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LYSINE-RICH HISTONES OF THE AMPHIBIANS

XENOPUS AND AMBYSTOMA

by

© GERALD, LESLIE BROWN, B.Sc. (Hons.), M.Sc.

A thesis submitted to the Faculty of
Graduate Studies and Research in partial fulfilment
of the requirements for the degree of
Doctor of Philosophy

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To my mother and my wife

ABSTRACT

The two most studied H1 satellites, H5 and H1o, are presumed to have related yet distinct functions associated with tissue maturation. It has been shown that both these proteins are synthesized in cells in the absence of synthesis of DNA or the other histones. H5 is probably involved in transcriptional inactivation and chromatin condensation in nucleated erythrocytes, while H1o may have a role in limiting cell division and the control of differentiation in various mammalian tissues. Previous work has demonstrated the presence of H1 satellites in amphibian erythrocytes, though the tissue-specificity of these proteins has been disputed. A portion of this work demonstrates, by amino acid composition and electrophoretic and chromatographic properties, that the H1 satellites found in Xenopus erythrocytes and liver are homologous proteins which closely resemble H1o by these same properties. This suggests that their functions are not tissue-specific which may also be true of H5 and H1o. Recent work has shown that Xenopus H1 satellite also continues to be synthesized after the other histones.

Characterization of the lysine-rich histones from

erythrocytes or liver of the Mexican axolotl by electrophoresis, cation-exchange chromatography and amino acid analysis revealed no H1 satellites while the H1 components had protein characteristics similar to those of Xenopus H1 but not H1 satellite. The apparent absence of H1 satellites questioned the need for specialized lysine-rich histones in tissue maturation and initiated a study of axolotl lysine-rich histone metabolism during erythrocyte maturation accompanying cryptic metamorphosis.

Changes in the synthesis and accumulation of larval and adult axolotl globins, detected by densitometry of stained gels and fluorograms containing 14-C-lysine-labelled globins, were coincident with changes in the proliferative activity of the cell populations, determined by 3-H-thymidine incorporation into DNA and 14-C-lysine incorporation into core histones. Both electrophoretically identifiable H1 components demonstrated preferential synthesis, determined by densitometry of stained gels and accompanying fluorograms, between these waves of proliferative activity. The preferential synthesis of one H1 component (H1b) declined with the decline in activity of the larval erythroid population. The other H1 component (H1a) was preferentially synthesized throughout the transition and demonstrated a peak of 14-C-lysine incorporation after the last division of the

adult population. A third H1 component (H1c), present only occasionally through the transition, shared synthetic characteristics of both principal H1 components and probably represents a modified form of either, or both, of the predominant proteins depending on the developmental stage of the cell populations.

The existence of an H1 component showing preferential synthesis during the final stages of erythrocyte maturation in the axolotl is consistent with the proposed need for a specialized lysine-rich histone during the late stages of tissue maturation. The preferential synthesis after the last wave of adult cell division suggests a role more closely aligned with transcriptional inactivation and chromatin condensation than with the regulation of proliferative ability.

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LIST OF ABBREVIATIONS

CPM.....	counts per minute
CNBr.....	cyanogen bromide
DPM.....	disintegrations per minute
DTNB.....	di-thiobis-2-nitro-benzoic acid
GuCl.....	guanidinium chloride
H1, H1o, H5, H1 ^s	are respective lysine-rich histones
H2A, H2B, H3, H4.....	are the four core histones
MEM.....	minimum essential medium
MS222.....	tricainemethanesulfonate
PAGE.....	polyacrylamide gel electrophoresis
PCA.....	perchloric acid
PMSF.....	phenylmethylsulfonylfluoride
SDS.....	sodium dodecyl sulfate
sp. act.....	specific activity
TCA.....	trichloroacetic acid
TEMED.....	(NNN'N'-tetramethyl-1,2-diaminoethane)
Tris.....	tris(hydroxymethyl) aminomethane
^u Ci.....	microcurie
ug.....	microgram
ul.....	microlitre

I. INTRODUCTION

A. Histones and Their Involvement in Chromatin Structure

Histones are small basic proteins, soluble in dilute acids, which are intimately associated with eukaryotic chromatin (Lewin, 1974). Five major classes exist: lysine-rich histones, H2A, H2B, H3 and H4. The lysine-rich class can be separated into two distinct subclasses - H1 components and "H1 satellites" (Rutledge et al., 1981; Brown et al., 1981). The four other histone classes and the two lysine-rich histone subclasses all contain minor sequence variants, or subtypes, which may exist in the same tissue (Kincade, 1969), be differentially represented in distinct tissues (Kincade, 1969), or change with development (see below).

Eukaryotic chromatin is composed of a repeating 10 nm diameter subunit structure - the nucleosome - organized by one pair of each of the four core histone classes (Worcel, 1977; Belyavsky et al., 1980). One hundred and forty base pairs (bp) of DNA is consistently found coiled around the octameric core particle (for reviews see Worcel, 1977; Kornberg, 1977; Felsenfeld, 1978; McGee et al., 1980). The internucleosome linker DNA varies from 10-70 bp in length, is symmetrically coiled and interacts with the lysine-rich histones (Worcel, 1977; Belyavsky et al., 1980). Portions of these histone molecules also interact with the outside of the coiled DNA opposite the core histones (Belyavsky et al., 1980) and this class organizes the condensation of the nucleosome subunits into 20-30 nm diameter chromatin fibers (reviewed in Worcel, 1977; Worcel and Benyajati, 1977; McGee et al., 1980; Thoma and Koller, 1981).

It has long been recognized that at least some, and perhaps all, types of transcriptional controls in higher organisms have a structural basis, expressed as an altered DNA packaging pattern controlled by the population of associated proteins (Elgin and Weintraub, 1975). Recent nuclease digestion and electron microscopic investigations support this by showing that active and potentially active genes have a different

organization at the nucleosome level than does bulk chromatin (Weintraub and Groudine, 1976; Bloom and Anderson, 1979; Mathis et al., 1980). This hypothesis and these observations encourage more detailed studies of the proteins controlling chromatin structure in the continuing search for an understanding of eukaryotic gene expression.

B. Core Histones and Gene Expression

The development of Triton X-100 polyacrylamide gels (Zweidler, 1976) has allowed the detection of core histone variants which differ between adult tissues (Zweidler, 1976; Urban et al., 1979) and are systematically expressed during early development (Cohen et al., 1975; Newrock et al., 1978; Childs et al., 1979; Heiter et al., 1979). These studies suggest that the structural distinctions characterizing transcriptionally active chromatin may be due to core histone subtype variations. However, the high degree of similarity between core histones throughout evolution (Elgin and Weintraub, 1975), the lack of DNA sequence specificity by nucleosomes in general (Nelson et al., 1979) and the demonstration of sequential variation of subtypes only

during embryogenesis suggest a role more closely aligned with the repression of huge blocks of chromatin accompanying early development (Newrock et al., 1978; Weintraub et al., 1978); however such a role is still hypothetical and requires experimental support.

C. Lysine-Rich Histones and Gene Expression

1. Histone H1

The lysine-rich histone class, present at about one molecule per nucleosome (Bates and Thomas, 1981), is distinctly different from the core histone classes in that it has sufficient variability, and perhaps specificity, to mediate tissue-specific gene expression. The largest and most carefully characterized subclass of lysine-rich histones (H1) is composed of as many as 10 subtypes (Bustin and Cole, 1968, 1969), all having molecular weights in the 20,000 - 22,000 dalton range (Elgin and Weintraub, 1975). They are particularly rich in lysine, proline and alanine (Elgin and Weintraub, 1975), and their closely related primary structures are folded into three distinct structural domains (Cole, 1977; Isenberg, 1979; Yaguchi et al., 1977, 1979).

H1 subtypes qualitatively vary from species-to-species (Panyim et al., 1971), where the differences are found in the flexible, basic one-third of the molecules toward the carboxy-terminus (Cole, 1977). Amino acid substitutions in the basic one-fifth of the molecules toward the amino-terminus account for the quantitative tissue-specific variations within a species (Kincade, 1969; Cole, 1977). Both terminal domains interact with core histones (Boulikas et al., 1980) and DNA (Glotov et al., 1978) of the nucleosome and protect the outside of the DNA from nucleolytic attack at regularly spaced intervals (Belyavsky et al., 1980). The terminal domains play a critical role in initiating chromatin condensation participating in the formation of the 20-30 nm chromatin supercoils (Allan et al., 1980). The electrostatic interactions of the carboxy-terminal domains (Kumar and Walker, 1980) are chiefly responsible for DNA-binding by these histones (Bradbury et al., 1975). However, the central globular domain seems also to be important in DNA-binding since the two-thirds of the molecule towards the carboxy-terminus, which includes the central domain, can recognize and bind more DNA on an equimolar ratio than can the entire H1 molecule (Singer and Singer, 1976). As one might expect, the binding affinity of the central domain in the intact molecule is moderated by amino acid

substitutions and post-synthetic modifications in the amino terminus (Glotov et al., 1977). The trypsin-resistant core of this protein is highly conserved (Cole, 1977), can close and protect two full turns of the DNA around the core particle (Crane-Robinson et al., 1980) and distinguishes between supercoiled and relaxed DNA (Singer and Singer, 1976, 1978). Interestingly, supercoiled DNA has been shown to be an important prerequisite for efficient transcription in prokaryotes (Kano et al., 1981) and appears to be important for delineation of expressed genes in eukaryotes (Larsen and Weintraub, 1982).

Not surprisingly, H1 subtypes are immunochemically distinct (Bustin and Stollar, 1972), show similar but subfraction-specific responses to salt and pH changes in vitro (Smerdon and Isenberg, 1976) and afford distinctly characteristic abilities to protect DNA in chromatin against nucleolytic attack (Gorka and Lawrence, 1979). Furthermore, reconstitution experiments have shown that each subtype displays individual binding characteristics with both naked DNA (Welch and Cole, 1980; Liao and Cole, 1981a) and dinucleosome preparations (Liao and Cole, 1981b). H1 components demonstrate a preferential and cooperative binding affinity for adenine(A) and thymine (T) rich DNA (Sponar and Sormova, 1972; Sawecka et al., 1980) which

is dictated principally by the central globular regions of the molecules (Marekov et al., 1978). Interestingly, and perhaps importantly, AT-rich DNA has been shown to define functionally active domains in eukaryotic chromatin (Moreau et al., 1982) but an association between these and H1 binding sites has yet to be established.

Like the core histones, H1 subtypes are coordinately expressed through embryonic development (Newrock et al., 1978; Senger et al., 1978; Hohmann, 1978; Arceci and Gross, 1980; Hentschel and Birnstiel, 1981), but unlike the core histone relative proportions of H1 subtypes continue to change at later stages of development (Mitsui et al., 1980; Valkonen, 1980; Ajiro et al., 1981; Pehrson and Cole, 1982). Another characteristic feature of lysine-rich histones is the appearance of a distinct new subclass "H1 satellites", in cells of highly differentiated tissues exhibiting little proliferative activity.

2. Histone H1 Satellites

The most studied H1 satellites, H5 and H1o, having molecular weights of 18,000 - 20,000, are slightly shorter than H1 subtypes and are thus recognized by their characteristic electrophoretic

mobilities in SDS and acid/urea polyacrylamide gels (Rutledge et al., 1981; Brown et al., 1981). Like H1, H5 (Allan et al., 1980; Crane-Robinson et al., 1980) and H1o (Keppel et al., 1979; Smith and Johns, 1980b; Cary et al., 1981; Gjerset et al., 1981) are located in the internucleosome linker regions. Although both are relatively rich in lysine, alanine and proline compared to the core histones, H5 has elevated levels of serine and arginine and reduced lysine and alanine compared to H1 (Elgin and Weintraub, 1975). H1o also has somewhat lower alanine and slightly higher serine than H1 but lysine levels are equivalent to those of H1 (Smith and Johns, 1980a). Both H5 and H1o contain one molecule of methionine but few, if any, H1 components contain this residue. Two H1o subtypes appear to be distinguishable by electrophoresis (Varricchio, 1977; Medvedev and Medvedeva 1977; D'Anna et al., 1981) and cation-exchange chromatography (Smith and Johns, 1980a), although their peptide maps are almost identical (Smith and Johns, 1980a). Other workers have shown that H1o subtypes show minor sequence heterogeneity (Pehrson and Cole, 1981) in their N- terminal domains (D'Anna et al., 1981).

Like H1, H5 and H1o have three principal domains but they share primary (Smith et al., 1980; Pehrson and Cole, 1981; Yaguchi et al., 1979; Briand et al., 1980)

and secondary (Allan et al., 1980; Mura and Stollar, 1981; Cary et al., 1981) structural characteristics which are distinct from H1 subtypes. Comparison of the primary sequences of goose H5 with trout testis H1, has shown a greater than 40% homology between the two proteins with a particular tract in the central domain, being 65% homologous (Yaguchi et al., 1979). Comparison of a large portion of the central domains of bovine H1o and chick H5 indicates greater than 70% homology in this region with a 42% homology between H1o and H1 (Smith et al., 1980; Pehrson and Cole, 1981). These studies suggest that both H1 satellites, H1o and H5, are divergent forms of the H1 subclass which share a highly conserved region. This hypothesis has been previously advanced for H5 (Yaguchi et al., 1979). The great similarity between the central domains of all lysine-rich histones suggests that these regions mediate a common and essential function.

3. The Erythrocyte-Specific H5

H5 was first detected in chicken erythrocyte nuclei (Neelin et al., 1974), and is tissue-specific in both avian (Hnilica, 1967; Elgin and Weintraub, 1975) and fish (Miki and Neelin, 1975) species. Studies with H5 fragments, generated by limited proteolysis, have

shown that the trypsin-resistant core closes and protects the two ends of the DNA leaving the nucleosome (Allan et al., 1980; Cowman and Fasman, 1980) however unlike H1 subtypes, the entire molecule is important in DNA binding (Aviles et al., 1979). H5-DNA binding is primarily electrostatic (Kumar and Walker, 1980) and demonstrates a preference for AT-rich DNA (Hwan et al., 1975), but the binding affinity is 7 times stronger than it is with H1 subtypes (Kumar and Walker, 1980). These studies show that structurally, H5 and H1 subtypes have many common characteristics which suggest similar roles in chromatin structure and function. However, their behavior metabolically and developmentally shows clear distinctions (see below).

4. Chicken Embryo Erythropoiesis

a. Hemoglobin Gene Expression

Erythropoiesis has proven to be a convenient system for monitoring both changes in chromatin structure and the expression of specific genes. In general, development involves the maturation of three successive series of distinct erythropoietic cell lines which synthesize and accumulate specific hemoglobins (Hb) (Benz, 1981). The type of Hb synthesized is

transcriptionally regulated by the differential expression of particular "alpha-like" and "non-alpha" globin genes (Stalder et al., 1980; Benz, 1981; Groudine and Weintraub, 1981), and this changes or switches usually with the development of a new cell line (Benz, 1981). The two classes of globin genes are arranged in separate gene clusters and one pair of each globin class combines to form the tetrameric Hb macromolecule (Benz, 1981). The development of each cell line involves two distinct phases: (a) an early commitment phase where an erythroid stem cell is programmed such that its progeny produce a particular type of Hb and (b) a maturation phase where the stem cell progeny mature, progressively acquiring cell-type-specific morphological and biochemical features characteristic of the particular erythroid cell line (Benz, 1981).

In chickens, Hb mRNA in the embryonic primitive cell line is first detectable at about 35 hours of development (Stalder et al., 1980). This cell line matures as a reasonably homogeneous population, passing through six cell cycles in a five day interval, with the length of time in the G1 phase of the cell cycle increasing with maturity (Weintraub et al., 1971). There is a progressive increase in the concentration of embryonic Hb per cell which plateaus at days 5 and 6 and then gradually increases again from days 6 to 9.

(Campbell et al., 1971). This plateau is preceded by a drop in RNA synthesis (Billet and Hindley, 1972; Urban et al., 1980) and the increased accumulation of Hb after the plateau is presumably due to translation from stored mRNA even though uridine incorporation continues at low levels (Weintraub et al., 1971; Billet and Hindley, 1972). Interpretation at this point is difficult since the embryonic definitive cell line starts to develop during the final maturation stages of the primitive cell line (Chapman and Tobin, 1979). The embryonic definitive cell line persists through hatching (21 days) at which point a new adult definitive erythroid line appears (Stalder et al., 1980). Both definitive lines produce adult globins but the embryonic definitive line initially synthesizes both embryonic and adult globins with the level of embryonic Hb declining as the cells mature (Chapman and Tobin, 1979).

Studies with the thymidine analogue bromodeoxyuridine (BUdR) have shown that primitive embryonic stem cell commitment, or determination, occurs between 1 and 2 cell divisions prior to the appearance of the Hb producing cells (Miura and Wilt, 1971; Weintraub et al., 1972). Early application (days 1-3) of BUdR prevents the initiation of the developmental program without influencing the level or quality of either RNA or protein synthesis (Miura and Wilt, 1971;

Weintraub et al., 1972). Addition of the inhibitor after the appearance of the Hb-producing cells does not adversely influence the development and maturation of the population (Miura and Wilt, 1971; Weintraub et al., 1972). These results suggest that BUdR has an all-or-none, non-mutagenic effect which probably interferes with some alteration in chromatin structure or organization accompanying determination (Miura and Wilt, 1971; Weintraub et al., 1972).

Deoxyribonuclease I (DNase I) digestion of chromatin in isolated nuclei from primitive and definitive embryonic and mature adult erythrocytes demonstrated that in each cell type the beta-globin gene sequences, expressed in that cell line, were in an altered chromatin conformation (Weintraub and Groudine, 1976). For example primitive cells, which synthesize embryonic beta-globin chains, contain embryonic beta-globin genes in a DNase I-sensitive (open) conformation while the adult beta-globin genes are resistant to nuclease digestion (closed). On the other hand definitive cells, synthesizing adult beta-globins, only show heightened adult beta-globin gene sensitivity. This conformational distinction is not a consequence of transcriptional activity, since adult mature erythrocytes do not synthesize RNA but still exhibit DNase I-sensitivity of adult beta-globin genes.

(Weintraub and Groudine, 1976). However, the altered conformation appears to be necessary for transcriptional activity, since embryonic beta-globin mRNA is only detectable after an embryonic beta-globin gene conformational change has occurred in the previous cell division cycle of the erythrocyte precursor cells (Weintraub and Groudine, 1976; Groudine and Weintraub, 1981).

When assayed by blot hybridization, which detects the first cut introduced in a gene by DNase I rather than demonstrating sensitivity of the entire gene sequence (hybridization in solution), the primitive embryonic cells were found to contain both embryonic and adult globins in DNase I-sensitive conformations (Stalder et al., 1980). As before, the adult cells only demonstrated DNase I-sensitivity of adult globins (Stalder et al., 1980). This has interesting developmental implications because embryonic globin genes are located on the 5' side of the adult globin genes (Weintraub et al., 1981), and a conformation allowing their expression (in primitive embryonic cells) may be responsible for the DNase I-sensitivity of the adult globins genes located downstream (Stalder et al., 1980). Further work has shown the existence of DNase I-hypersensitive sites at the 3' and 5' ends of the expressed genes in both primitive and definitive cells

(Weintraub et al., 1981); in some cases these sites appear to be AT-rich (Moreau et al., 1982). The sensitivity of the embryonic globin-associated sites disappears when the genes are no longer expressed (Weintraub et al., 1981). This sensitivity appears to be dependent on chromatin supercoiling, and selective sensitivity to S1 nuclease suggests some degree of single-strandedness associated with these regions (Larsen and Weintraub, 1982).

