1

CANADIAN THESES ON MICROFICHE

I.S.B.N.

THÈSES CANADIENNES SUR MICROFICHE

National Library of Canada
Collections Development Branch

Canadian Theses on
Microfiche Service

Ottawa, Canada
K1A 0N4

Bibliothèque nationale du Canada
Direction du développement des collections

Service des thèses canadiennes
sur microfiche

NOTICE

The quality of this microfiche is heavily dependent upon the quality of the original thesis submitted for microfilming. Every effort has been made to ensure the highest quality of reproduction possible.

If pages are missing, contact the university which granted the degree.

Some pages may have indistinct print especially if the original pages were typed with a poor typewriter ribbon or if the university sent us a poor photocopy.

Previously copyrighted materials (journal articles, published tests, etc.) are not filmed.

Reproduction in full or in part of this film is governed by the Canadian Copyright Act, R.S.C. 1970, c. C-30. Please read the authorization forms which accompany this thesis.

THIS DISSERTATION
HAS BEEN MICROFILMED
EXACTLY AS RECEIVED

AVIS

La qualité de cette microfiche dépend grandement de la qualité de la thèse soumise au microfilmage. Nous avons tout fait pour assurer une qualité supérieure de reproduction.

S'il manque des pages, veuillez communiquer avec l'université qui a conféré le grade.

La qualité d'impression de certaines pages peut laisser à désirer, surtout si les pages originales ont été dactylographiées à l'aide d'un ruban usé ou si l'université nous a fait parvenir une photocopie de mauvaise qualité.

Les documents qui font déjà l'objet d'un droit d'auteur (articles de revue, examens publiés, etc.) ne sont pas microfilmés.

La reproduction, même partielle, de ce microfilm est soumise à la Loi canadienne sur le droit d'auteur, SRC 1970, c. C-30. Veuillez prendre connaissance des formules d'autorisation qui accompagnent cette thèse.

LA THÈSE A ÉTÉ
MICROFILMÉE TELLE QUE
NOUS L'AVONS REÇUE

146

0-315-12114-9

National Library
of CanadaBibliothèque nationale
du Canada

Canadian Theses Division

Division des thèses canadiennes

Ottawa, Canada
K1A 0N4

60027

PERMISSION TO MICROFILM — AUTORISATION DE MICROFILMER

• Please print or type — Écrire en lettres moulées ou dactylographier

Full Name of Author — Nom complet de l'auteur

ROBERT GRAY RUTLEDGE

Date of Birth — Date de naissance

SEPT 5, 1955

Country of Birth — Lieu de naissance

CANADA

Permanent Address — Résidence fixe

180 LEES AVE OTTAWA, ONT.

Title of Thesis — Titre de la thèse

HISTONE SYNTHESIS DURING ERYTHROPOIESIS
IN XENOPUS LAEVIS

University — Université

CARLETON UNIVERSITY

Degree for which thesis was presented — Grade pour lequel cette thèse fut présentée

MASTER OF SCIENCE

Year this degree conferred — Année d'obtention de ce grade

1982

Name of Supervisor — Nom du directeur de thèse

J.M. NEELIN

Permission is hereby granted to the NATIONAL LIBRARY OF
CANADA to microfilm this thesis and to lend or sell copies of
the film.The author reserves other publication rights, and neither the
thesis nor extensive extracts from it may be printed or other-
wise reproduced without the author's written permission.L'autorisation est, par la présente, accordée à la BIBLIOTHÈ-
QUE NATIONALE DU CANADA de microfilmer cette thèse et de
prêter ou de vendre des exemplaires du film.L'auteur se réserve les autres droits de publication, ni la thèse
ni de longs extraits de celle-ci ne doivent être imprimés ou
autrement reproduits sans l'autorisation écrite de l'auteur.

Date

May 25, 1982

Signature

HISTONE SYNTHESIS DURING ERYTHROPOIESIS IN XENOPUS LAEVIS

BY

© ROBERT GRAY RUTLEDGE, B.SC. (HONS.)

A thesis submitted to the Faculty of
Graduate Studies in partial fulfillment
of the requirements for the degree of
Master of Science

Biology Department
Carleton University
Ottawa, Ontario
May, 1982

The undersigned hereby recommend to the Faculty of
Graduate Studies acceptance of this thesis, submitted by
Robert Gray Rutledge, B.Sc. (Hons.), in partial
fulfillment of the requirements for the degree of
Master of Science.

.....*Gray Herrian*.....
Chairman, Department of Biology

.....*James T. Reelin*.....
Supervisor

.....*V. L. Selig*.....
Co-supervisor

ABSTRACT

An H1^O-like histone H1^S has been reported in erythrocytes of Xenopus laevis and other amphibians. In order to relate the metabolism of H1^S to cell maturity, the rates of protein and DNA synthesis were followed in vitro in erythroid cells from X. laevis recovering from severe anemia, by incorporation of ¹⁴C-lysine and ³H-thymidine. Although cessation of DNA replication in the mid-stages of erythroid maturation was accompanied by a loss of H1 and core histone synthesis, extensive H1^S synthesis was found to continue (as has been reported for cell-specific H5 during chicken erythropoiesis). Quantitation of lysine-rich histones also revealed a progressive increase in the amounts of H1^S relative to H1, similar to that reported for both avian H5 and mammalian H1^O. The distinctive characteristics shared by these three lysine-rich histones suggest a functional class, called herein satellite H1 or H1^S, which may play a role in restricting template activity of DNA during establishment of G₀.

To our new born child.

For without, this work would have no purpose.

ACKNOWLEDGEMENTS

For their invaluable advice and unfailing support I
am indebted to Dr. V. Seligy and Dr. J.M. Neelin.

TABLE OF CONTENTS

Title	i
Abstract	iii
Acknowledgements	iv
Table of Contents	v
List of Tables	vii
List of Figures	viii
List of Abbreviations	xi
I. INTRODUCTION	1
A. Chromatin Structure and the Control of Gene Expression	1
B. Lysine-rich Histones	6
C. H1 ^S Quantitation Studies	14
D. H1 ^O Accumulation	18
E. H5 Accumulation	22
F. Experimental Approach	24
II. Materials and Methods	28
A. Induction of Anemia	28
B. Incorporation of Radioactive Precursors	29
C. Measurement of Isotope Incorporation	31
D. Autoradiography of Blood Smears	32
E. Isolation and Metabolism of Nuclei and Histones	32
F. Polyacrylamide Gel Electrophoresis	34
G. Fluorography of Polyacrylamide Gels	36
H. Quantitation and Specific Radioactivity of	

Histones	36
III. Results	38
A. Induction of and Recovery from Aemia	38
Anemia	38
B. In Vitro Incubation of Blood Cells	47
C. Incorporation Rate Determinations	55
D. Electrophoretic Characterization	68
E. Histone Synthesis	71
F. Fluorography of ¹⁴ C-lysine Labelled Histones	76
G. Quantitation of Lysine-rich Histone	92
IV. Discussion	97
A. Erythropoiesis in <u>Xenopus laevis</u>	97
i) Metabolic Changes	97
ii) Histone Synthesis	99
B. Are H1 ⁰ and H5 Functionally Related Histones?	101
C. Characterization of Satellite H1	103
D. H1 ^S Function	107
i) Is H1 ^S a DNA Replication Inhibitor?	107
ii) H1 ^S in Chromatin	109
iii) The Mechanism and Potential Effects of H1 ^S Accumulation	111
iv) Control of H1 ^S Accumulation	119
D. Future Considerations	123
i) Tissue Specificity of the H1 ^S Histones ...	123
ii) Are the HMG Proteins Functionally Related Lysine-rich Histones?	126
V. References	133

LIST OF TABLES

Table 1. Differential Blood Cell Counts of Maturing Erythroid Populations	46
Table 2. Distribution of ^{14}C -lysine Among Cell and Protein Fractions from Regeneration Series I	51
Table 3. Distribution of ^{14}C -lysine Among Cell and Protein Fractions From Regeneration Series II	52
- Table 4. Distribution of ^3H -Thymidine Among Cell and Protein Fractions From Regeneration Series II'	53
Table 5. Distribution of ^{14}C -lysine in Lysine-rich Histones Separated Electrophoretically in Acid-urea	96

LIST OF FIGURES

- Figure 1. Blood Hematocrits of Phenylhydrazine
Treated Xenopus laevis at Various
Recovery Times 39
- Figure 2. Photomicrographs of Differentially
Stained Blood Smears of Anemic Xenopus
laevis at Various Recovery Times 42
- Figure 3. Photomicrographs of Cresyl Blue stained
Blood Smears of Anemic Xenopus laevis at
Various Recovery Times 48
- Figure 4. Incorporation of ^{14}C -lysine into
TCA-insoluble Material From Regeneration
Regeneration Series I 56
- Figure 5. Incorporation of ^{14}C -lysine into
TCA-insoluble Material from Regeneration
Series II 58
- Figure 6. Incorporation of ^3H -thymidine into
TCA-insoluble Material From Regeneration
Series II 60
- Figure 7. Photomicrographs of Cell Autoradiograms
of ^3H -thymidine-labelled Erythroid Cells 63

Figure 8. Thymidine Incorporation Rate and Hemoglobin Concentration Versus Lysine Incorporation Rate	66
Figure 9. SDS Polyacrylamide Gel Electrophoresis of Xenopus Histones From Unincubated Erythrocytes	69
Figure 10. ^{14}C -lysine Incorporation into Total Histone Versus Lysine Incorporation Rate	72
Figure 11. Ratio of Lysine-rich to Core Histone Synthesis Versus Lysine Incorporation Rate	74
Figure 12. SDS Gel Electrophoresis and Fluorography of Lysine-rich Histone From Regeneration Series I	78
Figure 13. Acid-urea Gel Electrophoresis and Fluorography of Lysine-rich Histone From Regeneration Series I	80
Figure 14. SDS Gel Electrophoresis and Fluorography of Core Histone From Regeneration Series I	83
Figure 15. Acid-urea Gel Electrophoresis and Fluorography of Core Histone From Regeneration Series I	85

Figure 16. SDS Gel Electrophoresis and Fluorography of Total Histone From Regeneration Series II	88
Figure 17. Acid-urea Gel Electrophoresis of Total Histone From Regeneration Series II	90
Figure 18. Changes in the relative amounts of H1 ^S During Erythroid Maturation	94

LIST OF ABBREVIATIONS

CPM	counts per minute
DMSO	dimethylsulfoxide
HMG	high mobility group
H1	histone 1
H1 ^S	satellite H1
H5	histone 5
H2A	histone 2A
H2B	histone 2B
H3	histone 3
H4	histone 4
MEM	Minimal Essential Medium
NSM	nuclear suspension medium
PCA	perchloric acid
PMSF	phenylmethanesulfonyl fluoride
MS222	tricainemethanesulfonate
sp. act.	specific activity
TCA	trichloroacetic acid
tHCl	total histones extracted with HCl

I. INTRODUCTION

A. Chromatin Structure and the Control of Gene Expression

A major goal of molecular biology today is characterization of the processes responsible for development and maintenance of cell specialization, which must have been central to the evolution of multicellular organisms. What then are the strategies employed by the eukaryotic cell to effect cell specialization? Early research into eukaryotic genetics revealed that each cell within an organism, irrespective of its phenotype, has within its nucleus a complete replica of all genetic information possessed by that organism. Combined with other biochemical evidence, this has led to a generally held concept that cell specialization stems from differential expression of a small but select group of genes that code for cellular functions specific to each cell type. Thus it appears that the key to understanding the processes of eukaryotic cell specialization lies within elucidation of the mechanisms involved in control of gene transcription, from which the ability for selective gene expression has evolved.

Although a general mechanism for control of eukaryotic gene expression has not been established, research into chromatin function over the past decade has produced information from which an understanding of the

molecular basis for gene regulation is slowly emerging. This research has revealed that DNA of the nucleus is complexed with highly basic proteins called histones (for review see Hnilica 1967, Elgin and Weintraub 1975, Isenberg 1979), which supercoil DNA to form the structural basis of chromatin (for review see Kornberg 1977, Felsenfeld 1978). Experimentation has shown that chromatin structure likely plays an important role in the transcriptional activity of DNA, in that transcriptionally active or potentially active genes possess a structural organization different from that of repressed genes. Electron microscopic examination and nuclease probing of chromatin structure has revealed that transcriptionally active genes generally have a "disperse" conformation (Weintraub and Groudine 1976, Bloom and Anderson 1979, Mathis et al. 1980) in contrast to repressed genes which possess a highly condensed tertiary conformation.

This experimental evidence has led to the proposal that control of gene expression is achieved in part through regulation of chromatin structure (Alberts et al. 1977). This hypothesis provides for possible mechanisms for both accurate inheritance and modification in the patterns of genetic expression, which are believed necessary for differentiation and development of specialized cells within multicellular eukaryotic organisms (Holtzer et al. 1975). Differentiation is

3

proposed to be effected partly through "generation and propagation of variegated chromatin structures" (Weintraub et al. 1977) or "chromatin remodelling" (Newrock et al. 1977), in which chromatin structure is modulated in a programmed sequence. These changes in chromatin structure are supposed to be initiated by incorporation of "modified" chromosomal proteins, particularly histone, into chromatin during cell differentiation.

To test these hypotheses, it will be important to elucidate: 1) the molecular architecture of chromatin, 2) the mechanism of modulating chromatin structure, and 3) the precise biological role or roles of chromatin structure in dictating gene activity.

A breakthrough in understanding the molecular basis of chromatin structure was initiated by electron microscopic and nuclease investigations. These studies revealed that chromatin possesses a basic repeating unit, produced by periodical supercoiling of DNA around a histone core particle (for review see Elgin and Weintraub 1975, Kornberg 1977, Felsenfeld 1978). This repeating unit, called the nucleosome, consists of two regions or domains, the nucleosome core and the nucleosome linker (McGhee and Felsenfeld 1980). The nucleosome core is composed of 146 bp of DNA supercoiled around the outside of a histone octamer, comprised of two each of the 4 core histones H2A, H2B, H3, and H4 (Isenberg 1979). Periodical

supercoiling of DNA around these histone cores produces a primary chromatin fibre seen in the electron microscope as "beads on a string", in which the repeating nucleosome structure was first visualized. The nucleosome linker or linker DNA is comprised of the DNA connecting one nucleosome core to the next and is the site of association for the fifth class of histone, the lysine-rich or linker histones (Allan et al. 1980). Lysine-rich histones participate in the supercoiling of linker DNA between adjacent core particles during folding of the primary chromatin fibre into higher order structures.

Since discovery of the nucleosome, many models for higher order structures of chromatin have been put forward (Finch and Klug 1976, Weintraub et al. 1976, Worcel and Benyajati 1977, Bak et al. 1977, Worcel 1977, Olins 1977, Thoma et al. 1979, McGhee et al. 1980). The most recent models have stemmed from studies employing electron microscopic examination of fragmented chromatin in solutions of varying ionic strengths (Thoma et al. 1979, McGhee et al. 1980). It is thought that ionic charges within DNA become progressively neutralized with increased ionic strength, which induces condensation of chromatin into structures believed to possess a conformation similar to that of the tertiary chromatin fibre in vivo. At very low ionic strength, chromatin fragments take on a dispersed conformation recognized as a relaxed filament of

nucleosomes. Thoma et al. (1979) and Moyne et al. (1981) observed that DNA within these filaments enters and leaves the nucleosome cores at approximately the same location, producing a "zigzag" positioning of the nucleosome cores. This fibre conformation is ~~proposed~~ proposed by Thoma et al. (1979) and Allan et al. (1980) to be a result of lysine-rich histone binding at or close to the site of DNA entrance to the core, supposedly "locking" the supercoiled DNA around the histone cores. Increased ionic strength is proposed to induce the relaxed nucleosome filament to twist (Worcel et al. 1981) or coil (Thoma et al. 1979) into a helical structure, reported to have a solenoid conformation of 25-30 nm in diameter, with 5-7 nucleosomes per turn. In such a structure, linker DNA is supercoiled in a manner very similar to that of core DNA (McGhee et al. 1980, Allan et al. 1981).

Although these models provide a detailed structural picture of chromatin, they suggest little as to the mechanism employed by the cell for modulating chromatin structure or of its role in control of gene expression. However, one central conclusion derived from these studies provides an important clue to the mechanism for modulating chromatin structure, that is that lysine-rich histones play a critical role in chromatin condensation. Specifically, Thoma et al. (1979) and Allan et al. (1980) demonstrate an absolute requirement for lysine-rich

histone in the formation of ordered tertiary chromatin structures; although lysine-rich histone depleted chromatin did condense with increasing ionic strength, these condensed structures were highly disorganized, forming irregular clumps or tangles. Recently Thoma and Koller (1981) have confirmed that in fact it is the lysine-rich histones and not nonhistone chromosomal proteins, that are responsible for directing the ordered condensation of chromatin with increased ionic strength. This was also confirmed by Allan et al. (1981), who not only demonstrated that ordered condensation of depleted chromatin could be achieved with increased ionic strength in the presence of purified lysine-rich histone, but that fibre condensation could be induced at low ionic strength by addition of progressively greater amounts of lysine-rich histone. This illustrates that lysine-rich histones not only direct, but can induce condensation of the chromatin fibre in vitro, suggesting that lysine-rich histones likely play an important role in initiating and coordinating condensation of chromatin in vivo. This is in part the basis for today's intensive research into lysine-rich histone function, from which this project stems.

B. Lysine-rich Histones

The lysine-rich histones are primarily characterized by their solubility in 5% PCA. Possessing a molecular

weight of 20,000-22,000 (Elgin and Weintraub 1975), they are a diverse but functionally related group of proteins (Hohmann 1978) showing interspecies variation in amino acid content; all are rich in lysine, alanine, and proline, with H5 also rich in serine and arginine (Hnilica 1967). Lysine-rich histones possess much greater sequence heterogeneity than core histones (Isenberg 1979) and are the most accessible of all histones, being the first removed by salt or acid and the most susceptible to proteolytic degradation. Unlike core histones, lysine-rich histones do not self-aggregate in solution and form homopolymers during treatment of chromatin with bi-functional cross-linking reagents (Nikolaev et al. 1981, Pospelov et al. 1981).

Primary sequencing of lysine-rich histones from a large variety of organisms has shown them to be a structurally related family of proteins (Isenberg 1979, Von Holt et al. 1979, Yaguchi et al. 1977, 1979). They possess three distinct domains, with basic lysine and arginine residues clustered in the amino and carboxy terminal domains, with the central domain rich in hydrophobic amino acids and possessing sequences typical of a globular protein. The N- and C-terminal domains show large sequence variations, in contrast to the central domain which contain long stretches of highly conserved sequences. It is sequence divergency of the terminal

domains that is believed to be a major factor in the evolution of H1 variants, first characterized during early studies of lysine-rich histones (for review see Hohmann 1978).

Studies of lysine-rich histone tertiary structure have produced results that correlate very well with the sequence data (Bradbury et al. 1975, Chapman et al. 1976, 1978, Hartman et al. 1977, Allan et al. 1980, Aviles et al. 1978, Crane-Robinson et al. 1980, Cary et al. 1981). These studies have shown that lysine-rich histones have a conformation in solution which consists of three structural regions, with only the central domain possessing a stable tertiary structure. The N- and C-terminal regions possess random structures, making them highly susceptible to proteolytic digestion, unlike the central globular region of about 80 residues (Cary et al. 1981) which is resistant to protease action. The central domain is thought to interact in a specific manner with the nucleosome core in vivo (Crane-Robinson et al. 1980, Cary et al. 1981), thus suggesting a possible explanation for the observed sequence conservation within this central domain. Function of the terminal regions is not clear, although Allan et al. (1980) have demonstrated that they play a critical role in initiating chromatin condensation. In addition, Bradbury et al. (1975) has shown the C-terminal domain to be the principal DNA binding region

of the molecule.

Research into lysine-rich histones has led to the supposition that they are comprised of a single class of proteins, called H1. Comparison of H1 histones has revealed them to consist of several primary structure variants (Hohmann 1978) with many studies attempting to elucidate their function. Kinkade (1969) has shown that the same H1 variants are present in each tissue within an animal, but that the relative quantities of each H1 variant is tissue-specific. In addition, developmental changes in the relative proportions of H1 variants within tissues have been demonstrated (Hohmann 1978, Ajiro et al. 1981, Hentschel and Birnstiel 1981), and species- and cell-specific expression of H1 variants has been characterized in several types of cultured cells (Hohmann 1980). This has been interpreted to suggest an important role of H1 variants in the diversity of chromatin structure, thought to be central to differential gene expression (Cole 1977). However, no definitive study into the biological function of H1 variants has been developed, and therefore their role in chromatin function is still largely speculative.

