite consists of tandemly repeated copies of a unique 359 bp sequence; it is found in heterochromatin, is A-T rich and constitutes pure A-T tracts up to 13 bp long (Hsiesh and Brutlag, 1979). It has been proposed that this protein binds sequence specifically to satellite sequences (Levinger and Varshavsky, 1982).

**Protein D2**

Protein D2 was first detected as a contaminant of a preparative electrophoretic fractionation of \textit{Drosophila} histones (Rodriguez-Alfageme et al., 1974), and later purified by Bio-Gel-P-100 chromatography. Its amino acid composition is histone-like, but with major differences between D2 and H2A in mole percentage of several amino acids, especially threonine, serine, methionine, and histidine (Palmer, 1979). Comparison of D2 with the other core histones revealed similar distinct differences in amino acid content. Tryptic mapping demonstrated that D2 is not a simple sequence variant of any major histone. D2 was also found to co-sediment with mononucleosomes in sucrose gradients (Palmer, 1979), but its chromosomal location has yet to be elucidated.
Protein Al3

The identification, isolation, and characterization of Al3 was first reported by Bassuk and Mayfield (1980). The protein was first detected in 2% PCA and 0.35 M NaCl extracts of isolated nuclei. The purification was achieved by BioRex 70 cation exchange chromatography. The amino acid composition indicated a high proportion of basic residues (12.2% lysine) and acidic residues (11.5% aspartate and 13.1% glutamate). Based on these observations, Al3 was judged to be a NHC HMG-like chromosomal protein.

When homogenized nuclei were digested with increasing amounts of micrococcal nuclease, a protein that co-migrated in one-dimensional SDS-PAGE with Al3 was observed to be rapidly released from chromatin free of nucleosomes (Bassuk, 1980). The identity of the protein released by micrococcal nuclease was not established until this dissertation.

Active chromatin specific proteins

During the last several years, methods have been developed to study the distribution of chromosomal proteins on the polytene chromosomes of Drosophila salivary glands by immunofluorescence techniques. With the use of such an approach, it has been observed that enzymes involved in gene transcription (such as RNA polymerase) and RNA packaging proteins are found preferentially associated with the active loci (puffs) (Jamrich et al., 1977; Elgin et al., 1978; Silver and Elgin, 1977). In contrast, antibodies prepared against certain subfractions of the NHC proteins give a staining pattern indicating that some such proteins are preferentially associated with both the active loci and those scheduled
to be active (puff) at some time during the third instar larval and prepupal stages (Silver and Elgin, 1977; Mayfield et al., 1978). Such proteins may play an important role in chromatin structure related to the active state.

When *D. melanogaster* NHC proteins are fractionated by SDS-PAGE and lightly stained bands (molecular weight range from 80,000 - 110,000 daltons) are excised, lyophilized, minced, pulverized, mixed with Freund's adjuvant, and injected into rabbits, the resultant antisera is puff specific (Silver and Elgin, 1977). However, the definitive antigen has not been reported.

When proteins released from *D. melanogaster* nuclei by DNAase I are fractionated by SDS-PAGE, and bands ranging from 55,000 - 70,000 daltons (the "band 2" fraction) are used to immunize rabbits, the antisera is puff specific (Mayfield et al., 1978). The principal protein present in band 2 was A63 (D1). However, antisera raised against purified A63 does not specifically stain puffs (unpublished results from the laboratories of Dr. John E. Mayfield, Dept. of Zoology, Iowa State University, and Dr. Sarah C. R. Elgin, The Biological Laboratories, Harvard University), but instead displays a general distribution consistent with the observations of Rodriguez-Alfageme et al. (1976, 1980).

Monoclonal antibodies have also been prepared against the band 2 antigen (Howard et al., 1981) and the staining pattern observed for clone 28 stained puffs much like the anti-band 2 polyclonal sera. The crucial
experiment, however, demonstrated that both the mono- and poly-clonal antibodies reacted with a 5% PCA insoluble protein, as assayed by western blotting. This observation ruled out A63 (D1) as the antigen responsible for the highly specific staining pattern.

Thus, these techniques represent a powerful tool in the investigation of the mechanisms responsible for the gene regulation in D. melaongaster. Undoubtedly, the techniques will be exploited further.
MATERIALS AND METHODS

Experimental Protocol

Embryos

6-18 hour *Drosophila melanogaster* Oregon R embryos were obtained frozen from Dr. S. C. R. Elgin, Washington University, St. Louis, MO, and kept at -70°C until needed.

Isolation of nuclei

Nuclei were prepared essentially as described (Elgin and Hood, 1973; Hewish and Burgoyne, 1973; Mayfield et al., 1978; Bassuk, 1980).

Briefly, embryos were thawed, dechorionated, washed, homogenized, filtered, and centrifuged at 500 x g for 10 minutes. The supernatant was made 0.1% in Shell Nonidet P-40 detergent and centrifuged at 4,400 x g for 10 minutes. The pellet was resuspended and centrifuged through a discontinuous sucrose gradient (final concentration = 1.9 M sucrose) at 42,000 x g for 20 minutes. The pellet, containing purified nuclei, was resuspended in appropriate buffer and used immediately. All solutions were kept at 4°C and contained 2 mM PMSF and 1 ug/ml Aprotinin (Sigma).

Extraction of histones

Histones were obtained from homogenized nuclei in 10 mM Tris-HCl, pH 7.5, by the addition of an equal volume of cold 0.8 N H₂SO₄. After the mixture was stirred on ice for 30 minutes, it was centrifuged for
30 minutes at 15,000 \times g, and the pellet re-extracted once with cold 0.4 \text{ N} \text{ H}_2\text{SO}_4. Four volumes of cold ethanol were added to the combined supernatants, and the histones allowed to precipitate overnight at -20°C. The precipitate was collected by centrifugation for 15 minutes at 15,000 \times g. The histone pellet was washed twice with ethanol and dried under vacuum.

**Extraction of HMG-like proteins**

HMG-like proteins were extracted from homogenized nuclei (DNA at 10-15 mg ml\(^{-1}\)) by the dropwise addition of 70% PCA to a final concentration of 2%. The resulting suspension was gently stirred on ice for 30 minutes and then centrifuged for 30 minutes at 15,000 \times g. The clear supernatant was neutralized to pH 7 with 50% KOH.

**Purification of A13**

A13 was purified essentially as described (Bassuk and Mayfield, 1982). Briefly, the 20% PCA soluble proteins were chromatographed twice on BioRex 70 (Na\(^+\) form, 50-100 mesh, BioRad, previously equilibrated with 6 M urea and 50 mM Tris-acetate, pH 7.8) and fractions corresponding to A13 were desalted on Sephadex G-25 (equilibrated with 0.01 \text{ N} \text{ HOAc}) and lyophilized. The total protein content was determined by the method of Lowrey et al. (1951) using bovine serum albumin as a standard.
Preparation of anti-A13

Approximately 200 μg of purified A13 was electrophoresed into a preparative SDS-containing polyacrylamide (22%) slab gel (9x15x0.5 cm) according to the method of Laemmli (1970). The gel was stained for 1 minute with 0.15% Coomassie Brilliant Blue R, 45% ethanol, and 10% HOAc. The band corresponding to A13 was excised, dried, ground to a powder, suspended in 0.5 ml of phosphate-buffered saline (0.01 M sodium phosphate, pH 7.6, 0.015 M NaCl), and emulsified with 0.5 ml of Freund’s complete adjuvant (Perrin's modification, Calbiochem). After acquiring 20 ml of blood from the large ear vein, five fractions of 0.1 ml each were injected subcutaneously into the back of the neck of an adult rabbit. After four weeks, 10 ml of blood was collected from the large ear vein, and five booster injections of 0.1 ml each were given. After an additional four weeks, 40 ml of blood was collected. Blood was immediately centrifuged for 10 minutes at 1,000 x g and the upper serum layer was frozen at -70°C until needed.

Immunoprecipitation of A13

One hundred microliters (roughly 200 μg) of a 2% PCA-nuclear extract was mixed with 100 μl of either undiluted preimmune or anti-A13 serum. After incubation overnight at 4°C, 2 mg of protein-A Sepharose (Sigma) was added, and the mixture was agitated gently for two hours at room temperature. After centrifugation at 8,000 x g for 5 minutes, the pellet was washed five times with 25 mM Tris-Tricine (Sigma), pH 8.5, and the supernatants were pooled. The precipitates and
supernatants were separately mixed with equal volumes of 2X sample buffer (1.28 M Tris-HCl, pH 6.8, 0.02 M dithiothreitol, 20% glycerol, and 4% SDS) and electrophoresed on a SDS-containing polyacrylamide (10%) slab gel. After electrophoresis for 700 volt-hours, the gel was silver stained (Wray et al., 1981), photographed, cleared, and stained with Coomassie Brilliant Blue R.

Immunofluorescence

The distribution patterns of anti-A13 on polytene chromosomes from squash preparation of formaldehyde-fixed D. melanogaster salivary glands were obtained and analyzed by Liz Steiner in the laboratory of Dr. S. C. R. Elgin, Washington University, St. Louis, MO, according to published procedures (Silver and Elgin, 1976, 1977, 1978; Silver et al., 1978). Squashes were incubated with a 1:20 dilution of anti-A13 serum in TBS (0.85% NaCl, 10 mM Tris-HCl, pH 7.7) at 25°C for 30 minutes, washed, and incubated with a 1:20 dilution of fluorescein-conjugated goat anti-(rabbit IgG) antibodies (Miles) in TBS at 25°C for 30 minutes. After washing and mounting, slides were viewed with phase-contrast and incident ultraviolet illumination.

Squash preparations of chromosomes from heat-shocked larvae were obtained by incubating larvae at 35°C for 15 minutes immediately before removal and fixation of the salivary glands.