Taken together, these results show a strong dependency of globin gene expression on chromatin structure. A chromatin conformational change in primitive embryonic precursor cells allows embryonic globin gene expression and perhaps alters the conformation of adult globin genes making them more accessible than is bulk chromatin to nuclease digestion (Weintraub and Groudine, 1976; Stalder et al., 1980). Open conformations are not dependent on continued transcriptional activity (Weintraub and Groudine, 1976) and are correlated with the presence of flanking regions hypersensitive to both DNase I and S1 nuclease (Weintraub et al., 1981; Larsen and Weintraub, 1982) which are probably AT-rich (Moreau et al., 1982).

b. Changes in Chromatin Ultrastructure

In addition to changes in chromatin structure at the level of the gene, a number of studies have looked at changes in chromatin ultrastructure and nuclear organization during erythropoiesis. Kernell et al. (1971) noted a 45% decrease in the nuclear dry mass, probably due to decreased transcriptional activity, and a 65% decrease in nuclear area accompanying erythrocyte maturation during recovery from anemia. These changes were coincident with increased chromatin compaction judged by a decrease in the ability to incorporate the intercalating dye acridine orange and an increased stability to heat denaturation. The results were interpreted as involving a change in the relative proportion of euchromatin (considered to be transcriptionally active) and heterochromatin (considered to be transcriptionally inert) (Kernell et al., 1971). Subsequent work demonstrated similar trends when the areas of extended and condensed chromatin were expressed as a percentage of total chromatin (Dardick and Setterfield, 1976; Brasch et al., 1977). However, when the nuclear, euchromatin and heterochromatin volumes were calculated and compared throughout development the results indicated that all compartments decreased in size with maturation (Dardick and Setterfield, 1976; Brasch et al., 1977). Euchromatin

demonstrated the largest decrease, again presumably due to decreased transcriptional activity, which had a substantial influence on the reduction in mean nuclear volume (Dardick and Setterfield, 1976). As one might expect, nuclear volume changes were coordinated with transcriptional and translational activity, judged by the number of cytoplasmic ribosomes (Brasch et al., 1977). Collectively, these results show that erythropoiesis is accompanied by alterations in chromatin structure well above the level of specific genes and nucleosomes.

This discussion of erythropoiesis has suggested two major developmental periods where changes in lysine-rich histones may be involved in controlling gene expression and/or chromatin structure. The first period is the commitment or determination event. The possibility of lysine-rich histone involvement is suggested by the dependency of commitment on a change in chromatin organization (Miura and Wilt, 1971; Weintraub et al., 1972; Weintraub and Groudine, 1976; Groudine and Weintraub, 1981), the presence of AT-rich regions at either end of the genes being expressed (Weintraub et al., 1981; Moreau et al., 1982) and the altered chromatin structure of the genes when they are 'available' for expression (Weintraub et al., 1972;

Weintraub and Groudine, 1976; Stalder et al., 1980).

The second period occurs while the cell lines are going through their last division cycle, when transcriptional activity shows a precipitous decrease (Weintraub et al., 1971; Billet and Hindley, 1972; Urban et al., 1980) and the gross chromatin organization begins to undergo further condensation (Kernell et al., 1971; Dardick and Setterfield, 1976; Brasch et al., 1977).

Early synthesis of a lysine-rich histone during the development of a cell line would support the first possibility. The second would be supported by synthesis and accumulation of a lysine-rich histone late in the maturation process or a dependency of mature erythrocyte chromatin organization on the presence of a particular lysine-rich histone. However, these alternatives are not mutually exclusive.

5. The Role of Lysine-Rich Histones in Avian Erythropoiesis

Neelin et al. (1964) and later Purkayastha and Neelin (1966) suggested a relationship between the presence or absence of H5 and the cessation of macromolecular synthesis during the maturation of avian erythroid cells. Cell populations enriched with cells at different stages of maturity by density gradient

centrifugation, were found to contain the same total amount of H1 or core histones (Billet and Hindley, 1972). However, one H1 subtype was diminished in mature erythrocytes and the proportion of H5 relative to H1 increased 2.7 fold beginning with the decline in uridine incorporation prior to the final cell division cycle (Billet and Hindley, 1972). The correlation between H5 appearance and decreased transcriptional activity was also observed by Appels et al. (1971) and Urban et al. (1980) while coincident H5 and DNA synthesis was demonstrated by combined immunofluorescent and autoradiographic studies (Mura et al., 1978). Moss and co-workers (1973) observed similar lysine-rich histone quantitative changes in both the primitive and definitive embryonic cell lines, where the total amount of H1 did not change, a particular H1 subtype was under-represented in the mature cells and H5, appearing late in the development of the populations, accumulated with maturation. Their results indicated that the absolute amount of H5 in mature cells was specific to the cell line, with the primitive cell line having lower levels than those of the definitive cell line. They also confirmed the earlier work of Appels and Wells (1972), who demonstrated selective synthesis of H5 in maturing cells, in the absence of synthesis of other histones or DNA. This selective synthesis has been

confirmed by a number of other studies (Seligy et al., 1973; Tobin and Seligy, 1975; Sung et al., 1977; Urban et al., 1980; Ruiz-Vazquez and Ruiz-Cardillo, 1982), as has the cell-line-specific accumulation of H5 (Urban et al., 1980). Urban et al. (1980) have also demonstrated a slight decrease in the total amount of H1 histone which is characteristic of each cell line, but all studies show an increase in H5 such that the total amount of lysine-rich histone increases up to 1.8 fold with maturation (Weintraub, 1978; Schelegel et al., 1980).

Numerous biochemical studies, using selective extraction of avian erythrocyte histones in acid (Seligy and Neelin, 1970) and salt (Seligy and Miyagi, 1974) solutions, have shown substantial increases in chromatin template activity with the removal of H5. Similar studies with polystyrene sulfonate, which binds and removes H5 (Miller et al., 1972), and mammalian-avian heterokaryons, which progressively lose H5 (Appels et al., 1974), show an inverse relationship between H5 content and chromatin template activity.

Studies of H5 phosphorylation have extended these observations to show a direct relationship between the degree of H5 phosphorylation and the ease with which H5 can be selectively extracted (Brasch et al., 1974). This post-synthetic modification appears to have a role

in development since a reduction in H5 phosphorylation accompanies chromatin condensation associated with erythrocyte maturation (Bräsch et al., 1974).

All these studies support a role of H5 in either the cessation of transcriptional activity and/or progressive chromatin condensation during the final maturation phase of erythropoiesis.

Ultrastructural analysis of immature and mature goose erythrocytes has shown that chromatin structure is dependent on both H1 and H5, but that H5 alone causes a more condensed, or congealed, configuration moderated by H1 subtypes to cause a uniformly condensed structure in the mature erythrocyte (Brasch et al., 1972). This change in chromatin structure is detectable at the molecular level by partial digestion of linker DNA with micrococcal nuclease and estimation of the linker length between nucleosomes from the higher oligonucleosome particles. Weintraub (1978) has shown a progressive increase in linker length with H5 accumulation (from 0.2 to 1 H5 molecules per nucleosome), from the primitive erythrocytes through both definitive series into the mature adult erythrocytes in the chicken. A closer look at the adult definitive series results in a similar correlation and has also shown that late, but still immature, erythrocytes have both higher H5 levels and longer linker lengths than do the mature circulating

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erythrocytes (Schelegel et al., 1980).

These observations are inconsistent with those reported by Wilhelm et al. (1977) where the repeat lengths of hen ~~liver~~ and 3-day old chick erythroid cells were shown to be identical even though the immature erythrocytes contained substantial amounts of H5. Both however, had repeat lengths shorter than that of mature erythrocyte chromatin (erythrocytes from 3-day old chicks are a mixture of immature definitive cells). If the goose and chicken erythroid systems are similar, the shorter repeat-lengths of the immature chicken erythrocytes (at day 3), compared to those of the mature chicken erythrocytes, may be due to high levels of H5 phosphorylation which declines only with maturation and is coincident with chromatin condensation (Brasch et al., 1974).

An interesting observation has been reported by Miki and Neelin (1980) where H5 content and linker lengths were examined in mature erythrocytes of different fish species which contain different amounts of H5 (Miki and Neelin, 1977). Though all species demonstrated identically longer linker lengths in mature erythrocyte chromatin than in liver chromatin, the large variations in the relative amount of H5 suggested that it was not directly involved in linker length determination (Miki and Neelin, 1980). This apparent

contradiction is eliminated if one postulates that the linker length is dependent on total lysine-rich histone content, which was constant among the fish species, rather than the relative proportion of one H1, or satellite H1, component. Interestingly the total lysine-rich histone content of chick erythrocyte nuclei was equal to that of the three species of fish (Miki and Neelin, 1980), allowing the existence of inter-species tissue-specific levels of lysine-rich histones.

The dependency of chromatin structure on both H1 subtypes and H1 satellites is consistent with the chicken erythrocyte results (Weintraub, 1978; Schelegel et al., 1980) and is supported by both the ultrastructural studies (Bräsch et al., 1974), and the light microscopic evidence, showing decreased availability of DNA to intercalating dyes and increased thermal stability with development (Kernell et al., 1971).

The indirect involvement of H5 in linker length determination is supported by Mazen et al. (1978) who reported erythrocyte linker lengths in the lower eukaryote Spinculus nudus which were shorter than those reported for most other eukaryotic tissues. Unfortunately, the authors did not determine the repeat lengths in other tissues of this animal so that intraspecies tissue-specific differences in lysine-rich

histone content and repeat length can not be determined. Large differences in the core histones H2A and H2B of this animal (Mazen et al., 1978) may be partially responsible for the differences in repeat lengths between the erythrocytes of this species and those of the erythrocytes in higher eukaryotes.

The only studies to date which look at the relationship between DNase I-sensitive globin genes and lysine-rich histones have dealt with mature erythrocyte populations. Weintraub and Groudine (1976) have shown that nucleosomes (which do not contain linker DNA, H1 or H5) generated by micrococcal nuclease digestion (which under the conditions employed does not distinguish between open and closed DNA sequences) still retain DNase I-sensitivity of genes being expressed. Similarly, erythrocyte chromatin depleted of H1 or H1 and H5 by selective extraction (Villeponteaux et al., 1978) continues to maintain an open conformation only in expressed genes. These studies show that the open conformation is inherent in the nucleosome core particle but an essential role of lysine-rich histones in the initiation or propagation of an altered nucleosome conformation within the globin transcriptional unit is not ruled out (Villeponteaux et al., 1978). However, the experiments showing selective synthesis and

accumulation of H5 after initiation of hemoglobin synthesis makes globin gene activation an unlikely role for H5.

In general, the studies reviewed support the proposed role of H5 in transcriptional inactivation and/or chromatin condensation coincident with the late stages of erythrocyte maturation. The results of Weintraub and Groudine (1976) reduce these two possibilities by demonstrating continued adult globin gene DNase I-sensitivity in mature, transcriptionally inactive, adult erythrocytes. Thus, available evidence suggests that the accumulation of lysine-rich histones, usually achieved by selective synthesis and accumulation of H5 independent of cell proliferation, is associated with, and may be responsible for, further chromatin condensation and increased linker length associated with the final stages of erythrocyte maturation.

6. The Mammalian H1o

H1o was isolated initially from mammals, with its relative amounts being inversely related to the proliferative activity of the individual tissues (Panyim and Chalkley, 1969a, 1969b; Shaw and Huang, 1970; Russanova et al., 1980; Smith and Johns, 1980b). It

appears and accumulates during postnatal development of both pancreas (Varricchio, 1977) and liver (Balhorn et al., 1972b; Piha and Valkonen, 1979; Seyedin and Kistler, 1979; Valkonen, 1980; Smith and Johns, 1980b) coincident with the decline in proliferation rate of the tissues. Experimentally induced tissue damage results in the gradual loss of H1o at the onset and an accumulation near the completion of tissue regeneration (Benjamin, 1971; Balhorn et al., 1972a; Marsh and Fitzgerald, 1973; Garrard and Bonner, 1974). The direct relationship with proliferative ability was strengthened by reduced quantities of H1o in hepatomas in vivo (Lea et al., 1974; Medvedev and Medvedeva, 1980a) compared to that of normal liver preparations. However, Lea et al. (1974) also noted an inverse correlation between growth rate and the degree of differentiation of the individual hepatomas and suggested a role of H1o in differentiation.

As was the case with H5, H1o accumulation causes a substantial increase (up to two fold) in the total amount of lysine-rich histone which was accompanied by a slight reduction in the quantity of one H1 subtype (Valkonen, 1980). During pancreas ontogeny (Varricchio, 1977) and regeneration (Marsh and Fitzgerald, 1973), the H1o accumulation occurs after a decline in DNA synthesis which is also the case with rat liver development

(Balhorn et al., 1972b). Interestingly, Marsh and Fitzgerald (1973) found that a maximum of methyl-14-C-methionine incorporation into the presumed Hlo occurs earlier in the initial stages of regeneration than does the maximum in thymidine incorporation.

Some authors studying cells in culture support an Hlo association with proliferative ability (Pehrson and Cole, 1980; D'Anna et al., 1980a) while most suggest a relationship with differentiation (see below). Chemical induction of Friend erythroleukemia cells demonstrated Hlo accumulation associated with differentiation and declining proliferation (Keppel et al., 1977, 1979). Similar results were obtained with neuroblastoma cells induced to differentiate in culture by density inhibition, serum deprivation or n-butyrate, where a 2-7 fold increase in the concentration of Hlo was observed without change in the total level of H1 subtypes (Pehrson and Cole, 1980, 1982; Pieler et al., 1981). However, one H1 subtype decreased in quantity (Pehrson and Cole, 1982). Hlo accumulation occurred after a reduction in DNA synthesis and could be gradually reversed by the removal of the inducer though an immediate increase in DNA synthesis was seen (Pieler et al., 1981). Thus, the accumulation of Hlo and cessation of DNA synthesis are only loosely associated, as are the converse events. An association with differentiation is

further supported by the observation that the levels of accumulation of H10 paralleled the efficiency of the treatments causing differentiation (Pieler et al., 1981).

All studies are consistent with H10 involvement in an event that occurs during the final stages of tissue maturation and is associated with chromatin condensation, in analogy with H5 involvement in erythrocyte maturation. Tissue-specific H10 levels which are closely tied to the degree of differentiation of a particular tissue (Lea et al., 1974; Pehrson and Cole, 1980, 1982; Pieler et al., 1981) implies an involvement of H10 in the control of differentiation. In fact, the one observation of selective H10 synthesis before differentiation (Marsh and Fitzgerald, 1973) suggests that H10 may be necessary for differentiation.

7. Other H1 Satellites

Surveys of animal species have demonstrated the presence of electrophoretically identifiable H1 satellites in amphibian and reptile erythrocytes which were reported to be tissue-specific (Edwards and Hnilica, 1968; Vendrely and Picaud, 1968; Tsai and Hnilica, 1975; Destree et al., 1979). Other reports indicated the presence of similar H1 satellites in

tissues other than erythrocytes of these same species (Nelson and Yunis, 1969; Panyim et al., 1971; Alder and Gorovsky, 1975). The reported amino acid compositions of the erythrocyte H1 satellites from bullfrog (Edwards and Hnilica, 1968), turtle (Tsai and Hnilica, 1975) and Xenopus (Destree et al., 1979) more closely resembled H5 than they did H1 or H10. Similar studies have not been done with amphibian satellites isolated from other tissues; however, recent work with turtle H1 satellites has shown the presence of nearly identical components in both erythrocytes and liver which more closely resembled H10 in amino acid composition and electrophoretic and chromatographic properties than they did H5 (Rutledge et al., 1981).

The possibility exists that the amphibian species contain two H1 satellite subtypes, an erythrocyte-specific subtype analagous to H5, and an H10-like subtype specific to other differentiated tissues. Such a situation does appear to exist in trout kidney where the satellite H1 has an amino acid composition very similar to H5, while an electrophoretically and chromatographically identifiable H1 subtype has an amino acid composition more closely aligned with that of H10 (Seyedin and Cole, 1981). This situation would suggest that the functions of these two H1 satellite subtypes are different, which could also be

the case if both occur in anurans. Alternatively, if anuran H1 satellites show no tissue specificity as in turtles, these histones may perform a common function in all differentiated tissues.

D. Scope of the Thesis

Although H5 and H1o share many characteristics, many researchers consider them to have distinct functions - H5 being associated with transcriptional inactivation and the control of chromatin condensation while H1o is presumed to have a role in limiting cell proliferation and controlling tissue differentiation. Amphibians and reptiles have, in some cases, been reported to contain erythrocyte-specific H1 satellites, while others have suggested that these satellites are common to all differentiated tissues in these species. This study attempts to eliminate the latter controversy by detailed characterization of the H1 satellites from Xenopus laevis erythrocytes and liver, as well as partial characterization of erythrocyte and liver H1 satellites from bullfrogs and leopard frogs.

Vendrely and Picaud (1968) reported the presence of an H1 satellite in the erythrocytes of Pleurodeles; however, preliminary analysis of axolotl (Ambystoma mexicanum) erythrocytes did not detect an electrophoretically identifiable H1 satellite in this urodele (unpublished observations). This initial result prompted a brief survey of animal species for the presence of H1 satellites and questioned the necessity of these proteins in the establishment of mature erythrocyte populations.

As outlined previously, erythropoiesis provides a convenient developmental system for studying changes in both gene expression and cell proliferation. In Rana catesbeiana (Riggs, 1951; Herner and Friesen, 1961; Moss and Ingram, 1965, 1968a,b; Just and Atkinson, 1972; Benbassat, 1974), Xenopus laevis (Herner and Friesen, 1961; Jurd and Maclean, 1970), Ambystoma tigrinum (Edwards and Justus, 1969; Ducibella, 1974a) and Pleurodeles waltlii (Flavin et al., 1978a,b, 1979, 1982), metamorphosis is accompanied by erythropoiesis and a switch in hemoglobin subtypes. Although detailed studies have not been done with axolotls, the well-studied amphibian and early avian switches involve the development of new erythroid populations which undergo successive alterations in DNA and total protein synthesis (Grasso and Woodard 1967; Weintraub et al.,

1971; Just and Atkinson, 1972). The development of erythroid populations in animals recovering from anemia follow similar stages (Maclean and Jurd, 1971; DeWitt et al., 1972; Thomas and Maclean, 1975; Grasso et al., 1977; Chegini et al., 1979; Casale et al., 1980), involving a switch in globin mRNA synthesis (Hentschel et al., 1979) and the preferential synthesis of H1 satellites during final maturation stages (Rutledge, 1982).

Though sexually mature, the neotenuous adult axolotl (A. mexicanum) has not undergone the gross anatomical alterations other amphibia experience during metamorphosis. However, complete metamorphosis can be induced by either thyroid hormone injection (Prahlad and DeLanney, 1965; Prahlad, 1968; Edwards and Justus, 1969; Maclean and Jurd, 1971) or immersion (Ducibella, 1974a,b; Tompkins and Townsend, 1977). Initial work demonstrated no difference between the hemoglobins present in neotenuous adults and completely metamorphosed adults induced by exogenous thyroid hormone (Edwards and Justus, 1969). However, comparison of young axolotl tadpole Hb with that of the neotenuous adults did show altered Hb subtypes similar to those seen in other amphibia (Maclean and Jurd, 1971). Ducibella (1974a) further characterized this cryptic metamorphic transition, including the stage-specific globin

subtypes, in the axolotl. Early induction of the transition in the axolotl by exogenous thyroid hormones (Ducibella, 1974b) is also a characteristic feature of other amphibian Hb switches. This study concentrates on the changes in synthesis of H1 components and globin subtypes which occur during the development of a new erythroid cell line accompanying cryptic metamorphosis in the axolotl.

II MATERIALS AND METHODS

Leopard frog (Rana pipiens) and Necturus maculosus were obtained from Boreal Laboratories Ltd. (Mississauga, Ontario). Xenopus laevis were obtained from Boreal Laboratories or provided by Dr. J. Armstrong (Dept. of Biology, Univ. of Ottawa, Ottawa, Ontario). Adult axolotls were obtained from either Dr. J. Armstrong or Dr. W. Forbes (Indiana Univ. of Pennsylvania, Indiana, Penn.). All axolotls in the metamorphic transition experiments were provided by Dr. J. Armstrong, had been maintained at 18-20°C in 25% Holtfreter's solution on a diet of sliced beef heart and timed from the date of spawning. Bullfrogs (Rana catesbeiana) were collected locally and used the day after collection. Adult white Leghorn chickens were obtained from McEwen's Poultry Farm (North Gower, Ontario).

Chemicals used were reagent grade and obtained from either BDH (Toronto) or Fisher Scientific (Toronto), unless otherwise specified. Low speed centrifugations were done in a Sorval RC-2 high speed centrifuge equipped with an SS-34 rotor. A Beckman L5-50 ultracentrifuge equipped with an SW27 rotor was used for high speed centrifugations. Ultraviolet absorbance and optical density measurements of chromatographic fractions were measured in a Beckman Acta C-III spectrophotometer.