Two other types of lysine-rich histones, H5 and H1⁰, have also been characterized and are believed to be highly divergent variants of H1 (Yaguchi et al. 1977, 1979, Panyim and Chalkley 1969a). Histone H5 was originally isolated

from chicken erythrocytes (Neelin et al. 1964) and proposed to be specific to nucleated erythrocytes (for review see Hnilica 1967). Although its function is unknown, H5 is thought to be involved in the progressive nuclear condensation during erythropoiesis, which is related to the transcriptional inactivity of erythrocyte chromatin (Seligy and Neelin 1970, Billet and Hindley 1972, Medvedev and Kirpicheva 1972, Seligy and Miyagi 1974, Tobin and Seligy 1975, Sung 1977, Gasaryan 1978). Despite tremendous advances in elucidation of chromatin structure and function over the last decade, a definitive understanding of H5 function has not been achieved.

H1^o was first characterized by Panyim and Chalkley (1969a), who found it in greatest relative amounts in mammalian tissues possessing low levels of cell replication. Sequencing of the central domain of bovine H1^o has revealed at least 70% homology with chicken erythrocyte H5, as compared to 42% homology between H1^o and H1 (Smith et al. 1980, Pehrson and Cole 1981). Conformational studies of H1^o have also revealed structural similarity with chicken erythrocyte H5 (Cary et al. 1981), which also revealed that H1^o and H5 possess structural characteristics distinct from H1. Structural homology is further illustrated by the immunological homology of H1^o and H5 (Mura and Stoller 1981). All of these studies suggests that H1^o likely possesses a

function closely related to chicken erythrocyte H5.

Reports of an avian erythrocyte-specific H5 prompted investigation into lysine-rich histones of other nucleated erythrocytes. This subsequently resulted in reports of erythrocyte-specific lysine-rich histones in turtle (Tsai and Hnilica 1975), the bullfrog Rana catesbeiana (Nelson and Yunis 1969), the South African clawed toad Xenopus laevis (Destree et al. 1979) and several species of fish (Miki 1976, Miki and Neelin 1975, 1977a,b). However, conflicting reports indicated that turtle and bullfrog possess no erythrocyte-specific histone (Panyim et al. 1971, Alder and Gorovsky 1975). This prompted reevaluation of the existence of an erythrocyte-specific histone in the red-eared turtle (Rutledge et al. 1981), and the frogs, Rana catesbeiana, Rana pipiens, and Xenopus laevis (Brown et al. 1981). These studies revealed that the supposed H5 histones were not erythrocyte-specific in these animals, a fact that has been confirmed for Xenopus laevis (Risley and Eckhardt 1981). In addition, the supposed H5 histone of turtle and frog possessed little resemblance to avian H5, although its electrophoretic and chromatographic characteristics differed distinctly from H1. This lysine-rich histone of turtles and frogs did however closely resemble the mammalian histone H1⁰ in amino acid composition as well as in their electrophoretic and chromatographic characteristics. The structural

homology of Rana catesbeiana erythrocyte "H5" with mammalian H1⁰ has been further demonstrated by Shimada et al. (1981) using peptide mapping. Demonstration of an H1⁰-like histone in the erythrocytes and liver of turtles and frogs, combined with the sequence homology of H1⁰ with chicken H5 (Smith et al. 1980, Pehrson and Cole 1981), suggests that the functional role of H5 may not be restricted to nucleated erythrocytes but could be shared with other higher eukaryotic cells. This has led to the proposal of a new, functionally distinct lysine-rich histone sub-class, which includes turtle and frog H1^S, H1b or H5, avian and fish H5, and mammalian H1⁰.

This lysine-rich histone sub-class is called H1^S to stand for "satellite H1", signifying their distinct electrophoretic mobility in SDS and acid-urea relative to H1 (Rutledge et al. 1981, Brown et al. 1981). H1 satellites share many characteristics with H1, including species-specific differences in primary structure.

Varricchio (1977) observed two electrophoretic bands of rat pancreas H1⁰. Medvedev and Medvedeva (1980b) observed at least two H1⁰ variants in electrophoretic profiles of rat liver histones. Smith and Johns (1980a) identified three H1⁰ variants by ion-exchange chromatography of bovine liver lysine-rich histones, each having very similar amino acid compositions and peptide maps. Pehrson and Cole (1981) also saw primary sequence

microheterogeneity during sequencing of bovine liver H1⁰. Rutledge et al. (1981) and Brown et al. (1981) observed doublets during electrophoresis of liver H1^S from turtle and toad respectively. Risley and Eckhardt (1981) not only confirmed the presence of two H1^S variants in Xenopus laevis employing 2-D electrophoresis, but also clearly demonstrated the presence of H1^S doublets in several tissues other than liver. Finally, D'Anna et al. (1981) reported isolation and characterization of two H1⁰ variants from Chinese hamster ovary cells. Amino acid analysis of these H1⁰ variants indicated they are very closely related histones. Interestingly, D'Anna et al. (1981) observed that cleavage of the two H1⁰ variants with cyanogen bromide produced a large fragment from each variant which was homogeneous and indistinguishable electrophoretically. From this it was suggested that the structural differences between the two H1⁰ variants were located in the small N-terminal fragment produced during cyanogen bromide cleavage. A similar conclusion has been drawn for two H1 variants of calf thymus by Nikolaev et al. (1981) after chymotryptic digestion. Cary et al. (1981) conclude that the differences in tertiary structure of H1, H1⁰ and H5 are due primarily to differences in the terminal regions of these histones. In conclusion, these studies illustrate that each type of satellite H1, like H1, is composed of several closely related variants, with

differences in structure related to differences in sequence of the terminal domains.

The main support presented so far for the existence of satellite H1 as a functionally homologous sub-class of lysine-rich histone has come from physical characterization of these proteins. Although this approach has proven valuable in development of the satellite H1 concept, it does not provide conclusive evidence for functional homology between these histone types, nor does it allow evaluation of satellite H1 as a functionally distinct form of lysine-rich histone. The rest of this introduction has therefore been devoted to an examination of the functional characteristics of H1 satellites, in order to assess their functional homology and their relationship to H1 histone function.

C. H1^S Quantitation Studies

Tissue-specific quantitative differences in satellite H1 content were first reported by Panyim and Chalkley (1969a,b) for calf, rat, and mouse H1^O, with the amount of H1^O relative to the other histones inversely correlated with the proliferative activity of each tissue. Tissues of low proliferation activity such as lung, liver, and brain were found to have high amounts of H1^O, whereas in tissues having high rates of proliferation such as thymus or tumour cells, H1^O was absent or in very low amounts. Several other investigators have reported similar results.

For example the relative amounts of Hl^O in rat tissues (Seyedin and Kistler 1979), including rat liver hepatomas (Lea et al. 1974), and mouse tissues (Smith and Johns 1980b), as well as Hl^S in Xenopus laevis tissues (Risley and Eckhardt 1981) have generally been found inversely correlated with the proliferative activity of the tissues from which they were isolated.

This inverse relationship with cell proliferation suggests that satellite Hl may function as an inhibitor of cell replication, but there are reports which contradict this relationship. Rutledge et al. (1981) observed approximately twice the relative amounts of Hl^S in turtle liver than in erythrocytes, and Brown et al. (1981) also found approximately twice the amounts of Hl^S in the liver of Xenopus laevis than in erythrocytes. Since erythrocytes possess a much lower proliferative activity than liver, the relative content of satellite Hl is not consistent with its putative function as an inhibitor of cell replication.

This lack of correlation between the content of satellite Hl and cell proliferation can be extended to erythrocyte H5, with Miki and Neelin (1977a, 1980) reporting large differences in the relative amounts of H5 from erythrocytes of various animal species. Chicken erythrocytes were found to possess the highest amounts of H5 of all animals studied, amounting to 21% of total

histone, with carp erythrocytes possessing only 6% of total histones as H5 and white sucker little or none. Differences in H5 content have also been reported in the primitive and definitive erythrocytes of chicken embryos. Moss et al. (1973) and Urban et al. (1980) report that primitive erythrocytes possess lower relative amounts of H5 (13% of total histone) than adult erythrocytes (21% of the total histones) with definitive erythrocytes possessing an intermediate H5 content (17% of total histone). These studies therefore demonstrate that the relative content of the supposed erythrocyte-specific H5 of avians and fish varies greatly between species, despite an absence of cell proliferation within these erythrocyte populations.

Thus it appears that a simple correlation of satellite H1 content with inhibition of cell replication is not be valid. In this context it is interesting to note that early studies into H5 lead to the proposal that H5 functions as a DNA transcriptional inhibitor and not as a replication inhibitor. Other proposals for the function of H1⁰ have also been presented. For example Medvedev and Medvedeva (1980a) studied the relative content of H1⁰ in spontaneous hepatomas in aging mice, in which they observe an increase in the amounts of H1⁰ with age. They suggest that the relative amounts of H1⁰ may be reflective of tissue age. Lea et al. (1974) suggest that H1⁰ may be

more closely related to the degree of differentiation of tissues rather than growth rate per se. Piha and Valkonen (1979) and Valkonen (1980) report appearance of liver H1^O during the bovine postnatal period, and suggest that this increase may reflect an age-related condensation of chromatin, associated with reduction of RNA synthesis and DNA template activity. The possibility that satellite H1 may play a presently uncharacterized role in tissue development is further supported by the results of Varricchio (1977) who reports a similar postnatal increase in H1^O in rat pancreas, and by Risley and Eckhardt (1981) who report the presence of Xenopus laevis H1^S only in adult tissues.

If the precise function of satellite H1 is not apparent, quantitation studies of H1 suggests that lysine-rich histones as a whole likely function in a more complex manner than is now presently recognized. This comes from demonstration that tissue-specific differences in histone content are not restricted to satellite H1. For example, Wright and Olins (1975) report that lysine-rich histone content relative to core histone varies considerably from tissue to tissue within several animal species, including mammalian, avian, reptile, amphibian, and fish. Unger-Ullmann and Modak (1979) also report differences in the total relative content of lysine-rich histone in study of chicken embryonic tissues.

Piha and Valkonen (1979) and Valkonen (1980) report that the proportion of H1 relative to core histone, nearly doubles in amount during bovine prenatal period and continues to increase throughout ontogeny of bovine liver.

In summary, differences in the amounts of both H1 and satellite H1 relative to core histones have been demonstrated for a variety of animal species and tissues. In view of the central role played by the lysine-rich histones in chromatin condensation, this variability has major implications in our present understanding of chromatin function.

D. $H1^O$ Accumulation

Several studies have reported marked increases in the relative amounts of $H1^O$ in cultured cells during changes in proliferation rates. Pehrson and Cole (1980) compared the $H1^O$ content of rapidly growing HeLa cells and mouse neuroblastoma cells to these cells whose growth had been inhibited by high cell density or serum deprivation. They observed an increase in the ratio of $H1^O$ to H1 from 0.08 to 0.30 for neuroblastoma cells and from 0.05 to 0.20 for HeLa cells and proposed that variation in $H1^O$ levels is associated with a functional change within the cell, possibly related to repression of chromatin activity.

That changes in the relative content of $H1^O$ is not an artifact produced by induced inhibition of cell growth is demonstrated by other studies involving in vitro

differentiation of cell cultures. During erythroid differentiation induced in mouse Friend virus-transformed erythroleukemia cells by DMSO or HMBA, Keppel et al. (1977) observed a progressive increase in content of a histone IP25, from less than 5% to 40% relative to H1. Although IP25 has not been isolated in pure form for characterization, it has been tentatively identified as an H1⁰ histone (Keppel et al. 1979, Gjerest et al. 1981). Zlatanova et al. (1980) report accumulation of H1⁰ from 10% to 30% of H1 during n-butyrate-induced differentiation of erythroleukemic mouse cells. D'Anna et al. (1980a) also observed an increase in content of an H1-like protein during treatment of CHO cells with sodium butyrate. This protein, called BEP (butyrate enhanced protein) and identified as an H1⁰ histone (D'Anna et al. 1980b, 1981), was found to increase progressively from 6% to greater than 32% of that of H1, as a function of butyrate concentration and time. Pieler et al. (1981) have studied in vitro differentiation of mouse neuroblastoma cells induced by either n-butyrate, DMSO, HMBA, or 1,6-dibutyryl-adenosine 3',5'-monophosphate (Bt₂cAMP). They too observed an accumulation of a lysine-rich histone identified as mouse H1⁰ by peptide mapping. This accumulation of H1⁰ was accompanied by shutoff of DNA synthesis and transgression of the cells into G1-phase resting state, similar to that observed for accumulation

of IP25 and BEP (Keppel et al. 1977, D'Anna et al. 1980a). In contrast to the observations of Keppel et al. (1977), the level of $H1^O$ accumulation was dependent upon the type of agent used for induction of mouse murine neuroblastoma differentiation. Control levels of 12% $H1^O$ relative to total lysine-rich histones ($H1 + H1^O$) increased to 25%, 16%, 30%, and 20% during induction by n-butyrate, DMSO, HMBA, and Bt_2 CAMP respectively. Furthermore, resumption of cell proliferation upon removal of n-butyrate was accompanied by a decrease in $H1^O$ content, indicating that accumulation of $H1^O$ is reversible. This was implied by Pehrson and Cole (1980), but no other study on accumulation of $H1^O$ has tested reversibility of $H1^O$ accumulation directly.

Although these studies have all demonstrated modulation of $H1^O$ content, it is still conceivable that the observed accumulation of $H1^O$ in these systems is artifactual in nature, possibly due to inducing agents, in vitro conditions or peculiarities of transformed cells. However, studies of normal developing tissues have also demonstrated $H1^O$ accumulation. For example, Varricchio (1977) observed and quantitated the appearance and subsequent accumulation of $H1^O$ during postnatal development of rat pancreas. This postnatal increase was found to be progressive, reaching an $H1^O$ to $H1$ ratio of 0.27 and was accompanied by a decrease in DNA synthesis.

Marsh and Fitzgerald (1973) followed changes in $H1^O$ content during degeneration and regeneration of rat pancreas following ethionine treatments. They observed a decrease in $H1^O$ content during degeneration from control levels of about 27% $H1^O$ relative to total lysine-rich histone ($H1 + H1^O$), to a minimum of about 12% before the onset of DNA synthesis during regeneration. This low content of $H1^O$ persisted throughout the regeneration period until DNA synthetic rates had decreased to near control levels at which time $H1^O$ progressively increased in content reaching normal levels by the end of regeneration. Garrard and Bonner (1974) observed a similar decrease in the relative content of $H1^O$ during initiation of rat liver regeneration, reporting a 50% loss in the relative content of $H1^O$ relative to total histone. Block and Atkinson (1979) observed the appearance of an $H1^S$ -like protein, during the G1-arrest accompanying quail myogenesis. Gordon et al. (1981) also observed appearance of an $H1^S$ -like histone during development of chick myogenic tissue.

All of these studies clearly demonstrate several important characteristics of $H1^O$ histones. First, it is apparent that the relative amounts of $H1^O$ are found to change dramatically during environmental or developmental modulation of the cell. Secondly, these alterations in $H1^O$ content are accompanied reciprocally by large changes

in cell proliferation rates. Thirdly, the fact that increases in $H1^O$ occur during decreases in DNA replication suggests that $H1^O$ may be synthesized outside of S-phase, which is uncharacteristic of H1 and core histones (Borun 1975, Elgin and Weintraub 1975, Pederson 1976).

These characteristics not only serve to distinguish $H1^O$ from H1 but also illustrate the close relationship of $H1^O$ with avian H5. This comes from studies which have shown H5 to accumulate in erythroid cells during erythropoiesis in a manner similar to $H1^O$.

E. H5 Accumulation

Billet and Hindley (1972) report that the amount of H5 increases approximately 2.5 fold during adult chicken erythropoiesis, consistent with the results of Appels et al. (1972). Ruiz-Carrillo et al. (1974) observed at least a 2-fold increase in H5 during duck erythropoiesis. Similar results were reported by Sung (1977) who observed the ratio of H4 to H5 to increase from 1:1 in erythroblasts to about 1:2 in mature chicken erythrocytes. Accumulation of H5 during chick embryonic erythropoiesis has been reported by Moss et al. (1973), who observed a progressive increase in H5 content of developing primitive erythrocytes from very low levels in 2.5 day old embryos, to 13.5% of all histone in 19 day old embryos. A second wave of H5 accumulation was also noted during the erythropoiesis of the definitive line of erythrocytes,

subsequently resulting in an H5 content of 21% of total histone in adult erythrocytes. Weintraub (1978) also reports observing an increase in chicken H5 content from 0.2 molecules per nucleosome to 0.6 molecules per nucleosome during primitive erythropoiesis to 1 molecule per nucleosome in adult chicken erythrocytes. Urban et al. (1980) reported that chicken H5 accumulation occurs in three steps, with the molar ratio of H5 increasing to about 0.27 in primitive erythrocytes, 0.48 in definitive embryonic erythrocytes, and 0.83 in adult definitive erythrocytes.

In addition to investigation of chicken H5 accumulation, studies into the synthetic characteristics of H5 have also been reported. After incubation of immature chicken erythrocyte populations with radioactively labelled amino acids and electrophoretic separation of labelled histones, Ruiz-Carrillo et al. (1976), and Appels and Wells (1972) report that H5 was synthesized throughout erythropoiesis. However, unlike H1 and the core histones, H5 synthesis continued after DNA synthesis had ceased. This selective synthesis of H5 during late stages of chicken erythropoiesis (Seligy et al. 1973, Tobin and Seligy 1975) was also demonstrated by Sung et al. (1977) employing gel-filtration chromatography

of labelled histone, who also observed synthesis of H5 along with H1 and core histones, during the early stages of chicken erythropoiesis.

Despite striking similarities between H1^O and chicken H5, from which functional homology has been suggested, there are still several discrepancies. For example, the major distinguishing characteristic of H5 has been its supposed specific association with the nucleated erythrocyte. As well, despite some homology in sequence and tertiary structure, H5 is still divergent in structure from H1^O. Differences in chromatographic and electrophoretic characteristics, combined with differences in amino acid analysis also challenge a close functional homology between H1^O and H5. In addition, although the accumulation of H1^O suggests its synthesis outside of S-phase, no definitive studies into H1^O synthesis have been conducted to date. Therefore it has not been possible to correlate this aspect of chicken H5 metabolism with that of mammalian H1^O. Thus it is possible that H5 is a highly specialized H1^S histone whose structure and function may have diverged during its evolution, a function that may be specific to nucleated erythrocytes of birds and possibly fish.

F. Experimental Approach

Xenopus laevis erythrocyte H1^S is one of only a few recognized erythrocyte-associated satellite H1 that has

been shown also to be present in non-erythroid tissues (Brown et al. 1981, Risley and Eckhardt 1981, Rutledge et al. 1981). In addition, Xenopus erythrocyte H1 possesses structural characteristics very similar to H1^S (Brown et al. 1981, Risley and Eckhardt 1981); thus it may serve as a bridge between non-erythroid H1^S and avian erythrocyte-specific H5.

An equally attractive reason for study of this histone is the availability of a well characterized Xenopus laevis erythropoietic system, allowing detailed metabolic studies of frog H1^S during erythrocyte development. It is possible to completely eliminate all existing mature erythrocytes from the circulatory system of Xenopus laevis by injections of phenylhydrazine (Thomas and Maclean 1975). Chegini et al. (1979) have reported that after phenylhydrazine treatments all existing erythrocytes are damaged and eliminated from circulation by the liver and spleen, at which time the frogs respond to their severe anemia by release from the liver of a large number of erythroblasts into circulation, where they mature as a relatively homogenous cohort of cells. Thomas and Maclean (1975), who studied erythroid morphology during this wave of erythropoiesis induced by phenylhydrazine, reported a morphological sequence similar to chicken (Lucas and Jamroz 1961). They also demonstrated a decrease and final cessation of protein,

RNA, and DNA synthesis during the maturation of these erythroid cells.

Maclean et al. (1973) demonstrated that the nucleus of the *Xenopus* erythrocyte makes the nearest approach to being metabolically inert of any known nucleus, according to the extremely low levels of RNA synthesis in mature *Xenopus* erythrocytes. This was confirmed by Hilder and Maclean (1974), who found that isolated *Xenopus* erythrocyte nuclei possess extremely low basal rates of RNA synthesis, reflective of its highly restricted template activity. Studies on RNA polymerase activity also confirm restriction of DNA template activity during *Xenopus* erythrocyte maturation (Hentschel and Tata 1978). This is not only an extraordinary erythropoietic system making available the isolation of developmentally homogenous populations of immature erythrocytes, but as well *Xenopus* erythropoiesis furnishes an example of extreme nuclear inactivation. Metabolism of nuclear nonhistone proteins (Hilder et al. 1975) and globins (Hentschel et al. 1979) has been characterized during *Xenopus* erythropoiesis induced by phenylhydrazine, but no study of histone metabolism has been reported to date. Thus, this erythropoietic system provides an excellent opportunity for study of frog H1^S metabolism during the programmed shutdown of metabolic activity with erythrocyte maturation.

The objective of this project was first to develop this regenerating erythropoietic system of Xenopus laevis, to permit isolation of erythroid populations at various stages of maturity. The rates of protein and DNA synthesis within the populations would then be determined by following the incorporation of ^{14}C -lysine and ^3H -thymidine respectively into TCA-insoluble material. Electrophoretic separation and fluorography of histones isolated from ^{14}C -lysine labelled nuclei then permitted investigation into histone synthesis, with quantitation of electrophoretically separated histones allowing determination of the relative amounts of H1^{S} . From this it was hoped to obtain a better understanding of the metabolic and synthetic characteristics of frog H1^{S} , and its relationship to changes in DNA and protein synthesis during erythrocyte maturation.