Preparation of nucleosomes

Nuclei were prepared as described above but were further washed
by centrifuging once through ice cold 10 mM Tris-HCl, pH 7.5, 10 mM NaCl, 
1 mM CaCl₂. Nuclei were resuspended in the same buffer at a DNA concentra-
tion of 2-3 mg ml⁻¹ (40-60 A₂₆₀ units ml⁻¹), preincubated for 5 
minutes at 37°C, and digested for 5 minutes at 37°C with varying amounts 
of micrococcal nuclease (Sigma). The reaction mixture was then centri-
fuged for 15 minutes at 8,000 x g. The supernatant was designated S₁. 
The pellet was resuspended in 5 mM EDTA, pH 7.5, and incubated for 30 
minutes at 0°C. After centrifugation for 5 minutes at 8,000 x g, an 
aliquot of the supernatant (termed S₂) was electrophoresed in a standard, 
horizontal 1% agarose gel (or alternatively in a mini-gel) to visualize 
the electrophoretic profile of the polynucleosomal population.

**Fractionation of nucleosomes in preparative agarose gels**

**Method 1** Nuclear digestion products at 2-5 A₂₆₀ absorbing units 
were applied as a 0.001% bromphenol blue, 15% Ficoll-sucrose, 0.5 ug ml⁻¹ 
ethidium bromide solution to a 5 mm thick 1% agarose gel containing 
0.05 ul ml⁻¹ ethidium bromide and TBE buffer (89 mM Tris-HCl, pH 8.3, 
89 mM boric acid, 3 mM EDTA). The horizontal gel apparatus was con-
structed out of Plexiglass with quartz windows (Ames Laboratory, U. S. 
Department of Energy, original supplier is George Behm and Sons, 
Industrial Glass Division, Dayton, OH) cemented into the bottom plate 
so as to give a smooth, uniform surface on which to cast the gel. The 
overall dimensions of the cast gel was 8x35 cm and the quartz windows 
were located from 8 to 15.5 cm and 19 to 26.5 cm from the sample well. 
The gel was run submerged in TBE buffer containing 0.5 ug ml⁻¹
ethidium bromide. Electrophoresis was at 100 V and the progress was monitored by placing an ultraviolet transilluminator (Ultra-Violet Products, Inc., San Gabriel, CA) under the apparatus. Once the desired separation was achieved, a rectangular trough 2 mm wider than the band was cut in front of each desired band and a washed rectangle (2x5 cm) of dialysis membrane (BioRad) was inserted into the trough and samples collected by continued electrophoresis. The current was reversed for 2 minutes to free any samples adhering to the dialysis membrane. The samples were then removed from the trough with a pasteur pipet. Typically the samples were divided into two equal portions, and processed for DNA or protein electrophoretic analysis as described below.

Method 2 Alternatively, if the quartz bottomed gel box was not employed, the sample was fractionated on a standard agarose gel system and stained with ethidium bromide (1 ug ml⁻¹). Selected bands were excised, transferred to washed 3,500 Mₑ cut-off dialysis tubing (Spectropor) containing TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) and placed between two platinum wires fastened along opposite walls in either a small (25x10x10 cm) or large (18x18x18 cm) plexiglass container containing TE buffer. Electroelution was routinely performed overnight at 35 mA constant current. The voltage was reversed for 2 minutes to remove any nucleohistone adhering to the dialysis membrane. The agarose slices were removed and the sample was divided into two portions to be processed as described below.

Comments on the two methods No appreciable difference was observed in the recovery of samples between the two methods. If many
samples are to be processed, then Method 1 can be extremely arduous, and Method 2 is preferable. However, if time is of the essence, then the recovery from troughs, as described in Method 1, would be the most advantageous.

Analytical agarose gel electrophoresis

DNA or polynucleosomes were routinely visualized by electrophoresis in "Mini-gels" (7.6x5.1x0.3 cm) or in standard horizontal gels (23x13x 0.5 cm). Agarose (BRL) concentrations varied from 1-2%. All gels and running buffers contained TBE buffer. Samples were loaded in 0.001% bromphenol blue, 15% Ficoll and electrophoresed for 150 Volt-hours for mini-gels and 600 Volt-hours for the larger gels.

Polyacrylamide gel electrophoresis

Triton/acid/urea first dimension tube gels Polyacrylamide gels were poured in 0.5x15 cm glass tubes. The gel solution consisted of 12% acrylamide, 0.08% bisacrylamide, 0.67% TEMED, 6 M urea, 5% HOAc, 0.7% Triton DF-16, and 0.0067% ammonium persulfate. After thoroughly degassing the gel solution prior to the addition of ammonium persulfate and detergent, the solution was gently pipetted into the glass tubes, taking care not to introduce any air bubbles. After carefully over-layering with H₂O, 1 hour was allowed for polymerization. Pre-electro-electrophoresis was routinely performed overnight at 150 V. 100 ul of 1 M cysteamine was electrophoresed into the gel for 1 hour at 200 V to remove persulfate anions and hence avoid methionine oxidation. If
the sample volume was greater than 50 ul, a 1-1.5 cm stacking gel was cast on top of the pre-run and pre-scavenged resolving gel. The stacking gel consisted of 6% acrylamide, 0.16% bisacrylamide, 1.5% TEMED, 6 M urea, 0.375 M potassium acetate, pH 4.0, and 0.2% riboflavin 5'-phosphate. The stacking gel was photo-polymerized with ultraviolet light from two GE F6T5 bulbs located 1 cm from the gel tubes. Alternatively, standard 4 foot long fluorescent bulbs were used. The samples were then run in a water-jacketed tube gel apparatus attached to a cold water faucet for 1 hour at 100 V and then at 2.5 hours at 400 V.

**Acid-soluble nuclear protein samples** These samples were routinely obtained in a lyophilized form and simply resuspended in an appropriate volume of T/A/U sample buffer (6 M urea, 5% HOAc, 1% protamine sulfate, 5% β-mercaptoethanol, and 0.001% pyronin Y) so that less than 50 ul of sample solution could be loaded onto the resolving gel.

**Agarose fractionated nucleosomal samples** Electroeluted nucleosomal samples were adjusted to 0.25 M sodium acetate, pH 4.2. 1 ug ml⁻¹ of tRNA (Sigma) was added and the samples precipitated with three volumes of 100% ethanol for 1 hour at -70°C. After centrifugation for 30 minutes at 20,000 x g, the pellet was vacuum dried, dissolved in 200 ul of T/A/U sample buffer, and kept at room temperature for 30 minutes with occasional vortexing. Samples were then electrophoresed as described above.
Samples were electrophoresed as described above for T/A/U tube gels but a 9x15 cm slab gel was employed.

SDS slab gels SDS-PAGE was performed on 1.5 mm thick slab gels. The 9x15 cm resolving gel was either 12% (30:0.8 acrylamide to bis-acrylamide) or 22% (30:0.2 acrylamide to bisacrylamide) acrylamide. The polymerization conditions and gel compositions were as previously described (Laemmli, 1970; Bassuk, 1980; Bassuk and Mayfield, 1982).

For second dimensional electrophoretic analysis, the gel tubes were removed, transferred to test tubes and equilibrated for 2 hours with several changes of 62.5 mM Tris-HCl, pH 6.8, 10% glycerol, with 5% β-mercaptoethanol added the last 30 minutes. The tubes were then adhered to the top of a 22% SDS-containing polyacrylamide gel with 1% agarose in 62.5 mM Tris-HCl, pH 6.8 electrophoresed for 18 hours at 70 V.

Staining of proteins Gels were stained either with Coomassie Brilliant Blue R or by various silver procedures.

Coomassie Brilliant Blue R staining The staining mixture consisted of 0.15% Coomassie Brilliant Blue R (BioRad), 45% ethanol, and 10% HOAc. The gels were destained with 10% HOAc.

Silver staining Various procedures included those described by Wray et al. (1981), Morrissey (1981) and Guevara et al. (1982). The gels were processed in various sizes of Tupperware or freezer containers with gentle agitation throughout. The development of the image was visualized by placing the gel container on a light box. The gels were cleared with Kodak Rapid Fix, washed with Hypo
clearing agent, and finally with H\textsubscript{2}O. The gel could then be stained with Coomassie Brilliant Blue R.

**DNA electrophoresis** DNA was purified by extraction with freshly distilled phenol, followed by subsequent extractions with a 1:1 mixture of phenol:chloroform (24:1 chloroform:isoamyl alcohol), and chloroform. The resultant aqueous phase was made 0.3 M in sodium acetate, 1 \text{ug ml}^{-1} in tRNA, and mixed with 3 volumes of 100% ice cold ethanol. The DNA was allowed to precipitate at -70°C for at least 1 hour. After collecting the DNA by centrifugation, the sample was dissolved in 0.001% bromphenol blue and 15% Ficoll.

The sample was electrophoresed on a 9x15 cm slab gel consisting of 4.25% acrylamide (29:1 acrylamide to bisacrylamide), 0.17% bis-acrylamide, 0.02% ammonium persulfate, 0.027% TEMED, and TBE. The running buffer was TBE. Electrophoresis was for 2 hours at 150 V, and staining was with ethidium bromide.

**Photography** Ethidium bromide gels were photographed using a Polaroid camera and Royal Pan Film, utilizing a red filter. Coomassie gels used a yellow filter, while silver gels needed no filter. All films were developed in D-76 developer (Kodak) and processed by standard procedures.