Amphibians were anaesthetized in MS 222 (Sigma Chem. Co., St. Louis, Mo.) at room temperature at a concentration of 0.2% for adults and 0.1% for axolotls undergoing the metamorphic transition. Following a rinse in distilled water to remove excess MS 222, animals were blotted dry and the desired tissues isolated. All extraction and isolation procedures were done at 0-4°C unless otherwise specified.

A. Histone Characterization Studies

1. Isolation of Erythrocyte Nuclei

The body cavity, exposed by a ventral midline incision, was rinsed once with citrate-saline (0.14M NaCl containing 1.14% sodium citrate and the protease inhibitors 1 mM phenylmethanesulfonyl fluoride (Sigma, from a stock 50 mM solution in isopropanol) or 0.2 mM di-thiobis-2-nitro-benzoic acid (Sigma, from a stock 5mM solution in 0.01M Tris, pH 6.8). Blood was collected from the severed hepatic portal vein and the abdominal cavity, immediately mixed with citrate-saline and centrifuged at 800g for 10 minutes. The supernatant and the white cells were removed by aspiration and, after two washes in saline (0.14M NaCl containing 1mM PMSF or 0.2mM DTNB), the pelleted cells were suspended in saline to 1/2 the volume of the initial cell suspension. Nucleated cell ghosts were prepared by lysis in saponin. The volume of fresh 5% saponin (in 0.01M sodium phosphate, pH 6.8) added and the incubation time (on ice

with intermittent agitation) were selected to achieve greater than 95% cell lysis without the generation of chromatin clumps characteristic of nuclear lysis (Table 1). (One batch of saponin was used to avoid variation between saponin batches.) Nucleated ghosts were then freed of major cytoplasmic contaminants by successive washes in saline at 1500g for 5 minutes until the nuclear pellet appeared white and the supernatant clear.

2. Isolation of Liver Nuclei

Isolated livers were perfused with saline and, after removal of the gall bladder, immersed in 20-50 ml solution A (2.0mM calcium chloride, 1.0mM magnesium chloride, 10.0mM Tris pH 6.8, 0.32M sucrose and 1mM PMSF or 0.2mM DTNB). The tissue was minced with a Sorval Omnimixer operating at full speed for two 30-second intervals separated by a 30-second pause to allow larger pieces to settle. Following filtration through 2 layers of cheesecloth, the tissue was homogenized by 7 strokes at 900rpm in a 30-ml glass homogenizer equipped with a motor-driven teflon pestle. The homogenate was centrifuged at 1300g for 10 minutes and the resulting pellet suspended in 10-30 ml of solution A. This was mixed with 4 volumes of solution B (solution A containing 2.2M sucrose) by inversion and the resulting

Table 1: The final saponin concentration and incubation times used to prepare erythrocyte ghosts with greater than 95% cell lysis without nuclear lysis.

Animal	Final Saponin Concentration	Incubation Time (minutes)
leopard frog:	0.05%	11
bullfrog:	0.05%	11
<u>Xenopus:</u>	0.05%	7-10
<u>Necturus:</u>	0.05%	7
axolotl:	0.05%	7
chicken:	0.25%	12

suspension equally distributed into 30-ml centrifuge tubes. Each tube was underlaid with 10 ml of solution B and the suspensions were centrifuged at 130,000g for 1 hour. Nuclear pellets were combined and washed twice in 20-50 ml of solution A (3,000g, 10 minutes). Melanin granules, always apparent in liver nuclear preparations from the amphibians, could be reduced by repeated low speed centrifugations in solution A. However, their presence did not interfere with histone extraction and melanin did not appear as a contaminant in histone samples separated by SDS polyacrylamide gel electrophoresis.

3. Histone Extractions

Nuclear pellets were suspended in 0.01N HCl in 0.14M NaCl to 1/4 the volume of the initial tissue suspensions. Extraction of the lysine-rich histones was achieved by the addition of an equal volume of 10% perchloric acid (w/v) and incubation for 15 minutes with constant agitation. After centrifugation at 12,000g for 15 minutes, the pellets were resuspended and washed twice in 5% PCA, 0.005N HCl and 0.075M NaCl. The supernatant extracts were pooled. Core histones were solubilized in 0.25N HCl from the residual nuclear pellets using an identical extraction procedure.

When total histone samples were required, the nuclear pellets were suspended in 0.25N HCl to 1/2 the initial tissue suspension volume, incubated and washed as described above.

Solubilized histones were recovered from the pooled supernatants by incubation in 20% trichloroacetic acid (added as 80% TCA) for 30-60 minutes and centrifugation at 12,000g for 15 minutes. Protein pellets were stored overnight at -20°C in acidified acetone (1 ml conc. HCl in 500 ml acetone), repelleted (12,000g, 15 minutes), washed once more in acidified acetone and air dried. The proteins were lyophilized from distilled water and stored in air-tight vials at -20°C .

4. Bio-Gel P-150 Exclusion Chromatography

PCA extracts (7-50 mg), dissolved in 0.5 ml of 8M urea containing 5% (v/v) 2-mercaptoethanol, were applied to a 100 x 2.5 cm glass column containing Bio-Gel P-150 (Bio-Rad Laboratories Ltd., Mississauga). Proteins were eluted with 0.01N HCl containing 0.01% sodium azide at a flow rate of 20 ml per hour. Five-ml fractions were collected and monitored for protein content by their

absorbance at 230 nm. Pooled fractions were dialyzed against three changes of distilled water, frozen and lyophilized.

5. Amberlite CG-50 Cation-Exchange Chromatography

Guanidinium chloride (Sigma, 60% in distilled water) was filtered through a slurry of 33% activated charcoal and 66% celite. Sodium phosphate was added to the eluant to 0.1M, the pH adjusted to 6.8 with HCl and the resulting GuCl concentration determined with a Zeiss refractometer. All dilutions of GuCl were made with 0.1M sodium phosphate buffer at pH 6.8 and measured by refractive index.

PCA extracts (3-37 mg) were dissolved in 0.5-2.0 ml of 7-8% GuCl and applied to either a 1 x 60 cm or 0.5 x 50 cm column containing Amberlite CG-50 resin (Bio-Rad) previously regenerated with 50 ml of 40%, and equilibrated with 8% GuCl. Proteins were eluted with a linear gradient from 8% GuCl (100 ml) to 15% GuCl (100 ml) regulated by a Gilson Minipule II peristaltic pump (Mandel Scientific, Montreal) to flow at 5 ml per hour. Two-ml fractions were collected and the proteins located by determining the optical density at 400nm of a 200 μ l sample of each fraction added to 1 ml of 21.75% TCA. GuCl concentrations were monitored at 5-fraction

intervals across the gradient. Histones in pooled fractions were precipitated by adding 1/3 volume of 80% TCA. Residual GuCl and phosphate buffer were removed by three washes of the precipitated proteins in acidified acetone. After air drying, the proteins were dissolved in distilled water, frozen, lyophilized and stored in air-tight vials at -20°C .

6. SDS Polyacrylamide Gel Electrophoresis

SDS slab gels ($0.15 \times 20 \times 12$ cm or $0.07 \times 20 \times 12$ cm) were poured between the plexiglass and glass plates of an apparatus built by Science Workshops, Carleton University, Ottawa. Appropriate sized spacers and combs were cut by hand from teflon sheets and lubriseal (Fisher Scientific) was applied to all spacer-plate surfaces to prevent leakage. Comb teeth, spaced at 2.5-3 mm intervals, were approximately 3 mm wide.

The separating gel contained 0.1% SDS, 1 mg/ml ammonium persulfate (fresh), 0.5 ul/ml TEMED, 0.4M Tris (pH 8.8) and either 12.5% or 15% acrylamide (containing 2% bisacrylamide). The stacking gel consisted of 7.5% acrylamide (2% bisacrylamide), 0.1% SDS, 1 mg/ml ammonium persulfate, 1 ul/ml TEMED and 0.12M Tris pH 6.8. A flat interface between the separating and

stacking gels was achieved by either allowing the separating gel to polymerize against the flat edge of the comb or by layering the separating gel solution with distilled water. Running buffer (0.2M glycine, 0.1% SDS and 25mM Tris pH 8.3) was added to the upper chamber before the comb was removed and after the stacking gel had polymerized. The slots were rinsed with running buffer and samples, dissolved in sample buffer (1.5% SDS, 20% sucrose and 0.62M Tris pH 6.8) were applied with a 10- μ l Hamilton syringe (Chromatographic Specialties, Brockville). Lysine-rich histones were dissolved at approximately 2 mg/ml, while core histone and total histone preparations were adjusted to 10 mg/ml.

Electrophoresis was conducted at 0-5°C (maintained by running ice water through a central cooling chamber in the apparatus) with a constant voltage of 300V for thick gels, and 350V for thin gels. The run was stopped when the leading edge of the tracking dye (0.1% bromophenol blue) reached the bottom of the gel. Gels were warmed to room temperature before removal from the apparatus and stained (6 hours to overnight, with shaking) in either 0.25% Coomassie blue R 250 or 0.1% Amido black in 5% acetic acid and 40% ethanol. Destaining was accomplished by successive washes in 5% acetic acid and 20% ethanol.

7. Cyanogen Bromide Cleavage

Lyophilized proteins, dissolved in 35 μ l of distilled water, 100 μ l ammonium carbonate (0.2M) and 1.5 μ l of 2-mercaptoethanol per mg protein, were incubated for 18 hours at room temperature. The reduced proteins were precipitated in 20% TCA, washed three times in acidified acetone and air-dried. Samples were then incubated with 5 mg cyanogen bromide (CNBr) per mg protein (in 70% formic acid, v/v) for 18 hours at room temperature with constant agitation. Cleavage products were diluted with 10 volumes of distilled water, frozen, lyophilized and stored at -20°C .

In one instance an attempt was made to isolate the major CNBr-cleavage product from the other Xenopus erythrocyte lysine-rich histones. The protein mixture was dissolved in 0.5 ml of 8M urea containing 5% 2-mercaptoethanol and applied to a Sephacryl S-200 column (superfine, Pharmacia, 100 x 2.5 cm) equilibrated with 0.01N HCl. The flow rate was 60 ml/hour and 3-ml fractions were collected and monitored for protein at 230nm. Small polypeptides only were eluted with 0.01N HCl. The major cleavage product and uncleaved satellite H1 were subsequently co-eluted at two times the void volume with 1M NaCl in 0.01M sodium phosphate, pH 6.8. The remaining H1 subtypes were not detected in the fractions collected (see RESULTS).

8. Amino Acid Analysis

Pooled fractions were hydrolyzed in 6N HCl for 22 hours at 110°C in evacuated and sealed tubes. After drying the hydrolysates under vacuum, the amino acids were separated and quantitated by a Beckman 119BL amino acid analyzer (Beckman Instruments, Palo Alta, Calif.) equipped with a peak integrator (Beckman model 126). Chromatograms were repeated at least three times and the pooled fractions from each chromatogram were analyzed at least twice. The reported values are averages of at least six analyses expressed as the percentage of total recovered amino acids not corrected for losses during hydrolysis.

9. Amino Acid Sequencing

Protein sequencing, kindly performed by Drs. D. Watson and M. Yaguchi (Division of Biological Sciences, National Research Council, Ottawa), was determined by automatic degradation with a Beckman model 890C protein sequenator using a 0.5M quadrol program (no.122974) (Nazar et al., 1974). Thiazolinone and phenylthiohydantoin derivatives were hydrolyzed in 6N

HCl with or without 0.1% selenium chloride at 130°C for 20 hours. The resulting amino acids were analyzed with a Durrum D-500 amino acid analyzer.

B. Metamorphic Transition Studies in the Axolotl

1. Incubation with Radioactive Precursors

Erythrocytes from the peripheral circulation of 2-10 animals (in order to reduce individual variation and provide sufficient cell numbers for analysis) were collected by Pasteur pipette from the hepatic portal vein and body cavity in lysine-free incubation medium containing 5 mg/ml heparin. The incubation medium was that described by Maclean et al., (1973) and consisted of 10 ml of frog Ringer's solution plus 12 mg/ml bovine serum albumin (Fraction V, Sigma), 10 ml of a concentrated amino acid mixture (containing 105 mg arginine, 24 mg cysteine, 31 mg histidine, 52 mg isoleucine, 15 mg methionine, 32 mg phenylalanine, 48 mg threonine, 10 mg tryptophan, 45 mg valine and 35 mg thymidine per litre of distilled water), 1 ml MEM Vitamin solution (Grand Island Biological Co., 100 x conc.), penicillin (100 U/ml), streptomycin (100 mg/ml)

and 1 mg/ml sucrose. The pH was adjusted to 7.4 with 5% bicarbonate solution and the medium was stored at -20°C after filter sterilization.

Cell suspensions were diluted to 12 ml with heparin-free incubation solution and centrifuged at 800g (Sorval model GLC-1 centrifuge) at room temperature for 5 minutes in a graduated conical centrifuge tube. Supernatants and the buffy coat containing white blood cells were aspirated off and the cell pellets suspended to 10 ml in incubation solution. Samples were taken to determine total cell number (Fusch Rosenthal Haemocytometer) and the cell suspension was centrifuged at 800g for 10 minutes. The resulting cell pellet was resuspended in incubation solution at 2×10^8 cells/ml. A 0.5 ml aliquot was incubated in a 1.5 ml microfuge tube (Beckman) with 10 uCi (methyl-3-H)-thymidine (sp.act. 80.3 Ci./mmol., New England Nuclear, previously lyophilized and dissolved in incubation medium at 50 uCi/100 ul). A 1.4 ml aliquot was incubated in a 1.5 ml microfuge tube with 20 uCi/ml 3-H-thymidine and 20 uCi/ml L-(14-C(U))-lysine (sp. act. 331.0 Ci./mmol., New England Nuclear, lyophilized and dissolved in incubation medium at 50 uCi/100 ul). Suspensions were maintained at $18-20^{\circ}\text{C}$ with intermittent agitation.

2. Liquid Scintillation Counting

Since isotope incorporation was expressed as a function of cell number which was dependent upon sample volumes, dilution effects were minimized by air-drying TCA-precipitated samples prior to hydrolysis and counting. Dried samples were hydrolyzed in 100 μ l 70% PCA and 200 μ l 30% hydrogen peroxide overnight at 80°C. Three 50- μ l samples of each was mixed with an equal volume of fresh 15% ascorbic acid in 15-ml plastic scintillation vials to which 5 ml of Ready Solv HP (Beckman, Toronto) was added. Counts were determined by either a Beckman LS 8000 scintillation counter or a Beckman LS 7500 scintillation counter equipped with a data reduction system (Beckman, Toronto). Counts per minute (CPM) realized from the LS 8000 were, in most cases, converted to disintegrations per minute (DPM) and corrected for channel crosstalk with quench curves calculated with quenched standards for both tritium and carbon-14 (Beckman, Toronto) at two-month intervals throughout the study period. The data reduction system of the LS 7500 automatically constructed quench curves with each run, corrected for channel crosstalk and calculated CPM and DPM for each sample.

3. Incorporation Profiles and Fractionation of Cellular Components

All centrifugations were done at room temperature in a Beckman Microfuge B attached to a Powerstat (model 3PM116, The Superior Electric Co., Bristol, Connecticut). Cells were pelleted at 45 V for 1 minute, nuclei at 80 V for 1 minute and TCA precipitates at 110 V for 2 minutes. Supernatants were aspirated by Pasteur pipette. A simplified flow chart of the fractionation described below is presented in Figure 1. Pellets after each centrifugation were washed 3 times with fresh suspension medium appropriate to that step.

In order to establish the continued viability of cells during the incubation period and the presence of excess labelled precursors, 20-ul samples were taken at 10-minute intervals and precipitated by the addition of 20 ul of 40% TCA (A, Figure 1). After incubation for 30-60 minutes the cells were pelleted, washed three times in 5% TCA and air-dried in preparation for scintillation counting. Populations often demonstrated a 10-15 minute lag in the uptake of labelled precursors but the incorporation rate usually became linear throughout the rest of the incubation period. Populations which did not show linear incorporation were discarded. The total amount of isotope in the

Figure 1: In vitro labelling and cell fractionation scheme for axolotl erythrocytes isolated from animals at different stages of cryptic metamorphosis.

suspensions was determined by counting three air-dried 20-ul samples of the suspension. In no case did the amount of label incorporated into the macromolecules in the washed cells approach the amount of label present during the incubation period (Appendix I). This indicated a large excess of labelled precursors during the incubation which ensured that precursor availability did not interfere with isotope uptake and incorporation. This should have minimized amino acid pool size differences between preparations, though these were probably small because the axolotl does not exhibit substantial catabolic activity during cryptic metamorphosis (Ducibella, 1974a).

Incubation was terminated by centrifugation and three 1-ml washes in ice-cold, unlabelled incubation medium. Suspensions labelled with tritiated-thymidine alone were resuspended in incubation medium and samples were used to make blood smears for autoradiography (B, Figure 1; see 5. Autoradiography and Staining of Blood Smears). Cells incubated with both labelled precursors were suspended in 1.5 ml of saline and three 20-ul samples were precipitated in 20% TCA, washed three times in 5% TCA and air-dried (C, Figure 1). The remaining cells were pelleted and resuspended in 1 ml of saline. Cells were lysed by the addition of 10 ul of 5% saponin (in 0.01M sodium phosphate buffer, pH 6.8) and incubated

for 7 minutes with intermittent agitation. Nucleated ghosts were pelleted and three 20-ul samples of the saponin supernatants (D, Figure 1) were precipitated in 20% TCA, washed in 5% TCA (3X), air-dried and counted. A 100 ul sample of the saponin supernatant was mixed with SDS/PAGE sample buffer and stored with the remainder of the supernatant at -20°C .

Nuclei were washed twice in one ml of saline and three 20-ul samples of nuclei in the third wash (containing 1.5 ml of saline), were precipitated in 20% TCA, washed in 5% TCA (3X) and air-dried (E, Figure 1). A 300-ul aliquot of the nuclear suspension was centrifuged and the nuclei were suspended in 200 ul of 5% PCA in 0.005N HCl and 0.075M NaCl. This extraction was repeated once and three 20-ul samples of the combined supernatants, containing the lysine-rich histones, were precipitated in 20% TCA, washed in 5% TCA (3X), air-dried and counted (F, Figure 1). The pellet was extracted twice with 200 ul of 0.25N HCl and three 20-ul samples of the combined supernatants, containing the core histones, were precipitated in 20% TCA, washed in 5% TCA (3X), air-dried and counted. The remainder of the supernatants from both extractions were precipitated in 20% TCA, washed in acidified acetone, lyophilized and stored at -20°C .

The remaining nuclear suspension (1.04 ml) was

centrifuged and the nuclei suspended in 0.5 ml of 0.25N HCl. This was repeated twice and three 20-ul samples of the combined supernatants, containing both core and lysine-rich histones, were precipitated in 20% TCA, washed in 5% TCA (3X), air-dried and counted (H, Figure 1). The remainder of the supernatants was precipitated in 20% TCA, washed in acidified acetone and stored at -20°C. The residual nuclear pellet was suspended in 0.5 ml of 0.25N HCl, and three 20-ul samples were precipitated in 20% TCA, washed in 5% TCA (3X), air-dried and counted (I, Figure 1).

4. DNA Isolation

In one instance DNA was isolated from 154-day post-spawning animals to test for precursor incorporation into DNA. One ml of cold 10% TCA was added to a 2-ml sample of previously labelled cells suspended in unlabelled medium and the mixture was incubated for 1 hour at room temperature. Following two washes in 5% TCA (1 ml each), the precipitate was suspended in 1 ml of 5% TCA and three 50-ul samples were air-dried and counted. After centrifugation, the precipitates were successively washed in 1 ml volumes of ethanol, ethanol-ether (1:1) and ether. Three 50-ul samples of these supernatants were saved and counted.

The precipitate was then extracted twice in 1 ml of hot 10% TCA (in a boiling water bath for 5 minutes), and three 50-ul samples of the combined supernatants, containing purified DNA, were counted. The residual pellet was hydrolyzed in 100 ul 70% PCA and 200 ul 30% hydrogen peroxide (overnight at 80°C), and three 50-ul samples were combined with an equal volume of fresh 15% ascorbic acid and 5 ml of scintillation fluor before counting.