II. MATERIALS AND METHODS

A. Induction of Anemia

Large female (75-100g) Xenopus laevis were obtained from Boreal Laboratories, Mississauga, Ont., kept in holding tanks at room temperature (20-25 °C) and fed fresh beef heart or dried food (Boreal Laboratories) twice weekly. Severe anemia was induced by phenylhydrazine treatments as described by Thomas and Maclean (1975). Weighed animals were injected subcutaneously on 2 successive days with a 12.5 mg/ml neutralized phenylhydrazine hydrochloride solution (250 mg in 18.3 ml water, plus 1.7 ml 1.0 M NaOH) to give final dosages of 30 mg/kg body weight. This treatment resulted in loss of greater than 95% of circulating erythrocytes within 6 days after the last injection and produced no apparent distress in the animals.

Before collection of blood, frogs were anesthetized by immersion in a solution of 0.2% MS222 (Sigma Chemical Co., St. Louis, Mo.) for at least 30 minutes. Anesthetized animals were then rinsed in water to remove excess MS222, before the body cavity was carefully opened and immediately flushed with incubation media containing heparin (Fischer Sci. Co., 0.2 mg/ml, 146,000 U/g). After removing excess solution, the hepatic portal vein was severed, hematocrit samples and blood smears quickly

taken, and 1-2 ml of blood per animal collected with a Pasteur pipette while the body cavity was simultaneously flushed with heparinized incubation media, allowing efficient collection of small blood volumes. Blood cells were then washed twice at room temperature with incubation media, centrifuged at 150 Xg for 5 minutes.

Hematocrits were determined using heparinized micro-hematocrit tubes and spun at 150 xg for 5 minutes. Blood smears were air dried and stained differentially with May-Grunwald and Giesma stains (Lucas and Jamroz 1961). Cytoplasmic reticulum was visualized by addition of one drop of 1.0% brilliant cresyl blue (Baker Chem. Co.) in 0.9% NaCl to about 4 ul of blood cells suspended in 1.0 ml of incubation media and the mixture held at room temperature for 10-20 minutes before microscopic examination (Lucas and Jamroz 1961). Photomicrographs were taken using a Zeiss microscope employing bright field optics.

B. Incorporation of Radioactive Precursors

Incubation of Xenopus laevis erythrocytes in either MEM or frog Ringer's solution produced extensive cytoplasmic vacuolation as determined from phase contrast microscopic examination, but an incubation medium described by Maclean et al. (1973) showed no cytoplasmic vacuolation during incubation up to 6 hours. This medium

consisted of frog Ringer's solution supplemented with 12 g/ml bovine serum albumin (Fraction V, Sigma Chemical Co.), 20 common amino acids minus lysine, MEM Vitamin solution (Grand Island Biological Co.), penicillin (100 U/ml), streptomycin (100 mg/ml), and 1.0 mg/ml glucose. This solution was adjusted to pH 7.4 with 5% HCO_3 and millipore filtered before storage at -20°C .

Vials of 1.0 mCi (methyl- ^3H)-thymidine (sp. act. 80.3 Ci/mmol, New England Nuclear) in sterile water and 0.25 mCi L-(^{14}C (U))-lysine (sp. act. 331.0 mCi/mmol, New England Nuclear) in 0.01 M HCl were lyophilized and incubation media directly added to each vial to give 500 uCi/ml and 200 uCi/ml of ^3H -thymidine and ^{14}C -lysine respectively.

200 ul of washed cells were suspended in incubation media to a final volume of 3.6 ml. The cell suspension was then stirred and 400 ul of a 200 uCi/ml ^{14}C -lysine solution added, to give a final concentration of 20 uCi/ml. This mixture was then incubated with continuous stirring for 60 minutes at room temperature ($21-24^\circ\text{C}$). For thymidine incubations, 100 ul of washed cells were suspended in incubation media to a final volume of 1.9 ml, to which 120 ul of a 500 uCi/ml solution of ^3H -thymidine was added with stirring, to give a final concentration of 30 uCi/ml, and incubated with continuous stirring for 60 minutes.

C. Measurement of Isotope Incorporation

In order to monitor isotope incorporation during incubation, 20 μ l samples of the incubation mixture were taken at 10 minute intervals. Each sample was placed into a 1.5 ml microfuge tube, 1.0 ml of 5% TCA was added immediately and mixed. No difference in the results was found either by the addition of 10% rather than 5% TCA or by chilling the TCA precipitation mixtures on ice. After all samples were taken, the TCA precipitates were washed in 1.0 ml of 5% TCA, using medium speed on a Beckman microfuge B to pellet the precipitate. Each pellet was then suspended in 200 μ l of 30% hydrogen peroxide, followed by addition of 100 μ l 80% PCA and the mixture incubated overnight at 60 $^{\circ}$ C to solubilize the precipitate. After cooling, three 50 μ l aliquots of each time sample were taken and placed directly into 20 ml plastic scintillation vials. To reduce quench, 50 μ l of fresh 15% ascorbic acid was mixed with each sample before addition of 5.0 ml of Ready-Solv HP (Beckman, Toronto). The samples were then counted for radioactivity and the average CPM plotted as a function of incubation time. Incorporation rates for lysine and thymidine were determined directly by least squares analysis, with no correction for isotope or cell concentration and is expressed as CPM incorporated per minute incubation.

D. Autoradiograms of Blood Smears

Autoradiograms of ^3H -thymidine labelled cells were done according to Chegini et al. (1979). Air dried smears of incubated cells were fixed in methanol, brought to water through a graded series of methanol concentrations, and exposed to 5% TCA for 2 hours at room temperature. The slides were then washed, dried, and coated with Ilford L:4 emulsion (size A, Ilford Ltd.), diluted 1:1 with distilled water. Coated slides were incubated at 4 °C for 2 weeks and developed in Kodak Dektol.

E. Isolation and Metabolism of Nuclei and Histones

All operations during nuclear isolation were performed at 0 °C and all solutions made up to 1.0 mM PMSF from a 50 mM stock solution in isopropanol in order to inhibit protease activity. In early experiments, incubated cells were washed in NSM (0.24 M sucrose, 50mM Tris, pH 7.2, 100 M KCl, 5.0 mM $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$; Hilder et al. 1975) containing 1.0 mM PMSF, but cell clumping was sometimes observed. Since washing in 0.9% NaCl containing 1.0 mM PMSF reduces cell clumping, it was used in further experiments. The washed cells were then resuspended in 5.0 ml of either NSM or 0.9% NaCl containing 1.0 mM PMSF, and 1/50 volume of fresh 5% saponin in 0.1 M sodium phosphate (pH 6.8) was added to lyse the cells. After stirring on ice for 5 minutes, nuclei were pelleted by

centrifugation at 300 Xg for 10 minutes and the supernatant carefully removed. The nuclei were then washed free of hemoglobin with either NSM or 0.9% NaCl containing 1.0 mM PMSF. Hemoglobin concentrations were estimated from the A_{540} of the cell lysates and expressed as $A_{540}/\mu\text{l}$ packed cell.

Total histones were extracted three times by homogenizing nuclei with 3.0 ml of 0.25 M HCl. At first histones were precipitated by addition of nine volumes of acetone to the combined extracts, but later total HCl extracts were precipitated by addition of 1/3 volume of 80% TCA to improve histone recovery.

Lysine-rich histones were selectively extracted with 5% PCA. Washed nuclei were suspended in 1.5 ml of 0.01 M HCl, 1.5 ml of 10% PCA was added with mixing, the mixture was homogenized, and the insoluble material reextracted with 5% PCA. The lysine-rich histones were precipitated from the combined extracts by addition of 1/3 volume of 80% TCA. Core histones were extracted three times from the 5% PCA insoluble residue by homogenization in 1.0 ml 0.25 M HCl. The core histones were precipitated from the combined HCl extracts by the addition of either nine volumes of acetone or 1/3 volume of 80% TCA. All homogenizations were done with a loose fitting A pestle in a Dounce-type homogenizer (Kontes Glass, Vineland, N.J.). Precipitated histone samples were washed with acidified

acetone (1.0 ml 12 M HCl per 500 ml acetone), air-dried, dissolved in distilled water, lyophilized and stored at -20°C .

To allow monitoring of the uptake and incorporation of labelled precursors into the above fractions, three 20 μl samples were taken during cell incubation, nuclear isolation, and histone preparation and placed directly into 20 ml plastic scintillation vials. To reduce quenching, 50 μl of 30% hydrogen peroxide was mixed with each sample for about 20-30 seconds, followed by 50 μl of fresh 15% ascorbic acid and then by 5.0 ml of Ready-Solv HP. After counting, the average CPM for each sample was determined and the total CPM for each fraction calculated.

F. Polyacrylamide Gel Electrophoresis

Electrophoresis was done in 0.15x20x12 cm slab gels or 0.6x15 cm tube gels. SDS slab-gel electrophoresis used a separation gel of 12.5% acrylamide (40:1 bis-acrylamide), 0.1% SDS, 1.0 mg/ml ammonium persulphate, 0.5 $\mu\text{l}/\text{ml}$ TEMED, 0.4 M Tris (pH 8.8) and a stacking gel of 7.5% acrylamide (40:1 bis-acrylamide), 1.0 mg/ml ammonium persulphate, 1 $\mu\text{l}/\text{ml}$ TEMED, 0.12 M Tris (pH 6.8). Electrophoresis was conducted with a running buffer of 0.2 M glycine, 0.1% SDS, 25 mM Tris (pH 8.3) for 4-5 hours, 350 V at 0.5°C . Acid-urea gels consisted of

3.0 M urea, 5% acetic acid, with 12.5% acrylamide (40:1 bis-acrylamide) for slab and 15.0% acrylamide (150:1 bis-acrylamide) for tube gels. Tube gels were polymerized for about 1-2 hours, using 1.0 ul/ml TEMED, 1.0 mg/ml ammonium persulphate and pre-electrophoresed overnight at 2.0 mA per tube. Electrophoresis was carried out with a 5% acetic acid running buffer at 2.0 mA per tube for 6 hours at room temperature. Acid-urea slab gels were polymerized for 1-2 hours with 10.0 ul/ml TEMED, 1.0 mg/ml ammonium persulphate, and proteins were electrophoresed without pre-electrophoresis, using a 5% acetic acid running buffer at 350 V for about 7 hours at 0.5 °C. Electrophoretic samples were prepared by dissolving weighed lyophilized histone to a concentration of about 5 ug/ul in 20% sucrose for acid-urea gels, or in 2.5% SDS, 10.0% glycerol, 0.62 M Tris (pH 6.8) for SDS gels. When the amounts of protein were limiting, SDS samples were sometimes prepared by addition of an equal volume of SDS sample buffer to residual samples prepared for acid-urea gels.

Acid-urea tube gels were stained overnight in 0.1% amido black, 5% acetic acid, 40% ethanol and destained in 5% acetic acid, 40% ethanol. Slab gels were stained overnight with shaking in 0.25% Coomassie blue (R), 5% acetic acid, 20% ethanol and destained in 5% acetic acid, 20% ethanol.

G. Fluorography of Polyacrylamide Gels

Destained slab gels were first washed with shaking in distilled water for 30 minutes to remove acetic acid. The gel was then treated with shaking for 30 minutes in 1.0 M sodium salicylate (Sigma) pH 6.3 (Chamberlain 1979), and dried onto Whatman 3M chromatographic paper using a slab gel drier (Bio-Rad, model 224) with a mylar sheet. It should be noted that, although problems of the dried gels sticking to the mylar sheet were not experienced, it was necessary to allow cooling before removal of the mylar sheet. The dried gel was then exposed to Kodak X-Omat XAR-5 film (cat. 1651496) for two weeks at -80°C and developed for 5 minutes with agitation in Kodak liquid X-ray developer and replenisher (cat. 1465335). Since no qualitative differences were observed with preflashed X-ray plates (Laskey and Mills 1975), preflashing of the X-ray plates was not done when optimal quality of reproduction was desired.

H. Quantitation and Specific Radioactivity of Histones

To monitor changes in the relative proportions of the lysine-rich histones, 20-60 ug of 5% PCA extracted histones were electrophoresed in acid-urea tube gels, stained with amido black and the histone bands sliced out of each gel using a single-edged razor blade. Each slice was cut into several pieces, placed into a 1.5 ml glass

vial and 1.0 ml DMSO added. The vials were then incubated overnight at 60 °C during which time the DMSO quantitatively leached out the amido black dye (Chiu and Irvin 1980). The relative amounts of each lysine-rich histone was then determined from the A_{600} of the DMSO solution and the relative content of $H1^S$ expressed as the fraction of the total A_{600} .

To determine the relative ^{14}C -lysine content of each lysine-rich histone, 1.0 ml of 30% hydrogen peroxide was added to each vial after removal of the DMSO. Incubation overnight at 60 °C effectively dissolved the polyacrylamide; 100 μ l of acetic acid was added, left overnight, and 15 ml of Ready-Solv HP was added before counting.

III. RESULTS

A. Induction of and Recovery from Anemia

Anemia was induced in large female Xenopus laevis with two successive daily injections of phenylhydrazine. To monitor relative changes in blood cell number, blood hematocrits of individual phenylhydrazine-treated animals were determined at various times of recovery from 6 to 35 days to reveal the pattern shown in Figure 1. Untreated frogs were found to have blood hematocrits of about 45% that varied little from animal to animal. The 8% hematocrit observed in animals allowed to recover for 6 days is therefore indicative of massive losses of circulating erythrocytes induced in X. laevis by phenylhydrazine, similar to that described by Thomas and Maclean (1975) and again by Chegini et al. (1979). This nearly complete loss of blood cells was followed by a regeneration period in frogs allowed to recover for times greater than 7 days, when hematocrits increased as erythrocytes were replaced. After a recovery period of about 15 days animals had nearly normal hematocrits, with longer times having little further effect. As can be seen in Figure 1, rates of erythroid replacement varied greatly from one animal to another, but the trend was consistent.

Erythroid cell types present in circulation during

Figure 1. Blood hematocrits of X. laevis recovering from phenylhydrazine-induced anemia. The blood hematocrit of individual frogs at various recovery times was determined in order to follow changes in blood cell number.

erythrocyte regeneration were identified by differential staining of blood smears (Lucas and Jamroz 1961) taken from animals sacrificed at various recovery times. Photomicrographs of several representative blood smears are shown in Figure 2. Mature erythrocytes obtained from untreated X. laevis were morphologically similar to chicken erythrocytes, possessing an oval shape and a highly condensed nucleus (Fig. 2A). Erythrocytes in phenylhydrazine-treated animals were observed to be badly damaged, likely resulting in their rapid removal from circulation and the severe anemia observed at DAY 6. At DAY 7 (Fig. 2B) blood cells appeared to be a mixture of residual abnormal erythrocytes, new erythroblasts, and surviving white cells. By DAY 8 (Fig. 2C) large numbers of basophilic erythroblasts with many mitotic figures were found in the circulation just as hematocrits began to increase. This suggests that increases in red blood cell number during recovery is at least partly a result of erythroid mitosis in circulation. This was later confirmed by monitoring DNA synthesis by labelling erythroid populations in vitro with ³H-thymidine. With increased recovery time, erythroid cells accumulated hemoglobin, and nuclei became progressively more condensed (Fig. 2D to 2H), as is characteristic of erythropoiesis in nucleated erythrocytes (Lucas and Jamroz 1961).

Basophilic erythroblasts were never observed in the

Figure 2. Photomicrographs of stained blood smears taken from individual X. laevis allowed to recover for various times after phenylhydrazine treatment. Air dried blood smears were stained with May-Grunwald and Giesma stains (Lucas and Jumroz 1961) and photographed at x 2000 mg. A) untreated, B) day 7, C) day 8, D) day 10, E) day 15, F) day 20, G) day 26, H) day 35.

circulation of untreated X. laevis. This is consistent with the report of Thomas and Maclean (1975), that only mature erythrocytes are released into circulation during normal erythropoiesis in adult X. laevis. The observed appearance of circulating basophilic erythroblasts is thus an emergency response to severe anemia (Thomas and Maclean 1975, Chegini et al. 1979). It is apparent from Figure 2 and from differential blood cell counts (Table 1) that as recovery progressed, erythroid populations contained higher proportions of ever more mature stages of erythropoiesis. Consistent with the reports by Thomas and Maclean (1975) and Chegini et al. (1979) these observations suggest that during recovery from anemia, erythroblasts observed at DAY 8 mature as a cohort in circulation to produce a sequence of erythroid populations representing all stages of erythropoiesis. However, individual animals were observed to vary greatly in their rate of erythrocyte regeneration, thus making recovery time unreliable for predicting the precise developmental stage of individual erythroid populations.

Examination of stained blood smears (Fig. 2) combined with differential counts (Table 1) indicated considerable developmental homogeneity with all but the earliest populations consisting predominantly of erythroid cells at a common stage of development. In addition, cells out of phase or synchrony consisted of cell types adjacent in

Table 1

Differential Blood Cell Counts* of Maturing
Erythroid Populations

DAYS OF RECOVERY	WC**	EB	EP	P	E	MF	# CELLS COUNTED
UNTREATED	1.4%	0	0	0	98.6%	0	1241
8	55.2%	10.2%	14.8%	0.5%	18.4%	0.9%	1041
10	12.9%	2.3%	11.8%	59.7%	12.5%	0.8%	1093
10	10.7%	0	0.6%	79.4%	9.2%	0.1%	1195
12	5.8%	0	0	7.7%	86.5%	0	1193
15	3.1%	0	0	17.4%	79.5%	0	2019
15	10.3%	0	0	11.5%	78.1%	0.1%	1110
20	4.4%	0	0	3.6%	92.0%	0	2201
20	5.8%	0	0	0.6%	93.6%	0	1311
26	7.4%	0	0	4.2%	88.4%	0	1071
26	2.2%	0	0	1.8%	96.0%	0	1110
35	4.6%	0	0	0.7%	94.7%	0	1097
35	3.9%	0	0	0	96.1%	0	1043
85	7.6%	0	0	0	92.4%	0	1405

* Air dried blood smears were stained with May-Grunwald and Giesma stains (Lucas and Jamroz 1961); ** WC, white cells; EB, erythroblast; EP, early polychromatic; P, polychromatic; E, erythrocyte; MF, mitotic figure.

developmental stage to the mode of the erythroid population, with more developmental homogeneity as erythroid populations matured. Erythrocyte development was further examined by vital staining of cytoplasmic reticulum with cresyl blue. As shown in Figure 3, extensive reticulation in young populations decreased in density as erythroid maturation progressed. Moreover, erythroid cells within each population showed similar degrees of reticulation, again indicative of developmental homogeneity within these erythroid populations.

B. In Vitro Incubation of Blood Cells

Two major groups of incubation experiments were conducted on regenerating erythroid populations with some differences in procedure. In the first group of eight experiments (called herein Regeneration Series I), protein synthesis was monitored by in vitro incubations with ^{14}C -lysine. In the second group of five experiments (called Regeneration Series II), in addition to the ^{14}C -lysine incubation, a separate aliquot of cells was incubated with ^3H -thymidine and hemoglobin concentrations were also determined. For all incubation experiments, individual phenylhydrazine-treated animals were sacrificed at various recovery times and their blood was collected. After washing with incubation media, blood cells were incubated in vitro for 60 minutes with radioactively labelled precursors, as described in Materials and

Figure 3. Photomicrographs of frog blood cells stained with cresyl blue to show cytoplasmic reticulum (Lucas and Jamroz 1961). A) day 10, B) day 15, C) day 20, D) day 35 after induction of anemia.

Methods. After incubation, nuclei were isolated and histones extracted from ^{14}C -lysine-labelled nuclei. To follow uptake and utilization of labelled precursors, samples were taken from each fraction during nuclear isolation and total CPM of each fraction was determined (Tables 2, 3, and 4).

Tables 2, 3, and 4 are compilations of data of 13 experiments. In each Table the experiments are ordered from left to right by decreasing incorporation rate of lysine into total protein (Tables 2 and 3) or of thymidine into nuclei (Table 4), reflecting maturation of the erythroid populations. The tabulated data consists of two types of information; data describing the blood prior to incubation, and data indicating radioactivity contained within each cell and protein fraction obtained during nuclei isolation and histone extraction after the 60 minute incubation. In Regeneration Series I (Table 2), lysine-rich histone was first selectively extracted from isolated nuclei with 5% PCA, followed by extraction of core histone from the PCA-insoluble residue with 0.25 M HCl. In Regeneration Series II (Table 3), histones were extracted totally from isolated nuclei with 0.25 M HCl.