**Determination of \%A\textsubscript{260} released values** Bassuk (1980) showed that the percent of DNA rendered acid soluble by digestion of homogenized nuclei with DNAase I or micrococcal nuclease could be calculated from the following equation:
\[
\%A_{260} \text{ released} = \frac{(A_{260} \text{ of } S1)}{(A_{260} \text{ of } S1) + (A_{260} \text{ of } S2) + (A_{260} \text{ of } P)}
\]

where \( S1 \) = the first supernatant, \( S2 \) = the second supernatant, and \( P \) = the residual nuclear pellet. The equation holds if the denominator approximates the concentration of \( A_{260} \) absorbing material present in the original reaction mixture. Additionally, when the \( S1 \) fraction is made 5.8% in perchloric acid, the resultant \( A_{260} \) approximates the acid-free \( S1 \). The \( \%A_{260} \) values given in this study are derived from the above equation.

**Materials**

**Acrylamide**

All acrylamide used in this study was obtained from either Bethesda Research Laboratories, BioRad Laboratories, or from Accurate Chemical Co. Occasionally, acrylamide was recrystalized from chloroform, collected by filtration, and air-dried.

**Other reagents**

All chemicals were obtained from standard chemical warehouses except were specifically noted.
RESULTS 1. ELECTROPHORESIS OF ACID-SOLUBLE NUCLEAR PROTEINS

The control of gene expression is presumed to involve the association of regulatory protein molecules with recognition sites on DNA. By definition, any such species must be included in the nonhistone proteins. In addition, RNA molecules may play a regulatory role. So little is known about the control of eucaryotic gene expression that it is not possible to estimate how many such proteins there may be or in what amount each should be present. Peterson and McConkey (1976), using a two-dimensional polyacrylamide gel analysis, observed 450 NHC protein species from Hela cells. Because of the obvious difficulties in studying this heterogenous protein population, it is important to design an experiment to investigate a subset of these NHC proteins. My approach has been to utilize various reagents which are known to extract histones or basic NHC proteins from the nuclei from many species. These reagents included several concentrations of either TCA or PCA, 0.35 M NaCl followed by 2% TCA, and 0.4 N H₂SO₄.

One dimensional polyacrylamide gel electrophoresis of D. melanogaster acid-soluble proteins has been shown to be inadequate in resolving these proteins from each other (Bassuk, 1980). SDS-PAGE is satisfactory for the analysis of histones, but nonhistone proteins co-migrate with the core histones. Acid/urea polyacrylamide gel electrophoresis (A/U-PAGE) is also incapable of sufficient resolution. Even when these two gel systems are combined to yield a two-dimensional electrophoretogram, the resolution is inadequate. Only when a nonionic detergent, such as
Triton DF-16 is incorporated into the A/U-PAGE (T/A/U = Triton/acid/urea) first dimension is the electrophoretic separation highly discriminating. Figure 1 shows such a two-dimensional electrophoretic separation of the acid-soluble proteins from *D. melanogaster* embryonic nuclei.

Figure 1A demonstrates that the histones are dominant proteins extracted by 0.4 N H$_2$SO$_4$, a fact that has been known for many years. The HMG-like proteins, A63 and A13 (Bassuk, 1980; Bassuk and Mayfield, 1982) and the histone-like protein D2 (Palmer, 1979) are clearly identified in this electrophoretogram. The resolution of A13 and D2 from the histones is possible only when detergent is incorporated into the first electrophoretic dimension. Triton DF-16 (a polyoxyethylene ether) is a non-ionic detergent that has a preferential affinity for the hydrophobic residues of histone H2A (Rodriguez-Alfageme et al., 1974). While the mechanism for this affinity is unknown, the electrophoretic mobility of H2A is substantially reduced. The fact that H2A possesses more hydrophobic residues than the other three core histones probably accounts for this phenomenon. The extent of the reduction of electrophoretic mobility is dependent on the concentration of the detergent and urea (Rodriguez-Alfageme et al., 1974). Without Triton DF-16, A13 and H2A, and D2 and H2B, co-migrate in the A/U system. Therefore, T/A/U followed by SDS-PAGE is a necessary requirement for the electrophoretic analysis of *D. melanogaster* basic nuclear proteins.

The major technical disadvantage of this system is the time-consuming procedures which are necessary to prepare the A/U gel for sample application. After casting the first dimension tube gel, the polymerized gels
Figure 1. Two-dimensional electrophoretic polyacrylamide gels of the acid soluble proteins extracted from nuclei.

(A) Proteins solubilized by 0.4 M H$_2$SO$_4$.
(B) Proteins solubilized by 2% PCA.

Samples were first run in 12% polyacrylamide tube gels containing 6 M urea, 0.9 M acetic acid, and 0.7% Triton DF-16 with the cathode to the right. The proteins were then separated in a second dimension by using 22% polyacrylamide slab gels containing SDS with the anode at the bottom. The proteins were stained with Coomassie Brilliant Blue R.
frequently slip out of the tube due to the presence of Triton Of-16. Therefore, it is necessary to secure gauze around the bottom of each tube to hold the gel and to meticulously remove all air bubbles from between the gauze and the gel with a syringe. The tube gels must be pre-electrophoresed with a degassed running buffer to remove any undesirable ions other than protons and acetate ions. Pre-electrophoresis did not completely remove persulfate ions. Therefore, it was necessary to scavenge free radicals (e.g. $S_2O_8^{-2}$) by electrophoresing 0.1 ml of 1 M cysteamine through the gel for 30 minutes at 150 V, thus preventing oxidation of histone methionine during the subsequent electrophoretic run. Sample volumes of less than 50 ul can be routinely applied without any appreciable decrease in resolution. At sample volumes greater than 50 ul smearing of the electrophoresed proteins is routinely observed. This problem can be solved by employing a stacking gel cast onto the top of the pre-run and pre-scavenged T/A/U gel. This allows sample volumes of up to 300 ul to be electrophoresed. Initial experiments utilizing the stacking gel encountered another problem, the oxidation of histone methionine was observed. Figure 12 displays the artifacts induced by the formation of the oxidation of products of H2A, H2B and H3. H3 and H2A each contain 1 methionine residue and appear as two spots. H2B has two residues and appears as three spots. This observation therefore confirms the published number of methionine residues for these three proteins (Rodriguez-Alfageme et al., 1974; Elgin et al., 1979). This artifact was eliminated by utilizing low levels of riboflavin 5'-phosphate in place of ammonium persulfate, and by photo-polymerizing the stacking gel.
with intense fluorescent light.

Also present in the 0.4 N H$_2$SO$_4$ extract (Figure 1A) are two proteins which have been observed to possess reproducible electrophoretic mobility from many extracts. Based on the characteristics of electrophoretic mobility, solubility, and association with isolated nucleosomes (See Figure 18), the proteins are tentatively identified as the ubiquitin conjugates of H2A and H2B (Dr. Leonard H. Cohen, personal communication, Institute for Cancer Research, Fox Chase Cancer Center, Philadelphia, PA). Interestingly, based on their staining properties with Coomassie Brilliant Blue R, these proteins seem to be present in equal amounts; whereas, six times more uH2A than uH2B is found in mouse L1210 leukemia cells (West and Bonner, L980a,b).

In mammals, there are four sequence variants of H2A (West and Bonner, 1980a,b). Both the two major variants and the two minor variants are ubiquinated. The mammalian species are clearly resolved on two-dimensional gels utilizing T/A/U followed by acid-urea-cetyl ammonium bromide PAGE. However, only one species of uH2B has been reported. About 10% of the major mammalian H2A variants are ubiquinated (Goldknopf and Busch, 1977), and these ubiquitin adducts are found in nucleosomes (Martinson et al., 1979). It is suggested here that uH2A and uH2B are found in acid extracts of D. melanogaster embryonic nuclei and confirm the observation of Levinger and Varshavsky (1982) of polynucleosomal uH2A (See Figure 18). It is unclear if there is more than one protein species of uH2A or uH2B in D. melanogaster, because of the inability to resolve or recognize them. No free ubiquitin has been recognized. It is interesting that uH2A and uH2B are detected only in polynucleosomes larger than 465 bp.
(See Figure 18). The function of these ubiquitin adducts is currently unknown.
RESULTS II. PURIFICATION OF PUTATIVE HMG PROTEINS

It is clear from this and earlier studies that there are two major nonhistone nuclear proteins which are consistently extracted by low concentrations of both TCA and PCA. These proteins are also extracted by 0.35 M NaCl and therefore satisfy the solubility requirements established by Goodwin et al. (1973) as defining HMG proteins from mammalian tissues. Figure 1A demonstrates that A63 and A13 are solubilized by 0.4 N H$_2$SO$_4$. Figure 1B demonstrates that 2% PCA is a far more selective solvent. However, it was observed over many extractions that there was a variability in the number of proteins solubilized by 2% PCA, especially in the molecular weight range of A13. Therefore, an extraction series was performed (by J. E. Mayfield) on homogenized nuclei to clarify this observed variability. Figure 2 compares the proteins extracted by various concentrations of PCA and TCA. Since it was the goal of this research to define HMG proteins in D. melanogaster, and 20% PCA appeared to extract more proteins, including A13, (Figure 2, Lane 5), this concentration of PCA was used as a source of material for the purification of putative HMG proteins.

100 g of 6-18 hour embryos were used to prepare nuclei, and proteins solubilized by 20% PCA were chromatographed as described in Figure 3. Aliquots of selected fractions were visualized by 10% SDS-PAGE (Figure 4) and it was observed that A13 eluted at 3.2% guanidine hydrochloride (GuCl) (Figure 4A, Lane 2) and protein X, of interest because of its possible
Figure 2. Proteins extracted from nuclei by various reagents.

Each selected fraction was made 0.1% in SDS and dialyzed overnight vs. 1 mM Tris-HCl, pH 7.5, lyophilized, resuspended in sample buffer, electrophoresed by 12% SDS-PAGE and stained with Coomassie Brilliant Blue R. Lanes 1-12 represent proteins solubilized by the indicated acid.