5. Autoradiography and Staining of Blood Smears

Smears of tritiated-thymidine-labelled cells were fixed in 95% methanol. After air-drying, the slides were coated with Ilford L:4 emulsion (size A, Ilford Ltd.) diluted 1:1 with distilled water. Following a two week exposure at 4°C, the autoradiograms were developed in Kodak Dektol for 4 minutes at 18°C. Air-dried autoradiograms were differentially stained with May-Grunwald and Giemsa stains (Lucas and Jamroz, 1961). For each experiment a minimum of 500 cells was counted and the proportion of cells containing label was calculated (400X magnification, Zeiss photomicroscope).

6. Hemoglobin Purification

Hemoglobins were isolated from some cell lysates by exclusion chromatography in order to conclusively identify globin chains by SDS/PAGE and determine the extent of saponin supernatant contamination by other cytoplasmic proteins. One-tenth volume of 5% potassium ferricyanide was immediately mixed with the isolated saponin supernatant and the mixture was incubated for 5 minutes at room temperature. One volume of the dialysis buffer was added (0.01% potassium cyanide in 0.05M Tris, pH 8.0) and the solution was dialyzed against three 2-litre changes of this buffer over 36 hours at 4°C. The dialysate was layered onto a Sephadex G-100 column (Pharmacia, Montreal, 1.3 x 50 cm), previously equilibrated with dialysis buffer, and run at 10 ml per hour at 4°C. Three-ml fractions were collected and monitored for both hemoglobin (Abs. at 415nm) and total protein (Abs. at 280nm).

7. Fluorography of Polyacrylamide Gels

Destained slab gels were washed in distilled water for 30 minutes, soaked in 1.0M sodium salicylate pH 6.3 with agitation for 30 minutes and dried onto Whatman 3M chromatographic paper using a slab gel drier

(Bio-Rad, model 224) with a mylar sheet. After cooling, dried gels were removed from the apparatus, trimmed and mounted between glass plates. Kodak X-Omat XAR-5 film (cat.# 1651496) was preflashed (to OD=0.15 at 540nm, Laskey and Mills, 1975) and positioned on the dried gel between the glass plates. Following a two to six week exposure at -80°C , the film was developed for 6 minutes at 18°C in Kodak liquid X-ray developer and replenisher.

8. Densitometry of Stained Gels and Fluorograms

Positives of the stained gels were printed on Kodak X-Omat XAR-5 film and developed for 6 minutes at 18°C in Kodak Dektol diluted 1:2 with distilled water. Care was taken to keep the background density of the prints constant in an attempt to limit exposures to the same portion of the photographic response curve. Both fluorograms and prints of stained gels were scanned at 570nm on a Beckman model DU 8 spectrophotometer equipped with a slab gel scanning platform and an area integrator. Since the background staining was variable and irregular, particularly between closely spaced bands, most peaks were integrated manually using the formula: Area = peak height x width at 1/2 height. A densitometric standard (Beckman, Toronto) was scanned with each gel under each set of scanning conditions

employed and the area calculated by the integrator and by hand. Manually determined peak areas for protein bands were then converted to 'integrator' values by a conversion factor calculated from the densitometric standard scans. This allowed comparison within and among gels or fluorograms independent of background variations.

Each gel contained a serially diluted set of standards added approximately two hours before the end of the electrophoretic run to allow the construction of standard curves for protein or DPM vs peak area (after correction for band width) for each gel. These curves were independent of gel staining/destaining or length of fluorographic exposure, even though these parameters were kept as constant as possible.

The protein concentrations for globin standards were estimated by comparing OD at 400nm of axolotl erythrocyte lysates, following precipitation in 20% TCA, with a standard curve constructed from a serial dilution of sheep hemoglobins (Type III, Sigma) in the same SDS sample buffer. The amounts of radioactivity present were determined by liquid scintillation counting.

Amido black staining produced standard curves which varied considerable about the means (Figure 2a), while standard curves from Coomassie blue-stained gels demonstrated a linear relationship between protein

Figure 2: The relationship of protein concentration (a and b) and isotope level (c) to densitometric area for globin standards separated by SDS/PAGE and stained with either Amido black (a) or Coomassie blue (b). The protein concentrations (a and b) were determined by TCA turbidity at 400 nm and the isotope levels (c) by liquid scintillation counting. Peak areas of bands were calculated manually from tracings of fluorograms (c) or photos of stained gels (a and b) scanned at 570 nm.

concentration and peak area (Figure 2b, regression coefficient = 0.997). Although data from globin gels stained with either dye could be used, since standards were included in each gel, Coomassie blue staining produced the most consistently reliable results. The relationship between peak area and the amount of isotope present (DPM) was essentially linear for globins (Figure 2c) and for histones (Figure 5b); as has been reported for other proteins (Laskey and Mills, 1975).

Axolotl histone standards were quantitated by comparison with chicken erythrocyte lysine-rich and core histone extracts after SDS/PAGE, Coomassie blue staining and densitometry. The serially-diluted chicken samples (Figure 3, lanes 1-10) contained a mixture of 1 mg/ml 5% PCA extract (lysine-rich histones) and 2 mg/ml core histones. The relative amount of each histone class was calculated using the data of Bates and Thomas (1981). There was a linear relationship between the amount of histone and Coomassie blue dye-binding (peak area) for all chicken histones (Figure 4). A mature axolotl erythrocyte histone sample (1 mg/ml PCA extract and 2 mg/ml core histones) was serially diluted and loaded in slots 11-15 (Figure 3) at the beginning and two hours before the end of the run. The band labelled 'X' is an unknown, slower moving band from the samples added at the beginning of the run.

Figure 3: Chicken and axolotl erythrocyte histone standards separated by 12.5% acrylamide SDS/PAGE and stained with Coomassie blue. Lanes 1-10 contain chicken histones ranging from 0.4 to 5 ug lysine-rich and 0.8 to 10 ug core histones. Axolotl histones (lanes 11-15), from 0.4 to 2 ug lysine-rich and 0.8 to 4 ug core histone, were loaded at the beginning of the electrophoretic run (lower 2/3rds of the gel) and 2 hours prior to the end of the run (upper 1/3 of the gel). The band labelled 'X' is a slower moving component of the samples added at the beginning of the run.

Figure 4 : The relationship between the quantity of individual chicken histone components separated by 12.5% acrylamide SDS/PAGE (Figure 3) and Coomassie blue dye-binding (measured by densitometry at 570 nm). The amounts of each histone component were calculated from the data of Bates and Thomas (1981). Peak areas were calculated by hand. The numbers in brackets () adjacent to each histone component label are the calculated regression coefficients for each curve.

○ H1, x H2A, ⊙ H2B, △ H3, ▣ H4, ■ H5

Since axolotl core histones have an identical SDS mobility to chicken core histones (Figure 3), the molecular weights of the axolotl core histones were assumed to be the same as those of the chicken core histones. Because core histone stoichiometry rarely deviates (Bates and Thomas, 1981), one can estimate the relative weight of each of the axolotl core histones in the 2 mg/ml stock solution. Since densitometric measurements show that the axolotl H1 subtypes were present in equal amounts in this standard, their quantities could be estimated from their concentration in the sample and the quantity added to each gel slot. The Coomassie blue dye-binding and histone weight relationship was also linear for axolotl histones (Figure 5a) although the slopes were not identical to those of the chicken histones (Figure 4).

The differences in slopes were unexpected, since Coomassie blue dye-binding depends upon amino acid composition (Bradford, 1976, Bio-Rad Protein Assay Kit), and histones show little variation in composition among species (Elgin and Weintraub, 1975; Von Holt *et al.*, 1979; Kincade, 1969). This variation is not due to differences in batches of staining solution or extents of destaining, because it is also observed among samples within the same gel (Figures 3, 4 and 5a). Neither are these distinctions attributable to interspecific

Figure 5: The relationships of protein concentration and isotope level to band peak areas after (a) densitometry of a serially diluted axolotl erythrocyte histone sample, separated by 12.5% SDS/PAGE and stained with Coomassie blue or (b) fluorography of gels containing a serially diluted axolotl erythrocyte histone standard. Numerical values are the regression coefficients for the respective curves.

a) The histone sample is from 146d axolotl erythrocytes after in vitro labelling with ^{14}C -lysine. Core histone quantities were calculated from the data of Bates and Thomas (1981) assuming their molecular weights were equal to chicken erythrocyte core histones (Figure 3). Both H1 components appear to be present in equal proportions (note double points at each quantity estimate). $\circ$ H1, $\times$ H2A, $\otimes$ H2B, Δ H3, $\square$ H4

b) The curves reflect the differences in slopes between peak area and DPM for axolotl histone samples over similar concentration ranges from seven different electrophoretic runs. The high regression coefficients indicate that a linear relationship between band density and isotope level is maintained.

differences between axolotl and chicken histones, since gels containing axolotl histones only showed significant slope variations between gels, although the slopes themselves remained linear. A more likely explanation is that the concentration of the axolotl histone standards changed during the course of the densitometric analyses. The observed development of precipitates in the standard sample vials, and the occasional appearance of faint heterogeneous bands below the standards in some gels, provide likely explanations for protein concentration changes. This is consistent with the similar slope variability from the fluorographic standard curves (Figure 5b). A difference in hydration of the chicken and axolotl standards (and hence weight /mg protein) is probably not involved since the total histone concentrations were checked by TCA turbidity as described for the globin standard curves.

The observed variability in the slopes used for histone and fluorographic quantitation within a given gel and between successive gels allows the estimate of relative quantities only within a given sample. Comparison of specific activity ratios between total H1 components and total core histones throughout the developmental transition demonstrated consistent trends between gels which concurred with the trends observed by

scintillation counting of the histone samples selectively extracted immediately after the labelling periods (see RESULTS). Encouraged by this observation, ratios of specific activities of individual histones and H1 components to core histone specific activities were calculated and normalized to the values obtained from one gel which contained representative samples throughout the metamorphic transition (Appendix II).

Similar quantitative problems were encountered with globin gels but not to the same degree, presumably because globin SDS samples were not as prone to precipitation as were the histone samples. Averages of the values for ug of globin/band and DPM/band were used in specific activity calculations without normalization to any one set of values (Appendix III). As was the case with histones, the resulting trends from both densitometric measurements and scintillation counts were in agreement.

III. RESULTS

A. Tissue-Specificity of Amphibian H1 Satellites

1. Anuran H1 Satellites

Since one of the principle characteristics distinguishing the two H1 satellites (the mammalian H1o and the nucleated erythrocyte-specific H5) is tissue specificity, a number of amphibian species were surveyed for both the presence and tissue-specificity of H1 satellites (Figure 6). As others have reported, the erythrocytes of Xenopus (Destree et al., 1979), leopard frog (Alder and Goróvsky, 1975) and bullfrog (Edwards and Hnilica, 1968; Nelson and Yunis, 1969; Panyim et al., 1971) contain a 5% PCA-extractable H1 satellite (Figure 6, lanes 3, 7 and 9 respectively). However, unlike others (Destree et al., 1979; Edwards and

Figure 6: Lysine-rich and core histones from erythrocyte (lanes 1 & 2, 3 & 4, 7 & 8 and 11 & 12) and liver (lanes 5 & 6, 9 & 10 and 13 & 14) nuclei of Xenopus (lanes 3-6), leopard frog (lanes 7-10) and bullfrog (lanes 11-14) compared to chicken erythrocyte histones (lanes 1 & 2) after electrophoresis in 12.5% acrylamide SDS gels.
H1^S = H1 satellites

1 2 3 4 5 6 7 8 9 10 11 12 13 14

H1
H1^S
H5

H3
H2B
H2A
H4

6470

Hnilica, 1968) H1 satellites were also observed in liver preparations carefully perfused with saline prior to nuclear isolation and histone extraction (Figure 6, lanes 4, 8 and 10, respectively). The proteins appear to be about 200 residues long by comparison of their electrophoretic mobilities in SDS/PAGE with that of chicken erythrocyte H5 (189 residues; Briand et al., 1980) and H1 subtypes (approximately 212 residues; Elgin and Weintraub, 1975) (Figure 6, lane 1). No additional bands with electrophoretic mobility similar to H1 satellites were realized by subsequent 0.25N HCl extraction of the PCA-extracted nuclear pellets from either erythrocytes or liver (Figure 6, lanes 4, 6, 8, 10, 12 and 14), nor were any found in total histone extracts (not shown).

When compared to erythrocyte preparations, the liver H1 satellites appear to be present in greater proportions relative to H1 components (Figure 6). Exclusion chromatography in Bio-Gel P-150 of Xenopus erythrocyte and liver PCA extracts substantiates this observation where the amount of H1 satellite (Figure 7, peak B) compared to the combined H1 components (peak A) is always greater from PCA extracts of liver nuclei. These results agree with those reported for turtle erythrocyte and liver H1 satellites (Rutledge et al.,

Figure 7: Bio-Gel P-150 exclusion chromatography of Xenopus erythrocyte (solid line) and liver (broken line) lysine-rich histones. Pooled fractions were analyzed by 12.5% acrylamide SDS/PAGE and compared with chicken erythrocyte lysine-rich histones (inset, lane 1) and the parent erythrocyte (lane 2) and liver (lane 5) extracts. Peak A fractions contained the H1 components of both erythrocytes (lane 3) and liver (lane 6), while peak B fractions contained the H1 satellite components (erythrocytes, lane 4; liver, lane 7). H1^S = H1 satellites

1981) and support the conclusion that the H1 satellites are not the result of erythrocyte contamination of liver preparations.

The Xenopus H1 satellites appear as a major and a minor band in erythrocytes (Figure 7, inset lane 4) and a nearly equivalent doublet in liver (Figure 7, inset lane 7). These quantitative differences may represent modified forms of a single satellite protein species or two distinct protein species, as is the case in some mammalian H1o fractions (Varricchio, 1977; Smith and Johns, 1980a; Medvedev and Medvedeva, 1980b; Gjerset et al., 1981). The faster H1 satellite does not appear to be a degradation product of the slower subtype, since its quantity is not correlated with proteolytic activity, judged by the presence of heterogeneous bands with core histone mobility typical of artifactual degradation (Miki and Neelin, 1977) which were observed only occasionally. Amino acid analysis of the proteins contained in both peaks from either erythrocytes or liver confirms the distinct identity of H1 satellites which always contain methionine, histidine, more arginine, isoleucine and tyrosine and much less alanine than do the H1 fractions (Table 2). Slight differences in amino acid composition of the liver and erythrocyte satellites may reflect the different relative proportions of the two satellite components where

Table 2: Amino acid compositions of H1 components and H1 satelllites from Xenopus erythrocytes and liver. Perchloric acid extracts of erythrocyte or liver nuclei were fractionated by either exclusion chromatography (Bio-Gel P-150, Figure 7) or cation-exchange chromatography (Amberlite CG-50, Figure 9). The values are averages of at least three independent chromatographic separations where the proteins in each peak were analysed at least twice. The columns marked (*) are from Destree et al. (1979). ND = not detected

Histones:	H1						H1 Satelllites						"H1 and H5"		
	RBC			Liver			RBC			Liver			RBC		
	A	I	II	A	I	II	B	III	CG-50	P-150	B	III	I*	II*	III*
Asx	2.9	3.1	3.1	3.1	4.3	4.4	3.8	4.2	4.7	4.9	3.9	3.8	3.9	3.8	5.0
Thr	3.4	3.2	4.1	3.9	3.0	3.7	3.5	3.0	3.8	3.4	3.6	4.6	3.6	4.6	3.7
Ser	8.0	8.2	7.6	8.4	10.1	11.4	9.6	9.4	10.3	10.6	9.9	10.0	9.9	10.0	13.7
Glx	5.2	5.8	5.3	5.8	8.4	8.3	6.5	6.6	7.3	7.2	7.9	7.1	7.9	7.1	9.8
Pro	9.5	9.5	10.5	10.2	7.9	7.7	8.5	8.2	8.2	8.3	8.2	9.3	8.2	9.3	6.8
Gly	4.3	4.4	5.4	4.9	8.7	10.3	5.1	6.3	5.9	7.2	6.8	8.0	6.8	8.0	11.1
Ala	25.1	24.2	21.6	23.9	19.9	18.0	17.3	16.5	17.0	15.3	22.6	20.4	22.6	20.4	13.7
Val	5.0	4.6	5.7	5.4	4.6	5.2	5.9	5.8	5.7	5.1	4.6	5.4	4.6	5.4	4.7
Met	ND	ND	ND	trace	ND	ND	0.3	trace	0.4	0.2	ND	ND	ND	ND	0.4
Ile	0.3	0.4	0.7	0.4	0.9	1.0	2.3	2.1	2.3	1.8	0.6	0.7	0.6	0.7	2.8
Leu	5.5	5.9	5.1	5.8	5.2	4.3	4.6	3.4	4.4	3.3	5.7	5.0	5.7	5.0	4.0
Tyr	0.6	0.7	0.7	0.5	1.3	1.2	1.3	1.7	1.3	1.5	0.3	0.3	0.3	0.3	0.9
phe	0.5	0.6	0.6	0.5	1.3	1.0	0.7	0.9	0.7	1.0	0.7	0.7	0.7	0.7	1.5
His	0.1	0.2	0.2	0.1	0.5	0.7	0.7	0.7	0.6	1.0	trace	trace	trace	trace	trace
Lys	28.7	28.5	28.6	26.4	22.7	21.6	26.9	28.0	25.7	26.7	23.8	23.9	23.8	23.9	18.8
Arg	1.0	1.0	1.1	1.0	1.3	1.4	2.8	2.7	2.9	2.7	1.5	1.2	1.5	1.2	3.2

the slower satellite, predominant in erythrocytes, would be richer in lysine and alanine and poorer in aspartate, serine, glutamate and glycine than the faster one (Table 2).

The amino acid composition of the Xenopus erythrocyte satellite H1 differs substantially from the H5-like protein reported in the literature (Destree et al., 1979; Table 2). In an attempt to resolve this discrepancy, P-150 peak A and peak B proteins from PCA extracts of Xenopus erythrocytes were subject to Amberlite CG-50 cation-exchange chromatography (the procedure used by Destree et al., 1979) to be sure that the peak B proteins did not contain a similar component of coincident electrophoretic but different chromatographic properties. Peak A from gel filtration (Figure 8a) contained two H1-like components which separated into two peaks by cation-exchange chromatography (Figure 8b, peaks I and II, solid line), but peak B (Figure 8a) contained a single satellite H1 peak (Figure 8b, peak III, dashed line) contaminated by minor amounts of H1. Cation-exchange chromatography of 5% PCA extracts from Xenopus liver and erythrocytes resolved these same chromatographic peaks (Figure 9). Peaks I and II contained typical H1 bands (inset lanes 3 & 4 and 7 & 8) and peak III contained H1 satellite bands (inset lanes 5 and 9). Amino acid analysis of the CG-50

Figure 8: Cation-exchange chromatography (b) of lysine-rich histone fractions from Xenopus erythrocytes after exclusion chromatography (a).

a) Exclusion chromatography of a Xenopus erythrocyte PCA-extract (8.25 mg) in Bio-Gel P-150. Proteins were eluted in 0.01N HCl, 0.01% sodium azide. Fractions in peaks A and B were pooled, dialyzed against distilled water and lyophilized.

b) Cation-exchange chromatography of the lysine-rich components contained in peak A (solid curve) and peak B (broken curve) of Figure 8a. Proteins were eluted by a linear gradient of 8 - 15% GuCl (tangential line) and detected by TCA-turbidity at 400nm.

peaks of proliferative activity occurred in the early stages of the metamorphic transition (Figure 27). The faster H1 component (H1c) probably represents modified forms of the principal components, H1a and H1b, since its appearance is correlated with a reduction in the average proportion of either or both of these components (Figure 26; Table 8). Reduced H1b and the presence of H1c appear to be correlated with proliferative activity as judged by the high levels of incorporation into core histones (Figures 24a and 26). Samples which contained H1c and reduced levels of H1a usually demonstrated preferential synthesis of lysine-rich histones which were independent of proliferative activity (Figures 24c and 26). Neither proliferation nor preferential lysine-rich histone synthesis appear to require H1c, since samples exhibiting either of these characteristics did not necessarily contain H1c (Figures 24a, 24c and 26). Peaks of preferential synthesis of H1b gradually declined to low levels by 125d while peaks of H1a and H1c persisted between peaks of proliferative activity to 186 days after spawning (Figure 27b, c and d). These similarities between H1a and H1c suggest that H1c is probably a modified H1a component in the late developmental stages.