A large but variable proportion of counts remained soluble in 20% TCA during histone precipitation from the PCA-extracts of Regeneration Series I and HCl-extracts of

Table 2
Distribution of ^{14}C -lysine Among Cell and Protein
Fractions from Regeneration Series I

ANIMAL NUMBER:	#16	#21	#11	#17	#20	#15	#19	#7
DAYS OF RECOVERY	12	10	25	15	20	30	35	85
BLOOD HEMATOCRIT (%)	32.8	13.9	35.7	39.3	38.8	35.8	37.8	54.6
INCUBATION HEMATOCRIT (%)	5.5	4.8	5.2	5.1	3.8	4.4	5.6	5.3
INCUBATION VOLUME (ml)	4.7	4.0	5.0	4.0	3.9	4.3	4.0	4.0
INCUBATION CPM/ml*	24.7	27.5	22.6	40.3	32.8	32.3	35.0	34.8
TOTAL CPM ⁺								
WASHED CELLS*	29.7	18.5	---	22.4	12.3	---	12.0	7.2
LYSATE SUPERNATANT*	25.4	15.3	16.0	19.8	11.5	7.4	9.7	6.3
WASHED NUCLEI**	4800	2350	863	1060	237	179	106	34
1st PCA EXTRACT**	1560	902	310	182	63	67	43	18
2nd PCA EXTRACT**	176	101	42	26	19	20	5	4
PCA-TCA SUPERNATANT**	493	155	192	52	26	38	38	18
1st HCl EXTRACT**	778	395	107	63	20	24	NIL	NIL
2nd HCl EXTRACT**	486	245	86	39	10	10	NIL	NIL
3rd HCl EXTRACT**	193	84	25	22	11	NIL	NIL	NIL
INCORPORATION RATE ⁺⁺	223	127	112	110	38	35	10	7

* CPM $\times 10^{-6}$; ** CPM $\times 10^{-3}$; + Total CPM calculated for each fraction with no correction for free ^{14}C -lysine; ++ In units of CPM/min (see Figure 14 for explanation); ---- not determined.

Table 3
Distribution of ^{14}C -lysine Among Cell and Protein
Fractions from Regeneration Series II

ANIMAL NUMBER:	#36	#34	#37	#32	#35
DAYS OF RECOVERY	10	12	15	20	26
BLOOD HEMATOCRIT (%)	36.2	25.0	37.5	65.7	44.0
INCUBATION HEMATOCRIT (%)	5.4	5.8	4.6	4.4	4.3
INCUBATION VOLUME (ml)	4.0	4.0	4.0	4.0	4.0
INCUBATION CPM/ml*	43.4	37.1	43.5	36.0	36.8
	TOTAL CPM ⁺				
WASHED CELLS*	32.3	25.4	25.9	14.8	8.1
LYSATE SUPERNATANT*	21.8	18.7	23.4	13.4	0.9
WASHED NUCLEI**	5980	3600	1010	923	557
1st tHCl++ EXTRACT**	4530	2080	707	330	277
2nd tHCl++ EXTRACT**	504	272	126	37	67
3rd tHCl++ EXTRACT**	364	281	87	----	38
tHCl-TCA SUPERNATANT**	1160	329	150	59	72
INCORPORATION RATE ⁺⁺⁺	269	230	175	84	60

* CPM $\times 10^{-6}$; ** CPM $\times 10^{-3}$; + Total CPM calculated for each fraction with no correction for free ^{14}C -lysine;

++ tHCl is the 0.25 M HCl extract of total histone; +++ In units of CPM/min (see Figure 14 for explanation); ---- not determined.

Table 4
³
 Distribution of H-thymidine Among Cell Fractions from
 Regeneration Series II

ANIMAL NUMBER:	#36	#34	#37	#32	#35
DAYS OF RECOVERY	10	12	15	20	26
BLOOD HEMATOCRIT (%)	36.2	25.0	37.5	65.7	44.0
INCUBATION HEMATOCRIT (%)	5.3	6.9	4.4	4.6	4.2
INCUBATION VOLUME (ml)	2.0	3.0	2.0	2.0	2.0
INCUBATION CPM/ml *	18.0	11.7	16.8	16.5	19.9
	TOTAL CPM ⁺				
WASHED CELLS**	1079	1180	1170	285	509
LYSATE SUPERNATANT**	257	140	293	235	265
WASHED NUCLEI**	1050	686	532	34	132
INCORPORATION RATE ⁺⁺	131	91	43	1	5
S-PHASE NUCLEI (%) ⁺⁺⁺	27.3	11.0	3.3	0	0
# OF CELLS COUNTED ⁺⁺⁺	10 ³	10 ³	10 ³	10 ⁴	10 ⁴

* CPM x 10⁻⁶; ** CPM x 10⁻³; + Total CPM calculated for each fraction with no correction for free ³H-thymidine;

++ In units of CPM/ml (see Figure 6 for explanation); +++ Counts of autoradiograms (Fig. 7).

Series II. Dialysis (MW cutoff 6,000 d) of this TCA-soluble, acid-extracted radioactivity against distilled water resulted in loss of greater than 90% of the radioactivity (data not shown). Bio-gel P-2 gel filtration (exclusion MW 2,000 d) revealed that most of the ^{14}C -lysine in this supernatant was associated with a molecule of about 1,000 d (data not shown), but SDS-gel electrophoresis in 20% acrylamide did not reveal a low molecular weight peptide (data not shown). Further characterization of this low molecular weight component was not possible, but it may have been adventitious ^{14}C -lysine or degraded lysine-containing oligopeptide in the PCA extract. Evidently further purification of protein by chromatography or electrophoretic separation is necessary to assess histone synthesis precisely.

A general decrease in uptake and utilization of ^{14}C -lysine (Tables 2 and 3) and ^3H -thymidine (Table 4) was observed for progressively more mature erythroid populations, consistent with the well characterized decrease in rates of protein and DNA synthesis during erythropoiesis (Seligy et al. 1973, Thomas and Maclean 1975, Grasso et al. 1977). Although the factors controlling erythrocyte number in X. laevis circulation are unknown, it is apparent from Tables 2 and 3 that increases in blood hematocrit are not coordinated with loss in ^{14}C -lysine utilization. Thus effective analysis

of these trends was difficult due to large individual differences in the relative rates of anemia recovery, combined with a lack of an independent, quantifiable parameter of population maturity.

C. Incorporation Rate Determinations

To further monitor erythroid activity, incorporation of radioactive precursors into TCA-insoluble material was followed during incubations of these erythroid populations, sampled at 10 minute intervals, and TCA-insoluble CPM was plotted as a function of incubation time (Fig. 4, 5 and 6). Linear incorporation of lysine was observed for all successful experiments, thus permitting least squares quantitation of the ¹⁴C-lysine incorporation rate and demonstrating that each erythroid population possessed a characteristic lysine incorporation rate (Tables 2 and 3), which is likely related to the average rates of protein synthesis within the erythroid populations. More importantly, a decrease in lysine incorporation rate with increasing population maturity is observed, consistent with the decrease in precursor utilization seen in Tables 2 and 3, as well as with the reported decrease in rates of protein synthesis during erythropoiesis (Seligy et al. 1973, Thomas and Maclean 1975, Grasso et al. 1977). These results therefore

Figure 4. Incorporation of ^{14}C -lysine during the 60 minute in vitro uncubations of the first group of eight experiments (henceforth called Regeneration Series I). Samples of the incubation mixtures were taken at 10 minute intervals, and the TCA-insoluble CPM determined as described in Materials and Methods. Numbers on the right refer to animal number (Table 2). Linear incorporation of ^{14}C -lysine allowed quantitation of the lysine incorporation rate using least squares determination of the slope, which decreased with increased erythroid maturity.

Figure 5. Incorporation of ^{14}C -lysine during the 60 minute incubations of the second group of five experiments (henceforth called Regeneration Series II). Sample preparation and counting identical to Figure 4. Numbers on the right refer to animal number (Table 3).

Figure 6. Incorporation of ^3H -thymidine into TCA-insoluble material during the incubations of erythroid cell from Regeneration Series II (Fig. 5). Numbers on the right refer to animal number (Table 4). Since slowing of thymidine incorporation for animals #36 and #34 at late stages of incubation was likely an artifact due to the incubation conditions, the thymidine incorporation rate was determined for these two populations from the first 20 minutes of incubation (initial rate). Thymidine incorporation rates for the last three populations were determined like the lysine incorporation rates (Fig. 4).

suggest an inverse relationship between lysine incorporation rate and erythroid maturity, explored more thoroughly in later sections.

Thymidine incorporation rates were determined in a similar manner as were the lysine incorporation rates. However as seen in Figure 6, incorporation by the two youngest populations were found to fall at late stages of incubation. Although the reason for slowing in ^3H -thymidine incorporation was not identified, thymidine incorporation rates during for the first 20 minutes of incubation (initial rate) was taken as an estimate of thymidine incorporation rate for these two youngest populations. Like lysine incorporation, the thymidine incorporation was found to decrease and finally cease with increasing population maturity.

To establish that thymidine incorporation is truly reflective of DNA replication, and to further examine decreases in thymidine incorporation during erythroid maturation, autoradiograms of ^3H -thymidine-labelled cells were made (Fig. 7). Representative autoradiograms demonstrate that incorporated ^3H -thymidine was localized in the erythroid nuclei. It is important to note that heavy grain density was observed over labelled nuclei even in the three most mature populations (Fig. 7C and 7D), demonstrating that the low rate of thymidine incorporation in these populations was not due to inability of S-phase

Figure 7. Photomicrographs of autoradiograms of erythroid cells from X. laevis after the 60 minute incubation with ^3H -thymidine (as described in Materials and Methods). A) #36, B) #34, C) #37, D) #32, numbers refer to animal numbers (see Table 4 and Fig. 5). Microscopic examination (x 1600 mg) revealed silver grains localized over the nucleus, confirming ^3H -thymidine incorporation into DNA. Although not clear in these reproductions, silver grains were evident over nuclei of 8, 5, 4, and 1 cells in A, B, C, and D respectively.

cells to take up and incorporate ^3H -thymidine or to dilution in intracellular pools. This is in contrast to the two youngest populations in which only light grain density was observed over labelled nuclei (Fig. 7A and 7B). There is a marked decrease in the number of labelled cells with increasing population maturity (Table 4), suggesting that the observed decrease in thymidine incorporation rate (Table 4) was entirely due to the number of cells in S-phase. Indeed the decline in incorporation rate with prolonged incubation likely resulted in the weaker grain intensity in labelled nuclei of young populations, leading to an under-estimation of the proportion of replicating cells in these populations.

The progressive reduction in lysine incorporation rate during population maturation (Fig. 4 and 5, Tables 2 and 3) suggests that there may be an inverse relationship between the rate of protein synthesis and erythroid maturity. If this were so, hemoglobin accumulation would be expected to be correlated with changes in lysine incorporation rate. Figure 8 demonstrates such a correlation, with hemoglobin accumulation paralleling a decrease in lysine incorporation rate. Decreases in thymidine incorporation rate (Fig. 8) also was correlated with the reduction in lysine incorporation rate. These results give supporting evidence that the lysine

Figure 8. Thymidine incorporation and hemoglobin concentration versus lysine incorporation rate.

Decreasing lysine incorporation rates from left to right reflect increasing population maturity.

Thymidine incorporation rate reflects total DNA synthesis within the erythroid population. Relative hemoglobin concentration were estimated from A_{540} of cell lysates after pelleting nuclei.

incorporation rate is in fact directly related to erythropoietic maturation and could be utilized as a quantitative parameter of erythroid maturity.

D. Electrophoretic Characterization

Histones were extracted from isolated erythrocyte nuclei, either totally with 0.25 M HCl or by first selectively extracting lysine-rich histones with 5% PCA, followed by extraction of core histones with 0.25 M HCl. Isolated histones were then characterized by polyacrylamide gel electrophoresis in SDS or acid-urea slab gels and stained with Coomassie blue, as described in Materials and Methods. SDS-gel electrophoresis of histone isolated from unincubated erythrocytes of phenylhydrazine-treated frogs at 12 and 26 days of recovery are shown in Figure 9, along with histones of normal erythrocytes obtained from an untreated animal. Chicken erythrocyte histones were included as standards to which erythrocyte histones of X. laevis could be compared. As seen in Figure 9, frog erythrocyte H1^S is a lysine-rich histone possessing an electrophoretic mobility intermediate between that of H1 and chicken erythrocyte histone H5. H1 and core histones of X. laevis possess electrophoretic mobilities similar to those of chicken erythrocyte histones in SDS. Little difference between histones from populations in the early stages of regeneration (DAY 12) and populations in the late stages

Figure 9. SDS polyacrylamide gel electrophoresis of histones from unincubated erythroid cells X. laevis. Histones were extracted either totally with 0.25 M HCl (lanes 3, 6, and 9) or selectively by first extracting the lysine-rich histones with 5% PCA (lanes 1, 4, 7, and 10), followed by extraction of the core histones with 0.25 M HCl (lanes 2, 5, 8, and 11). Lanes 1 and 2, chicken erythrocyte histones; lanes 3, 4, and 5, *Xenopus* erythrocyte histones at 12 days of recovery. Lanes 6, 7, and 8, *Xenopus* erythrocyte histones at 26 days of recovery. Lanes 9, 10, and 11, histones of normal erythrocytes from untreated X. laevis.

1 2 3 4 5 6 7 8 9 10 11

H1—
H5—

— H1
— H1s

COPIES

of regeneration (DAY 26) or normal histones was evident (Fig. 9). No differences between the histone profiles of nonincubated and incubated erythroid cells were observed (not shown). These electrophoretic patterns are consistent with those reported by Brown et al. (1981) and Destree et al. (1979) for normal cells of X. laevis. Although histone degradation during isolation was not usually a problem, less histone degradation was observed during sequential extractions than during total extraction of histone.

E. Histone Synthesis

Figure 10 compares ^{14}C -lysine incorporated into histones extracted with 0.25 M HCl to thymidine incorporation rate obtained from Regeneration Series II (Table 4). A good correlation between histone synthesis and DNA replication was evident in early to middle stages of erythroid maturation. However at late stages, when DNA synthesis had all but stopped, significant levels of ^{14}C -lysine incorporation into acid-soluble protein persisted. This result suggests a lack of coupling between histone and DNA synthesis in late stages of frog erythropoiesis.

Lysine-rich histone synthesis was also compared to core histone synthesis from Regeneration Series I. In Figure 11 the ratios of PCA-soluble CPM to HCl-soluble CPM is plotted as a function of lysine incorporation rate.

Figure 10. ¹⁴C-lysine incorporation into total histone versus thymidine incorporation rate for Regeneration Series II. After 0.25 M HCl extraction of total histone from ¹⁴C-lysine labelled nuclei from Regeneration Series II (Table 3, Fig. 5), total CPM was determined and plotted against thymidine incorporation rate of each erythroid population (Table 4, Fig. 6) to reflect increasing population maturity from left to right.

Figure 11. Ratio of lysine-rich histone synthesis to core histone synthesis versus lysine incorporation rate, from Regeneration Series I. After 5% PCA extraction of lysine-rich histone from ¹⁴C-lysine labelled nuclei, core histone was extracted with 0.25 M HCl and total CPM calculated for each extract (Table 2). The ratio of PCA/HCl-soluble CPM reflects the rates of lysine-rich histone relative to core histone for each erythroid population. The lysine incorporation rate is used as a quantitative determinant of erythroid maturity and plotted with increasing population maturity from left to right.

Even allowing for TCA-soluble counts in both fractions, the rates of lysine-rich histone synthesis relative to core histone synthesis increased during erythroid maturation. These results also suggest that synthesis of lysine-rich histone is responsible for persistence of histone synthesis in the absence of DNA synthesis during late stages of erythropoiesis (Fig. 10), a fact confirmed below by electrophoretic characterization of the labelled histones.

F. Fluorography of ¹⁴C-lysine-labelled Histones

To examine modulation in synthesis of histone subfractions during erythroid maturation, histone extracts from ¹⁴C-lysine-labelled nuclei were resolved by electrophoretic separation in SDS or acid-urea, and radioactivity detected by fluorography as described in Materials and Methods. Figures 12 through 15 are comprised of the electropherograms and fluorograms of lysine-rich and core histones from Regeneration Series I (Table 2), and Figures 16 and 17 are comprised of the electropherograms and fluorograms of total histone extracts from Regeneration Series II (Table 3). Histone samples for each group of experiments are ordered in the electropherograms according to decreasing lysine incorporation rate, to give a developmental sequence of increasing population maturity from left to right. Histone types in both SDS and acid-urea electropherograms

are identified in accord with Brown et al. (1981).

It is apparent that resolution of X. laevis histones differs electrophoretically in SDS and in acid-urea. Most evident was resolution of erythrocyte H1 into two subfractions H1a and H1b in acid-urea, but differences in core histone mobilities were also observed. More importantly, these two electrophoretic systems allowed satellite H1 to be distinguished conclusively from HMG proteins and putative globin chains, which differ greatly in their relative electrophoretic mobilities in SDS and acid-urea. Thus, combination of SDS and acid-urea electrophoresis allows conclusive identification of histone types.

Figures 12 and 13 show that the vast majority of radioactivity in the PCA-soluble proteins is contained in H1 and H1^S. In addition, SDS electrophoresis (Fig. 12) reveals synthesis in the two earliest populations of nonhistone proteins possessing electrophoretic mobilities greater than H1^S. Staining of the SDS electropherogram with Coomassie blue reveals that these proteins are present in very low quantities, suggesting that they are rapidly synthesized in early stages of erythropoiesis. Identification of these proteins as nonhistone and not degradation peptides of H1 and H1^S, is based primarily on their absence in acid-urea electropherograms (Fig. 13), in which only basic proteins migrate. Besides the expected

Figure 12. SDS gel electrophoresis and fluorography of 5% PCA extracted lysine-rich histone from Regeneration Series I (Table 2). A) electropherogram, B) fluorogram. Lane 1, chicken erythrocyte lysine-rich histone. Lane 2, #16 (day 12); lane 3, #21 (day 10); lane 4, #11 (day 25); lane 5, #17 (day 15); lane 6, #20 (day 20); lane 7, #15 (day 30); lane 8 #19 (day 35); lane 9, #7 (day 85); lane 10, chicken erythrocyte core histone. Approximately equal quantities of histone were applied to each lane, with samples ordered with decreasing lysine incorporation rate from left to right. The fluorogram band at the H1 position in the empty lane adjacent to lane 2 is an artifact due to protein leakage during electrophoresis, which is just visible in the electropherogram. This was not experienced in any other electropherogram of labelled histones. The dark area in the core histone region of lane 4 is extraneous exposure that occurred during X-ray plate development, not radioactivity in this area.

A

1 2 3 4 5 6 7 8 9 10

B

2 3 4 5 6 7 8 9

H1
H1s

CORE

Figure 13. Acid-urea gel electrophoresis and fluorography of 5% PCA extracted lysine-rich histone from Regeneration Series I. A) electropherogram, B) fluorogram. Samples are identical to those described in Figure 12. The very low histone quantities seen in lane 6 of the electropherogram was due to the limited supply of this sample, which was exhausted. H1 is resolved in two subfractions, H1a and H1b, in addition to H1^S.

A

1 2 3 4 5 6 7 8 9 10

H1a
H1b
H1s

CORE

B

2 3 4 5 6 7 8 9

decrease in synthesis of H1^S and H1 with increasing erythroid maturation, it is clear that H1 synthesis ceases before that of H1^S, resulting in the labelling of H1^S in the absence of H1 labelling during late stages of erythrocyte maturation, even though both proteins are present throughout. These apparent differences in H1 and H1^S synthesis is not due to the slight differences of lysine content within these proteins (Brown et al. 1981); any bias in ¹⁴C-lysine labelling would favour H1.

To monitor core histone synthesis in Regeneration Series I, these histones were extracted from the PCA-insoluble residue. Figures 14 and 15 demonstrate that the radioactivity within these extracts was confined almost exclusively to core histone bands. It is evident that core histone synthesis diminishes and finally stops as erythroid maturation progresses. The differences in labelling among the four core histones, (more evident in the fluorogram of Fig. 15) is likely not due to differential synthesis but rather to differences in the respective lysine content of each core histone. The radioactive bands migrating electrophoretically mobilities like the core histones in SDS (Fig. 14B, lane 5) are suspected to be contaminating hemoglobin, absent from acid-urea electropherograms (Fig. 15). Most significant is the loss of core histone synthesis (Fig. 14 and 15, lanes 6 to 10) in erythroid populations which have ceased

Figure 14. SDS gel electrophoresis and fluorography of core histones from Regeneration Series I.

A) electropherogram, B) fluorogram. After extraction of lysine-rich histones, core histones were extracted from the PCA-insoluble residue with 0.25 M HCl (Table 2). Lanes 1 and 2, chicken erythrocyte lysine-rich and core histones respectively; lane 3, #16 (day 12); lane 4, #21 (day 10); lane 5, #11 (day 25); lane 6, #17 (day 15); lane 7, #20 (day 20); lane 8, #15 (day 30); lane 9, #19 (day 35); lane 10, #7 (day 85). Approximately equal quantities of histone were applied to each lane, with samples ordered with decreasing lysine incorporation rate from left to right. Radioactive proteins seen in lanes 6, 7, and 8 and which possess a mobility similar to core histone, are suspected to be contaminating hemoglobin.