<table>
<thead>
<tr>
<th>Lane</th>
<th>Acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2% PCA</td>
</tr>
<tr>
<td>2</td>
<td>5% PCA</td>
</tr>
<tr>
<td>3</td>
<td>10% PCA</td>
</tr>
<tr>
<td>4</td>
<td>15% PCA</td>
</tr>
<tr>
<td>5</td>
<td>20% PCA</td>
</tr>
<tr>
<td>6</td>
<td>2% TCA</td>
</tr>
<tr>
<td>7</td>
<td>5% TCA</td>
</tr>
<tr>
<td>8</td>
<td>10% TCA</td>
</tr>
<tr>
<td>9</td>
<td>15% TCA</td>
</tr>
<tr>
<td>10</td>
<td>20% TCA</td>
</tr>
<tr>
<td>11</td>
<td>0.4 M H₂SO₄</td>
</tr>
<tr>
<td>12</td>
<td>45% HOAc</td>
</tr>
<tr>
<td>13</td>
<td>0.35 M NaCl soluble followed by 2% TCA soluble</td>
</tr>
<tr>
<td>14</td>
<td>2 M NaCl soluble followed by 0.25 M HCl soluble</td>
</tr>
<tr>
<td>15</td>
<td>2 M NaCl soluble followed by 0.25 M HCl soluble, followed by 20% TCA soluble</td>
</tr>
<tr>
<td>16</td>
<td>20% TCA insoluble pellet from Lane 15</td>
</tr>
</tbody>
</table>
Figure 3. Fractionation of proteins solubilized by 20% PCA on a BioRex 70 cation exchange chromatography column.

(A) Nuclei were extracted with PCA (final concentration = 20%) by stirring on ice for one hour, centrifuging for 30 minutes at 15,000 x g, and neutralizing the clear supernatant with 50% KOH. The sample was made 6 M in urea and 50 mM in Tris-acetate, pH 7.8, and applied to a 1.5x16 cm BioRex 70 column column (Na+ form, 50-100 mesh, BioRad) previously equilibrated with 6 M urea, 50 mM Tris-acetate buffer, pH 7.8. The sample was eluted with a linear, 0-10%, gradient of guanidine hydrochloride (GuCl) at a flow rate of 50 ml h⁻¹, and 2.0 ml fractions were collected at room temperature. The GuCl concentration was monitored with a conductivity meter. Aliquots (0.1 ml) were withdrawn from selected fractions for SDS-PAGE.

(B and C) The peaks eluting at 3.2 and 6.5% GuCl (Figure 3A) were each separately rechromatographed. The peaks eluted at 3 and 6% GuCl, respectively.

The electrophoretogram for selected column fractions is shown in Figure 4.
Figure 4. Electrophoretogram demonstrating the purification of Al3 and protein X.

(A) Aliquots from the chromatographed 20% PCA soluble proteins were made 0.1% in SDS, dialyzed, lyophilized, resuspended in sample buffer and visualized by 12% SDS-PAGE. Lanes correspond to the designated fraction from the elution profile in Figure 3. Lanes 1, 11, and 12 are 20% PCA soluble proteins and represent the sample applied to the BioRex 70 column.

Lane 2 Fraction 30 (3.2% GuCl peak) Lane 3 Fraction 36
3 36 4 39
5 44 6 51 (6.5% GuCl peak)
7 53 8 56
9 62 10 69

(B) The 3% GuCl peak (Figure 3B) and the 6% GuCl peak (Figure 3C) were collected and desalted in 5 ml batches on Sephadex G-25 column. Aliquots of the pooled material were visualized by 12% SDS-PAGE. Lanes 1 and 2 are the desalted protein fractions from Figures 3B and 3C, respectively.

(C) An aliquot of desalted protein X from the 6% GuCl peak (Figure 3C) was electrophoresed on a 12% T/A/U slab gel.

All gels were stained with Coomassie Brilliant Blue R.
classification as an HMG protein) eluted at 6.5% GuCl (Figure 4A, Lanes 6 and 7). Pooled fractions were individually rechromatographed (Figure 3A and 3B), and fractions were again pooled and desalted separately on a Sephadex G-25 column. Figure 4 shows the results of the purification procedure. Based on its initial elution from the first BioRex 70 column (Figure 3A and Figure 4A, Lane 2), its elution from the second ion-exchange column (Figure 3B) and because it ran as a single band on SDS-PAGE, A13 was judged to be in pure form. Because protein X electrophoresed as a diffuse band on SDS gels (Figure 4B, Lane 2), an aliquot was also electrophoresed on a 12% acid-urea slab gel (Figure 4C). In this electrophoretic system it ran as a single sharp band, so protein X was also judged to be relatively pure.

The concentration of each purified protein was determined by the method of Lowry et al. (1951). The standard curve was prepared from a bovine serum albumin (BSA fraction V) dissolved in water, assuming an extinction coefficient of 0.6 \( \text{OD}_{280} \) for a 1.0 mg ml\(^{-1}\) solution. The total yield of A13 and protein X from 100g of embryos was 0.25 mg and 1.0 mg, respectively.

A13 and protein X were hydrolyzed in triplicate for 24, 48 and 72 hours in 6 N HCl and their amino acid composition was determined by Bill Harris in the laboratory of Dr. Donald Graves at Iowa State University. The values obtained for serine and threonine were corrected for degradation by extrapolating to zero hydrolysis time (for protein X, see Figure 5). The results (Table 2) show that the composition of A13 prepared in this way closely resembles the previous analysis of less pure material.
Figure 5. Destruction of serine and threonine during hydrolysis of protein X in 6 N HCl.

The nmols of threonine (●—●—●) and serine (○—○—○) hydrolyzed at varying times was extrapolated to zero hydrolysis time. The data were fitted by a computerized linear regression program.
X-INTERCEPTS
SER = 134
THR = 106

nmol

0  24  48  72
HOURS
Table 2. Amino acid compositions of proteins purified from a 20% PCA extract. Mole %

<table>
<thead>
<tr>
<th></th>
<th>A13^a</th>
<th>H2B^b</th>
<th>H2B^c</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asx</td>
<td>9.7</td>
<td>6.6</td>
<td>5.7</td>
</tr>
<tr>
<td>Thr</td>
<td>3.8^d</td>
<td>7.9^d</td>
<td>8.2</td>
</tr>
<tr>
<td>Ser</td>
<td>11.2^d</td>
<td>9.9^d</td>
<td>9.8</td>
</tr>
<tr>
<td>Glx</td>
<td>13.3</td>
<td>8.4</td>
<td>7.4</td>
</tr>
<tr>
<td>Pro</td>
<td>3.9</td>
<td>3.9</td>
<td>3.3</td>
</tr>
<tr>
<td>Gly</td>
<td>13.6</td>
<td>6.7</td>
<td>4.1</td>
</tr>
<tr>
<td>Ala</td>
<td>10.6</td>
<td>11.6</td>
<td>11.5</td>
</tr>
<tr>
<td>Val</td>
<td>4.6</td>
<td>5.5</td>
<td>4.9</td>
</tr>
<tr>
<td>Cys</td>
<td>trace</td>
<td>0.1</td>
<td>0.0</td>
</tr>
<tr>
<td>Met</td>
<td>1.2</td>
<td>1.4</td>
<td>1.6</td>
</tr>
<tr>
<td>Ile</td>
<td>2.2</td>
<td>5.8</td>
<td>7.4</td>
</tr>
<tr>
<td>Leu</td>
<td>3.7</td>
<td>5.0</td>
<td>4.9</td>
</tr>
<tr>
<td>Tyr</td>
<td>1.6</td>
<td>3.3</td>
<td>4.1</td>
</tr>
<tr>
<td>Phe</td>
<td>1.3</td>
<td>1.5</td>
<td>1.6</td>
</tr>
<tr>
<td>Lys</td>
<td>10.4</td>
<td>15.8</td>
<td>17.2</td>
</tr>
<tr>
<td>His</td>
<td>1.1</td>
<td>2.1</td>
<td>2.5</td>
</tr>
<tr>
<td>Arg</td>
<td>4.3</td>
<td>4.7</td>
<td>5.7</td>
</tr>
<tr>
<td>Trp</td>
<td>3.7^e</td>
<td>0.7^e</td>
<td>0.0</td>
</tr>
<tr>
<td>Lys/Arg</td>
<td>2.4</td>
<td>3.4</td>
<td>3.0</td>
</tr>
<tr>
<td>Basic/acidic</td>
<td>0.7</td>
<td>1.4</td>
<td>1.7</td>
</tr>
<tr>
<td>Hydrophobic^f</td>
<td>14.6</td>
<td>25.3</td>
<td>24.5</td>
</tr>
<tr>
<td>Basic + acidic</td>
<td>38.8</td>
<td>35.5</td>
<td>36.0</td>
</tr>
</tbody>
</table>

^a Purified from the 3% GuCl peak of Figure 3B.
^b Purified from the 6% GuCl peak of Figure 3C.
^c Mole% from the primary sequence of Elgin et al. (1979)
^d Extrapolated to zero hydrolysis time.
^e Estimated spectrophotometrically.
^f Val, Met, Ile, Leu, Tyr, and Phe.
(Bassuk, 1980). Protein X is apparently histone H2B, based on its residue composition. Moreover, the electrophoretic mobility of protein X is identical with H2B prepared from a 0.4 N H$_2$SO$_4$ extract. Thus, H2B is apparently slightly soluble in 2% PCA and very soluble in 20% PCA. This is not a totally unexpected result since H2B from plants is soluble in 5% PCA (Spiker et al., 1976; Fazal and Cole, 1977).
RESULTS III. IMMUNOFLUORESCENCE STUDIES OF A13