The erythrocyte populations prior to 125 days consisted of cells synthesizing either larval globins or a mixture of larval and adult globins (Figure 23b). The

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Figure 9: Cation-exchange chromatography of Xenopus erythrocyte (solid curve) and liver (broken curve) lysine-rich histone components. Proteins were eluted in a linear gradient of 7 - 15% GuCl (tangential curve) and detected by TCA turbidity. Fractions in peaks I, II and III from both tissues respectively were pooled, precipitated in 20% TCA, washed in acid-acetone and lyophilized from distilled water. The proteins contained in the peaks were compared to chicken erythrocyte lysine-rich histones (inset, lane 1) and parent erythrocyte (lane 2) and liver (lane 6) extracts after 12.5% acrylamide SDS/PAGE. Lanes 3, 4 and 5 contain erythrocyte peak I, II and III components respectively. The liver peak I, II and III components are in lanes 7, 8 and 9. H1^S = H1 satellites

peaks confirmed that the erythrocyte and liver H1 satellite components are identical to those achieved by exclusion chromatography (Table 2). The proteins in peaks I and II did not differ significantly from each other or from fraction A separated by exclusion chromatography (Table 2). The minor peak between peaks I and II contained mixtures of both H1 and satellite H1 components (not shown).

The presence of methionine, which is typical of H1 satellites but not H1 components (see INTRODUCTION), and the specificity of cyanogen bromide cleavage at this residue, afford the opportunity of determining the amino acid sequence in the central "globular" region of this protein (Smith and Johns, 1980a; Cary et al., 1981). After treating a 5% PCA extract of Xenopus liver or erythrocyte nuclei with CNBr (see MATERIALS AND METHODS), a shorter primary cleavage product was generated at the expense of the H1 satellites (Figure 10, lanes 4 and 6). The liver H1 satellite doublet is reduced to a single band after cleavage (lane 4) indicating that the variability between the two liver subtypes resides in the lost amino-terminal arms of the molecules. The major cleavage product from both the erythrocytes and liver had a mobility in SDS between that of chicken erythrocyte H5 and its major cleavage product (lane 2), suggesting that about 30 amino acids

Figure 10: CNBr-cleaved (lanes 2, 4 and 6) and parent (lanes 1, 3 and 5) lysine-rich histones from chicken erythrocytes (lanes 1 and 2) and Xenopus liver (lanes 3 and 4) and erythrocytes (lanes 5 and 6) electrophoresed in 12.5% acrylamide SDS gels. H1^S = H1 satellites, CP = major CNBr-cleavage products.

had been removed. The positioning of the methionine at approximately residue 30 agrees well with its location in bovine H1o (Smith et al., 1980) and in H5 from chicken (Briand et al., 1980) and goose (Yaguchi et al., 1979). The persistent difference between Xenopus H1 satellite and H5 CNBr cleavage products suggests that they differ in the C-terminal 2/3rds of the molecules. These results are similar to those reported for H1 subtypes, where variations in the C-terminal arms occur between species but not between subtypes of a given species while variations in N-terminal arms account for intra-species differences (Cole, 1977).

Chromatography of the CNBr-cleaved, PCA extracted histone of Xenopus erythrocytes on Sephacryl S-200 permitted the separation of both major and minor cleavage products from the uncleaved H1 proteins (Figure 11). Elution with 0.01N HCl resolved two polypeptide peaks (Figure 11a), which contained a single, high mobility protein - presumably the minor cleavage product (inset lane 3), and an unidentified protein doublet (inset lane 4). Subsequent elution with 1M NaCl in 0.01M sodium phosphate (pH 6.8), released the major cleavage product and uncleaved satellite H1 (Figure 11b, inset lane 3) together, at twice the void volume of the column (Figure 11a, arrow). The H1 components were not detected in fractions collected after elution with

Figure 11: A Xenopus erythrocyte PCA extract (lane 1 in both insets) was subject to CNBr cleavage and the products (lane 2 in both insets) were separated by Sephacryl S-200 exclusion chromatography and identified by SDS gel electrophoresis. The minor cleavage product (a; peak A, inset lane 3) and an unidentified doublet (a; peak B, inset lane 4) were eluted in 0.01N HCl. The major cleavage product and uncleaved H1 satellite (b; inset lane 3) co-eluted in 1M NaCl (b) at approximately twice the void volume of the column (arrow in a). H1 components were presumably contained in the broad, early-eluting high-salt fractions (b).

either buffer though high background suggests they were probably contained in the early eluting high salt fractions (Figure 11b).

The satellite/major cleavage product mixture was subject to Edman degradation and amino acid sequencing. The small quantity allowed resolution of only a few residues, but presumed masking of the intact satellite H1 avoided the necessity of eliminating intact protein. Comparison of the sequence generated with that of bovine H10 (Smith et al., 1980), chicken H5 (Briand et al., 1980), goose H5 (Yaguchi et al., 1979) and bovine H1 (Cole, 1977) from their methionine residues towards the C-termini of the molecules (Table 3) clearly shows the sequence similarity of the H1 satellites. In this region, Xenopus erythrocyte satellite is nearly identical to bovine H10, and both are similar to avian H5 histones, but all satellites are distinct from H1. The apparent homogeneity of the residues released in sequence indicates release of a single peptide by CNBr and thus confirms that the N-terminus of the uncleaved satellite H1 is blocked. This is consistent with reports for mammalian H10 and chicken erythrocyte H5 (Smith and Johns, 1980a).

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Table 3: Partial amino acid sequences of H1 satellites and rabbit H1 aligned to show maximum homology from the methionine residue towards the C-terminal ends of the molecules. Numbers over the first residues of H5 and H1 sequences indicate their positions within their respective whole molecules. H1^s = H1 satellites; (1) Smith and Johns, 1980; (2) Briand et al., 1980; (3) Yaguchi et al., 1979; (4) Cole, 1977.

<u>Xenopus</u> erythrocyte H1 ^s :	- met - ileu - leu - ala - ala - val - gln - ala -
Bovine liver H1o (1):	- met - ileu - val - ala - ala - ileu - gln - ala -
Chicken erythrocyte H5 (2):	- ³² met - ileu - ala - ala - ala - ileu - arg - ala -
Goose erythrocyte H5 (3):	- ³² met - ileu - ala - ala - ala - ileu - arg - ala -
Rabbit thymus H1 (4):	- ⁴⁵ leu - ileu - thr - lys - ala - val - ala - ala -

When considered collectively, the chromatographic and electrophoretic properties along with the amino acid compositions and partial sequence analysis lead to the conclusion that the anuran H1 satellites are not tissue-specific and more closely resemble the mammalian H1₀ subclass of lysine-rich histones than they do the nucleated erythrocyte-specific H5.

2. Urodele Lysine-Rich Histones

Preliminary analysis of axolotl and Necturus PCA extracts of erythrocyte nuclei indicated the absence of electrophoretically identifiable H1 satellites, but unlike anurans both urodele extracts contained an H1 doublet (Figure 12, lanes 8 and 10 respectively), while the turtle contained both an H1 doublet and an H1 satellite (lane 12). These extracts were subject to CNBr cleavage (lanes 9, 11 and 13) and compared to similarly treated extracts from Xenopus, leopard frog, bullfrog and chickens (lanes 2 & 3, 5, 7 and 15 respectively). Principle cleavage products of the anuran, reptilian and avian H1 satellites and the slower axolotl H1 component (H1a) were generated. None of the other H1 components were CNBr-sensitive. The proportion of axolotl H1a cleaved, quantitated by densitometry of

Figure 12: SDS/PAGE of lysine-rich histones isolated from the erythrocytes of Xenopus (lanes 1-3), leopard frog (lanes 4 and 5), bullfrog (lanes 6 and 7), axolotl (lanes 8 and 9), Necturus (lanes 10 and 11), red-eared turtle (lanes 12 and 13) and chicken (lanes 14 and 15). Parent extracts appear in the left lane of each group while the CNBr-cleaved samples occupy the other lanes to the right. The Xenopus erythrocyte sample in lane 3 was cleaved in the presence of 3M urea. The major cleavage products (CP) are indicated by bars (-) to their left.
H1^S = H1 satellites

H1
H1s

CP

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

the stained gel (without correction for dye-binding), was 81% (Table 4). When this was compared to the total amount of intact Hla after cleavage, relative to the amount of Hlb (the faster axolotl H1 component), the difference (uncleaved Hla + Hlb) - (Hla + Hlb + major cleavage product) was in the same proportion as the difference seen with Xenopus cleaved and uncleaved fractions. This indicated that the axolotl Hlb component was not reduced by cleavage and that all of the fragment was generated from Hla.

A series of treatments with 2, 5 and 10 mg CNBr/mg protein were tested with Xenopus erythrocyte PCA extracts which demonstrated that quantitative differences in the extent of cleavage, observed in different experiments, were not dependent on the concentration of CNBr during cleavage (not shown). Nor were they dependent on the tertiary conformation of the proteins, since incubation in the presence of 3M urea did not increase the proportion of satellite cleaved (Table 4). The variability may depend on the amount of oxidized methionine present in the respective samples, since oxidized methionine is insensitive to CNBr cleavage (Croft, 1980).

Exclusion chromatography of the adult axolotl erythrocyte PCA extract produced only one peak (Figure 13) containing both H1 components (not shown), while

Table 4: Quantitative estimates of the sensitivity of various H1 satellites to CNBr-cleavage. PCA extracts from isolated erythrocyte nuclei of the indicated species were reduced and separated into two aliquots. Both were treated identically in subsequent steps except for the addition of CNBr to one aliquot only. The products were separated by SDS/PAGE (Figure 12) and protein bands were quantitated by densitometry of the Coomassie blue-stained gel (not corrected for dye-binding). H1^S = H1 satellites except in the axolotl where it refers to the slower moving, CNBr-sensitive H1 component. CP = major cleavage product

Sample:		Area H1	Area H1 ^S	Area CP	% H1 ^S cleaved
<u>Xenopus</u>	uncleaved	2.079	0.459	-	-
	cleaved	3.074	0.169	0.223	75.1
	cleaved(urea)	8.856	0.555	0.702	71.6
leopard frog	uncleaved	3.225	0.504	-	-
	cleaved	5.316	0.319	0.107	61.6
bullfrog	uncleaved	4.420	0.573	-	-
	cleaved	7.510	0.595	0.320	38.9
axolotl	uncleaved	4.210	3.107	-	-
	cleaved	2.466	0.352	0.425	80.7
<u>Necturus</u>	uncleaved	8.555	-	-	-
	cleaved	7.351	-	-	-
turtle	uncleaved	6.382	0.552	-	-
	cleaved	6.993	0.042	0.198	93.1
chicken	uncleaved	1.452	1.278	-	-
	cleaved	1.216	0.146	0.429	84.5

Figure 13: Bio-Gel P-150 exclusion chromatography of axolotl erythrocyte lysine-rich histones. All H1 components were eluted in a single peak.

separation of both erythrocyte and liver PCA extracts by Amberlite CG-50 cation-exchange chromatography resolved three principle peaks (Figure 14), as it had for Xenopus erythrocyte and liver PCA extracts (Figure 9). Peak I contained two protein bands (H1a and H1b; Figure 14, inset lanes 4 and 9) with mobilities identical to the bands in the parent extracts (lanes 3 and 8). Occasionally this peak was partially resolved into two overlapping peaks and recent work has shown that it can be entirely eliminated if PCA extracts are treated with 8M urea and 5% 2-mercaptoethanol prior to TCA precipitation, acid-acetone washes, air-drying and column application in 7% GuCl. Peak II contained one major electrophoretic component with mobility equal to that of H1a in the parent extracts (Figure 14, inset lanes 5 and 10). Peak III also contained a single component but with electrophoretic mobility similar to that of H1b (Figure 14, inset lanes 6 and 11).

CNBr cleavage of these three erythrocyte fractions demonstrated that only proteins in the second CG-50 fraction (peak II) contained methionine (Figure 15, lanes 6, 7 and 8). The uncleaved proteins of fraction II, after CNBr treatment, appeared as a doublet with mobilities identical to those of the parent extract and approximately identical in intensities (Figure 15, lane 7). Careful examination shows the presence of both

Figure 14: Cation-exchange chromatography of axolotl erythrocyte (solid curve) and liver (broken curve) lysine-rich histones. Proteins were eluted in a linear gradient from 7 - 14% GuCl (tangential line) and detected by TCA turbidity at 400 nm. The indicated fractions were pooled and H1 components in peaks I, II and III from both erythrocytes (inset; lanes 4, 5 and 6 respectively) and liver (inset; lanes 9, 10 and 11 respectively) were compared to the parent erythrocyte (lane 3) and liver (lane 8) extracts following 15% acrylamide SDS/PAGE. Chicken erythrocyte lysine-rich histones (inset; lane 1) and axolotl erythrocyte (lane 2) and liver (lane 7) core histones were included for reference. The presence of one liver H1 component (inset; lane 8 arrow) was always correlated with the presence of heterogeneous bands having core histone mobility (lane 7). Polypeptides in liver peak 'i' were not detectable in samples after gel electrophoresis (not shown).

1 2 3 4 5 6 7 8 9 10 11
H1 H5

Figure 15: SDS gel electrophoresis (12.5% acrylamide) of CNBr-treated (lanes 5-8) and untreated (lanes 1-4) axolotl erythrocyte H1 components. Lanes 1 and 5 contain total lysine-rich histone extracts before (lane 1) and after (lane 5) CNBr-cleavage. Lanes 2 and 6 are from peak I, 3 and 7 are from peak II and 4 and 8 are from peak III fractions after cation-exchange chromatography (Figure 14). Chicken erythrocyte lysine-rich histones before (lane 9) and after (lane 10) CNBr-treatment are included for reference. CP = major cleavage product

H1a
H1b

H5

CP[

1 2 3 4 5 6 7 8 9 10

components in peak II fractions before cleavage though the protein with H1b mobility is poorly represented (Figure 14, inset lane 5; Figure 15, lane 3). This faster protein may be a contaminating protein from peak I and/or peak III. The apparent increase in relative proportion after CNBr cleavage (Figure 15, lane 7) is likely due to a reduction in the H1a component caused by cleavage and the increased sample concentration necessary to visualize the shorter primary cleavage product.

Since the parent methionine-containing H1a of axolotl is larger than the satellite H1 of Xenopus and since the major cleavage products of the two preparations have nearly identical mobilities (Figure 9), the size of the axolotl CNBr-cleaved, N-terminal fragment is slightly larger than that generated from Xenopus satellite H1. This suggests that the methionine in the CNBr-sensitive axolotl H1a component is approximately 40 amino acid residues from the N-terminus of the protein. Given that the globular regions of H1 satellites are conserved (Yaguchi et al., 1979; Smith et al., 1980; Pehrson and Cole, 1981) and that variations occur in the two terminal domains (Cole, 1977; Yaguchi et al., 1979), the single methionine residue in the axolotl peak II component may be located in a similar location, with respect to the globular region, as it is

in H1o, H5 and Xenopus satellite H1. The differences in mobility of the major cleavage products suggests species-specific variations in the carboxy-terminal arms of these molecules, as is the case with H1 subtypes (Cole, 1977). The high degree of evolutionary conservation between H1 subtypes and H1 satellites makes it very unlikely that the CNBr is cleaving a fragment off the C-terminal end of the axolotl H1a component, though my data cannot argue this interpretation.

Comparison of the amino acid compositions of proteins contained in the axolotl erythrocyte CG-50 fractions (Table 5) demonstrated that the CNBr-sensitive fraction (peak II) had a slightly elevated level of methionine and serine and a slightly reduced level of valine and aspartate. The other two peaks contained proteins with identical amino acid compositions. Although their compositions indicated traces of methionine, electrophoresis of CNBr-cleaved PCA extracts after cation exchange chromatography demonstrated that they were not CNBr-sensitive (Figure 15) and the trace amounts may have been due to some peak II contamination during fractionation.

The liver peak II fraction contained reduced levels of aspartate, glutamate and glycine residues with elevated levels of alanine and lysine, compared to the peaks I and II (Table 5). These differences are not

Table 5: Amino acid compositions of lysine-rich histones from axolotl erythrocytes (RBC) and liver after CG-50 cation-exchange chromatography. Values are averages of at least three independent chromatographic separations where the proteins in each peak were subject to amino acid analysis at least twice. Xenopus erythrocyte and liver H1 (P-150, peak A) and H1 satellite (P-150, peak B) compositions are from Table 2. ND = not detected

Animal:	Axolotl			Axolotl			Xenopus		Xenopus	
	RBC			Liver			RBC		Liver	
	CG-50	CG-50	RBC	CG-50	CG-50	CG-50	P-150	P-150	P-150	P-150
Separation:	I	II	III	I	II	III	A	B	A	B
Peak:	I	II	III	I	II	III	A	B	A	B
Asx	3.5	2.9	3.4	6.0	4.7	5.5	2.9	3.8	3.1	4.7
Thr	2.5	2.3	2.5	3.1	2.6	2.5	3.4	3.5	3.9	3.8
Ser	5.5	7.5	5.6	6.6	5.8	6.2	8.0	9.6	8.4	10.3
Glx	4.9	5.1	4.7	11.8	9.9	9.1	5.2	6.5	5.8	7.3
Pro	9.2	9.9	9.3	6.1	6.4	6.6	9.5	8.5	10.2	8.2
Gly	6.8	7.0	6.7	11.0	9.5	12.1	4.3	5.1	4.9	5.9
Ala	25.7	25.5	25.5	13.0	21.7	19.6	25.1	17.3	23.9	17.0
Val	7.9	5.2	7.2	5.7	6.3	6.1	5.0	5.9	5.4	5.7
Met	trace	0.5	0.1	1.2	0.5	0.6	ND	0.3	trace	0.4
Ile	0.6	0.6	0.6	2.3	1.4	1.4	0.3	2.3	0.4	2.3
Leu	4.2	4.1	4.1	4.5	4.0	3.8	5.5	4.6	5.8	4.4
Tyr	0.6	0.7	0.6	2.5	1.2	1.5	0.6	1.3	0.5	1.3
Phe	0.8	0.7	0.7	3.0	1.4	1.5	0.5	0.7	0.5	0.7
His	0.3	0.2	0.3	1.5	0.9	0.7	0.1	0.7	0.1	0.6
Lys	26.1	26.8	26.9	20.0	21.7	20.2	28.7	26.9	26.4	25.7
Arg	1.5	1.7	1.8	3.3	2.2	2.0	1.0	2.8	1.0	2.9

consistent with the trends demonstrated by the erythrocyte H1 fractions (Table 5). All axolotl liver H1 components demonstrated considerable difference from their erythrocyte counterparts, but this variation may be due to contaminating polypeptides from the broad early eluting peak (i, Figure 14); trailing through the low levels of H1-containing fractions. The consistently high levels of methionine, aspartate, glutamate and glycine with lower levels of alanine and lysine between the liver fractions and their erythrocyte counterparts (Table 5), supports this hypothesis. Of the three liver fractions, peak II proteins show the closest similarity to their erythrocyte counterparts, which may be a reflection of their higher relative proportion (Figure 14), and hence decreased susceptibility to contaminating polypeptides. Comparison of the amino acid compositions of the polypeptides contained in the early eluting peak (i, Figure 14, Table 5) with those of the liver H1 fractions demonstrated higher levels of methionine, acidics, serine, histidine, phenylalanine, tyrosine, isoleucine and glycine and lower levels of lysine and alanine and mirrored the consistent differences between the liver and erythrocyte counterparts (Table 5). Repeated extractions and chromatography failed to

substantially reduce the size of this early peak or change the amino acid composition of the axolotl liver H1 fractions.

Taking erythrocyte and liver fractions together, it appears that all axolotl lysine-rich histones more closely resemble H1 components than they do H1 satellites of Xenopus (Table 5). The high alanine and low isoleucine and arginine levels, common to fractions from both tissues, are characteristic of H1 subtypes (Kincade, 1969; Bustin and Cole, 1968), as are the lower serine, glutamate, tyrosine, histidine and higher proline levels, obvious in the erythrocyte fractions, which appear to contain little polypeptide contamination (Figure 14).