22
OF / DE

A

1 2 3 4 5 6 7 8 9 10

B

3 4 5 6 7 8 9 10

H1
H2

CORE

Figure 15. Acid-urea gel electrophoresis and fluorography of core histones from Regeneration Series I. A) electropherogram, B) fluorogram. Samples are identical to those of Figure 14. The protein of mobility similar to H1^S in lane 3 is of unknown origin, although it is evidently not H1^S because of its absence after SDS electrophoresis (Fig. 14). The suspected contaminating hemoglobin observed in Figure 14 is absent in this fluorogram, confirming the nonhistone origin of these radioactive proteins.

B
3 4 5 6 7 8 9 10

A
1 2 3 4 5 6 7 8 9 10

H1a
H1b
H1s

CORE

H1 synthesis (Fig. 12 and 13, lanes 5 to 9), even those in late stages of erythropoiesis in which H1^S synthesis persists (Fig. 12 and 13, lanes 5, 6 and 7).

Correlation between reduction in the rates of H1 and core histone synthesis during erythroid maturation is most evident in Figures 16 and 17, showing total histone extracts of Regeneration Series II. These results confirmed the observations in Series I, namely a progressive decrease in histone synthesis, with continued synthesis of H1^S in the two most mature populations. Because H1^S is synthesized in all populations, it is likely that the observed selective synthesis of H1^S is a result of its sustained synthesis during late stages of erythropoiesis. Radioactive bands possessing mobilities similar to core histone (Fig. 16, lanes 4 and 5) are again suspected to be due to hemoglobin contamination, (as in Fig. 14) because of their absence from this region in acid-urea gels (Fig. 17). Overexposure of the fluorogram of Figure 17 demonstrates that the only histone synthesized in the two most mature populations was H1^S. Furthermore, incubation of these populations with ³H-thymidine (Table 4) indicates not only synthesis of H1^S in the absence of DNA replication, but cessation of H1 and core histone synthesis with cessation of DNA replication.

In conclusion, these fluorograms clearly demonstrate synthesis of H1^S in late stages of erythropoiesis after

Figure 16. SDS gel electrophoresis and fluorography of 0.25 M HCl extracts of total histone from Regeneration Series II. A) electropherogram, B) fluorogram.

Approximately equal quantities of histone were applied to each lane, with samples ordered with decreasing lysine incorporation rate from left to right. Lane 1, #36 (day 10); lane 2, #34 (day 12); lane 3, #37 (day 15); lane 4, #32 (day 20); lane 5, #35 (day 26). Radioactive proteins possessing a mobility similar to core histone seen in lanes 4 and 5 are believed to be due to hemoglobin contamination because of their absence from these regions in acid-urea electropherograms (Fig. 17, lanes 5 and 6).

A

1 2 3 4 5

H1
H1s

CORE

B

1 2 3 4 5

Figure 17. Acid-urea gel electrophoresis and fluorography of 0.25 M HCl extracted total histones from Regeneration Series II. A) electropherograms, B) fluorograms. Lane 1, chicken erythrocyte lysine-rich histones; lane 2, #36 (day 10); lane 3, #34 (day 12); lane 4; #37 (day 15); lane 5, #32 (day 20); lane 6, #35 (day 26); lane 7, chicken erythrocyte core histones. Fluorogram is overexposed to demonstrated the absence of synthesis of any other acid-soluble proteins during late stages of erythropoiesis.

A

1 2 3 4 5 6 7

B

2 3 4 5 6

H1a
H1b
H1s

CORE

DNA replication had ceased. This selective synthesis of $H1^S$ was confirmed using both SDS and acid-urea electrophoresis, of both total histone extracts and 5% PCA extracts of lysine-rich histones. It was also observed that the synthesis of $H1$ and core histone progressively decreased, correlating well with loss in thymidine incorporation into DNA. This suggests that coupling between histone and DNA replication observed earlier (Fig. 10) involves primarily $H1$ and core synthesis, and that loss in correlation of DNA and histone synthesis at late stages is apparently due to continued synthesis of $H1^S$. Figure 12 through 15 are likely the most dramatic examples of using lysine incorporation rate as a quantitative determinant of erythroid maturity. As seen in Table 2, population maturity based on either days of recovery or blood hematocrit would have produced erratic results during analysis of the experimental data. The lysine incorporation rate has thus proved to be a convenient and effective measurement of population maturity.

G. Quantitation of Lysine-rich Histones

If $H1^S$ synthesis persists while $H1$ synthesis stops, one might expect an evident accumulation of $H1^S$ relative to total $H1$ or other histones. To monitor changes in the relative proportions of $H1^S$, 5% PCA-extracted histones were electrophoresed on acid-urea tube gels and

lysine-rich histones quantitated as described in Materials and Methods. These results are presented in Figure 18, in which the fraction of $H1^S$ relative to total lysine-rich histone is plotted as a function of decreasing lysine incorporation rate (ie. relative erythroid maturation). $H1^S$ constitutes only about 12% of the lysine-rich histones early in erythropoiesis and progressively increases during erythroid maturation, reaching a final level of about 31% of total lysine-rich histone in mature erythrocytes. The populations in order of decreasing incorporation rate correspond to the samples in lanes 2, 3, 4, 5, 7 and 9 respectively (Fig. 12 and 13) in which the first two are synthesizing both $H1$ and $H1^S$, the next three much more $H1^S$ than $H1$ (little or none), and the last essentially no histone. Thus accumulation of $H1^S$ is a direct consequence of the continued synthesis of $H1^S$ as $H1$ synthesis declines, and $H1^S$ is found in greatest proportions in populations synthesizing little or no $H1$, core histone and DNA. This has two major implications. First, relative $H1^S$ accumulation occurs mostly after erythroid cells have ceased proliferation. Secondly, synthesis of $H1^S$ in progressively greater relative proportions is likely directly correlated with increases in the amounts of chromatin bound $H1^S$ relative to $H1$. Support for this last conclusion comes from quantitation of lysine-rich histone radioactivity.

Figure 18. Changes in the relative amounts of H1^S during erythroid maturation. PCA extracted lysine-rich histones from Regeneration Series I (Table 2) were electrophoresed in acid-urea tube gels and the lysine-rich histones quantitated as described in Materials and Methods. The relative content of H1^S is expressed as the fraction of total lysine-rich histone and plotted against the lysine incorporation rate with increasing population maturity from left to right. Due to limiting amounts of histone, quantitation of histones from #20 and #19 was not possible.

After protein determination, each gel slice was counted for radioactivity as described in Materials and Methods. Table 5 demonstrates a shift in the proportion of $H1^S$ synthesis during erythropoiesis, confirming the trends observed in the fluorograms.

Table 5
¹⁴C-lysine in Acid-urea Gel Slices

ANIMAL* NUMBER	H1a	H1b CPM/BAND	H1 ^S	%H1 ^S CPM ⁺ (A)	%H1 ^S PROTEIN (B)
#16	12,748	7023	6959	32.3%	11.9%
#21	4420	4671	7666	45.7%	17.0%
#11	339	389	2195	56.6%	22.0%
#17	339	339	2195	75.3%	21.1%
#15	132	157	505	63.6%	30.9%

*for details of cell populations see Table 2; + %H1^S relative to total H1a, H1b and H1^S.

IV. DISCUSSION

A. Erythropoiesis in *Xenopus laevis*

i) Metabolic Changes

In this study nearly complete destruction of preexisting erythrocytes was induced in *Xenopus laevis* by phenylhydrazine treatments. The resulting anemic frogs were found to initiate erythroid regeneration within about 7 days, characterized by release of basophilic erythroblasts into circulation for a limited period (Table 1, Fig. 1 and 2). Mitosis and maturation of the new cohort within the circulatory system produced erythroid populations possessing a high degree of developmental homogeneity, resulting in complete erythrocyte replacement over a period of some 10-20 days. Chegini et al. (1979) demonstrated that the source of erythroblasts observed at early stages was primarily the liver, and found that this release, once initiated, persisted only for a period of one or two days. This ON/OFF mechanism was reported to effectively produce developmentally synchronous erythroid populations similar to those characterized in this study.

In these experiments the rates of ^3H -thymidine incorporation progressively decreased during erythroid maturation, with cell replication ceasing at about the late polychromatic stage of erythropoiesis.

Autoradiography of ^3H -thymidine-labelled cells revealed that loss in the rate of total DNA synthesis was due to a decrease in the fraction of cells in S-phase, likely because of an increase in erythroid generation time similar to that reported in chicken erythropoiesis (Weintraub et al. 1971). It is probable that this increase in generation time gives rise to the observed increase in the degree of developmental homogeneity (Table 1, Fig. 2 and 3), resulting in populations of essentially homogenous erythroid cells 3 to 5 days after initiation of erythrocyte regeneration.

A decline in the rate of ^{14}C -lysine incorporation during erythropoiesis was also observed, reflecting a progressive decrease in the rate of total protein synthesis which is likely related to a decrease in uptake of ^3H -thymidine and ^{14}C -lysine (Tables 2, 3 and 4). Although changes in the rate of RNA synthesis were not investigated in this study, Thomas and Maclean (1975) report the RNA synthesis to decrease and ceases at about the late polychromatic stage of erythropoiesis in X. laevis. However a more definitive study is necessary before a precise understanding of the modulation of RNA synthesis during *Xenopus* erythropoiesis can be established.

ii) Histone Synthesis

Studies into chromatin replication has established that histone synthesis in somatic cells is closely coupled to DNA replication (reviewed by Borun 1975, Elgin and Weintraub 1975, Pederson 1976). This was developed from repeated demonstration that histone synthesis only occurs during S-phase, supported by demonstration that cytoplasmic histone mRNA is present only in cells replicating DNA (Melli et al. 1977, Stein et al. 1978). In addition, inhibition of DNA synthesis with such agents as cytosine arabinoside or hydroxyurea results in rapid inhibition of histone synthesis and degradation of histone mRNA (Gallwitz 1975, Marashi et al. 1982). Recently Groppi and Coffino (1980) have challenged coupling between histone and DNA synthesis, presenting evidence that mouse lymphoma and hamster ovary cells synthesized histones at equivalent rates during both G1 and S-phase. They contend that histones synthesized in G1 remain in the cytoplasm and only become associated with chromatin after the onset of S-phase. However subsequent studies have not supported these conclusions (Delegeane and Lee 1982, Marashi et al. 1982). Although explanations for this discrepancy have been put forward (Wu and Bonner 1981, Delegeane and Lee 1982, Marashi et al. 1982), it is difficult at this time to properly assess the validity or extent of coupling between histone and DNA synthesis. Some clarification has

come however from Wu and Bonner (1981), who propose that histone synthesis consists of two distinct forms, S-phase synthesis and basal synthesis. S-phase histone synthesis, which makes up 90% of total histone synthesis, is closely coupled to DNA replication, being absent during G1 and inhibited by hydroxyurea treatment. Basal histone synthesis occurs during both G1 and S-phase and is insensitive to hydroxyurea treatment, amounting to about 10% of S-phase histone synthesis. Contrary to the results of Groppi and Coffino (1980), basal histone synthesis makes up only a small fraction of total histone synthesis, and basally synthesized histones become rapidly associated with chromatin (Wu and Bonner 1981).

Tarnowka and Baglioni (1978) have reported lysine-rich histone synthesis in G1 by BHK cells, using synchronized cell populations. However, due to S-phase cell contamination they were only able to demonstrate quantitative increases in the proportion of H1 synthesis in G1-synchronized cell populations. More definitive studies of uncoupled lysine-rich histone synthesis comes from studies employing hydroxyurea inhibition of DNA replication. Ruiz-Carrillo et al. (1976) report that H5 synthesis in immature duck erythroid cells is insensitive to hydroxyurea treatment, unlike H1 and core histone synthesis. Zlatanova (1980) report similar results for H1⁰ synthesis, with hydroxyurea treatment of Friend cells

resulting in the inhibition of H1 and core synthesis but having no effect on H1^O synthesis. These results suggest that like the basal histone synthesis characterized by Wu and Bonner (1981), chicken H5 and mammalian H1^O synthesis is not coupled to DNA replication.

The parallel reduction of H1 and core histone synthesis with DNA replication, observed herein during erythropoiesis in *X. laevis*, suggests but does not prove coupling between these processes. This is in contrast to the continued synthesis of frog H1^S after cessation of erythroid replication. It is unlikely that this selective synthesis of frog H1^S can be attributed solely to the small number of S-phase erythroid cells observed during autoradiographic analysis after ³H-thymidine labelling, but rather that it is due to synthesis of frog H1^S in cells inactive in DNA replication. This suggests that synthesis of frog erythrocyte H1^S is independent of DNA replication, as has been shown for H5 and H1^O (Ruiz-Carrillo et al. 1976, Zlatanova 1980). This probably accounts in large part for accumulation of these histones in the nuclei of nonreplicating cells.

B. Are H1^O and H5 Functionally Related Histones?

Homology in primary sequence (Smith et al. 1980, Pehrson and Cole 1981) and tertiary structure (Cary et al. 1981) of the central domains, immunological homology (Mura

and Stollar 1981), relative increases during decreased cell replication (Sung et al. 1977, Weintraub 1978, Urban et al. 1980, Pehrson and Cole 1980), and insensitivity of synthesis to hydroxyurea (Ruiz-Carrillo et al. 1976, Zlatanova 1980), all present compelling evidence for functional homology between H5 and H1^O. Despite these facts, the supposed erythrocyte-specificity of H5 implies that it possesses a function specific to the nucleated erythrocyte. This, combined with sequence differences in the terminal ends of H5 and H1^O (Cary et al. 1981), challenges functional homology and has led to the suggestion they may possess separate and distinct functions (Smith and Johns 1980b, Smith et al. 1981). Unfortunately it is not possible to study mammalian H1^O function directly in the erythrocyte, due to the fact that mammals do not possess nucleated erythrocytes. However, characterization of a non-erythrocyte-specific, H1^O-like histone in the erythrocytes of frogs (Brown et al. 1981, Shimada et al. 1981) presents the possibility to study metabolic characteristics of this histone during erythropoiesis, and allows definitive evaluation of functional homology with avian H5. The study conducted for this thesis revealed that frog H1^S possesses metabolic characteristics very similar to chicken erythrocyte H5, progressively accumulating during erythrocyte maturation (Fig. 18). Most convincing was the demonstrated selective

synthesis of Xenopus H1^S in the absence of DNA replication during the final stages of erythropoiesis, which has until now been demonstrated only for H5 during avian erythropoiesis.

H1^O accumulation in a variety of non-erythroid cell types during decline in the rate of cell proliferation has suggested its synthesis outside of S-phase (Pehrson and Cole 1980), but this is the first direct demonstration of synthesis in the absence of DNA synthesis of a satellite H1 not specific to the erythrocyte. Although it is not possible at this time to demonstrate conclusively, it is unlikely that erythrocyte association of frog H1^S is attributable for its selective synthesis. Rather, synthesis outside of S-phase is probably characteristic of all H1 satellites, irrespective of cell type, including H1^O and H5 in mammalian and avian cells. In conclusion, these results present evidence that H5, H1^O and frog H1 are functionally homologous histones, sharing common structural and metabolic characteristics that are distinct from H1, justifying their classification into a new sub-class of lysine-rich histone called satellite H1 or H1^S.

C. Characterization of Satellite H1

From the information presented in this thesis, several structural and metabolic characteristics can be

described for H1 satellites. Mammalian H1^O was first recognized by Panyim and Chalkley (1969a, 1969b) and Panyim et al. (1971) using polyacrylamide gel electrophoresis, in which H1^O possesses an electrophoretic mobility slightly greater than H1. H1^S of turtles (Rutledge et al. 1981) and frogs (Brown et al. 1981, Risley and Eckhardt 1981) possess mobilities very similar to H1^O. Electrophoretic mobility of H5 from various species of fish differ considerably (Miki 1976, Miki and Neelin 1975, 1977a, 1977b), possessing mobilities greater than H1 but less than H3. H1t is a mammalian testis-specific histone (Seyedin and Kistler 1980, Seyedin et al. 1981), with a slightly greater mobility than H1^O in SDS, but identical to H1 in acid-urea. It should be noted that this is the first example of a lysine-rich histone possessing greatly differing mobilities in SDS and acid-urea.

Although electrophoretic mobility is valuable for tentative identification of H1 satellites, it can by no means provide a conclusive identification. Rather it is necessary to isolate in pure form and obtain amino acid composition, for reliable identification of a satellite H1. H1 satellites have been purified by ion-exchange chromatography eluted with guanidinium chloride, or by gel filtration in dilute acid. H1^O was first isolated from calf lung using Amberlite CG-50 cation-exchange resin

(Panyim and Chalkley 1969a), which was also used to purify turtle (Rutledge et al. 1981) and frog (Brown et al. 1981) erythrocyte and liver Hl^S, and fish erythrocyte H5 (Miki 1976, Miki and Neelin 1975, 1977a, 1977b). Bio-Rex 70 cation-exchange has been used to isolate bullfrog erythrocyte Hl^S (Shimada et al. 1981), bovine liver Hl^O (Smith and Johns 1980a), chinese hamster ovary Hl^O (D'Anna et al. 1980b, 1981), and rat testis Hlt (Seyedin and Kistler 1980). Gel filtration using Bio-Gel P-100 has proven more effective in isolating Hl satellites and has been used to purify turtle (Rutledge et al. 1981) and frog (Brown et al. 1981) erythrocyte and liver Hl^S, mouse and rat Hl^O (Medvedev and Medvedeva 1980b), mouse neuroblastoma Hl^O (Pehrson and Cole 1980), chicken H5 (Sung et al. 1977) and mammalian testis Hlt (Seyedin et al. 1981).

Amino acid analysis of Hl satellites has revealed similar trends in amino acid content (see also Hnilica 1967), in that relative to Hl, all Hl satellites so far analyzed possess a lower content of alanine and lysine, and higher content of serine, arginine, aspartic acid, histidine and isoleucine. In addition, all contain at least one methionine residue which is absent in Hl (Smith and Johns 1980a), so that Hl satellites may be cleaved selectively into a small and large fragment by treatment with cyanogen bromide (D'Anna et al. 1981).

Physical characterization of H1 satellites has proven valuable in demonstrating structural homology within this sub-class of lysine-rich histone, although it does not provide direct evidence for functional homology, and may not provide a conclusive identification. However, due to the distinct metabolic characteristics of satellite H1, a conclusive demonstration of functional homology and identification of H1 satellite can be obtained from metabolic studies. The most distinctive characteristic is independence of H1 synthesis from that of DNA replication. This is characterized by synthesis of H1^S outside of S-phase, as for example during the late stages of erythropoiesis. As well, the insensitivity of H1^S synthesis to inhibitors of DNA replication, such as hydroxyurea, provides a simple and rapid method for identifying H1 satellites. Increases in the relative amount of H1^S during decreases in the rate of cell metabolism most commonly associated with G1-arrest, is another characteristic which should prove valuable in identifying H1 satellites. Finally, a report that chicken erythrocyte H5 mRNA is polyadenylated, unlike H1 or core histone mRNA (Molgaard et al. 1980), suggests that polyadenylated mRNA is another distinctive characteristic of satellite H1.

D. $H1^S$ Function

i) Is $H1^S$ a DNA Replication Inhibitor?

$H1$ satellites have been characterized in all major species of higher eukaryotes, including fish, reptiles, amphibians, avians, and mammals. Thus satellite $H1$ likely evolved prior to, or early in vertebrate evolution, with the evolutionary conservation of these histones suggesting they play an important but presently uncharacterized role in chromatin function. Because of the demonstrated role of lysine-rich histones in controlling and maintaining higher-order chromatin structure and the implications of chromatin structure in regulation of gene expression, it is of great interest to elucidate the precise function of satellite $H1$ and its relationship to regulation of gene expression.

The observation of Panyim and Chalkley (1969a) that mammalian tissues of low proliferative activity also possess highest content of $H1^O$ led to the suggestion that $H1^O$ functions as an inhibitor of DNA replication (Marsh and Fitzgerald 1973, Smith and Johns 1980b, Smith et al. 1981). If satellite $H1$ is involved in inhibiting DNA replication, changes in its relative content would be expected to correlate with changes in the rates of DNA replication. The few studies that have directly examined this question report little consistent correlation. Marsh

and Fitzgerald (1973) observed a progressive decrease in the relative amounts of $H1^O$ during initial stages of pancreas degeneration, but no evidence of an increase in the rates of DNA synthesis. The rates of DNA synthesis increased significantly only after $H1^O$ had reached its minimum level, and the relative content of $H1^O$ changed very little if at all during regeneration despite large changes in the rates of DNA replication. Initiation of $H1^O$ accumulation occurred only after DNA replication had all but ceased, and it continued to accumulate long after DNA synthesis had stopped all together.