A13, purified as described in the previous section, was further purified by preparative SDS-PAGE and used to immunize an adult rabbit. The antigen was administered by subcutaneous injection with a booster injection after 4 weeks. Immune sera was collected after an additional 4 weeks. Undiluted preimmune or immune sera were incubated with 2% PCA soluble proteins and then with protein-A conjugated to Sepharose beads. The sepharose was then washed five times by centrifugation through TT buffer (25 mM Tris-Tricine, pH 8.5), boiled for one minute in SDS-PAGE sample buffer and electrophoresed by standard SDS-PAGE. The results are shown in Figure 6. Panel A shows the gel stained with ammoniacal silver by the method of Wray et al. (1981). This same gel was cleared with Kodak Rapid-Fix and restained with Coomassie Brilliant Blue R. The Coomassie Blue stained gel is shown in panel B. Protein-A is a surface glycoprotein from Staphylococcus aureus which specifically binds to the IgG class of antibodies (Forsgren and Sjoquist, 1966). Antigen-antibody complexes are also efficiently bound so the anti-A13 sera should remove A13 from a 2% PCA extract of D. melanogaster nuclei and A13 should be found bound to the protein-A-sepharose beads in this procedure. In Figure 3B, lanes 2, 3 and 4 show the proteins present in a 2% PCA nuclear extract; lanes 8 and 9 show the proteins present in the protein-A-sepharose fraction after treatment of the 2% PCA protein solution with preimmune sera; lanes 12 and 13 show the proteins present in the protein-A-sepharose fraction after treatment of the 2% PCA solution with immune
Figure 6. Immunoprecipitation of anti-A13 bound to A13 by protein-A.

A 2% PCA extract was prepared and used as the source of antigen for anti-A13. After incubation overnight at 4°C, 2.0 mg of protein-A sepharose was added and the mixture was gently agitated for two hours. After centrifugation, the pellet was washed 5 times and the supernatants were pooled.

(A) Silver stain, Wray et al. (1981)
(B) The same gel was cleared with Kodak Rapid-Fix and stained with Coomassie Brilliant Blue R.
Lanes 2-4 2% PCA soluble proteins
Lanes 8-9 Precipitate from 2% PCA, preimmune sera, protein-A
Lanes 12-13 Precipitate from 2% PCA, immune sera, protein-A
Lane 15 Supernatant from Lanes 8-9
Lane 16 Supernatant from Lanes 12-13

Note the discrepancy between the staining patterns of the silver and Coomassie gel. A13, A63 and IgG light chain do not stain with the silver stain of Wray et al. (1981).

SP = serum protein, which is a contaminant throughout. When sera only is electrophoresed, the SP is a dominant contaminant (data not shown).
sera; and lanes 15 and 16 show the proteins which remain in the 2% PCA supernatant following treatment with the two sera and extraction with protein-A-sepharose. The only major differences which can be seen between the immune and preimmune sera is the presence of A13 in the immune sera protein-A selected sample (compare lanes 8 and 9 with lanes 12 and 13). Thus, the antisera prepared clearly binds to A13. Whether or not the sera is monospecific or also has specificity for other minor contaminants present in the A13 preparation is not answered by this experiment.

A frustrating observation is the negative silver staining of several proteins: A13, A63 and IgG light chain (compare lanes 8, 9, 12, and 13). While Coomassie Brilliant Blue R readily stains these proteins, the basis for this discrepancy with ammoniacal silver is unknown and is discussed later in the Results section.

The anti-A13 antisera was examined for its ability to bind *D. melanogaster* polytene chromosomes by use of an indirect immunofluorescence assay (Silver and Elgin, 1976, 1977, 1978; Silver et al., 1978). Salivary glands from third instar larvae were dissected free, lightly fixed with formaldehyde, and squashed in 45% acetic acid. The resultant chromosome spreads were then incubated with the anti-A13 sera or preimmune sera followed by fluorescein conjugated anti-rabbit IgG fraction goat antisera. The resultant stained preparations were then observed in a fluorescence microscope. The preimmune sera did not react with the chromosomes. While staining the chromosomes specifically, relative to cytoplasmic debris, the anti-A13 sera showed a general antigenic distribution. The patterns were, however, nonrandom, and may be investigated
in detail in the future. Figure 7A and 7B displays the phase-contrast and fluorescent micrographs, respectively, of the polytene chromosomes obtained from a larvae grown at room temperature. This figure is representative from many squashes and indicates an intensely staining band located at 60°C, which is mapped at the distal end of the right arm of chromosome 2, and a less intense pattern over the remainder of the chromosomes.

Figure 8A and 8B displays a chromosome squash from larvae grown at room temperature, and then heat shocked at 35°C for 15 minutes. The notable feature of this figure is the lack of reactivity of anti-A13 with the nucleolus, and the prominent fluorescent band at 60°C. Figure 9A and 9B shows another squash from a heat shocked larva. The antisera noticeably failed to react with the chromocenter, indicating a lack of specificity for heterochromatin. Upon close examination of Figures 7 and 9, it is clear that anti-A13 has no special preference for puffs whether developmentally or heat shock induced. Some are stained brighter than others, but the pattern suggests no obvious explanation. Interestingly, the bright staining observed at 60°C with room temperature larvae is maintained during heat shock. This portion of the chromosome is asynapsed in Figure 9 yielding a pair of intense bands (Figure 9B). Whether or not this locus retains this strong reactivity throughout different periods of development will be investigated during this author's postdoctoral work.

Thus, it is apparent that A13 is not preferentially localized in active chromatin, in heterochromatin, or in the nucleolus. The intense staining at 60°C may indicate a concentration of A13 at this locus or,
Figure 7. Staining pattern obtained using anti-A13 serum.

Glands were obtained from a later third instar larva grown at 25°C, dissected out, and processed through the formaldehyde fixation technique. Anti-A13 serum was used for staining at a 1:20 dilution.

(A) Phase contrast micrograph
(B) Fluorescence micrograph. After reaction with anti-A13 and washing, the chromosomes were incubated with anti-rabbit IgG fluorescein conjugated goat antisera, washed, and photographed under a fluorescent microscope.
Figure 8. Staining pattern obtained following heat shock using anti-A13 serum

Parameters as in Figure 7, except larva were heat shocked for 15 minutes at 35°C prior to dissection. Note the lack of reactivity with the nucleolus and the prominent fluorescent band at the distal tip of chromosome arm 2R.
Figure 9. Staining pattern obtained following heat shock using anti-A13 serum.

Parameters as in Figure 7. Heat shock loci 33B, 63C, 87AC, and 93D can be seen in this full chromosome set from a male fly. Other labeled loci are developmentally active sites. Note that in this particular squash chromosome are 2R is asynapsed (split) at the distal tip. CC = chromocenter, HS = heat shock.
more likely, it may indicate that the sera used possesses determinants which react strongly with some other band specific protein. Perhaps this protein was a minor contaminant of the A13 preparation but was more antigenic than A13 itself.
RESULTS IV. ASSOCIATION OF DROSOPHILA HMG-LIKE PROTEINS WITH CHROMATIN

There is little doubt that A13 and A63 are present in cell nuclei, since the methods of extraction involve isolating nuclei as a preliminary step. Furthermore, anti-A63 (Mayfield and Elgin, unpublished results) and anti-A13 react with isolated salivary gland polytene chromosomes. The observation that micrococcal nuclease initially attacks linker regions in chromatin and releases first oligo- and then mono-nucleosomes (Noll and Kornberg, 1977) indicate that this enzyme may be a useful tool for probing the chromosomal location of A63 and A13. D. melanogaster nuclei were treated with increasing concentration of micrococcal nuclease and fractionated as described in detail under Materials and Methods.

Briefly, following enzyme digestion, the nuclei were pelleted from the digestion buffer. This supernatant is called S1 and should contain proteins which were released free from the nuclei by the digestion. This fraction is always free of nucleosomes and contains only acid soluble DNA fragments (Noll and Kornberg, 1977; Bassuk, 1980). The pelleted nuclei were then resuspended in 5 mM EDTA, pH 7.5, and pelleted again. This second supernatant is called S2 and generally contains nucleosomal fragments.

Since the action of micrococcal nuclease on isolated nuclei or chromatin releases nucleosomes which contain intact core DNA there is little argument that the linker regions of DNA which connect nucleosomes together are preferentially digested by this enzyme. Thus, it is
reasonable to suppose that proteins released by moderate levels of digestion with this enzyme were associated in some manner with this linker DNA.

A protein, which co-migrated with A13 on one-dimensional SDS-PAGE was repeatedly detected in the S1 fraction and its concentration rose with increasing digestion with micrococcal nuclease (Bassuk, 1980). No evidence was offered to better establish the identity of this protein. Figure 10 displays a mixing experiment in which the identity of this protein is confirmed as A13. A two-dimensional separation of the proteins released into S1 (21% digestion) are shown in Figure 10A, and a preparation of D. melanogaster histones are shown in Figure 10B. When these two samples are co-electrophoresed, the major protein seen in panel A is seen to have the exact electrophoretic mobility of purified A13. Thus, A13 is the major protein released from chromatin by micrococcal nuclease, and it is surmised that the binding of A13 to chromatin involves linker DNA.

If this is true for all A13 molecules, then one would expect to find at very high digestion levels that most or all of A13 would be released into the S1 fraction. However, even when 24% of the DNA is rendered acid soluble, A13 was still found in the S2 nucleosomal fraction (See Figure 15D). At lower levels of digestion, A13 is progressively released into the S2 fraction and depleted from the pellet fraction. Figure 11 is a 2% agarose electrophoretic gel which displays the DNA fragment sizes which resulted from an experiment in which 6, 8 and 16% of the input DNA was rendered acid soluble by micrococcal nuclease action on isolated nuclei. The S2 fraction represents the fraction containing solubilized
Figure 10. Mixing experiment of S1 proteins and D. melanogaster histones.