Statistical comparison of the amino acid compositions of the axolotl erythrocyte H1 components with Xenopus erythrocyte H1 and satellite H1 components (Table 6) confirms this relationship showing a high degree of similarity between the Xenopus peak A and all three axolotl H1 components and a low level of similarity between the Xenopus erythrocyte H1 satellites (peak B) and either all axolotl components or Xenopus H1 peak A (Table 6). However, the axolotl erythrocyte peak II proteins are as related to the Xenopus erythrocyte H1 satellite components as the latter are to the Xenopus erythrocyte H1 components. That is, the axolotl peak II

Table 6: Statistical comparison of amino acid compositions of Xenopus erythrocyte H1 and H1 satellite components (isolated by P-150 exclusion chromatography) with axolotl erythrocyte H1 components fractionated by CG-50 cation-exchange chromatography (Table 5).

$$DI = \frac{\sum |X_A - X_B|}{2}, \quad S4Q = \sum (X_A - X_B)^2 \text{ and } D = 0.01 \cdot S4Q^{1/2} \text{ where } X_A \text{ and } X_B \text{ represent}$$

the percent composition of amino acid X in proteins A and B respectively (Cornish-Bowden, 1980).

Protein A : <u>Xenopus RBC P-150 peak A</u>	<u>Xenopus peak A</u>		<u>Xenopus RBC P-150 peak B</u>	
	vs		vs	
Protein B : axolotl RBC CG-50 peaks:	<u>Xenopus peak B</u>		axolotl RBC CG-50 peaks:	
	I	II	I	II
		III		III
$\Sigma X_A - X_B $:	15.8	10.6	14.4	22.7
$\Sigma (X_A - X_B)^2$:	31.5	15.7	24.0	80.6
DI :	7.9	5.3	7.2	11.4
S4Q :	32	16	24	81
D :	0.056	0.040	0.049	0.090

According to Cornish-Bowden (1980) the critical values for the difference index (DI), the compositional divergence (D) and the index of Marchalonis and Weltman (1971; S4Q) for proteins containing 200 amino acid residues are 10.3, 42.0 and 0.0648 respectively. Tabulated values below these represent near certainty of a relationship between the two proteins under comparison.

proteins are as closely related to the Xenopus satellite components as these satellites are to their homologous H1 components. This suggests that the axolotl peak II proteins and the Xenopus satellite components share a 'loose' relationship (Cornish-Bowden, 1980), as indicated by values below a second set of 'critical values' of 15.2, 93.0 and 0.0964 for DI, S4Q and D respectively (Table 6).

These results indicate that the axolotl contains an H1 component which in some respects resembles H1 satellites (a loose statistical relationship and the presence and probable location of a single methionine residue) while its size (determined by SDS/PAGE and exclusion chromatography), cation-exchange chromatographic properties and amino acid composition more closely resemble H1 subtypes. Although electrophoretic mobility is the most common method used for identifying H1 satellites, the amino acid composition is the most sensitive parameter for confidently distinguishing between H1 subtypes and H1 satellites. In the case of the axolotl, both parameters suggest that the erythrocytes, and probably the liver, contain no satellite H1 components.

This situation has interesting developmental and functional implications for other nucleated erythrocytes contain H1 satellites (Elgin and Weintraub, 1975) as do

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most differentiated tissues of higher eukaryotes (Panyim and Chalkley, 1969a,b). One wonders how the adult axolotl can exist without these specialized histones and, if one of the H1 components behaves like an H1 satellite during a developmental transition. In order to further characterize the axolotl erythrocyte H1 components, a study of hemoglobin switching and accompanying histone and DNA synthetic changes was undertaken through cryptic metamorphosis.

B. Hemoglobin Synthesis, Cell Division Cycles and
Histone Metabolism During Cryptic Metamorphosis in the
Axolotl

I. Cryptic Metamorphosis

The axolotl undergoes a biochemically characterized cryptic metamorphic event which includes changes in both the hemoglobin and serum protein composition of the blood and is not accompanied by gross anatomical alterations (Ducibella, 1974a). Preliminary work in this study demonstrated the presence of two distinct larval globin subunits which persisted in

Figure 16: Globin subunits released by saponin lysis of axolotls 154 days (lane 2), 163 days (lane 3), 178 days (lane 4), 192 days (lane 5) and approximately 2 years (lane 6) after spawning, and separated by 15% acrylamide SDS/PAGE. An axolotl core histone sample was included for reference (lane 1). The molecular weights of the fast larval band (13,800) and the slow adult band (24,500) are from Ducibella (1974a).

animals older than 154 days after spawning (Figure 16, lanes 2-5). Judging from their mobility relative to that of an axolotl core histone sample (lane 1), these polypeptides correspond to the 13,800 and 19,500-dalton bands previously described (Ducibella, 1974a). With advancing age the three adult globin subunits evident as traces in the 154d animals progressively increased relative to the larval subunits (Figure 16, lanes 2-5). The adult globin subunits (lane 6) correspond to the 15,000, 19,000 and 24,500-dalton bands reported by Ducibella (1974a). The accumulation of adult globin subunits indicated that, in the colony studied, axolotl cryptic metamorphosis started prior to 154 days after spawning and continued past 192 days of development. This transition occurred in a similar time frame to that of previous studies where the accumulation of the 13,800-dalton adult globin reached 30% of the total globin by 150 days after hatching (168 days after spawning) (Ducibella, 1974a).

In order to extend these observations and consider the relationships among cell division, globin subunit synthesis and accumulation, and histone synthesis, pooled extracts of erythrocytes from 2-10 animals were incubated in vitro in the presence of excess tritiated-thymidine alone or in conjunction with 14-C-lysine. Samples were taken at 10-minute intervals

during this incubation to ensure cellular viability and excess of precursor, as judged by linear incorporation of isotope into cells. The few populations which did not show linear incorporation were discarded. Labelling was terminated by washing the cellular suspension in ice-cold unlabelled medium at which point smears of the tritiated-thymidine-labelled cells were prepared for autoradiography. TCA-precipitable incorporation of both labelled precursors was determined for washed whole cell samples, while incorporation of ^{14}C -lysine into cytoplasmic lysates, nuclear pellets, lysine-rich histones, core histones, total histone extracts and the residual nuclear pellet after histone extraction was measured (Appendix I). Cytoplasmic protein and histone samples were separated by SDS/PAGE, and individual protein bands were quantitated by densitometry of the stained gels. Estimates of the specific activities of the protein bands were achieved by quantitative fluorography.

2. Cell Division Cycles

Since histone and DNA synthesis are in most cases tightly coupled (Lewin, 1974; Elgin and Weintraub, 1975), and the number of cells incorporating labelled-thymidine (determined by autoradiography) and

incorporation of labelled-thymidine into cells (determined by scintillation counting) measure the same parameter, relative changes in these three sets of data represent relative changes in the same cellular phenomenon - proliferation. With this in mind, the curves through the data points of these parameters (Figure 17) are drawn in consideration of the trends observed by all three parameters. This necessitated the exclusion of certain data points but, I believe, allows an accurate interpretation of the results. Replicate populations of cells taken at selected times after spawning showed comparable levels of activity (Appendix IV) and successive samples from a common spawning also showed these typical waves of activity.

The incorporation of labelled-thymidine into axolotl erythrocytes during the metamorphic transition was cyclical with peaks occurring at approximately 20-day intervals from 60d to 150d after spawning (Figure 17a, Appendix I). Autoradiography demonstrated that the labelling was confined to the cell nucleus (Figure 18a, b, c, e and f). The mature adult erythrocytes are smaller and their oval nuclei appear to be in a more condensed state than are the large rounded nuclei of the larval erythrocytes (Figure 18 e & f vs a & b). The 154d population contains a mixture of both cell types (Figure 18d). This is consistent with a previous report

Figure 17: Incorporation of tritiated-thymidine (a and b) into erythrocytes and ^{14}C -lysine (c-e) into erythrocytes and cellular fractions through cryptic metamorphosis in the axolotl. Thymidine incorporation was determined by liquid scintillation counting of cell samples (a), and by determining the proportion of cells incorporating label by autoradiography of blood smears (b). Lysine incorporation was determined by liquid scintillation counting of cell (c), saponin-supernatant (d) and total histone (e) samples taken after in vitro labelling. The data for curves a, c, d and e is contained in Appendix I. NA = neotenus adult

Figure 18: Blood smears from erythrocyte populations of 100d (a and b), 146d (c), 154d (d) and 190d (e and f) animals after in vitro labelling with tritiated-thymidine, autoradiography and May-Grunwald and Geimsa staining. m = mitotic figure; E = eosinophile; L = lymphocyte; scale bar = 10 um

(Ducibella, 1974b), even though crosslinking fixatives were not employed in the present study. Distinct mitotic figures (m) were observed in all populations from 70d to 224d but were difficult to quantitate due to the large amount of dense chromatin in each nucleus (Figure 18b, d and e). White blood cells (E= eosinophile and L= lymphocyte) constituted less than 0.5% of the populations and seldom demonstrated incorporation, even when neighbouring erythrocytes were densely labelled (Figure 18a and f).

When DNA was isolated from a 154d erythrocyte population by ethanol, ether and hot TCA extraction, 92% of the labelled thymidine was incorporated into the deoxyribonucleic acid fraction, 0.08% was released by the ethanol/ether washes and 7% remained in the residual protein pellet (Table 7). These data indicate that the incorporated 3-H-thymidine (Figure 19) was being synthesized into the DNA of the erythrocytes.

When the proportion of cells incorporating tritiated-thymidine (determined by autoradiography) was plotted against animal age (Figure 17b), there was a close correspondence with peaks of incorporation into DNA realized by liquid scintillation counting (Figure 17a), with the possible exception of the first peak containing the least mature cells. This indicated that variations in thymidine incorporation were the result of

Table 7: Axolotl erythrocytes, isolated from animals 154 days after spawning, were incubated in vitro with tritiated-thymidine (20 uCi/ml of cell suspension). Samples were taken at 10-minute intervals during the one hour incubation to ensure continued cell viability and excess labelled precursor indicated by linear isotope incorporation (Figure 19). DNA was isolated by ethanol/ether washes and hot TCA extraction. The amount of label in each fraction (CPM/ 4×10^6 cells) was determined by liquid scintillation counting and expressed as the percent of label incorporated per fraction compared to that incorporated into the washed cell sample after the labelling period (% incorp.).

Fraction :	Cells	ETOH Wash	ETOH Ether	Ether Wash	Hot TCA Soluble	Residual Pellet
<hr/>						
CPM						
4×10^6 cells:	23665	12	5	3	21763	1624
% incorp. :	100	0.05	0.02	0.01	92	7

Figure 19: Tritiated-thymidine incorporation profile for the axolotl erythrocyte population described in Table 7. A brief 10-15 minute lag at the beginning of the incubation is followed by nearly linear precursor incorporation during the remaining 45-50 minutes of in vitro labelling.

differences in the number of proliferating cells rather than a reflection of the speed at which cells in the populations were traversing the cell cycle. No consistent differences were found in the intensity of autoradiographic labelling between cell populations from different stages of the transition.

The profiles of ^{14}C -lysine incorporation into cells, cytoplasmic lysates (mostly hemoglobin; see later) and histones during the developmental transition (Figure 17c, d and e respectively; Appendix I) paralleled the tritiated-thymidine incorporation profiles (Figure 17a). These consistent trends, independent of cellular compartmentalization or the type of labelled precursor suggest that intracellular pool sizes of these precursors are negligible compared to the levels made available, and support an interpretation of non-random fluctuations in cellular proliferation and precursor uptake and incorporation into protein during cryptic metamorphosis.

3. Hemoglobin Switching During the Metamorphic Transition

By separating the ^{14}C -lysine-labelled proteins contained in the saponin supernatants in SDS/PAGE followed by fluorography, I was able to 1) demonstrate

that the only components present in sufficient quantities to allow analysis were globins, 2) determine the type and specific activities of the globins synthesized and 3) plot the accumulation of adult and decline of larval globins during the metamorphic transition.

When the cytoplasmic lysate was reduced, partially purified by Sephadex G-100 exclusion chromatography, and the fractions monitored for both hemoglobin and total protein (by absorbance at 415 and 280nm, respectively), over 90% of the protein co-eluted with the hemoglobin-containing fractions (Figure 20). Electrophoresis of these fractions in SDS/PAGE beside SDS-solubilized saponin-supernatants demonstrated that no other components were contained in the lysates in sufficient quantities to interfere with the visualization of the globin chains (Figure 21). However, some of the 13,800 and 15,000-dalton subunits appear to be lost on chromatography (Figure 21, lanes 2, 4 and 6).

Lysates taken from animals during the metamorphic transition contained both larval and adult globin chains (Figure 21, lanes 2, 3 and 4) but the 19,000-dalton adult and the 19,500-dalton larval subtypes were difficult to distinguish and appeared to occur in all stages. Comparison of the saponin supernatants through

Figure 20: Sephadex G-100 exclusion chromatography of saponin lysates from erythrocytes of animals 100 days after spawning. Fractions were monitored for both hemoglobin (Absorbance at 415nm, ●) and total protein (Absorbance at 280nm, x). Fractions in the center of the hemoglobin peak (15-20) were typically pooled for analysis by SDS/PAGE.

Figure 21: SDS/PAGE (in 15% acrylamide) of axolotl globin components from saponin lysates before (lanes 1, 3 and 5) or after (lanes 2, 4 and 6) partial purification by exclusion chromatography (Figure 20). Samples were from erythrocytes of animals 114 days (lane 1), 118 days (lane 2), 154 days (lanes 3 and 4) and 166 days (lanes 5 and 6) after spawning. The molecular weight designations are from Ducibella (1974a).

19,500
13,800

24,500
19,000
15,000

1 2 3 4 5 6

Figure 22: SDS/PAGE (in 12.5% acrylamide) of globins from animals 75, 90, 110, 120, 140, 163, 190, and 224 days after spawning. Protein bands were stained with Coomassie blue (a). The bands in (b) are after fluorography of the same gels shown in (a).

a.

Age: 75 90 110 120 140 163 190 224

b.

the metamorphic transition demonstrated that the 15,000 and 24,500-dalton adult globin bands become apparent at 110d, and persisted with the 13,800-dalton larval band (Figure 22a). The 13,800-dalton larval band ceased to be detectable between 190 days and 224 days after spawning (Figure 22a).

The fluorograms demonstrated that incorporation into adult globins was first detectable at 90d while incorporation into larval globins persisted until after 163 days (Figure 22b). To obtain more precise comparisons, three sets of replicate gels were run containing samples of 14-C-lysine-labelled saponin supernatants from 60 to 224d animals and including lysates from mature adult erythrocytes. Specific activities of larval and adult globin bands were calculated by densitometry of the stained gels and their accompanying fluorograms (Appendix III), ignoring the poorly resolved 19,000 and 19,500-dalton globin bands. When the data was plotted against animal age (Figure 23b), peaks of activity between 100 and 160d matched with those measured by scintillation counting of saponin supernatants of the same cell populations (Figure 23a). Labelled precursor was specifically incorporated into larval globins in 60-84d samples and then continued at much reduced levels until approximately 125d (Figure 23b). Adult globins demonstrated peaks of incorporation

Figure 23: The behavior of axolotl globins through the metamorphic transition.

a) ¹⁴C-lysine incorporation into saponin lysates determined by liquid scintillation counting (data in Appendix I).

b) The specific activities of globin subunits determined by densitometry of stained gels and their accompanying fluorograms (Figure 22; data in Appendix III).

c) The relative amounts of one larval globin (13,800 daltons) and two adult globins (24,500 and 15,000 daltons), calculated as percent of total globins in each sample (including the 19,500 and 19,000 dalton subtypes which are not resolved), determined by densitometry of stained gels (data in Appendix III). NA = neotenuous adult

in 90, 110, 134 and 154d samples with low level incorporation from 166d to the adult (Figure 23b). When the protein quantitation data was plotted as percent of total globin (Figure 23c), there is a sharp but temporary rise and decline in larval bands between 100 and 130d. This trend was subsequently reversed to be replaced by a gradual accumulation of the adult globins at the expense of the larval globins (Figure 23c).

Collectively, these results show successive peaks of incorporation of radioactive precursors into both DNA and globins, with a switch in globin subtype synthesis and accumulation through the metamorphic transition. The question addressed at this point is "Are any of the modulations of incorporation into DNA or specific globins accompanied by alterations of precursor incorporation into particular axolotl erythrocyte H1 subtypes?"

4. Histone Metabolism During the Cryptic Metamorphic Transition

Peaks of incorporation of 14-C-lysine into histones (Figure 17e) at progressive stages of development coincide with peaks of 3-H-thymidine incorporation into DNA (Figure 17a), as do peaks of 14-C-lysine into hemoglobin (Figure 17c). Core (Figure

24a) and lysine-rich (Figure 24b) histones show similar changes in incorporation of 14-C-lysine with development. However, the synthesis of these functionally different histones is not uniform as shown by the ratio of 14-C-lysine incorporated into lysine-rich histones relative to core histones through the metamorphic transition (Figure 24c). The horizontal line indicates the expected ratio (0.37) given the synthesis of 1.34 molecules (Bates and Thomas, 1981) of H1 (26% lysine, Table 5) for every eight molecules of core histone having an average lysine content of 12% (Elgin and Weintraub, 1975). Lysine-rich histones show preferential incorporation of labelled precursor after transient rises in incorporation into core histones (Figure 24a) and globin subtypes (Figure 23 a and b). Early in development there is a 10-day lag in this uncoupled incorporation, while the last core histone incorporation wave is followed by preferential incorporation into H1 components at 10 and 30-day intervals (Figure 24c). The first peak in the 65d sample could either be following a previous core histone incorporation peak or may indicate preferential incorporation into H1 components before the subsequent peak in core histone incorporation at 75d (Figure 24a).

Studies of erythropoiesis in Xenopus (Rutledge, 1982) and chickens (reviewed in INTRODUCTION) have shown

Figure 24: Incorporation of ^{14}C -lysine into erythrocyte core (a) and lysine-rich (b and d) histones through axolotl cryptic metamorphosis. The values for curves (a) and (b) were determined by liquid scintillation counting of histone samples taken immediately after in vitro labelling (data in Appendix I). Curve (c) represents values used to construct curve (b) divided by the values used to construct curve (a). The dashed horizontal line (c) is the expected ratio given 1.34 molecules of lysine-rich histone per octamer of core histones (Bates and Thomas, 1981). The specific activity values for lysine-rich histones (d) were estimated by densitometry of stained gels and their accompanying fluorograms (data in Appendix II). NA = neoténous adult

Animal Age (days after spawning)

60 80 100 120 140 160 180 200 220 NA

a. Core Histones

b. Lysine-Rich Histones

c. Lysine-Rich Core Histones

d. Lysine-Rich Histones Specific Activity

Animal Age (days after spawning)

preferential synthesis of H1 satellites during the late stages of erythrocyte maturation. In order to determine whether the peaks of preferential precursor incorporation into lysine-rich histones during the metamorphic transition in the axolotl (detected by liquid scintillation counting) reflected the preferential incorporation into particular axolotl H1 components, attempts were made to determine the specific activities of the total and individual H1 components through cryptic metamorphosis by densitometry of stained gels (Figure 25a) and their fluorograms (Figure 25b).

Visual analysis of the protein bands suggested little differences in the relative proportions of the core histones through cryptic metamorphosis, while there appeared to be some variation in the relative proportion of the individual H1 components (Figure 25a). Consideration of the accompanying fluorogram (Figure 25b) indicated two clear phases of ¹⁴C-lysine incorporation into histones (75d and 120d; lanes 3 and 7 respectively) with preferential incorporation into various H1 components throughout the transition. For example most samples had low levels of H1a components compared to H1b (Figure 25a) but the density of these bands on the fluorogram appeared equal to, or greater than, that of the accompanying H1b bands (Figure 25b, especially lanes 3, 4, 5, 9, 11 and 14).