The continued accumulation of satellite $H1$ after cessation of DNA replication was also observed by Varricchio (1977) during the postnatal accumulation of $H1^O$ in rat pancreas. These observations are consistent with accumulation of frog $H1^S$ during erythropoiesis in X. laevis which was most extensive after cessation of DNA replication (Fig. 12, 13 and 18). Similar results were obtained by Pieler et al. (1981) during studies into chemically induced differentiation of murine neuroblastoma cells. These reports not only show satellite $H1$ accumulation after cessation of DNA replication, but that accumulation occurs to a much greater extent in the absence of DNA replication, as though cell replication in fact inhibits satellite $H1$ accumulation. Thus a direct

relationship between $H1^S$ content and the rate of DNA replication has not been established, and suggests that $H1$ satellites do not function to inhibit DNA replication.

ii) $H1^S$ in Chromatin

The metabolic characteristics of chicken erythrocyte H5, mammalian $H1^O$, and frog erythrocyte $H1^S$ illustrate that satellite $H1$ likely possesses a function distinct from $H1$. However, when the molecular functioning of lysine-rich histones is examined, it appears that satellite $H1$ behaves at a molecular level in a manner very similar to $H1$. Major support for this conclusion comes from Allan et al. (1981), in which fragmented chicken erythrocyte chromatin depleted of lysine-rich histones was reconstituted with either purified chicken erythrocyte $H1$, calf thymus $H1$, or chicken erythrocyte H5 at various lysine-rich histone to nucleosome ratios. This was followed by several experiments to investigate structural characteristics of the reconstituted chromatin.

In all experiments, similar results were obtained irrespective of the type of lysine-rich histone used in reconstitution. The relative extent of chromatin condensation, as measured by sedimentation analysis and DNA compaction ratio, was found to be directly dependent on the number of lysine-rich histones per nucleosome, but not upon the type of lysine-rich histone. Although minor

differences were in fact evident in the micrococcal cleavage patterns of linker DNA complexed with the various lysine-rich histones, they all showed a 10.4 bp periodicity in linker DNA. From this it was concluded that variations in micrococcal cleavage pattern was a result of differences in the frequency of cleavage, not in the presence or absence of cleavage sites directly. Therefore chicken H5, one of the most divergent of all H1 satellites, likely participates in chromatin structure very similar to that of H1.

Studies into the chromatin binding characteristics of chicken H5 have in fact revealed some differences relative to H1 (Kumar and Walker 1980, Lasters et al. 1981), but these differences apparently do not effect their role in chromatin structure in vitro. Support for the functional similarity of H1^o and H1 comes from the study of Smith and Johns (1980b), who used micrococcal nuclease digestion of chromatin to show that H1^o, like H1, is associated with the nucleosome linker. Cary et al. (1981) also demonstrated that while H1^o and chicken H5 share tertiary structural characteristics that differ from H1, they still possess significant structural homology with H1, consistent with the demonstrated primary sequence homologies between H1 and H5 (Yaguchi et al. 1977, 1979). Although it is necessary to test other types of satellite H1 before a definitive conclusion can be made,

it appears that lysine-rich histones do not possess large functional differences with respect to their role in condensation of chromatin. This leads to the conclusion that H1^S metabolism is likely related to initiating the functional effects of satellite H1, and not necessarily differences in its role in chromatin structure relative to H1.

iii) The Mechanism and Potential Effects of H1^S
Accumulation

On consideration of the mechanism of satellite H1 accumulation, it is clear that cellular control of H1^S synthesis must play a central role in this accumulation. That is, independent control of H1^S synthesis must be critical in allowing the freedom necessary to initiate changes in H1^S content independently from synthesis of DNA and other histones. This likely involves specific transcriptional and translational controls, as well as presently unknown mechanisms for H1^S transport to and assembly into nuclear chromatin. One testable aspect, which has major implications in evaluating the potential effects of satellite H1 accumulation, is the possible replacement of chromatin-bound H1 by H1^S. This would be reflected by changes in total lysine-rich histone content during H1^S accumulation.

In this regard Smith and Johns (1980b) may have

confused functional interchangeability in mature tissues with progressive accumulation during development. Specifically these investigators claimed that the relative content of $H1^O$ in various mouse tissues varied, while the total lysine-rich histone content ($H1+H1^O$) remained constant. From this they concluded that $H1^O$ "replaces" $H1$ in chromatin. They further make the erroneous conclusion the the results of D'Anna et al. (1980a) contradict this proposal, in that they report $H1^O$ accumulation without loss of $H1$ content. The apparent source of confusion by Smith and Johns (1980b) likely stems from their lack of appreciation that an invariant lysine-rich histone content of developmentally mature tissues implies only a interchangeability of $H1$ by $H1^S$. This concept is clearly presented by Miki and Neelin (1977a, 1980) who found constant lysine-rich histone content in various species of nucleated erythrocytes, despite large, reciprocal variations in $H5$ and $H1$ content.

That $H1^S$ accumulation does not involve replacement of $H1$ is further supported by Marsh and Fitzgerald (1973) who found no change in the relative amounts of $H1$, despite large changes in $H1^O$ content during the regeneration of rat pancreas. Weintraub (1978) and Urban et al. (1980) have also reported no change in the relative content of $H1$ during the $H5$ accumulation of chicken erythropoiesis. Ruiz-Carrillo et al. (1974) report extensive increase in

H5 content with little or no change in H1 content during duck erythropoiesis. Thus it appears that accumulation of satellite H1 may not be accompanied by a loss in H1 content. If so, it could be proposed that the effect of H1^S accumulation is to initiate an increase in the total lysine-rich histone content of chromatin. It should be noted that in this thesis project the quantitative relationship of H1^S or total lysine-rich histone to DNA or core histones during X. laevis erythropoiesis could not be precisely measured, due to difficulty in quantitative extraction of histones.

The potential effect of variation in the content of lysine-rich histone on modulating chromatin structure is clearly illustrated by Allan et al. (1981) who demonstrated that the extent of chromatin condensation is directly dependent on the stoichiometry but not the type of lysine-rich histone. Due to the potential effects of chromatin structure in regulating gene expression, changes in the lysine-rich histone content of chromatin has major implications for changes in vivo in the template activity of chromatin. Allan et al. (1981) show that each nucleosome possesses two lysine-rich histone binding sites, with chromatin reaching its greatest condensation at about 2 linker histone molecules per nucleosome. This is supported by the findings of Nelson et al. (1979), which reveal that exogenous H1 can be added to calf thymus

chromatin up to a total lysine-rich histone content of two molecules per nucleosome core. More importantly, Allan et al. (1981) demonstrate that reconstituted chicken erythrocyte chromatin, containing two lysine-rich histones per nucleosome core, possesses a structural integrity indistinguishable from native chicken erythrocyte chromatin. Furthermore they propose that increases in lysine-rich histone content of chromatin are associated with decreases in DNA template activity in vivo. This is partly supported by the observation of somewhat less than two but more than one molecule of lysine-rich histone per nucleosome in avian erythrocytes (Urban et al. 1980, Bates and Thomas 1981). Thus the genetic inactivity of the erythrocyte nucleus may be a result of maximum chromatin condensation induced by satellite H1 accumulation.

That the nucleosome in vivo possesses two lysine-rich histones has been challenged by Bates and Thomas (1981), who conducted histone quantitation studies of several tissues including chicken erythrocyte chromatin. They conclude that, except in chicken erythrocytes, the H1 stoichiometry is close to one molecule per nucleosome in all tissues studied. Their observation that erythrocyte chromatin possesses only 1.3 molecules of lysine-rich histone per nucleosome is in contradiction to the conclusions of Allan et al. (1981), and to the proposal that condensation of erythrocyte chromatin is induced by

binding of lysine-rich histone to the saturation level of about two lysine-rich histones per nucleosome. This discrepancy could be resolved in part by the possibility that other nuclear proteins that are presently not taken into consideration, bind to the same site as the lysine-rich histones. One such type are HMGl and 2, which have been shown by micrococcal nuclease studies to be associated with the nucleosome linker in a manner similar to H1 (Levy-Wilson et al. 1977,1979), and likely substitute for lysine-rich histones on the linker DNA (Jackson et al. 1979, Seyedin and Kistler 1979). Thus at present, interpretations concerning comparisons of in vivo and in vitro studies of histone stoichiometry must be viewed with some reservation. In addition, despite the fact that Bates and Thomas (1981) claim consistency of one lysine-rich histone per nucleosome in vivo, their data reveals that the total lysine-rich histone content per nucleosome core ranges from as little as 0.79 in rat liver chromatin to as much as 1.32 in chicken erythrocyte chromatin. Variations in the lysine-rich histone content of different tissues have also been demonstrated by Wright and Olins (1975), and Unger-Williams and Modak (1979). Thus it is apparent that the total lysine-rich histone content varies significantly from tissue to tissue. The concept of variable chromatin content of linker histone in vivo is discussed in detail by Altmann et al. (1981), who

believe it to be an intrinsic feature of chromatin.

If chromatin possesses less than a full complement of lysine-rich histone, there likely exist chromatin areas lacking lysine-rich histone. Mathis et al. (1980) discuss evidence for a lack of tertiary structure in actively transcribing areas of chromatin, which could reflect a deficiency of lysine-rich histone in these areas. This is supported by histone quantitation studies, that actively transcribing chromatin is deficient in lysine-rich histone (Mathis et al. 1980). In addition Gabrielli et al. (1981) recently found active chromatin to possess no HMG1 and HMG2, and only 72% of the lysine-rich histone content of non-transcribing chromatin. This suggests that if nucleosomes deficient in lysine-rich histone do exist in vivo they likely are associated with areas of actively transcribing chromatin.

A simple model can be presented in which chromatin could be envisaged to possess two lysine-rich histone binding sites (LHB sites). Each LHB site could potentially bind either H1 or H1^S. These LHB sites could also be unoccupied or could bind other linker proteins such as HMG1 and HMG2. Presently available information suggests that it is the number of LHB sites bound by lysine-rich histone that directly dictates such parameters as chromatin condensation and DNA template activity (Allan et al. 1981). From this it could be proposed that

it is the total lysine-rich histone content of chromatin that is functionally significant and not the relative content of H1^S per se. This indicates that the very characteristic which resulted in the recognition of H1 by Panyim and Chalkley (1969a), the tissue-specific differences in the relative content of H1^O, may not be as functionally important as is now believed. This might also explain the observation of Rutledge et al. (1981) and Brown et al. (1981) that turtle and frogs respectively possess greater relative amounts of H1^S in liver than in erythrocytes, even though the latter are even less capable of DNA synthesis. Specifically, it is likely not the content of H1^S that reflects tissue-specific differences, but rather it is the total lysine-rich histone content that is functionally important. Thus, the low proliferative and metabolic activity of erythrocyte chromatin as compared to liver chromatin, may reflect greater total lysine-rich and not H1^S content.

Although the proposal that satellite H1 accumulation initiates changes in the transcriptional activity of chromatin is largely speculative, evidence concerning the role of lysine-rich histone in controlling tertiary chromatin structure strongly supports such a function for H1^S accumulation. Further support for this concept comes from studies of changes in chromatin structure during chicken erythropoiesis which suggest that H5 plays a role

in nuclear condensation (Kernell et al. 1971, Billet and Barry 1974, Brasch et al. 1974). Although the precise contribution of H5 accumulation to nuclear condensation is of question (Dardick and Setterfield 1976, Brasch et al. 1977), nuclear condensation is likely involved in the transcriptional inactivity of erythrocyte chromatin (Seligy and Neelin 1970, Seligy and Miyagi 1974). In addition Darzynkiewicz et al. (1981) report changes in chromatin structure using flow cytometry during n-butyrate induced differentiation of mouse murine leukemic cells, as well as during induction of cell quiescence observed in a great variety cell types (Darzynkiewicz et al. 1980), during which a progressive loss in cellular RNA content is observed. Based on our understanding of H1^S function it seems probable that changes in H1^S content plays a role in these changes in RNA content and chromatin structure associated with modulation of cellular proliferation and metabolism.

This model does not explain tissue- and species-specific differences in satellite H1' content relative to H1. For example, Miki and Neelin (1977a, 1980) and Urban et al. (1980) report very large differences in the H5 to H1 ratio of mature erythrocytes of primitive and definitive cell lines, despite a constant total lysine-rich histone content (H1+H5). It is likely that two factors are involved in establishing satellite H1

to H1 ratio: a) the fraction of LHB sites bound by H1, and b) the total number of LHB sites bound by linker proteins which may be related to the template activity of chromatin. The content of H1 relative to core histones is likely established during cell determination, and is independent of nuclear activity. This is developed from the invariant content of H1 during satellite H1 accumulation, combined with tissue-specific content of H1. Demonstration that chromatin condensation is related to lysine-rich histone content and the proposed dependency of DNA template activity on chromatin structure suggests a relationship between transcriptional activity and lysine-rich histone content. In addition, that tissues likely possess characteristic levels of transcriptional activity indicates that lysine-rich histone content of chromatin is indirectly determined during tissue differentiation. Therefore, $H1^S$ to H1 ratio is likely dependent on presently uncharacterized but genetically determined factors which dictate H1 content and transcriptional activity of chromatin.

iv). Control of $H1^S$ Accumulation

It is apparent that $H1^S$ accumulation can be initiated under a variety of conditions. These include genetically programmed maturation of tissues such as during erythropoiesis, tissue regeneration, or ontogeny. As well

it can be induced in cultured cells using various chemicals such as DMSO, or by serum and amino acid deprivation. Under all of these conditions two common effects are seen: 1) decrease in cellular metabolism and 2) slowing and final cessation of cell proliferation associated with G₁-arrest and establishment of G₀.

It is important to note that decreases in the rate of protein synthesis in cultured cells has been found to occur under similar conditions in which accumulation of Hl^S is initiated. Specifically, Jagus et al. (1981) report decreases in protein synthesis during amino acid and glucose deprivation, as well as during increased cell density. It is likely that a similar mechanism is employed in the erythroid cells to implement reduction in the rates of protein synthesis during erythropoiesis. However, it is apparent that Hl^S accumulation is not directly involved in the loss of protein synthesis, in that it has been established that protein synthesis is regulated via control over the initiation of mRNA translation, but not on RNA synthesis directly (Ochoa and de Haro 1979, Ochoa 1979, Jagus et al. 1981). Thus it can be proposed that the control over cellular metabolic rates involves a process which initiates related but independent effects on protein, RNA, and DNA synthesis, and that associated changes in satellite Hl content is a response to but does not necessarily initiate changes in cellular

metabolic rates.

Although the precise role of developmental appearance and accumulation of satellite H1 is unknown, it may be accompanied by but not necessarily involved in the initiation of new proliferation and metabolic controls during differentiation and developmental maturation of tissues. This is derived from reports that within multicellular organisms, a G1 period appears to be absent in cells that are engaged in very rapid proliferation, a phenomenon which is particularly evident during early embryogenesis. Prescott (1976) reports that in most tissues, a G1 period is introduced in the course of tissue differentiation and development and is accompanied frequently by increases in the length of the cell cycle, as the rate of cell proliferation slows. This suggests that at some point during embryonic differentiation and development, new proliferative and metabolic controls are initiated during establishment of G1. That G1 in differentiated tissues is a developmentally established process, is supported by the observation that the G1 period continues to be present in cultured cells growing in rich media. Prescott (1976) suggests that even cells freed of any environmental restriction on growth must overcome in each cell cycle an inherent tendency to be transiently blocked in G1, and that this phenomenon is directly related to the control of cell proliferation via

G1-arrest.

This comes from the proposed control of cell proliferation by inhibition of S-phase initiation which normally occurs in G1 (G1-arrest), and implies that establishment of control over cell proliferation is dependent on the establishment of G1. (Prescott 1976, Pardee et al. 1978). I propose that establishment of G1-arrest is directly related to the developmental appearance of satellite H1. However, although satellite H1 is associated with G1-arrest, it is unlikely that this association is related to the loss of cell proliferation. Rather, it is likely that satellite H1 is associated with cellular control of the transcriptional activity of the nucleus, such that satellite H1 accumulation is directly related to the losses in DNA template activity during initiation of G1-arrest and establishment of G₀.

The observed developmental appearance and accumulation of satellite H1 during tissue maturation, might suggest a possible role in cellular differentiation. However, from the limited information available it can be tentatively concluded that H1 satellites are probably not involved in cellular determination directly. That H1 satellites and their accumulation has been found to occur in a vast variety of tissue and cell types, under both in vitro and in vivo conditions, suggests a non-specific, possibly universal function for these histones. The

extensive cell specificity generally thought necessary for such a function, has not been demonstrated for the H1 sub-class of lysine-rich histones. In addition, H1^S accumulation has also been demonstrated in at least one cell type to be reversible (Pieler et al. 1981). All of these facts suggest that satellite H1 does not play a direct role in cell differentiation.

E. Future Considerations

i) Tissue Specificity of Satellite H1

The potential for satellite H1 to be directly involved in control of chromatin activity suggests that satellite H1 plays an important role in function of the eukaryotic nucleus. In view of this, consideration of tissue specificity of these histones presents two important aspects for future investigation. The first involves elucidating the role of their structural diversity, such as seen in the supposed erythrocyte-specific H5 of birds and fishes, which are the most structurally divergent of all H1 satellites so far characterized. Seyedin and Kistler (1980) and Seyedin et al. (1981) have also reported the isolation of a mammalian testis specific histone H1t, consisting of 10% serine, 7.2% arginine, 15% alanine, and 1.5% methionine, remarkably like chicken H5. It is possible that H1t may

reflect an extreme variant of H1^o, possessing presently unknown specialized functions related somehow to testis function. It is important to note that the study of Allan et al. (1981) has shown that although chicken H5 has diverged in structure, its role in initiating chromatin condensation has not, at least with the present experimental resolution. Thus at present, the possible specialized function resulting from the structural divergence of H5 and H1t can not be evaluated.

A second important question to be considered is the universal presence of H1^s in higher eukaryotic cells. This brings forward the question, if H5 is in fact erythrocyte-specific, do the non-erythroid tissues of these animals possess other H1 satellites? Examination of chicken liver histones by Smith and Johns (1981) revealed no histone species identifiable as H1 other than H5. Miki (1976) also observed small amounts of H5 in liver histone preparations from several species of fish proposed to possess erythrocyte-specific H5. However, due to the high relative content of H5 in erythrocytes (about 80% of the total lysine-rich histones in chicken erythrocytes) presence of H5 in liver preparations has been assumed to be due to erythrocyte contamination. This poses a difficult technical problem in establishing whether or not H5 is in fact erythrocyte-specific in these animals. Partial resolution of this problem comes from a study done

by Block and Atkinson (1979) in which quail myogenesis was studied in vitro. These authors report the appearance of a histone of similar electrophoretic mobility to H5 during myogenesis in vitro where erythrocyte contamination during histone preparation seems unlikely. In addition the observed increase in amount of this histone during myogenesis also suggests its source to be non-erythroid. This may be the first conclusive report of the presence an avian H5 histone in a non-erythroid tissue. Gordon et al. (1980) also report accumulation of an H5-like histone in differentiating chicken skeletal muscle. Further evidence which suggests chicken H5 is not erythrocyte-specific comes from immunofluorescent studies of chicken tissues using antibodies specific to H5. Specifically Mura et al. (1982) observed chicken liver nuclei to produce significant fluorescence with anti-H5, suggesting the presence of H5 in chicken liver.

From this, it is apparent that the erythrocyte specificity of H5 must be reexamined in order to establish the existence of satellite H1 in the non-erythroid tissues of birds and fish. Investigation into the existence of satellite H1 in other types of eukaryotic cells, such as in lower eukaryotes, or of the existence of other histone types that may fulfill the functional role played by H1^S, will also be an important area of investigation in the establishment and elucidation of the precise role played

by these histones in the eukaryotic cell.

ii) Are HMG Proteins Functionally Related
to the Lysine-rich Histones?

Another important consideration, is the possibility that other nuclear proteins are functionally related to the lysine-rich histones. For example the high mobility group (HMG) proteins possess characteristics similar to the lysine-rich histones, suggesting a possible functional relationship. Unfortunately, little definitive research has been directed at elucidating either the metabolic characteristics of HMG proteins, or of their role in chromatin structure and function. Therefore, evaluation of a possible functional relationship between the HMG and histone proteins is difficult to establish at present. Despite this, the limited research into the HMG proteins reveals several important facts, that have tremendous implications for the future of research into chromatin function.

HMGs are a group of PCA-soluble proteins containing 25% basic and 30% acidic amino acid residues, first characterized in detail by Goodwin et al. (1973) and named for their high mobility in polyacrylamide gels. Like the histones, they possess very limited cell specificity (Rabbani et al. 1978a), and they are postsynthetically modified (Sterner et al. 1978b, Levy-Wilson 1981, Linnala-Kankkunen and Maenpaa 1981, Bhorjee 1981). Like

the lysine-rich histones, the HMG proteins have been shown to consist of several sequence variants, which differ significantly in their iso-electric points (Rabbani et al. 1978a,b; Brown et al. 1980).

The HMG proteins consist of four major types, HMG1, 2, 14, and 17. They can be considered to consist of two major functional groups, HMG1 and 2 being functionally related to each other, as are HMG14 and 17. Examination of the physical and functional characteristics, demonstrates that HMG14 and 17 bind to the nucleosome core (Mardian et al. 1980) near the linker DNA, possibly interacting with about 15 bp of linker DNA (Goodwin et al. 1979). All four have been found associated with actively transcribed chromatin (Goodwin et al. 1979) with HMG14 and 17 likely involved in establishing the DNaseI sensitivity of active chromatin (Weisbrod and Weintraub 1979). However, due to their association with the nucleosome core, and because they show no functional homology with the lysine-rich histones, HMG14 and 17 will not be discussed in detail, despite the fact that they obviously play a very important role in chromatin metabolism, perhaps in the binding of lysine-rich histones to the nucleosome (Goodwin et al. 1979).