(A) Proteins released from nuclei into S1 by micrococcal nuclease (21% digestion).
(B) D. melanogaster histones prepared from a 0.4 N H₂SO₄ extract.
(C) Samples from A and B mixed.

Samples were electrophoresed as in Figure 1, and stained with Coomassie Brilliant Blue R.
Figure 11. Nucleosomal agarose (2%) gel of S1, S2, and P fractions obtained after digestion with increasing levels of micrococcal nuclease.

Equal volumetric aliquots were withdrawn, electrophoresed, and stained with ethidium bromide. Fractions A, B, and C represent 6, 8 and 16% digestion levels. The protein components of each fraction are displayed in Figure 12. Mono-, di-, and tri-nucleosomes are indicated by 1°, 2°, and 3°, respectively.
<table>
<thead>
<tr>
<th>S1</th>
<th>S2</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>B</td>
<td>C</td>
</tr>
<tr>
<td>A</td>
<td>B</td>
<td>C</td>
</tr>
</tbody>
</table>

1°
2°
3°
nucleosomes, and the P fraction represents the residual nuclear pellet which contains insoluble material. Polynucleosome-sized fragments are absent in the S1 fraction. At the 16% digestion level (Figure 11, lane S2-C) the S2 fraction contains primarily mono-, di-, and tri-nucleosomes. The pellet fractions also contain enzymatically cleaved polynucleosomes which were not solubilized by the procedure. Figure 12 shows two-dimensional gels of proteins present in the S2 and P fractions. A comparison of Figures 12A-S2, 12B-S2, and 12C-S2 shows that with increasing digestion there is an increase in the levels of A13 in the S2 fraction and a decrease in the residual nuclear pellet. Also present in all S2 and P fractions are histone H1 and A63.

The next set of experiments was designed to investigate the fractionation of chromatin at digestion levels approaching the limit digest. Nuclei were again incubated with increasing amounts of micrococcal nuclease and S1 and S2 fractions obtained. The percentages of the nuclear DNA rendered acid soluble are as follows: fraction A, 4%; fraction B, 7%; fraction C, 16%, fraction D, 24%. Figure 13 displays an acrylamide gel through which purified S2 fraction DNA from each of the samples was electrophoresed. DNA lengths were determined by comparison with standard molecular weights. In fraction A (lane 3, Figure 13), the DNA is mostly longer than 5000 bp indicating the presence of very long polynucleosomes. In fractions B and C (lanes 4 and 5, Figure 13), the DNA lengths range from very large to about 180 bp. Peaks of mono- and di-nucleosome DNA (200 and 400 bp) are clearly evident in these samples. Fraction D (lane 6, Figure 13) exhibits only small DNA fragments ranging from 200 to 160
Figure 12. Proteins associated with the S2 and pellet (P) fractions generated by micrococcal nuclease digestion below the limit digest.

A, B, and C represent 6, 8 and 16% digestion, respectively. An equal aliquot was withdrawn from each S2 and P fraction, transferred to a small plastic tube, lyophilized, resuspended in sample buffer in the same tube, and electrophoresed as described in Figure 1. In these and all subsequent two-dimensional gels, 1% protamine sulfate was included in the T/A/U sample buffer to effect the dissociation of nucleosomal proteins from DNA. Also, these and all subsequent gels contained a stacking gel that was photo-polymerized onto the pre-run and pre-scavenged T/A/U tube gel. Gels were stained with Coomassie Brilliant Blue R.

These gels correspond to the nucleosomal agarose gel displayed in Figure 11.
Figure 13. Acrylamide gel (4.25%) of purified DNA from various levels of solubilized chromatin obtained by digestion with micrococcal nuclease.

Lane 1  Eco R1 - Hind III double digest of lambda phage DNA.
Lane 2  Hpa II digest of pBR322.
Lane 3  DNA from a 4% digest. Corresponds to Figures 14 and 15A.
Lane 4  DNA from a 7% digest. Corresponds to Figures 14 and 15B.
Lane 5  DNA from a 16% digest. Corresponds to Figures 14 and 15C.
Lane 6  DNA from a 24% digest. Corresponds to Figures 14 and 15D.

The gel was stained with ethidium bromide. DNA lengths were calculated from a computer programmed linear regression analysis yielding a linear plot of log $M_r$ vs. distance migrated.
bp. These fragments are clearly smaller than the "monosome" fragments seen in lanes 4 and 5, yet are not as small (145 bp) or as uniformly sized as would be expected of the DNA from core particles.

The initial impression of the data shown in Figure 13 is of material being "chased" from the high molecular weight fractions in lane 3 through intermediate sizes seen in lanes 4 and 5 to the monosome fraction seen in lane 6. Although this is undoubtedly happening in the nucleus as a whole, the amount of DNA in the S2 fraction itself changes dramatically during the course of the digestion as seen in Figure 14. Thus, the amount of nucleosomal material in fraction D is about the same as in fraction A and substantially less than is seen in fractions B and C. Furthermore, the total amount of \( A_{260} \) absorbing material found in S2 is at no time greater than about 25%.

Thus, there is no real evidence that the nucleosomes in fraction D represents "typical" or "average" nucleosomes which have been set free from fractions A, B, and C. In fact it is just as likely that the nucleosomes in fraction D represent a discrete subset of the nucleosomes present in the undigested nucleus and which may or may not be present in the polynucleosomes released into the S2 fraction by light digestion with micrococcal nuclease. In fact, if the mononucleosomes found in fraction D did represent a chemically distinct subset of the total nucleosomal population, it would partially explain the shape of the digestion curve shown in Figure 14. As increasing amounts of DNA is rendered acid soluble by micrococcal nuclease, first increasing amounts of polynucleosomal material and then decreasing amounts are released into the S2 fraction.
Figure 14. Digestion plot of micrococcal nuclease treated nuclei.

Nuclei (DNA at 2.5 mg ml\(^{-1}\)) were incubated with varying concentrations of micrococcal nuclease for 5 minutes at 37°C. The reaction was stopped with cold 0.1 M EDTA, centrifuged, and the absorbance at 260 nm was determined for the supernatant (S1). The pellet was resuspended in 5 mM EDTA, allowed to sit in ice for 30 minutes, and centrifuged. The second supernatant was designated S2 and its absorbance determined (○―○ S1; ○―○ S2). For each S2 data point, the electrophoretic profiles of purified DNA (Figure 13) and protein composition (Figure 15) were determined.

<table>
<thead>
<tr>
<th>ug enzyme</th>
<th>Figure 13</th>
<th>Figure 15</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Lane 3</td>
<td>A</td>
</tr>
<tr>
<td>2</td>
<td>Lane 4</td>
<td>B</td>
</tr>
<tr>
<td>5</td>
<td>Lane 5</td>
<td>C</td>
</tr>
<tr>
<td>10</td>
<td>Lane 6</td>
<td>D</td>
</tr>
</tbody>
</table>
It would appear that under the conditions used, large polynucleosomal chromatin fragments are free to diffuse from the nuclei, whereas, at higher levels of digestion, small nucleosomal fragments are not. A subset of the mononucleosomes, however, seem to remain soluble. Histone modification, nonhistone chromosomal proteins and three dimensional configuration would all play a role in this phenomenon.

Figure 15 displays two-dimensional gels of protein components of each of the four fractions shown in Figure 13. Histone H1 is present in all fractions except D which is near the limit digest for D. melanogaster (about 30%, Palmer, 1979). Also present in all fractions are A63 and A13, indicating the presence of these proteins in both polynucleosomal as well as mononucleosomal fractions.

In order to investigate the distribution of nuclear proteins in more detail, the protein contents of purified mono-, di-, tri-, and oligonucleosomal fractions were investigated. Purified nuclei were digested to 4% acid solubility and the S1 fraction applied to a 15x35 cm preparative agarose (1%) gel containing 0.5 ug ml^-1 ethidium bromide. The gel box was constructed with quartz plates fitted into the bottom, permitting visualization of samples during electrophoresis. Selected nucleosomal fractions were recovered from troughs cut in front of each bond. An aliquot of each fraction was phenol extracted, ethanol precipitated, and the DNA electrophoresed on a 1% agarose gel. The remainder of each fraction was analyzed for protein content by two-dimensional PAGE. Figure 16 shows the electrophoretogram of purified DNA from the total S2 sample (lanes 2 and 8) and the purified DNAs from five nucleosomal
Figure 15. Two-dimensional gels of proteins associated with various digestion fractions described in Figures 13 and 14.

Equal volumetric aliquots from the four digestion fractions from Figures 13 and 14 were incubated with A/U sample buffer containing 1% protamine sulfate for 0.5 hours at room temperature. Following two-dimensional PAGE, the gels were stained with Coomassie Brilliant Blue R. Each gel corresponds to the electrophoretic profile of purified DNA shown in Figure 13, and to each S2 data point in Figure 14.

<table>
<thead>
<tr>
<th>Gel</th>
<th>Figure 13</th>
<th>Figure 14</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Lane 3</td>
<td>1 ug</td>
</tr>
<tr>
<td>B</td>
<td>Lane 4</td>
<td>2 ug</td>
</tr>
<tr>
<td>C</td>
<td>Lane 5</td>
<td>5 ug</td>
</tr>
<tr>
<td>D</td>
<td>Lane 6</td>
<td>10 ug</td>
</tr>
</tbody>
</table>
Figure 16. Agarose (1%) of purified DNA from nucleosomes fractionated on a preparative agarose gel.