Figure 25: SDS/PAGE (in 12.5% acrylamide) of erythrocyte total histone samples from axolotls through cryptic metamorphosis after in vitro labelling with ^{14}C -lysine.

a. The lower 2/3rds of the gel contains histones taken from animals at the indicated times after spawning (Age in days). The upper 1/3rd of the gel contains a serially diluted set of histone standards (from 0.2 to 12 ug of core histones) added 2 hours before termination of the 6 hour electrophoretic run. The gel was stained with Coomassie blue.

b. Bands are the result of fluorography of the dried gel shown in (a) after a 16-day exposure at -80°C .

a.

H1a
H1b
H1c

Core
Histones

Age :

60 70 75 96 100 114 120 126 140 146 155 166 178 186 224

b.

The ratios of the specific activity of the H1 components to core histone specific activity through the transition (determined by densitometry of stained gels and fluorograms; Appendix II) demonstrated peaks of preferential incorporation into lysine-rich histones (Figure 24d), which coincided with those realized by PCA/HCl histone fractionation and liquid scintillation counting (Figure 24c). The relative constancy of the individual core histone/total core histone specific activity ratios throughout the study period (Appendix II) is consistent with co-ordinate synthesis of all nucleosomal histones and serves as a control on specific activity ratios of H1 components/total core histones (Appendix II).

A previously undetected, faster migrating H1 component (H1c, Figure 25) was resolved in 12.5% but not 15% acrylamide SDS gels. Three sets of gels, each containing most histone samples taken through the transition period, contained this component in samples from 75d, 96d, 100d, 120d, 126d, 155d and 186d animals (Figures 25, 26 and 27). Comparison of the relative proportions of each H1 component to the total amount of lysine-rich histone in each sample (Table 8) indicated that the appearance of H1c (ranging from 13-19% of the total H1 components) corresponded to drops below average proportions of either or both of the other two H1

Table 8: The relative proportions of individual H1 components expressed as the percentage of total lysine-rich histone (tH1) in samples taken through axolotl cryptic metamorphosis. The values (+/- the standard error of the mean) are averages of independent estimates (the number of which appears in brackets after the values) determined by densitometry of stained gels and corrected for dye-binding by comparison with protein standards included in the same gel (Figure 25a). NA = neotenus adult

Age	% H1a/tH1	% H1b/tH1	% H1c/tH1
60	37 +/- 7 (4)	63 +/- 2 (4)	ND*
70	37 +/- 6 (4)	62 +/- 5 (4)	ND
75	31 +/- 5 (3)	49 +/- 3 (3)	19 +/- 3 (3)
79	44 +/- 4 (3)	57 +/- 4 (3)	ND
84	31 +/- 5 (4)	69 +/- 5 (4)	ND
90	34 +/- 6 (3)	66 +/- 6 (3)	ND
96	34 +/- 6 (3)	54 +/- 4 (3)	13 +/- 2 (3)
100	26 +/- 5 (3)	61 +/- 5 (3)	14 +/- 1 (3)
105	37 +/- 5 (3)	64 +/- 4 (3)	ND
110	36 +/- 3 (4)	64 +/- 3 (4)	ND
114	43 +/- 4 (5)	57 +/- 3 (5)	ND
120	25 +/- 1 (3)	60 +/- 2 (3)	15 +/- 3 (3)
126	19 +/- 2 (3)	64 +/- 3 (3)	17 +/- 2 (3)
130	25 +/- 2 (4)	74 +/- 2 (4)	ND
140	25 +/- 1 (5)	75 +/- 1 (5)	ND
146	35 +/- 5 (4)	65 +/- 5 (4)	ND
150	34 +/- 2 (4)	66 +/- 2 (4)	ND
155	31 +/- 4 (3)	55 +/- 5 (3)	14 +/- 1 (3)
166	37 +/- 5 (5)	63 +/- 5 (5)	ND
178	32 +/- 7 (4)	69 +/- 7 (4)	ND
181	32 +/- 1 (3)	68 +/- 2 (3)	ND
186	21 +/- 3 (3)	63 +/- 5 (3)	16 +/- 3 (3)
190	31 +/- 2 (3)	69 +/- 2 (3)	ND
224	30 +/- 4 (4)	71 +/- 4 (4)	ND
NA	35 +/- 2 (5)	65 +/- 3 (5)	ND

ND = not detected

Figure 26: The relative proportions of individual erythrocyte H1 components expressed as the percentage of total lysine-rich histones in samples taken through axolotl cryptic metamorphosis. The horizontal solid line is the mean level of H1b. The horizontal broken line is the mean level of H1a. Error bars represent the standard error of the mean for each value. (The data is contained in Table 8.) NA = neotenus adult

components (Figure 26, horizontal lines).

Interestingly, samples containing H1c and below-average levels of H1a also demonstrated preferential synthesis of lysine-rich histones (100d, 120d, 126d and 186d; Figure 24). Other samples showing preferential synthesis of lysine-rich histones (Figure 24c) did not contain H1c (60, 70, 84, 105, 114, 140, 146 and 181d; Figure 26). Samples containing both H1c and below-average H1b levels were, in most cases, involved in proliferative activity indicated by high levels of incorporation into core histones (75d, 96d, 100d and 120d; Figure 24a; the 155d sample is an exception). However 60, 70, 90, 114 and 130-day samples had high levels of proliferative activity (Figure 24a) and did not contain H1c (Figure 26). The 100 and 120d samples demonstrated preferential lysine-rich histone synthesis, high levels of incorporation into core histones, evident H1c and lower levels of both H1a and H1b (Figures 24c, 24a and 26 respectively). These data suggest that H1c is a modified form of the principle H1 components (H1a and H1b; Figure 25). Insufficient quantities were available to test for CNBr or alkaline phosphatase sensitivity; however, highly phosphorylated forms of H1 do have altered SDS mobility due to tertiary structural alterations (Billings et al., 1979).

Figure 27: Changes in the specific activity of erythrocyte H1 components (b, c and d) compared to the proliferative activity of the erythroid populations (a) through axolotl cryptic metamorphosis. Values in (a) were determined by liquid scintillation counting of core histone samples taken immediately after in vitro labelling with 14-C-lysine (data in Appendix I). Values in b, c and d were estimated by densitometry of stained gels and their accompanying fluorograms (data in Appendix II). NA = neotenus adult

In the early stages of the metamorphic transition peaks of preferential incorporation into all H1 components (Figure 27 b, c and d for H1a, H1b and H1c respectively) followed peaks of incorporation into core histones (Figure 27a). After 125d, preferential incorporation into H1 components was confined to H1a and H1c (Figure 27 b and d respectively).

The gradual decline in the preferential incorporation into H1b correlates well with both the decline in larval globin and increase in adult globin incorporation (Figure 23b). Preferential incorporation into H1a and H1c occurs with the accumulation of adult globins (Figure 23c) and is responsible for the peak of incorporation into lysine-rich histones (186d) which follows the last phase of high level incorporation into core histones (150-186d; Figure 27a).

IV. DISCUSSION AND CONCLUSIONS

A. Tissue-Specificity of Anuran H1 Satellites

Comparison of the amino acid compositions of Xenopus erythrocyte and liver H1 satellites demonstrated that the proteins are identical (Table 2 and Brown et al., 1981). The liver H1 satellites are unlikely the result of erythrocyte contamination since they are present in higher quantities, relative to the H1 components, than they are in erythrocytes (Figure 7). Recent results comparing peptide maps of Xenopus liver and erythrocyte lysine-rich histone fractions have confirmed the identity, and lack of tissue-specificity, of these H1 satellites (Risely and Eckhardt, 1981).

The faster Xenopus H1 satellite component, faintly detectable in erythrocyte preparations but nearly equal in proportion to the other satellite component in liver extracts (Figure 6), could be either

a modified form of the slower component or a slightly different H1 satellite subtype. Both explanations are possible since H1 satellites undergo postsynthetic modifications (D'Anna et al., 1980b) and nearly homologous satellite doublets are found in various tissue preparations (including liver) from mammalian species (Varricchio, 1977; Smith and Johns, 1980a; Medvedev and Medvedeva, 1980b; Gjerset et al., 1981; Pehrson and Cole, 1981; D'Anna et al., 1981). The cyanogen bromide cleavage studies (Figure 10) have shown no difference between the size of the major cleavage product of the erythrocyte and liver satellites, suggesting the absence of tissue-specific differences between Xenopus satellites in the 2/3rds of the molecules towards the carboxy-terminal ends. Both liver satellite components appear to give rise to the same major cleavage product. This indicates that the variation between the two liver components resides in the amino-terminal arms of these molecules. Both these features are characteristic of H1 subtype differences within a given tissue (Cole, 1977).

An explanation for the difference between our Xenopus H1 satellites and the erythrocyte-specific H1 satellite reported by Destree et al. (1979) remains elusive, even though experimental differences have been minimized. The agreement in amino acid compositions

between and among the H1 satellites from erythrocytes and liver of Xenopus (Brown et al., 1981), turtle (Rutledge et al., 1981), bullfrog (Brown et al., 1981; Shimada et al., 1981) and various mammalian H1o fractions (Smith et al., 1980; Smith and Johns, 1980a; Medvedev and Medvedeva, 1980b; Gjerset et al., 1981) is too great to be dismissed as coincidental. The electrophoretic mobility, chromatographic properties, lack of tissue-specificity and, at least in one case, partial amino acid sequence (Table 3) have also shown a closer homology of the Xenopus satellite H1 with the mammalian H1o than with the nucleated erythrocyte-specific H5.

These results are similar to previous reports where comparison of the amino acid compositions of bullfrog (Brown et al., 1981; Shimada et al., 1981) and turtle (Rutledge et al., 1981) erythrocyte and liver satellites suggested a closer resemblance to the mammalian H1o. The electrophoretic mobilities of both the whole satellites and the major CNBr-cleavage products from Xenopus, bullfrog, leopard frog, and turtle (Figure 12) suggest an H1 satellite homology within the anurans and the reptile, with slight differences in the carboxy-terminal 2/3rds of the molecules. This may be related to the variations in the carboxy-terminal arms of H1 subtypes which are commonly

seen between species (Cole, 1977).

These observations and comparisons support an anuran and reptilian H1 satellite homology with the mammalian H1o rather than the erythrocyte-specific H5 which has been proposed for Xenopus (Destree et al., 1979), bullfrog (Edwards and Hnilica, 1968) and turtle (Tsai and Hnilica, 1975). However, their similarity to H1 subtypes (indicated by their amino acid compositions, primary sequence analysis and subtype-specific patterns of N- and C-terminal variations) support a distant evolutionary relationship among all lysine-rich histone subclasses. Thus, we are not convinced that slight H1o/H5 distinctions in primary structure or tissue-specificity have functional significance and suspect that both types of H1 satellite have a similar role structurally and developmentally (Rutledge et al., 1981; Brown et al., 1981; Rutledge, 1982). This functional similarity is strengthened by the presence of identical H1 satellites in both erythrocytes and liver of Xenopus (Brown et al., 1981; Risley and Eckhardt, 1981), bullfrogs (Brown et al., 1981; Shimada et al., 1981) and turtle (Rutledge et al., 1981), even though satellite H1 functions remain to be clearly defined.

B. Urodele H1 Components

The protein characterization indicated that axolotl erythrocytes contained only H1-like components by their electrophoretic (Figure 12) and chromatographic (Figures 13 and 14) properties as well as by amino acid composition (Tables 5 and 6). However, one axolotl erythrocyte fraction (CG-50 peak II; Figure 14) shared a loose statistical relationship with Xenopus erythrocyte satellite H1 components (Table 6) and contained a single methionine residue, detectable by CNBr cleavage (Figure 12), which is probably located in a similar position relative to the conserved central domain as that in the Xenopus satellite. Axolotl liver probably contains H1 components similar to those found in erythrocytes, but persistent polypeptide contamination problems make the amino acid compositions of liver H1 components inaccurate.

The presence of a methionine-containing H1 subtype, in addition to an H1 satellite, has been previously reported in rat tissues (Kincade, 1969) and later confirmed (Medvedeva et al., 1975), but the role of this subtype in chromatin structure or in development has not been examined. Although an H1 doublet was extracted from the erythrocytes of Necturus, neither of these components appear to be sensitive to CNBr-cleavage (Figure 12) as was the case with the turtle erythrocyte

H1 doublet (Figure 12).

In an attempt to further characterize the axolotl erythrocyte H1 components and define their roles during a developmental event, a study of their behavior during the hemoglobin switch accompanying cryptic metamorphosis was undertaken. In other systems studied, H1 satellites demonstrate both selective synthesis and appreciable accumulation during differentiation of cell lines (reviewed in INTRODUCTION), while H1 subtypes remain relatively constant in proportion, or show a slight decrease only in the amount of one particular H1 subtype (Billet and Hindley, 1972; Moss et al., 1973; Urban et al., 1980; Valkonen, 1980; Pehrson and Cole, 1982). Since axolotl H1 components have typical H1 characteristics, they could be developmentally characterized by the maintenance of relatively constant synthetic rate and accumulated amount throughout the metamorphic transition. H1 components showing either selective synthesis or accumulation with the maturation of a new erythroid population would be exhibiting satellite H1 characteristics.

C. Cell Division, Hemoglobin Switching and Lysine-Rich
Histone Synthesis During Cryptic Metamorphosis

Analysis of thymidine incorporation into circulating erythrocyte populations during the metamorphic transition demonstrated waves of proliferative activity occurring at approximately 20-day intervals (Figure 17a). These peaks represented the proportions of cells in the populations (measured by autoradiography, Figures 17b and 18) incorporating labelled thymidine into DNA (Table 7). The relative amounts of incorporation probably accurately reflected DNA synthesis by the populations since precursor levels were always in large excess (Appendix I), fluctuations through the transition paralleled independent fluctuations in 14-C-lysine incorporation which were not dependent on precursor availability (Appendix IV), precursor was incorporated into macromolecular DNA (Table 7), and large pool size variations, evident in species undergoing gross anatomical alterations during metamorphosis (Just and Atkinson, 1972; Morris and Cole, 1978), probably do not occur during cryptic metamorphosis in the axolotl.

The waves of proliferative activity could be due to either 1) uncontrolled variations between animals or cell population samples, 2) a population of erythrocytes

passing through five essentially synchronous cell cycles of approximately 20 days duration or, 3) the successive stimulation of erythrocyte populations which pass through a proliferative series approximately 20 days in duration. The first alternative seems unlikely because replicate samples show the same activity (Appendix IV) and successive samples from the same spawning fit on the curves presented. The last interpretation is favoured since (1) the time required for the completion of a proliferative phase in maturing erythrocytes of amphibians is approximately 20 days (Ducibella, 1974a; Tompkins and Townsend, 1977; Thomas and Maclean, 1975; Chegini et al., 1979; Maclean and Jurd, 1971; Hentschel et al., 1979), (2) at least in chickens and geese, the synthesis of hemoglobins after the final cell division does not show a precipitous decrease co-ordinate with the decline in proliferative activity (Weintraub et al., 1971; Campbell et al., 1971; Chapman and Tobin, 1979; Urban et al., 1980) and (3) waves of erythroid lines are common during a transition from one hemoglobin type to another (Weintraub et al., 1971; Moss et al., 1973; Urban et al., 1980; Benz, 1981).

Globin subtype accumulation and synthesis - again accurately reflected by precursor incorporation because of precursor excess (Appendixes I and IV) and little catabolic activity - demonstrated synthesis of larval globins early in the transition, replaced by adult

globin synthesis (Figure 23b) and accumulation, which peaked at 125-130d (Figure 23c). The accumulation of adult globins after 140d (Figure 23) is in close agreement with reports in the literature (Ducibella, 1974a) but the sharp and temporary peak from 120-130d (Figure 23c) has not been previously described.

There are two possible mechanisms by which these profiles can be achieved. One requires that the stimulated adult erythroid line pass through a phase of synthesizing both larval and adult Hb before it becomes competent to synthesize adult globins exclusively. Early work, using single cell electrophoresis (Rosenberg, 1970) and antibodies directed against larval and adult globins (Maniatis and Ingram, 1971a,b), suggested that this does not occur during erythropoiesis accompanying bullfrog metamorphosis. However, subsequent immunochemical work using the more sensitive indirect assay, has indicated that 16% of the circulating bullfrog erythrocytes contain both larval and adult hemoglobins during this same erythropoietic stage (Benbassat, 1974). A similar situation has been reported for the chicken definitive line erythrocytes (Chapman and Tobin, 1979), where antibodies specific to embryonic and adult hemoglobins bind to all cells early in erythropoiesis, while more mature erythrocytes bind only antibodies against the adult globins. This transient expression of non-adult globins is also seen

in man (Roberts et al., 1972), rabbits (Clissold et al., 1977) and regenerating erythrocytes of anemic Xenopus (Maclean and Jurd, 1971). The specific activity plot (Figure 23b) supports this interpretation since larval globins were synthesized prior to the adult globins and their synthesis declines in concert with increased adult globin synthesis. However, the accumulation data demonstrated that the adult globin peak precedes the larval globin peak in the 130-150d period (Figure 23c).

An alternative explanation is that the stimulation of erythropoiesis during the transition causes proliferation of both larval and adult erythropoietic stem cells. Just and Atkinson (1972) found that the early induction of bullfrog metamorphosis by thyroid hormone injection caused an increase in the accumulation of larval hemoglobins which was closely followed by the detection and accumulation of adult hemoglobins. Similarly, studies involving recovery from anemia with Xenopus (Maclean and Jurd, 1971) and bullfrog and leopard frog (Meints and Forehand, 1977) have shown a partial reversion to larval hemoglobin synthesis followed by adult globin synthesis. The proliferation of both larval and adult cells in the axolotl would allow synthesis of both groups of globin subtypes, and their accumulation need not be in phase. This is consistent with a previous report demonstrating mixed populations of both larval and adult proliferating

erythrocytes during the early stages of cryptic metamorphosis (Ducibella, 1974b).

The observed results are probably due to circumstances where both larval and early adult erythrocytes proliferate asynchronously and both synthesize larval globins (prior to 125d). This would explain the transient accumulation of larval globins to a level higher than that seen in younger populations (Figure 23c), as well as the comparatively low level accumulation of adult globins (synthesized by the adult cells only) around the time of the switch. The adult cells would then switch to synthesizing adult globins only (around 125d, as Figure 23b indicates by the absence of detectable larval globin synthesis), and the larval cells would gradually be diluted to low levels in the circulating erythrocyte populations as adult globins accumulate with maturity (Figure 23c).

Though technical problems prevented the acquisition of histone accumulation data (see MATERIALS AND METHODS), I was able to obtain estimates of the synthetic activity of the histones through the metamorphic transition. Precursor incorporation is again assumed to accurately reflect synthesis because of the large labelled precursor excess (Appendices I and IV) and limited catabolic activity during the

transition. Peaks of total core and total lysine-rich histone synthesis were coincident with the proliferative activity of the erythrocyte populations when measured by liquid scintillation counting of extracts immediately after the in vitro labelling period (Figure 24). This was expected because of the tight coupling of DNA and histone synthesis (reviewed by Lewin, 1974; Elgin and Weintraub, 1975; recently confirmed by Delegeane and Lee, 1982; Marashi et al., 1982; Rickles et al., 1982). It is also unlikely that simultaneous fluctuations in precursor pools for both DNA and proteins could account for the coincidence of the curves. However, comparison of lysine-rich to core histone synthesis, with consideration of the relative lysine content of the two histone groups, demonstrated preferential synthesis of total lysine-rich histones (Figure 24c) between peaks of proliferative activity (indicated by high levels of core histone synthesis; Figure 24a). These peaks of uncoupled, preferential synthesis are coincident with peaks in total lysine-rich histone specific activity relative to core histone specific activity (Figure 24d) estimated from densitometry of stained electrophoretic gels (Figure 25a) and their accompanying fluorograms (Figure 25b). Such relative changes in protein synthesis could hardly be caused by fluctuations in precursor pools.