Unlike HMG14 and 17, HMG1 and 2 do show functional similarities with the lysine-rich histones. Although HMG1 and 2 possess little if any sequence homologies with the

lysine-rich histones (Walker et al. 1980), they do possess basic amino acid residues clustered to the N-terminal section of the protein, thought to be involved in DNA binding (Cary et al. 1976), and they possess a molecular weight of 26,000 (Goodwin et al. 1975). HMG1 and 2 possess great evolutionary conservation in their primary and tertiary structures (Bustin et al. 1978, Romani et al. 1979) similar to that displayed by histone H3 and H4. This suggests that their biological function is dependent on stringent structural requirements, consistent with their proposed role as structural proteins, being present in approximately one million molecules per nucleus (Romani et al. 1979), corresponding to as much as 10% of the mass of lysine-rich histones. Nuclease probing has shown HMG1 and 2 to be associated with transcribed DNA sequences (Levy-Wilson et al. 1977, 1979, Georgieva et al. 1981), although this has been challenged by the results of Gabrielli et al. (1981). HMG1 and HMG2 bind to the linker DNA (Levy-Wilson et al. 1977, 1979) possibly substituting or replacing lysine-rich histones (Jackson et al. 1979, Seyedin and Kistler 1979). They form homopolymers on treatment of intact chromatin with bifunctional reagents (Itkes et al. 1980), suggesting that chromatin arrangement of HMG1 and 2 is similar to that of lysine-rich histones. Thus it is apparent that HMG1 and 2 possess similar structural and functional characteristics to the

lysine-rich histones. Further evidence that HMG proteins may be functionally related to the H1^S histones comes from research into HMG metabolism.

Kuehl (1979) investigated synthetic characteristics of HMG proteins during rat liver regeneration and found that, like H1^S, HMG synthesis is not coupled to DNA replication. This was supported by the demonstrated insensitivity of HMG synthesis to hydroxyurea inhibition of DNA synthesis, unlike the H1 and core histone synthesis, which were reduced by more than 50%. The similarity of HMG metabolism with H1^S, is further developed from the demonstration that HMG mRNA is polyadenylated (Bustin et al. 1981), as is chicken H5 mRNA (Molgaard et al. 1980). Modulation in the relative amounts of HMG1 and 2 during tissue development have also been reported by Seyedin and Kistler (1979), Gordon et al. (1981), and Teng and Teng (1981). These results then show a potential functional relationship between the satellite H1 and HMG1 and 2.

✓ Definitive evidence for this comes from Seyedin and Kistler (1979) who demonstrated a reciprocal relationship between HMG2 and H1^O. Specifically, they found that the relative content of HMG2 paralleled the proliferative activity of several rat tissues and chicken skeletal muscle. Thus rat tissues possessing high levels of proliferative activity also possessed high levels of HMG2

and low levels of H1⁰. This inverse relationship between HMG2 and H1^S was further demonstrated by monitoring changes in the relative levels of HMG2 during loss of cell proliferation. The HMG2 content of adult rat testis was found to decrease dramatically during loss of proliferation, by surgical confinement to the abdomen. A similar loss in chicken HMG-E, a homologue of HMG2, was found during loss of proliferation accompanying maturation of chicken skeletal muscle. Thus, HMG2 apparently possesses a dynamic metabolic character, similar but inversely related to the H1^S histones. It is also very important to note that the relative levels of HMG1 did not show marked variability during loss in cell proliferation. The significance of this observation will be made apparent later. These results then present evidence for a possible functional interrelationship between H1^S and HMG2.

Evidence for direct molecular interactions comes from several studies directed at evaluating the interaction of HMG1 and 2 with the lysine-rich histones in solution. Specifically, HMG1 has been found to form complexes in solution with H1. These complexes have been studied using sedimentation analysis (Shooter et al. 1974, Smerdon and Isenberg 1976, Cary et al. 1979), NMR spectroscopy (Cary et al. 1979) and affinity chromatography (Yu and Spring 1977). From these studies has been proposed that H1 variants interact in a specific manner with HMG1 (Smerdon

and Isenberg 1976, Yu and Spring 1977), although Cary et al. (1979) claim lack of specificity, suggesting that the observed interaction of HMGl with Hl may be artifactual. Although, it is clear that further work is necessary before the specificity of interaction between these two proteins is established, it is also equally apparent that a lack of specificity has also not been properly demonstrated. Nonetheless several important observations come from these experiments. One of these is demonstration that HMG2 interacts only weakly, if at all, with Hl. This produces the untested possibility that HMG2^s interacts specifically with Hl^s, as suggested by Seyedin and Kistler (1979). Their demonstration of invariant levels of HMGl, combined with the previously demonstrated invariant levels of Hl during Hl^s accumulation, produces an interesting possibility for a functional interaction. In conclusion, I propose that there exists an interrelationship between the functioning of HMGl and Hl, as well as between HMG2 and Hl^s.

Although this proposal is certainly speculative, one final observation can be presented that is difficult to interpret, unless these proposed functional interrelationships are considered. This is developed from the studies of Sterner et al. (1978a) and Romani (1979) who claim that chicken possesses an erythrocyte-specific HMG-E, shown through peptide mapping and serological

analysis to be a divergent form of HMG2. In addition HMG-T, a protein related to mammalian HMG2, is present in the tissues of trout (Brown et.al. 1980, Christensen and Dixon 1981, Watson and Dixon 1981). If in fact H1^S histones do interact specifically with HMG2 proteins, one could predict that H5, due to its divergent structure, would be likely found in association with a structurally divergent form of HMG2. Demonstration of such a divergent HMG2 protein, HMG-E in chicken erythrocytes and HMG-T in trout, fulfills such a prediction and suggests that such functional relationships of the H1 satellites with HMG2 may in fact exist.

There is little doubt that these proposals are for the most part speculative. However, I feel that the information available concerning HMG function, combined with the establishment of the H1^S sub-class of lysine-rich histone, does provide supportive evidence for these proposals. The speculative nature of these proposals are likely due more to a lack of definitive research into HMG function, rather than to a lack of potential for such a functional relationship between the linker HMG proteins and the lysine-rich histones. Finally, the major purpose of presenting these proposals is an attempt to demonstrate the necessity of considering the function of HMG proteins in order to successfully elucidate function of the H1 and H1^S.

V. REFERENCES

- Ajiro, K., T.W. Borun, and D. Solter. 1981. Quantitative changes in the expression of histone H1 and H2B subtypes and their relationship to the differentiation of mouse embryonal carcinoma cells. *Devel. Biol.* 86,206.
- Alberts, B., A. Worcel, and H. Weintraub. 1977. On the biological implications of chromatin structure. In "The Organization and Expression of the Eukaryotic Genome", (eds. E.M. Bradbury and K. Javaherian) N.Y. Academic Press, pp 165-197.
- Alder, D., and M.A. Gorovsky. 1975. Electrophoretic analysis of liver and testis histones of the frog Rana pipiens. *J. Cell. Biol.* 64,389.
- Allan, J., P.G. Hartmann, C. Crane-Robinson, and F.X. Aviles. 1980. The structure of histone H1 and its location in chromatin. *Nature* 288,675.
- Allan, J., G.J. Cowling, N. Harborne, P. Cattini, R. Craige, and H. Gould. 1981. Regulation of the higher-order structure of chromatin by histones H1 and H5. *J. Cell. Biol.* 90,279.
- Appels, R., and J.R.E. Wells. 1972. Synthesis and turnover of DNA-bound histone during maturation of avian red blood cells. *J. Mol. Biol.* 70,425.
- Appels, R., J.R.E. Wells, and A.F. Williams. 1972. Characterization of DNA-bound histone in the cells of the avian erythropoietic series. *J. Cell. Sci.* 10,47.
- Aviles, F.J., G.E. Chapman, G.G. Kneale, C. Crane-Robinson, and E.M. Bradbury. 1978. The conformation of histone H5: Isolation and characterization of the globular segment. *Eur. J. Biochem.* 88,363.
- Bak, A.L., J. Zeuthen, and F.H.C. Crick. 1977. Higher order structure of human mitotic chromosomes. *Proc. Natl. Acad. Sci. USA* 74,1595.
- Bates, D.L., and J.O. Thomas. 1981. Histones H1 and H5: one or two molecules per nucleosome? *Nuc. Acid Res.* 9,5883.

- Bhorjee, J.S. 1981. Differential phosphorylation of nuclear nonhistone high mobility group proteins HMGl4 and HMGl7 during the cell cycle. *Proc. Natl. Acad. Sci. USA* 78, 6944.
- Billet, M.A., and J. Hindley. 1972. A study of the quantitative variation of histones, and their relationship to RNA synthesis, during erythropoiesis in adult chicken. *Eur. J. Biochem.* 28,451.
- Billet, M.A., and J.M. Barry 1974. Role of histones in chromatin condensation. *Eur. J. Biochem.* 49,477.
- Block, J.A., and B.G. Atkinson. 1979. Histones and histone phosphorylation during quail myogenesis in vitro. *Cell Differentiation* 8,413.
- Bloom, K.S., and J.N. Anderson. 1979. Conformation of ovalbumin and globin genes in chromatin during differential gene expression. *J. Biol. Chem.* 254,10532.
- Borun, T.W. 1975. Histones, differentiation, and the cell cycle. In "Results and Problems in Cell Differentiation", Vol.7, Cell Cycle and Cell Differentiation (eds. J. Reinert and H. Holtzer) N.Y.: Spring-Verlog. pp 249-290.
- Bradbury, E.M., G.E. Chapman, S.E. Danby, P.G. Hartman, and P.L. Riches. 1975. Studies on the role and mode of operation of the very-lysine-rich histone H1 (F1) in eukaryotic chromatin: The properties of the N-terminal and C-terminal halves of histone H1. *Eur. J. Biochem.* 57,521.
- Brasch, K., G.H.M. Adams, and J.M. Neelin. 1974. Evidence for erythrocyte-specific histone modification and structural changes in chromatin during goose erythrocyte maturation. *J. Cell Sci.* 15,659.
- Brasch, K., G.H.M. Adams, and J.M. Neelin. 1977. The morphology of erythroid cells separated by density gradient centrifugation through ficoll. *Cell Tiss. Res.* 176,373.
- Brown, E., G.H. Goodwin, E.L.V. Maynes, J.R.B. Hastings and E.W. Johns. 1980. Heterogeneity of proteins resembling high mobility group proteins HMG-t in trout testis nuclei. *Biochem. J.* 191,661.
- Brown, G.L., R.G. Rutledge, and J.M. Neelin. 1981. Anuran erythrocytes and liver both contain satellite histone H1^S. *Life Sciences* 28,2993.

- Bustin, M., R.B. Hopkins, and I. Isenberg. 1978. Immunological relatedness of high mobility group chromosomal proteins from calf thymus. *J. Biol. Chem.* 253,1694.
- Bustin, M., N.K. Neihart, and J.B. Fagin. 1981. mRNA of chromosomal proteins HMG-1 and HMG-2 are polyadenylated. *Biochem. Biophys. Res. Comm.* 101,893.
- Cary, P.D., C. Crane-Robinson, E.M. Bradbury, K. Javaherian, G.H. Goodwin, and E.W. Johns. 1976. Conformational studies of two nonhistone chromosomal proteins and their interactions with DNA. *Eur. J. Biochem.* 62,583.
- Cary, P.D., K.V. Shooter, G.H. Goodwin, E.W. Johns, J.Y. Olayemi, P.G. Hartman, and E.M. Bradbury. 1979. Does high mobility group nonhistone protein HMGI interact specifically with H1 subfractions? *Biochem. J.* 183,657.
- Cary, P.D., M.L. Hines, E.M. Bradbury, B.J. Smith, and E.W. Johns. 1981. Conformational studies of histone H1^o in comparison with histones H1 and H5. *Eur. J. Biochem.* 120,371.
- Chamberlain, J.P. 1979. Fluorographic detection of radioactivity in polyarylamide gels with the water-soluble fluor, sodium salicylate. *Anal. Biochem.* 98,132.
- Chapman, G.E., P.G. Hartman, and E.M. Bradbury. 1976. Studies on the role and mode of operation of the very-lysine-rich histone H1 in eukaryote chromatin: The isolation of the globular and non-globular regions of the histone H1 molecule. *Eur. J. Biochem.* 61,69.
- Chapman, G.E., P.G. Hartman, P.D. Cary, E.M. Bradbury, and D.R. Lee. 1978. A nuclear magnetic resonance study of the globular structure of the H1 histone. *Eur. J. Biochem.* 86,35.
- Chegini, N., V. Aleporou, G. Bell, V.A. Hilder, and N. Maclean. 1979. Production and fate of erythroid cells in anaemic Xenopus laevis. *J. Cell Sci.* 35,403.
- Chiu, M.L., and J.L. Irvin. 1980. Separation and quantitative analysis of rat testis histones by one-dimensional gel electrophoresis. *Anal. Biochem.* 109,102.

- Christensen, M.E., and G.H. Dixon. 1981. Comparison of high mobility group proteins and their chromatin distribution in trout testis and liver. *J. Biol. Chem.* 256,7549.
- Cole, R.D. 1977. Special features of the structures of H1 histones. In "The Molecular Biology of the Mammalian Genetic Apparatus", T'so, P., Ed. Elsevier North-Holland Biomedical Press. pp. 93-104.
- Crane-Robinson, C., L. Bohm, P. Puigdonemeh, P.D. Cary, P.G. Hartman, and E.M. Bradbury. 1980. Structural domains in histones. In "DNA-Recombination, Interactions and Repair" (FEBS) eds. S. Zadrozil, and J. Sponar. Pergamon Press Oxford pp293-300.
- D'Anna, J.A., R.A. Tobey, and L.R. Gurley. 1980a. Concentration dependent effects of sodium butyrate in Chinese hamster cells: Cell cycle progression, inner-histone acetylation, histone H1 dephosphorylation, and induction of an H1-like protein. *Biochemistry* 19,2656.
- D'Anna, J.A., L.R. Gurley, R.R. Becker, S.S. Barkam, R.A. Tobey, and R.A. Walters. 1980b. Amino acid analysis and cell cycle dependent phosphorylation of an H1-like, butyrate-enhanced protein (BEP;H1⁰;IP25) from chinese hamster cells. *Biochem.* 19,4331.
- D'Anna, J.A., L.R. Gurley, and R.R. Becker. 1981. Histones H1^{0a} and H1^{0b} are the same as CHO histones H1(III) and H1(IV): New features of H1⁰ phosphorylation during the cell cycle. *Biochemistry* 20,4501.
- Dardick, T., and G. Setterfield. 1976. Volume of condensed chromatin in developing primitive erythrocytes of chick. *Exptl. Cell Res.* 100,159.
- Darzynkiewicz, Z., F. Traganos, and M. Melamed. 1980. New cell cycle compartments identified by multiparameter flow cytometry. *Cytometry* 1,98.
- Darzynkiewicz, Z., F. Traganos, S. Xue, and M.R. Melamed. 1981. Effect of n-butyrate on cell cycle progression and in situ chromatin structure of L1210 cells. *Exptl. Cell Res.* 136,279.
- Delegeane, A.M., and A.S. Lee. 1982. Coupling of histone and DNA synthesis in the somatic cell cycle. *Science* 215,79.

- Destree, O.H.J., H.J. Hoenders, A.F.M. Moorman, and R. Charles. 1979. Histones of Xenopus laevis erythrocytes: Purification and characterization of the lysine-rich fraction. *Biochim. Biophys. Acta* 577,61.
- Elgin, S.C.R., and H. Weintraub. 1975. Chromosomal proteins and chromatin structure. *Ann. Rev. Biochem.* 44,725.
- Felsenfeld, G. 1978. Chromatin. *Nature* 271,115.
- Finch, J.T., and A. Klug. 1976. Solenoidal model for superstructure in chromatin. *Proc. Natl. Acad. Sci. USA* 73,1897.
- Gabrielli, F., R. Hancock, and A.J. Faber. 1981. Characterization of a chromatin fraction bearing pulse-labelled RNA. 2. Quantification of histones and high mobility group proteins. *Eur. J. Biochem.* 120,363.
- Gallwitz, D. 1975. Kinetics of inactivation of histone mRNA in the cytoplasm after inhibition of DNA replication in synchronized HeLa cells. *Nature* 257,247.
- Garrard, W.T., and J. Bonner. 1974. Changes in chromatin proteins during liver regeneration. *J. Biol. Chem.* 249,5570.
- Gasaryan, K.G., N.B. Andreeva, and T.Yu. Vishnevskaya. 1978. Existence of differences in repressor properties between serine-rich histone (H5, F2c) from immature and mature pigeon erythroid cells. *Differentiation* 10, 123.
- Georgieva, E.I., I.G. Pashev, and R.G. Tsanev. 1981. Distribution of high mobility and other acid-soluble proteins in fractionated chromatin. *Biochem Biophys. Acta* 652,240.
- Gjerest, R., F. Ibarrando, S. Saragosti, and H. Eisen. 1981. Distribution of IP25 in chromatin and its possible involvement in chromatin condensation. *Biochem. Biophys. Res. Comm.* 99,349.
- Goodwin, G.H., C. Sanders, and E.W. Johns. 1973. A new group of chromatin associated proteins with a high content of acidic and basic amino acids. *Eur. J. Biochem.* 38,14.

- Goodwin, G.H., K.V. Shooter, and E.W. Johns. 1975. Interaction of a non-histone chromatin protein (high mobility group 2) with DNA. *Eur. J. Biochem.* 54,427.
- Goodwin, G.H., C.G.P. Mathew, C.A. Wright, C.D. Venkov, and E.W. Johns. 1979. Analysis of the high mobility group proteins associated with salt-soluble nucleosomes. *Nuc. Acid Res.* 7,1815.
- Gordon, J.S., R. Kaufman, and B.I. Rosenfeld. 1981. Independent control during myogenesis of histone and high mobility group (HMGs) chromosomal protein heterogeneity. *Arch. Biochem. Biophys.* 211,709.
- Grasso, J.A., N.C. Chromey, and C.F. Moxey. 1977. Biochemical characterization of RNA and protein synthesis in erythrocyte development. *J. Cell. Biol.* 73,206.
- Groppi, V.E., and P. Coffino. 1980. G1 and S phase mammalian cells synthesize histones at equivalent rates. *Cell* 21,195.
- Hartman, P.G., G.E. Chapman, T. Moss, and E.M. Bradbury. 1977. Studies on the role and mode of operation of the very-lysine-rich histone H1 in eukaryote chromatin: The three structural regions of the histone H1 molecule. *Eur. J. Biochem.* 77,45.
- Hentschel, C.C., R.M. Kay, and J.G. Williams. 1979. Analysis of Xenopus laevis globins during development and erythroid cell maturation and the construction of recombinant plasmids containing sequences derived from adult globin mRNA. *Devel. Biol.* 72,350.
- Hentschel, C.C., and J.R. Tata. 1978. Template-engaged and free RNA polymerase during Xenopus erythroid cell maturation. *Devel. Biol.* 65,496.
- Hentschel, C.C., and M.L. Birnstiel. 1981. The organization and expression of histone gene families. *Cell* 25,301.
- Hilder, V.A., and N. Maclean. 1974. Studies on the template activity of "isolated" Xenopus erythrocyte nuclei. I. The effects of ions. *J. Cell Sci.* 16,133.
- Hilder, V.A., N. Thomas, and N. Maclean. 1975. The erythroid cells of anaemic Xenopus laevis. II. Studies on nuclear non-histone proteins. *J. Cell Sci.* 19,521.

- Hnilica, L.S. 1967. Proteins of the cell nucleus. *Progr. Nuc. Acid Res.* 7,25.
- Hohmann, P. 1978. The H1 class of histone and diversity in chromosomal structures. *Subcell. Biochem.* 5,87.
- Hohmann, P. 1980. Species and cell specific expression of H1 histones in tissue culture cells. *Arch. Biochem. Biophys.* 205,198.
- Holtzer, H., N. Rubinstein, S. Fellini, G. Heoh, J. Chi, J. Birnbaum, and M. Okayama. 1975. Lineages, quantal cell cycles and generation of cell diversity. *Quart. Rev. Biophys.* 8,523.
- Isenberg, I. 1979. Histones. *Ann. Rev. Biochem.* 48,159.
- Itkes, A.V., B.O. Glotov, L.G. Nikolaev, and E.S. Severin. 1980. Clusters of nonhistone chromosomal protein HMG 1 molecules in intact chromatin. *FEBS Lett.* 118,63.
- Jackson, J.B., J.M. Pollock Jr., and R.L. Rill. 1979. Chromatin fractionation procedure that yields nucleosomes containing near stoichiometric amounts of high mobility group nonhistone chromosomal proteins. *Biochemistry* 18,3739.
- Jagus, R., W.F. Anderson, and B. Safer. 1981. The regulation of initiation of mammalian protein synthesis. *Prog. Nuc. Acid Res. Mol. Biol.* 25,127.
- Keppel, F., B. Allet, and H. Eisen. 1977. Appearance of a chromatin protein during the erythroid differentiation of Friend virus-transformed cells. *Proc. Natl. Acad. Sci. USA* 74,653.
- Keppel, F., B. Allet, and H. Eisen. 1979. Biochemical properties and localization of the chromosomal protein IP25. *Eur. J. Biochem.* 96,477.
- Kernell, A.M., L. Bound, and N.R. Ringertz. 1971. Chromatin changes during erythropoiesis. *Exptl. Cell Res.* 65,1.
- Kinkade, J.M. 1969. Qualitative species differences and quantitative tissue differences in the distribution of lysine-rich histones. *J. Biol. Chem.* 244, 3375.
- Kornberg, R.D. 1977. Structure of chromatin. *Ann. Rev. Biochem.* 46,931.