Lane 1  Hpa II digested pBR322
Lane 2,8 DNA purified from total S2 (4% digestion) sample
Lanes 3-7 DNA purified from each fractionated nucleosomal class. The lane numbers correspond to the densitometric scan in Figure 16 and to the protein gel shown in Figure 17.
fractions (lanes 3 through 7). Lane 3 represents the fastest migrating sample, and lanes 4-7 represent the subsequent fractions obtained. Figure 17 displays a densitometric scan of the gel from Figure 16 with each scan labeled to correspond to its respective lane in Figure 16. Sample 3 clearly represents nearly pure nucleosomes. Sample 4 contains dinucleosomes and a high molecular weight subset of mononucleosomes. Sample 5 contains predominantly di-, tri-, and tetra-nucleosomes, sample 6 contains polynucleosomes ranging in size from 4 to 10 nucleosomes, and sample 7 contains very large polynucleosomal fragments.

Figure 18 displays the results of a two-dimensional gel analysis of proteins associated with each nucleosomal fraction. In each sample, all of the core histones are present. Histone H1 is not detected in samples 3 and 4 indicating that this histone is absent from mononucleosomes and probably dinucleosomes produced by light digestion with micrococcal nuclease. H1 is associated with larger nucleosomal oligomers (gels 5, 6, and 7). These observations are consistent with the hypothesis that H1 is involved in higher order chromatin structures (Igo-Kemenes et al., 1982). Protein D2 is found in all the higher nucleosomal fractions but is a minor component or is absent in samples 3 and 4. Gels 3 and 4 also display two proteins of unknown identity (see arrows). These proteins are absent from samples which contain the ubiquitin conjugates of H2A and H2B (gels 5-7).

Proteins A63 and A13 are detected in higher molecular weight polynucleosomal fractions though the staining characteristics of A63 and A13 are weak. Nevertheless, these proteins are capable of being detected with silver staining and their detection in higher molecular weight
Figure 17. Densitometric scans of the gel shown in Figure 16.

Scan 1  Hpa II digested pBR322
Scan 2  DNA purified from total sample
Scans 3-7 represent DNA purified from isolated fractions. Numbers correspond to the lane numbers in Figure 16.
Scan 3  Mononucleosomes of 230 bp
Scan 4  Mono- and di-nucleosomes of 280 and 460 bp
Scan 5  Di-, tri-, and tetra-nucleosomes of 465, 660, and 850 bp
Scan 6  Approximately 1200 bp
Scan 7  Greater than 2000 bp
Figure 18. Two-dimensional gels of proteins from nucleosomes fractionated as described in Materials and Methods.

The samples recovered from preparative agarose gel electrophoresis were precipitated with ethanol and dissolved in A/U sample buffer containing 1% protamine sulfate. The T/A/U tube gel also contained a stacking gel photo-polymerized on top of it. The numbers on each gel correspond to the lane numbers in Figures 16 and 17.
fractions of polynucleosomes is probably due to the concentration of the proteins in these fractions.
RESULTS V. SILVER STAINING OF PROTEINS IN POLYACRYLAMIDE GELS

The silver staining of proteins in polyacrylamide gels was first described by Hubbell et al. (1979) in their study of nucleolar proteins. Subsequently, there has been numerous reports of modified silver staining techniques, each claiming greater than 100 fold sensitivity than provided by staining with Coomassie Brilliant Blue E. Obviously, silver staining can be a tremendous advantage when investigating submicrogram amounts of proteins which have been fractionated in polyacrylamide gels.

At the most basic level, all silver staining procedures can be classified into the following two categories: (1) deposition of silver ions in a very alkaline solution; (2) deposition of silver ions in a slightly acidic solution. In the context used here, deposition is defined as the binding of silver cations with negatively charged moieties within the protein.

After electrophoresis, all techniques first employ extensive washing of the gel in various concentrations of either methanol or ethanol to remove SDS, glycine, Tris, etc. In the alkaline process, the preparation of the silver staining solution can best be described as arduous. A dilute solution of silver nitrate is added dropwise to a previously prepared solution containing ammonium and sodium hydroxide. Vigorous vortexing is required to maintain the solubility of silver oxide, which readily forms a brown precipitate. Equations 1 and 2 describe the chemical reactions involved in the formation of the colorless diammine silver ion (ammoniacal silver ion) which is the cation that is responsible for the disposition
of silver onto the protein.

\[ 2\text{Ag}^+ + 2\text{OH}^- = 2\text{Ag}_2\text{O(s)} + \text{H}_2\text{O} \]  

\[ \text{Ag}_2\text{O(s)} + 4\text{NH}_3 + \text{H}_2\text{O} = 2\text{Ag(NH}_3)_2^+ + 2\text{OH}^- \]  

The ammoniacal silver mixture can be explosive if the solution is allowed to evaporate.

After allowing the gel to sit in the staining solution, the gel is washed, developed with a solution of dilute citrate and formaldehyde, and stopped by lowering the pH. The mechanism of the alkaline procedure probably involves the association of ammoniacal silver ions with negatively charged moieties within the protein and the subsequent reduction of the silver cation to the colloidal form of metallic silver by the concurrent oxidation of formaldehyde.

\[ \text{HCHO} + 2\text{Ag(NH}_3)_2^+ + 3\text{OH}^- = \text{HCOO}^- + 2\text{Ag}^0 + 4\text{NH}_3 + 2\text{H}_2\text{O} \]  

The reaction is analogous to that of Tollens' reagent and, under alkaline conditions, the oxidation of formaldehyde provides electrons for the reduction of the silver ion. Citrate, an antioxidant, is required to avoid surface deposition of "black dust" and probably attenuates the rate of formaldehyde oxidation, thus slowing the rate of image development produced by the reduction of silver ions.

The acidic method involves fixing the protein with gluteraldehyde, extensive washing, reduction of protein side chains with dithiothreitol, and soaking the gel in 0.1% silver nitrate, developing with dilute formaldehyde in 3% sodium carbonate, and stopping by lowering the pH.

Figure 19 displays a 0.4 N H$_2$SO$_4$ extract that has been electrophoresed in two separate two-dimensional gels. Figure 19A has been
Figure 19. Comparison of Coomassie and the silver stain of Guevara et al. (1982).

A 0.4 N H$_2$SO$_4$ extract was electrophoresed in two separate two-dimensional gels.

(A) Coomassie Brilliant Blue R stained.
(B) Alkaline silver stain of Guevara et al. (1982).
stained with Coomassie Brilliant Blue R and Figure 19B has been stained with the alkaline procedure described by Guevara et al. (1982). Both A13 and A63 failed to stain with this procedure. This procedure is probably the most sensitive technique available. The alkaline procedure of Wray et al. (1981), which is the easiest and fastest technique, also failed to stain A13, A63, and IgG light chain (see Figure 6A).

Figure 20 shows a 2% PCA extract that has been fractionated on two separate two-dimensional gels. Figure 20A has been stained with Coomassie Brilliant Blue R and Figure 20B has been stained with the acidic silver process described by Morrissey (1981). Again, little or no detectable staining of A13 or A63 was observed.

Figure 21A shows proteins solubilized by micrococcal nuclease in the S2 fraction. This Coomassie Brilliant Blue R stained gel clearly shows A13 and A63. This same gel was then placed in 88% formic acid overnight, which resulted in the complete removal of the dye from the gel and from the proteins, as judged by visual inspection. After extensive washing with water, the gel was restained by the alkaline protocol of Wray et al. (1981). It was found that after this treatment A13 was readily stained. However, A63 negatively stained, as indicated by the halo.

These experiments demonstrate a major difficulty with the use of silver staining for the quantitative or even qualitative detection of individual proteins. Clearly, certain proteins stain very poorly or not at all with most of the recipes. Unfortunately, both A63 and A13 fall into this category although they do sometimes weakly stain with the method of Wray et al. (1981). It is doubtful that these findings
Figure 20. Comparison of Coomassie and the silver stain of Morrissey (1981).

A 2% PCA extract was electrophoresed in two separate two-dimensional gels.

(A) Coomassie Brilliant Blue R stained.
(B) Acidic silver stain of Morrissey (1981).
Figure 21. Effect of pretreating a gel with Coomassie and formic acid prior to silver staining.

(A) Proteins solubilized by micrococcal nuclease were run on a two-dimensional gel and stained with Coomassie.

(B) After photography, the gel was soaked with formic acid, washed extensively with water, and then stained with the alkaline procedure of Wray et al. (1981).
are a direct result of my inability to properly utilize the various protocols, for other laboratories have experienced difficulty in silver staining protein D1 (A63) (Dr. Candido Rodriguez-Alfageme and Dr. Leonard H. Cohen, personal communication, The Institute for Cancer Research, Fox Chase Cancer Center, Philadelphia, PA). These observations raise a major obstacle to the continued analysis of the proteins present in various nucleosomal subfractions. Gel electrophoretic separation of nucleosomes has far greater resolution than sucrose gradient separation but is difficult to scale up to provide the quantity of protein needed for Coomassie Brilliant Blue R staining of two dimensional gels. Resolution of this problem will be necessary before further analysis of the nucleosomal distribution of the acid-soluble nonhistone proteins of Drosophila will be possible.
DISCUSSION

*Drosophila melanogaster* contains $1.4 \times 10^8$ bp of DNA in its haploid genome, which is distributed among 4 chromosomes. During interphase, two levels of euchromatic organization are now widely accepted to exist. The lowest level is the 10 nm filament which is readily visualized in the electron microscope. The next higher level of eucaryotic nuclear organization appears to be best described by a winding of the 10 nm filament into a shallow supercoiled "solenoid" (Finch and Klug, 1976) with a pitch of 11 nm, a diameter of 30 nm and with 6 nucleosomes per solenoid turn. This 30 nm solenoid appears to be identical with the thick chromatin fiber seen in nuclei with the electron microscope. It is the configuration of the bulk of eucaryotic chromatin *in vivo* (Davies et al., 1974; Langmore and Schutt, 1980).

There is a difference in chromatin structure between transcriptionally competent and inactive genes. This is demonstrated by the preferential DNAase I sensitivity of transcriptionally competent sequences as originally demonstrated by Weintraub and Groudine (1976) and subsequently by other investigators. The different structures may be a consequence of one of five levels: (1) DNA nucleotide sequences; (2) modified DNA bases; (3) the interaction with small RNA molecules; (4) modified histones; (5) the absence, presence, or modification of non-histone chromosomal proteins. The same DNA sequences are found in all cell types, but genes containing these sequences are only active in specific tissues or cell types. Since the sequence does not change, this possibility may be ruled out. Modification of bases is widespread throughout the animal kingdom in the form of 5'-methyl cytosine (5-mC).
As a general rule, hypermethylated DNA is correlated with gene inactivation. However, *Drosophila* cells do not contain 5-mC at levels detectable by chemical methods, but there could be an exceedingly low amount of 5-mC in highly strategic positions. Furthermore, if the role of 5-mC is to promote structural alterations of DNA, e.g. by facilitating the transition from the B to the A or to the Z conformation, perhaps the presence of certain sequences alternating frequently in purine and pyrimidine bases might render the occurrence of 5-mC in large amounts unnecessary. Other, not yet identified modified bases, could play an important role in *Drosophila* DNA. Equally plausible is that *Drosophila* gene regulation may be different from that of other higher eucaryotes and may not require modified bases at all. The role of small RNA molecules is currently not known. That leaves nuclear proteins with the probable major role in the maintenance of the structure of transcriptionally competent sequences.

The evidence presented by Weintraub and his colleagues makes it difficult to escape the conclusion that HMG proteins play a major role in either the maintenance or regulation of gene expression in chick red blood cell nuclei. Because of the extensive use of *D. melanogaster* as an experimental organism for the study of eucaryotic gene expression, it is of major importance to ascertain whether or not this organism has nuclear proteins analogous to the vertebrate HMG proteins. The present study clearly shows that there are two and only two major nuclear proteins to be found in *Drosophila* embryonic nuclei with physical properties similar to those of HMG proteins found in higher organisms.
A63 (D1) is unusual because it is over twice the size of HMGs 1 and 2, the larger of the mammalian HMG proteins. The amino acid composition of this protein is rather typical of HMG proteins, with the acidic and basic residues each constituting about 20% of the total. A63 is known to be associated with micrococcal nuclease prepared polynucleosomes fractionated on sucrose gradients (Bassuk, 1980). A63 (D1) is most likely to be specifically bound to the 1.688 satellite chromatin in vivo as part of the nucleosomal structure of this satellite (Levinger and Varshavsky, 1982; Hsieh and Brutlag, 1979; Rodriguez-Alfageme et al., 1980).

One function of A63 (D1) may be to form the highly compact structure of the A-T rich satellite heterochromatin. A63 (D1) may be the first example of a sequence recognizing protein responsible for the highly compact state of tandemly repetitive heterochromatic regions in eucaryotic chromosomes.

A13, though clearly a protein of the HMG type, is somewhat less basic than most recognized HMG proteins. It is extracted from nuclei by both 2 and 20% PCA and also by 0.4 N H₂SO₄. It is rapidly released from nuclei by micrococcal nuclease, which implies an interaction with DNA sequences or proteins residing in the linker region which connect nucleosomes together. A13 cannot be completely released from nuclei by this nuclease, however, since even at very high digestion levels, the protein is detected in the S2 nucleosomal fractions. This observation suggests that there are at least two classes of A13 distributed throughout the nucleus. The general distribution of A13 throughout chromatin is substantiated by the immunofluorescent staining of
polytene chromosomes with antibodies prepared against purified Al3. The staining pattern indicates that Al3 is not in any simple manner associated with active chromatin, for anti-Al3 does not specifically react with puffs, nor is the protein preferentially associated with heterochromatin or nucleoli. The reactivity of the anti-serum indicates a more general distribution along the chromosomal fiber, but the strong reaction at 60°C of chromosomes 2R complicates the interpretation of the data. It is possible that the immunogen used to inject rabbits may have contained a contaminating antigenic determinant. The determination of the exact specificity of the anti-Al3 serum will probably be pursued in the future.

When polynucleosomes, prepared by light digestion of nuclei with micrococcal nuclease, are fractionated on preparative agarose gels, H1 is detected only in oligonucleosomal fractions. At high digestion levels, H1 is absent from mononucleosomes prepared by micrococcal nuclease; it is in these fractions that Al3 is most prevalent. It is therefore reasonable to suppose that Al3 and H1 are distributed in different "chromosomal domains". As micrococcal nuclease digests DNA, there is most likely a structural breakdown of chromatin organization and as a consequence H1-containing chromatin may aggregate and become insoluble in 5 mM EDTA. The only fragments that remain in solution are those which are free of H1. Some of these also contain Al3. It is possible that Al3 is located at strategic positions between H1 binding sites. Since H1 has been widely implicated in the conversion from the 10 nm filament to the 30 nm solenoid (for a review, see
Igo-Kemenes et al., 1982), it is possible that A13 serves in a manner where the 30 nm solenoid is converted to the 10 nm filament.

The data presented suggest that A13 does not play a role analogous to vertebrate HMGs 14 or 17; instead, the properties of A13 more closely resemble those possessed by HMGs 1 and 2. HMGs 1 and 2 are known to be rapidly released from rat nuclei by mild micrococcal nuclease digestion. They also partially associate with a population of monomeric nucleosomes depleted of H1 and containing a heterogenous distribution of DNA fragments (Seyedin and Kistler, 1979).

It is known that approximately one in two nucleosomes of the transcribed Drosophila copia and hsp 70 genes contain uH2A instead of H2A; whereas, less than one in 25 nucleosomes of the 1.688 satellite DNA contains uH2A (Levinger and Varshavsky, 1982). These results indicate that uH2A resides preferentially in transcribed chromosomal regions. Figure 21B displays a two-dimensional gel which had been previously been stained with Coomassie, (Figure 21A), cleared with formic acid, and then silver stained. The Coomassie gel is the identical electrophoretogram shown in Figure 15D. Both putative uH2A and uH2B are detected at this 24% digestion level, as well as the 16% level of Figure 15C. These gels indicate that uH2A, uH2B, and A13 are present in both H1-containing and H1-depleted chromosomal fragments. High resolution models of nucleosome structure strongly suggest that the ubiquitin moiety of uH2A is located on the surface of the octameric histone "disk (Klug et al., 1980). Since nucleosome-nucleosome interactions probably play a crucial role in higher order packaging, it
is likely that the presence of ubiquitin, or possibly A13, on either side of the nucleosome could weaken interactions of this nucleosome with its neighbors.
LITERATURE CITED


USA 77:127-131.

DNA in Drosophila melanogaster. Pages 35-46 in W. J. Peacock and
R. D. Brock, eds. The eucaryote chromosome. Australian National
University Press, Canberra.

Candido, E. P. M., R. Reeves, and J. R. Davie. 1978. Sodium butyrate


Chen, C. C., D. L. Smith, B. B. Bruegger, R. M. Halpern, R. M. Smith,
acid-labile histone phosphatases in Walker-256 carcinosarcome cell-

scope observations on the organization of the nucleus in chicken
erythrocytes and a superunit thread hypothesis for chromatin structure.

Calf and pea histone IV. III. Complete amino acid sequence of pea
seedling histone IV: Comparison with the homologous calf thymus

Dixon, G. H., E. P. M. Candido, B. M. Honda, A. J. Louie, A. R. Macleod,
and M. T. Snug. 1975. Histone acetylation. Pages 229-258 in The
structure and function of chromatin, CIBA Foundation Symposia 28.
Elsevier-Excerpta, Amsterdam, North Holland.


fluorescent studies of Drosophila chromosomal proteins. Cold Spring


Ellison, J. R., and H. J. Barr. 1972. Quinacrine fluorescence of


fluorescent techniques in the analysis of chromosomal proteins.
Pages 151-160 in G. Stein, J. Stein, and J. Kleinsmith, eds.

Simpson, R. T. 1978a. Structure of the chromatosome, a chromatin
particle containing 160 base pairs of DNA and all the histones.
Biochemistry 17:5524-5531.

Simpson, R. T. 1978b. Structure of chromatin containing extensively

from intact nuclei alters the digestion of nucleosome core DNA by

ribose) covalently bound to histone Fl in vivo. Biochem. Biophys.
Res. Commun. 54:297-300.

Smith, D. L., C. C. Chen, B. B. Bruegger, S. L. Holtz, R. M. Halpern,
acid-labile histone phosphates in Walker 256 carcinosarcoma cell

digestion of nuclei and chromatin by staphylococcal nuclease.
Biochemistry 14:2915-2920.

Spiker, S. 1980. A modification of the acetic acid-urea system for
use in microslab polyacrylamide gel electrophoresis. Analytical

Spiker, S., J. L. Key, and B. Wakim. 1976. Identification and
fractionation of plant histones. Arch. Biochem. Biophys. 176:
510-518.

90:391-395.

Sterner, R., G. Vidali, and V. G. Allfrey. 1979. Studies of
acetylation and deacetylation in high mobility group proteins-
identification of the sites of acetylation in HMG-1. J. Biol.


ACKNOWLEDGEMENTS

This dissertation is dedicated to the late John B. Balinsky, Professor and Chairman of the Department of Zoology, Iowa State University. The author is indebted to John for financial support during the author's graduate study and for helping out with supplies when grant funds ran low. The author extends his sympathy to John's family.