- Kuehl, L. 1979. Synthesis of high mobility group proteins in regenerating rat liver. *J. Biol. Chem.* 254,7276.
- Kumar, N.M., and I.O. Walker. 1980. The binding of histones H1 and H5 to chromatin in chicken erythrocyte nuclei. *Nuc. Acid Res.* 8,3535.
- Laskey, R.A., and A.D. Mills. 1975. Quantitative film detection of ^3H and ^{14}C in polyacrylamide gels by fluorography. *Eur. J. Biochem.* 56,335.
- Lasters, I.S., S. Muyldermans, L. Wyns, and R. Hammers. 1981. Differences in rearrangements of H1 and H5 in chromatin. *Biochemistry* 20,1104.
- Lea, M.A., L.A. Youngworth, and H.P. Morris. 1974. Acid soluble nuclear proteins of rat liver: differential absorbance of bound dyes and changes in neoplasia. *Biochem. Biophys. Res. Comm.* 58,862.
- Levy-Wilson, B., N.C.W. Wong, and G.H. Dixon. 1977. Selective association of the trout-specific H6 protein with chromatin in regions susceptible to DNaseI and DNaseII: Possible location of HMG-T in the spacer region between core nucleosomes. *Proc. Natl. Acad. Sci. USA* 74,2810.
- Levy-Wilson, B., W. Connor, and G.H. Dixon. 1979. A subset of trout testis nucleosomes enriched in transcribed DNA sequences contains high mobility group proteins as a major structural component. *J. Biol. Chem.* 254,609.
- Levy-Wilson, B. 1981. Enhanced phosphorylation of high mobility group proteins in nuclease sensitive mononucleosomes from butyrate treated HeLa cells. *Proc. Natl. Acad. Sci. USA* 78,2189.
- Linnala-Kankkunen, A., and P.H. Maenpaa. 1981. Phosphorylation of high mobility group protein HMG14 by a cyclic GMP-dependent protein kinase from avian liver nucleoli. *Biochem. Biophys. Acta* 654,287.
- Lucas, A.M., and C. Jamroz. 1961. *Atlas of Avian Hematology*. Department of Agriculture, Washington, D.C.
- Maclean, N., V.A. Hilder, and Y.A. Baynes. 1973. RNA synthesis in *Xenopus* erythrocytes. *Cell Differentiation* 2,261.

- Marashi, F., L. Baumbach, R. Rickles, F. Sierra, J.L. Stein, and G.S. Stein. 1982. Histone proteins in HeLa S3 cells are synthesized in a cell cycle stage specific manner. *Science* 215,683.
- Mardian, J.K.W., A.E. Paton, G.J. Bunick, and D.E. Olins. 1980. Nucleosome cores have two specific binding sites for nonhistone chromosomal proteins HMG14 and HMG17. *Science* 209,1534.
- Marsh, W.H., and P.J. Fitzgerald. 1973. Pancreas acinar cell regeneration. XIII. Histone synthesis and modification. *Fed. Proc.* 32,2119.
- Mathis, D., P. Oudet, and P. Chambon. 1980. Structures of transcribing chromatin. *Prog. Nuc. Acid Res. Mol. Biol.* 24,1.
- McGhee, J.D., and G. Felsenfeld. 1980. Nucleosome structure. *Ann. Rev. Biochem.* 49,1115.
- McGhee, J.D., D.C. Rau, E. Charney, and G. Felsenfeld. 1980. Orientation of the nucleosome within the higher order structure of chromatin. *Cell* 22,87.
- Medvedev, Zh.A., and N.D. Kirpicheva. 1972. Time of the erythrocyte-specific histone fraction F_{2c} synthesis during erythropoiesis in birds. *Molek. Biol.* 6,607.
- Medvedev, Zh.A., and M.N. Medvedeva. 1980a. High H1⁰/H1 histone ratio in spontaneous hepatomas in aging mice. *IRCS Med. Sci.* 8,431.
- Medvedev, Zh.A., and M.N. Medvedeva. 1980b. A group of H1 histone satellite acid-soluble non-histone chromatin proteins. *FEBS Lett.* 112,35.
- Melli, M., G. Spinelli, and E. Arnold. 1977. Synthesis of histone messenger RNA of HeLa cells during the cell cycle. *Cell* 12,167.
- Miki, B.L.A. 1976. The histones of fish erythrocytes. PhD. Thesis, Carleton University.
- Miki, B.L.A., and J.M. Neelin. 1975. The histones of rainbow trout erythrocytes include an erythrocyte-specific histone. *Can. J. Biochem.* 53,1158.
- Miki, B.L.A., and J.M. Neelin. 1977a. Comparison of the histones from fish erythrocytes. *Can. J. Biochem.* 55,1220.

- Miki, B.L.A., and J.M. Neelin. 1977b. Histone H5 from carp erythrocytes. *Can. J. Biochem.* 55,384.
- Miki, B.L.A., and J.M. Neelin. 1980. DNA repeats lengths of erythrocyte chromatin differing in content of histones H1 and H5. *Nuc. Acid. Res.* 8,529.
- Molgaard, H.V., M. Perucho, and A. Ruiz-Carrillo. 1980. Histone H5 messenger RNA is polyadenylated. *Nature* 283,502.
- Moss, B.A., W.G. Joyce, and V.M. Ingram. 1973. Histones in chick embryonic erythropoiesis. *J. Biol. Chem.* 248,1025.
- Moyne, G., R. Freeman, S. Saragosti, and M. Yaniv. 1981. A high-resolution electron microscopic study of nucleosomes from Simian Virus 40 chromatin. *J. Mol. Biol.* 149,735.
- Mura, C.V., and B.D. Stoller. 1981. Serological detection of homologies of H1^o with H5 and H1 histones. *J. Biol. Chem.* 256,9767.
- Mura, C.V., G. Setterfield, and J.M. Neelin. 1982. Immunofluorescent localization of lysine-rich histones in isolated nuclei from adult and embryonic chicken erythrocytes. *Can. J. Biochem.* 60,215.
- Neelin, J.M., P.X. Callahan, D.C. Lamb, and K. Murray. 1964. The histones of chicken erythrocyte nuclei. *Can. J. Biochem.* 42,1743.
- Nelson, P.P., S.C. Albright, J.M. Wiseman, and W.T. Garrard. 1979. Reassociation of histone H1 with nucleosomes. *J. Biol. Chem.* 254,11751.
- Nelson, R.D., and J.J. Yunis. 1969. Species and tissue specificity of very lysine-rich and serine-rich histones. *Exptl. Cell. Res.* 57,311.
- Newrock, K.M., C.R. Alfageme, R.V. Nardi, and L.H. Cohen. 1977. Histone changes during remodeling in embryogenesis. *Cold Spring Harbor Symp. Quant. Biol.* 42,421.
- Nikolaev, L.G., B.O. Glotov, A.V. Itkes, and E.S. Severin. 1981. Mutual arrangement of histone H1 molecules in chromatin of intact nuclei. *FEBS Lett.* 125,20.
- Ochoa, S. 1979. Regulation of protein synthesis. *CRC Critical Rev. Biochem.* 7,7.

- Ochoa, S., and C. de Haro. 1979. Regulation of protein synthesis in eukaryotes. *Ann. Rev. Biochem.* 48,549.
- Olins, A.L. 1977. Nu bodies are close-packed in chromatin fibres. *Cold Spring Harbor Symp. Quant. Biol.* 42,325.
- Panyim, S., and R. Chalkley. 1969a. A new histone found only in mammalian tissues with little cell division. *Biochem. Biophys. Res. Comm.* 37,1042.
- Panyim, S., and R. Chalkley. 1969b. The heterogeneity of histones. I. A quantitative analysis of calf histones in very long polyacrylamide gels. *Biochemistry* 8,3972.
- Panyim, S., D. Bilek, and R. Chalkley. 1971. An electrophoretic comparison of vertebrate histones. *J. Biol. Chem.* 246,4206.
- Pardee, A.B., R. Dubrow, J.L. Hamlin, and R.F. Kletzien. 1978. Animal cell cycle. *Ann. Rev. Biochem.* 47,715.
- Pederson, T. 1976. Cellular aspects of histone synthesis. In "Protein Synthesis", Vol. 2, E.H. McConkey, ed. (N.Y.: Marcel Dekker), pp. 69-123.
- Pehrson, J.R., and R.D. Cole. 1980. Histone H1⁰ accumulates in growth-inhibited cultured cells. *Nature* 285,43.
- Pehrson, J.R., and R.D. Cole. 1981. Bovine H1⁰ histone subfractions contain an invariant sequence which matches histone H5 rather than H1. *Biochemistry* 20,2298.
- Pieler, C., G.R. Adolf, and P. Sevetly. 1981. Accumulation of histone H1 during chemically induced differentiation of murine neuroblastoma cells. *Eur. J. Biochem.* 115,329.
- Piha, R.S., and J.H. Valkonen. 1979. Changes in liver nuclear histones during bovine ontogeny. *FEBS Lett.* 108,326.
- Pospelov, V.A., A.M. Jerkin, and A.T. Khachatrian. 1981. H1 and H5 histone arrangement in chromatin of pigeon erythrocytes. *FEBS Lett.* 128,315.
- Prescott, D.M. 1976. The cell cycle and the control of cellular reproduction. *Adv. Genet.* 18,99.

- Rabbani, A., G.H. Goodwin, and E.W. Johns. 1978a. Studies on the tissue specificity of the high mobility group proteins from calf. *Biochem. J.* 173,497.
- Rabbani, A., G.H. Goodwin, and E.W. Johns. 1978b. High mobility group non-histone chromosomal proteins from chicken erythrocytes. *Biochem. Biophys. Res. Comm.* 81,351.
- Risley, M.S., and R.A. Eckhardt. 1981. H1 histone variants in Xenopus laevis. *Devel. Biol.* 84,79.
- Romani, M., T.C. Rodman, G. Vidali, and M. Bustin. 1979. Serological analysis of species specificity in the high mobility group chromosomal proteins. *J. Biol. Chem.* 254,2918.
- Ruiz-Carrillo, A., L.J. Wangh, V.C. Littau, and V.G. Allfrey. 1974. Changes in histone acetyl content and in nuclear non-histone protein composition of avian erythroid cells at different stages of maturation. *J. Biol. Chem.* 249,7358.
- Ruiz-Carrillo, A., L.J. Wangh, and V.G. Allfrey. 1976. Selective synthesis and modification of nuclear proteins during maturation of avian erythroid cells. *Arch. Biochem. Biophys.* 174,273.
- Rutledge, R.G., C.E. Shay, G.L. Brown, and J.M. Neelin. 1981. The similarity of histones from turtle erythrocytes and liver. *Can. J. Biochem.* 59,273.
- Seligy, V.L., and J.M. Neelin. 1970. Transcription properties of stepwise acid-extracted chicken erythrocyte chromatin. *Biochem. Biophys. Acta* 213,380.
- Seligy, V.L., and M. Miyagi. 1974. Comparison of template property changes after salt extraction of avian erythrocyte and liver chromatin. *Eur. J. Biochem.* 46,259.
- Seligy, V.L., G.H.M. Adams, and J.M. Neelin. 1973. The role of histones in avian erythropoiesis. In *Proc. Int'l. Cong. on the "Biochemistry of Gene Expression in Higher Organisms"*. eds. J. Pollak and J.W. Lee, Australia and New Zealand Book Co. TTY, Ltd. 1973, pp 177-190.
- Seyedin, S.M., and W.S. Kistler. 1979. Levels of chromosomal protein high mobility group 2 parallel the proliferative activity of testis, skeletal muscle, and other organs. *J. Biol. Chem.* 254,11264.

- Seyedin, S.M., and W.S. Kistler. 1980. Isolation and characterization of rat testis H1t: An H1 histone variant associated with spermatogenesis. J. Biol. Chem. 255,5949.
- Seyedin, S.M., R.D. Cole, and W.S. Kistler. 1981. H1 histones from mammalian testis. The widespread occurrence of H1t. Exptl. Cell. Res. 136,399.
- Shimada, T., Y. Okihama, C. Murata, and R. Shukuya. 1981. Occurrence of a H1^o-like protein and protein A24 in the chromatin of bullfrog erythrocytes lacking histone 5. J. Biol. Chem. 256,10577.
- Shooter, K.V., G.H. Goodwin, and E.W. Johns. 1974. Interactions of purified nonhistone chromosomal protein with DNA and histone. Eur. J. Biochem. 47,263.
- Smerdon, M.J., and I. Isenberg. 1976. Interactions between the subfractions of calf thymus H1 and proteins HMG1 and HMG2. Biochemistry 15,4242.
- Smith, B.J., and E.W. Johns. 1980a. Isolation and characterization of subfractions of nuclear protein H1^o. FEBS Lett. 110,25.
- Smith, B.J., and E.W. Johns. 1980b. Histone H1^o: its location in chromatin. Nuc. Acid Res. 8,6069.
- Smith, B.J., J.M. Walker, and E.W. Johns. 1980. Structural homology between mammalian H1 subfractions and avian erythrocyte-specific histone H5. FEBS Lett. 112,42.
- Smith, B.J., Y. Cook, E.W. Johns, and R.A. Weiss. 1981. Absence of H1^o from quiescent chicken cells. FEBS Lett. 135,77.
- Stein, G.S., J.L. Stein, W.D. Park, S. Detke, A.C. Lichtler, E.A. Sheppard, R.L. Jansing, and I.R. Phillips. 1978. Regulation of histone gene expression in HeLa S3 cells. Cold Spring Harbor Symp. Quant. Biol. 42,1107.
- Sterner, R., L.C. Boffa, and G. Vidali. 1978a. Comparative structural analysis of high mobility group proteins from a variety of sources: Evidence for a HMG protein unique to the avian erythrocyte nuclei. J. Biol. Chem. 253,3830.

- Sterner, R., G. Vidali, R.L. Henrikson, and V.G. Allfrey. 1978b. Postsynthetic modification of high mobility group proteins: Evidence that high mobility group proteins are acetylated. *J. Biol. Chem.* 253,7601.
- Sung, M.T. 1977. Phosphorylation and dephosphorylation of histone V (H5): Controlled condensation of avian erythrocyte chromatin. *Biochemistry* 16,286.
- Sung, M.T., J. Harford, M. Bundmann, and G. Vidalakas. 1977. Metabolism of histones in avian erythroid cells. *Biochemistry* 16,279.
- Tarnowska, M.A., and C. Baglioni. 1978. Synthesis of H1 histones by BHK cells in G1. *Cell* 15,163.
- Teng, C.T., and C.S. Teng. 1981. Changes in the quantities of high mobility group protein 1 in oviduct cellular fractions after oestrogen stimulation. *Biochem. J.* 198,85.
- Thoma, F., Th. Koller, and A. Klug. 1979. Involvement of histone H1 in the organization of the nucleosome and of the salt-dependent superstructures of chromatin. *J. Cell Biol.* 83,403.
- Thoma, F., and Th. Koller. 1981. Unravelled nucleosomes, nucleosome beads and higher order structures of chromatin: Influence of non-histone components and histone H1. *J. Mol. Biol.* 149,709.
- Thomas, N., and N. Maclean. 1975. The erythroid cells of anaemic Xenopus laevis. I. Studies on cellular morphology and protein and nucleic acid synthesis during differentiation. *J. Cell Sci.* 19,509.
- Tobin, R.S., and V.L. Seligy. 1975. Characterization of chromatin-bound erythrocyte histone V (f2c): Synthesis, acetylation and phosphorylation. *J. Biol. Chem.* 250,358.
- Tsai, Y.H., and L.S. Hnilica. 1975. Tissue specific histones in the erythrocytes of chicken and turtle. *Exptl. Cell. Res.* 91,107.
- Unger-Ullmann, C., and S.P. Modak. 1979. Gel electrophoresis analysis of histones in lens epithelium, lens fibre, liver, brain, and erythrocytes of late chick embryos. *Differentiation* 12,135.

- Urban, M.K., J.M. Neelin, and T.W. Betz. 1980. Correlation of chromatin composition with metabolic changes in nuclei of primitive erythroid cells of chicken embryos. *Can. J. Biochem.* 9,726.
- Valkonen, K.H. 1980. Developmental changes in H1 histones from bovine liver. *Life Sciences* 27,1217.
- Varricchio, F. 1977. Postnatal increase in histone H1a in the rat pancreas. *Arch. Biochem. Biophys.* 179,715.
- Von Holt, C., W.N. Strickland, W.F. Brandt, and M.S. Strickland. 1979. More histone structures. *FEBS Lett* 100,201.
- Walker, J.M., K. Gooderham, J.R.B. Hastings, E. Mayes, and E.W. Johns. 1980. The primary structures of non-histone chromosomal proteins HMG1 and HMG2. *FEBS Lett.* 122,264.
- Watson, D.C., and G.H. Dixon. 1981. Amino acid sequence homologies between the high mobility group proteins, HMG-T from trout testis and HMG-1 and -2 from calf thymus: is the poly-aspartic-glutamic acid polypeptide within the main chain? *Bioscience Reports* 1,167.
- Weintraub, H. 1978. The nucleosome repeat length increases during erythropoiesis in the chick. *Nuc. Acid Res.* 5,1179.
- Weintraub, H., and M. Groudine. 1976. Chromosomal subunits in active genes have an an altered conformation. *Science* 193,848.
- Weintraub, H., G.Le.M. Campbell, and H. Holtzer. 1971. Primitive erythropoiesis in early chick embryogenesis I. Cell cycle kinetics and the control of cell division. *J. Cell Biol.* 50,652.
- Weintraub, H., A. Worcel, and B.A. Alberts. 1976. A model for chromatin based upon two symmetrically paired half-nucleosome. *Cell* 9,409.
- Weintraub, H., S.J. Flint, I.M. Leffak, M. Groudine, and R.M. Grainger. 1977. The generation and propagation of variegated chromosome structures. *Cold Spring Harbor Symp. Quant. Biol.* 42,401.

- Weisbrod, S., and H. Weintraub. 1979. Isolation of a subclass of nuclear proteins responsible for conferring a DNaseI sensitive structure on globin chromatin. *Proc. Natl. Acad. Sci. USA* 76,630.
- Worcel, A. 1977. Molecular architecture of the chromatin fibre. *Cold Spring Harbor Symp. Quant. Biol.* 42,313.
- Worcel, W., and C. Benyajati. 1977. Higher order coiling of DNA in chromatin. *Cell* 12,83.
- Worcel, A., S. Strogatz, and D. Riley. 1981. Structure of chromatin and the linking number of DNA. *Proc. Natl. Acad. Sci. USA* 78,1461.
- Wright, E.B., and D.E. Olins. 1975. Histone stoichiometry in chicken erythrocyte nuclei. *Biochem. Biophys. Res. Comm.* 63,642.
- Wu, R.S., and W.M. Bonner. 1981. Separation of basal histone synthesis from S-phase histone synthesis in dividing cells. *Cell* 27,321.
- Yaguchi, M., C. Roy, M. Dove, and V. Seligy. 1977. Amino acid sequence homologies between H1 and H5 histones. *Biochem. Biophys. Res. Comm.* 76,100.
- Yaguchi, M., C. Roy, and V.L. Seligy. 1979. Complete amino acid sequence of goose erythrocyte H5 histone and the homology between H1 and H5 histones. *Biochem. Biophys. Res. Comm.* 90,1400.
- Yu, S-H., and T.G. Spring. 1977. The interaction of nonhistone chromosomal proteins HMG1 and HMG2 with subfractions of H1 histone immobilized on agarose. *Biochem. Biophys. Acta* 492,20.
- Zlatanova, J.S. 1980. Synthesis of histone H1⁰ is not inhibited in hydroxyurea treated Friend cells. *FEBS Lett.* 112,199.
- Zlatanova, J.S., K. Oberhammer, and P. Swetly. 1980. Expression of histone H1⁰ during differentiation of erythroleukemic mouse cells. In "In vivo and in vitro Erythropoiesis: The Friend System" (ed.G.B. Rossi), Elsevier/North-Holland Biomedical Press pp 297-307.

END

1 0 1 0 9 8 4

FIN