Preferential synthesis of all H1 subtypes after

peaks of proliferative activity occurred in the early stages of the metamorphic transition (Figure 27). The faster H1 component (H1c) probably represents modified forms of the principal components, H1a and H1b, since its appearance is correlated with a reduction in the average proportion of either or both of these components (Figure 26; Table 8). Reduced H1b and the presence of H1c appear to be correlated with proliferative activity as judged by the high levels of incorporation into core histones (Figures 24a and 26). Samples which contained H1c and reduced levels of H1a usually demonstrated preferential synthesis of lysine-rich histones which were independent of proliferative activity (Figures 24c and 26). Neither proliferation nor preferential lysine-rich histone synthesis appear to require H1c, since samples exhibiting either of these characteristics did not necessarily contain H1c (Figures 24a, 24c and 26). Peaks of preferential synthesis of H1b gradually declined to low levels by 125d while peaks of H1a and H1c persisted between peaks of proliferative activity to 186 days after spawning (Figure 27b, c and d). These similarities between H1a and H1c suggest that H1c is probably a modified H1a component in the late developmental stages.

The erythrocyte populations prior to 125 days consisted of cells synthesizing either larval globins or a mixture of larval and adult globins (Figure 23b). The

gradual decline in the peaks of preferential synthesis of H1b correlates well with both the presumed inactivation of the larval erythrocyte population (at around 125 day after spawning, Figure 23b) and the increased synthesis of adult globins by the adult population (Figure 23b and c). The gradual decline in the levels of uncoupled synthesis of H1b (Figure 27b) also shows a direct relationship with the proliferative activity of the population judged by high levels of incorporation into core histones (Figure 27a).

Transient uncoupled synthesis of both H1a and H1c continued throughout the transition, apparently independent of cell line since it was found in cells synthesizing either larval globin only (60d and 70d, Figure 23b) or adult globin only (156d onward, Figure 23b); hence these proteins may have a developmental role in both cell lines which may not be restricted to proliferation. However, it is possible that the earlier peaks are associated with adult cells not yet synthesizing detectable amounts of adult globins. If this was the case, one would have expected the preferential synthesis to increase in proportion to an increase in the adult cell numbers, but this was not observed (Figures 27b and d, 21b and c). Despite the confusion generated by overlapping larval and adult cell populations, the data does clearly show preferential synthesis of both H1a and H1c after the last wave of

adult cell division (186d, Figure 27b and d).

The purpose of this series of experiments was to use the synthetic activity of the axolotl H1 components through a developmental transition as a tool to further characterize these components and study their roles during this transition. The results have shown that all components resolved by SDS/PAGE demonstrated peaks of synthetic activity which were not coincident with peaks of proliferative activity. This situation has been observed for total H1 subtypes in G1-arrested BHK cells (Tarnowska et al., 1978) and in mouse neuroblastoma cells (Pehrson and Cole, 1982). The characteristic feature of H1 satellites is the selective synthesis or accumulation above the levels observed for the other histones (either H1 subtypes or core histones) near the completion of tissue maturation. This condition is realized by the proteins contained in the bands H1a and H1c during the later stages of adult erythrocyte maturation (Figure 27).

Although I was unable to determine quantitative changes in axolotl H1 components relative to core histone levels during development, the data does allow for the establishment of an under-representation of an H1 component (H1b in the axolotl) and increased satellite H1 levels (H1a in the axolotl) with adult cell maturation, analogous to that seen in other

differentiating systems (reviewed in INTRODUCTION). In the axolotl, one might predict 1) a reduction in the final level of H1b, since uncoupled synthesis of this component is halted early in the metamorphic transition and 2) loading of H1a due to its continued uncoupled synthesis throughout maturation. These differences in the final axolotl H1 component levels are possible provided that the proliferation-associated synthesis of H1 is unchanged during the developmental transition. This appears to be the case since comparison of the lysine-rich/core histone incorporations through the transitions always shows at least stoichiometric synthesis when corrected for lysine-content (Figure 24c).

So, although the axolotl does not contain a clearly demonstrable H1 satellite by protein characterization methods, it does have an H1 component (H1a) which appears to behave like an H1 satellite - demonstrating synthesis independent of proliferation in the late stages of erythroid maturation. Another component (H1b) behaves like H1 subtypes being associated with proliferation of the cell populations in

the early stages of the transition. The third component (Hlc) shares synthetic characteristics of both principle H1 components and probably represents a modified form of either, or both, of the principle components depending on the developmental stage of the cell population.

D. Satellite H1 Functions

The homology between Xenopus erythrocyte and liver H1 satellites, demonstrated in this study (Brown et al., 1981) and confirmed by Risley and Eckhardt (1981), is in agreement with similar comparisons of bullfrog (Brown et al., 1981; Shimada et al., 1981) and turtle (Rutledge et al., 1981). These results allow the suggestion that anuran and reptilian H1 satellites perform similar, if not identical, functions in both tissues. The great structural similarity between anuran and turtle H1 satellites and the mammalian H1o and avian H5 suggest functional homology between these proteins. Recent work with Xenopus erythrocyte H1 satellites has shown developmental similarities between the anuran, avian and mammalian H1 satellites (Rutledge, 1982). As was the case with H1o and H5, Xenopus erythrocyte satellite H1 progressively accumulated with maturation and was selectively or preferentially synthesized, in

the absence of detectable DNA synthesis, during the final stages of tissue maturation (Rutledge, 1982).

The observation that the axolotl does not contain an obvious H1 satellite cast doubt on an essential regulatory role for these proteins. However, further work showing the presence of an H1 component, sharing some satellite H1 characteristics and exhibiting preferential synthesis between waves of proliferative activity and during the final maturation stages of erythropoiesis, adds support to the need for a specialized lysine-rich histone during tissue differentiation.

Ultimately, proliferation and differentiation appear to be mutually exclusive in that rapidly proliferating cells (embryonic cells, malignant tissues) seldom synthesize luxury gene products, while a differentiated cell rarely exhibits proliferative activity. The existence of these two extremes and the inverse relationship between proliferation and the degree of differentiation (Lea et al., 1974) suggests that these two processes are inversely related so that limiting proliferative ability allows the expression of a particular cellular phenotype. This progressive relationship is mirrored by the selective accumulation of H1 satellites thereby implying a possible role for these proteins in proliferation and/or differentiation.

Easily demonstrable DNA synthesis and mitotic condensation followed by nuclear division and cytokinesis has allowed the delineation of four cell-cycle phases - G₁, S, G₂ and M (reviewed in Prescott, 1976; Pardee et al., 1978). During S phase, histone and DNA synthesis are tightly coupled and inhibition of either usually stops the synthesis of the other (Elgin and Weintraub, 1975; Prescott, 1976). For instance, the inhibition of protein synthesis by media deficient in any amino acid except tryptophan (not found in histones; Elgin and Weintraub, 1975) results in a block in both histone and DNA synthesis (Weintraub, 1972). Hydroxyurea and cytosine arabinoside both stop or prevent DNA synthesis and cause the rapid disappearance of histone mRNA from cytoplasmic polysomes (Melli et al., 1977; Stein et al., 1978; Pardee et al., 1978). Further fluorographic (Marashi et al., 1982) and autoradiographic (Wilkes et al., 1978) work confirmed that histone synthesis was restricted to S phase and was sensitive to cytosine arabinoside (Marashi et al., 1982).

Other recent work has demonstrated that some basal histone synthesis (approximately 10% of S phase synthesis, Wu and Bonner, 1981) and most H1 satellite synthesis is insensitive to hydroxyurea inhibition. This points to the existence of a control mechanism for

H1 satellite synthesis which is distinct from that of other histone classes and H1 subtypes (Ruiz-Carrillo et al., 1976, Zlatanov, 1980). G2 and mitosis (M) do not have detectable levels of either DNA or histone synthesis while G1, or the extended G1 (Go), is the phase where basal histone (Wu and Bonner, 1981) and uncoupled satellite H1 synthesis occur (Appels and Wells, 1972; Appels and Ringertz, 1974; Sung et al., 1977; Pieler et al., 1981). The concept of controls for H1 satellite levels which are independent of those regulating most other histone levels is supported by the demonstration of different rates of satellite H1 turnover after induced differentiation (Pehrson and Cole, 1982) and G1 arrest (Gooderham and Naha, 1981).

Among phases in the cell cycle, G1 is the most variable in length, and much work has indicated the presence of at least two distinct sub-phases (Prescott, 1976; Pardee et al., 1978). For instance, density-dependent inhibition, limitation of serum or some amino acids, depletion of phosphate, glucose or lipid and treatment with certain drugs cause a block in early G1 which, in some cases, induces differentiation (Prescott, 1976; Pardee et al., 1978). Removal of the inhibiting condition results in a progression into S phase but only after a 4-5 hour lag in late G1 (reviewed in Pardee et al., 1978). Protein and RNA synthesis are

necessary for progression into S phase, and early studies suggested that the attainment of a cell 'critical mass' is necessary for this transition (Prescott, 1976). Recent work has convincingly shown the existence of G1-A and G1-B phases distinguishable by increased RNA levels and decreased chromatin compaction from G1-A to G1-B to S phase (Darzenkiewicz et al., 1980, 1981). G1-A is the variable portion of the cell cycle while G1-B, S, G2 and M are all relatively constant in length (Prescott, 1976; Darzynkiewicz et al., 1980, 1981). Factors which cause a block in G1 (n-butyrate or serum deprivation) stop cells before the G1-A/G1-B boundary, while removal of the blocking agents allows resumption of the cycle at its normal rate in G1-B. Hydroxyurea, however, blocks cells in G1-B or at the G1-B/S boundary (Darzenkiewicz et al., 1980). Since hydroxyurea specifically interferes with DNA synthesis by inhibiting gap-filling and elongation steps (Radford et al., 1972), and since inhibitors which induce G1 arrest and differentiation act at the G1-A/G1-B boundary, the control of proliferation is thought to be in the G1-A (or early G1) phase (Prescott, 1976; Pardee et al., 1978; Darzynkiewicz et al., 1980, 1981).

Interestingly, factors causing G1-A arrest and differentiation also cause satellite H1 accumulation (Pehrson and Cole, 1980, 1982; Zlatanova, 1980; Zlatanova et al., 1980; Pieler et al., 1981), but hydroxyurea, influencing DNA synthesis only, does not decrease satellite H1 synthesis or accumulation (Ruiz-Carrillo et al., 1976; Zlatanova, 1980). These results suggest a satellite H1 role in G1-A arrest or differentiation but negate a regulatory role in the mechanics of preparation for or the synthesis of DNA.

Other studies have shown no correlation between H1 satellite synthesis and accumulation and cell replication as originally proposed by Panyim and Chalkley (1969a,b). Marsh and Fitzgerald (1973) noted little change in satellite H1 concentration even though there were large changes in DNA replication rate during pancreas regeneration. Major satellite H1 accumulation occurs after DNA synthesis had stopped in pancreas ontogeny (Varricchio, 1977), differentiating mouse neuroblastoma (Pieler et al., 1981), Xenopus erythropoiesis during recovery from anemia (Rutledge, 1982) and in avian erythropoiesis (Billet and Hindley, 1972; Appels and Wells, 1972; Urban et al., 1980). Finally, Hugues and Osbourne (1981) have shown that murine erythroleukemia cells, induced to differentiate with hexamethylene bisacetamide, synthesized satellite

H1 (IP25) and hemoglobin while ceasing to proliferate. Treatment with dexamethasone (which interferes with the transcription of luxury gene products; Commer et al., 1979) after induction of these cells blocked hemoglobin synthesis and permitted proliferation to continue but did not stop subsequent satellite H1 synthesis and accumulation. This result in particular shows no satellite H1 involvement in the control of proliferation. This review indicates that satellite H1 synthesis and accumulation are associated with early G1 and the changes occurring with the commitment to differentiate.

The consistent result of preferential synthesis and accumulation of H1 satellites seems to be the augmentation of total lysine-rich histone content. Marsh and Fitzgerald (1973) show no loss of H1 subtypes despite large increases in H1o concentration during pancreas regeneration. This is also seen in erythropoietic systems where there may be a slight reduction in the amount of one H1 subtype while the total lysine-rich histone content increases with maturation (Billet and Hindley, 1972; Appels and Wells, 1972; Ruiz-Carillo et al., 1974; Weintraub, 1978; Urban et al., 1980). Tissue culture systems show similar results (D'Anna et al., 1980a; Pehrson and Cole, 1980, 1982) where the total amount of lysine-rich histone

increases with maturation. The most convincing evidence of this was reported by Miki and Neelin (1980) where the total lysine-rich histone content of fish erythrocytes was constant between species while the relative proportion of H1 satellites were extremely variable.

The further condensation of chromatin, found in erythrocytes with H5 accumulation (reviewed in INTRODUCTION) and characteristic of non-erythroid tissues in G1-arrest or G₀ (Darzynkiewicz et al., 1981) under conditions which exhibit satellite H1 accumulation (see above), suggest a role in chromatin structure which is dictated by the total lysine-rich histone content. Studies by Allan and co-workers (1981) support this proposition by showing the existence of two internucleosomal binding sites for lysine-rich histones per nucleosome in vitro which, when both are occupied, achieve maximal chromatin condensation. Their results show that the extent of chromatin condensation is directly dependent on the amount but not the type of lysine-rich histone and suggest that chromatin loading by lysine-rich histones may be responsible for limiting DNA template activity in vivo (D'Anna et al., 1980b; Allan et al., 1981). The enhanced DNA template binding affinity of H5 in comparison to H1 subtypes (Kumar and Walker, 1980) supports a role in template restriction, as do studies showing increased template activity after

the removal of H5 (Seligy and Neelin, 1970; Seligy and Miyagi, 1974; Miller et al., 1972). Although similar studies have not been done with other satellites, the increased basicity of mammalian, anuran and turtle H1 satellites (judging by their properties during cation-exchange chromatography) suggest a similar DNA-binding ability. An obvious extension to the present mammalian and amphibian H1 satellite data is a study of their influence on chromatin structure.

The other common feature between the axolotl H1a component and other H1 satellites (besides metabolism during tissue maturation) is the presence and location of a single methionine residue. Two lysine-rich histone features may be related to this. The first involves selective turnover and degradation. Both Appels and Ringertz (1974) and Pehrson and Cole (1982) found decreased turnover of satellites, compared to H1 subtypes, with maturation. Few studies have been done with site-specific proteases and lysine-rich histones, but one carefully characterized nuclear histone protease, has a high specificity for H1 subtypes (Hagiwara et al., 1980). It is possible that the presence of

a methionine molecule has some influence on the susceptibility of the protein to proteolytic degradation, especially considering the overall similarities in primary and secondary structure of all lysine-rich histones (reviewed in INTRODUCTION).

The other possibility involves lysine-rich histone phosphorylation. H1 subtypes undergo both N- and C-terminal phosphorylation at serine and threonine residues in late G1, which is correlated with, and may regulate to some degree, chromatin replication (Langan, 1978; Gurley et al., 1978). Superphosphorylation in mitosis appears to be involved with chromosome condensation (Gurley et al., 1978; Matsumoto et al., 1980). Another type of in vivo phosphorylation occurs in G1 on serine residue 37 which is not associated with replication (Langan, 1978). It too enhances chromatin condensation but not to the same degree as N- and C-terminal phosphorylation (Rattle et al., 1977; Mathews and Bradbury, 1978). The main influence of phosphorylation at this site seems to be to reduce the N-terminal stability of the H1-DNA interaction (Rattle et al., 1977). Studies with H5 in vitro (Aviles et al., 1978; Sung and Freeland, 1978) and H1o (D'Anna et al., 1981) have shown N- and C-terminal, mitosis-associated phosphorylation while there have been no reports of in vivo phosphorylation at the N-terminal end of the

central globular regions of these molecules, even though a serine residue is located at or near position 37 in the satellites when they are aligned with H1 for maximal homology (Von Holt et al., 1979; Pehrson and Cole, 1981). A recent in vitro study has shown H5 phosphorylation at this site with a cAMP-dependent protein kinase from rat pancreas (Martinage et al., 1981), but similar studies with H1 subtypes have shown phosphorylation at serine residue 107 which is never detected in vivo and is thought not to have functional significance (Langan, 1978). The same may be true of the H5 serine 37 site.

The selective synthesis of H1 satellites during the same cell cycle period as serine 37 is phosphorylated in H1 subtypes and the positioning of a single methionine residue in a similar location (30-40 amino acid residues from the amino terminal end of the molecules) may be more than a coincidence. Although the function of H1 serine 37 phosphorylation is unknown, it may be related to transcriptional activity (Langan, 1978), unlike H1 satellites which correlate with transcriptionally inactive (or condensed) chromatin (Johmann et al., 1973; Tobin and Seligy, 1975; Smith and Johns, 1980b; Gjerset et al., 1981).

In summary, H1 satellites are a distinct histone subclass whose synthesis can be controlled independently of the synthesis of other histones, particularly during the final stages of tissue maturation. They appear to have two interdependent functions. The first involves the increased chromatin compaction associated with tissue maturation which is probably the result of satellite H1 accumulation causing high total lysine-rich histone content. This condition does not appear to directly depend on unique structural characteristics of H1 satellites since in vitro studies have shown that an increase in lysine-rich histone content due to an increase in any one lysine-rich histone causes the same type and degree of chromatin compaction (Allan et al., 1981). The in vivo relationship with H1 satellites may simply be due their independent synthetic control, and perhaps to an increased resistance to proteolytic degradation. The second function involves the restricted template capacity of mature tissues which are alleviated by the removal of H1 satellites. This situation may depend more on the satellite H1 per se rather than lysine-rich histones generally, where the

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increased DNA-binding affinity of H1 satellites and their apparent lack of serine 37 phosphorylation may play an important role in augmenting transcriptional inhibition imposed by high lysine-rich histone contents and chromatin compaction.

V. APPENDIXES

Appendix I: Radioactive precursor (3-H-thymidine and 14-C-lysine) incorporation into cells and cell fractions after in vitro labelling for one hour. All values are expressed as DPM/10⁶ cells. NA = neonatous adult

Age	14-C-lys 3-H-thy 3-H-thy			14-C-lysine in cells and cell fractions						
	in medium	in medium	in cells	cells	saponin super.	nuclei	lysine rich	core histone	total histone	residual pellet
60d	497367	1283227	162560	5620	2469	2207	368	181	926	1582
64d	411092	1903848	215018	9759	3984	1933	349	43	3158	1141
70d	418696	1769839	46657	8190	5861	1781	351	213	1625	532
75d	376570	1562936	295323	11967	4911	4353	582	794	2170	1768
79d	152383	347982	140504	10595	3709	4413	299	857	2115	3393
84d	87322	169052	43046	3053	2156	1246	184	259	701	1004
90d	391858	681337	222199	12442	4031	6120	400	1059	2316	5559
96d	371243	553944	224393	11812	4236	4413	242	536	1217	3790
100d	246210	214158	8998	1814	830	636	111	193	566	235
105d	155189	317985	36262	5474	3124	1069	202	236	939	292
110d	338999	305983	26163	6986	2773	1027	205	509	1026	1208
114d	394345	294345	70342	11891	8151	3985	551	1084	1947	1954
120d	325163	275779	29226	9522	4140	809	86	448	1477	1155
125d	43319	43019	889	165	82	44	9	7	24	4
126d	125109	117306	3983	1802	1160	437	86	113	300	113
130d	87443	271234	49042	3714	1791	1280	173	439	428	996
134d	165495	229131	34345	4101	2139	1117	263	374	-	733
140d	129281	127008	2009	1809	1040	501	142	129	364	181
146d	141657	232746	6983	2749	1773	509	133	194	445	224
150d	203635	705660	43940	4549	2310	1514	232	497	862	843
155d	142626	187292	32954	2590	1197	660	63	107	254	678
163d	112499	249630	182371	3930	1973	1536	349	566	-	2803
166d	412074	650164	27216	3288	1631	1045	44	135	254	901
178d	566669	-	-	2808	1748	475	-	-	37	277
181d	183555	270287	28194	1959	1100	726	120	113	207	473
186d	135133	280988	16811	2069	1031	262	39	41	185	100
190d	21345	31046	1308	267	162	88	11	18	42	34
224d	49198	44687	3508	1109	781	352	41	102	219	95
NA	33505	55655	3781	309	126	144	6	22	-	133

Appendix II: Specific activities of erythrocyte histones isolated from axolotls during cryptic metamorphosis. The values from eight electrophoretic runs were normalized to values obtained from one run which contained representative samples from all stages of the transition. The normalized average (n.a.) for each histone component was then divided by the total core histone specific activity (cores) to test for preferential synthesis of particular histone components. NA = neotenuous adult; ND = not detected

Histone:	H1a				H1b				H1c				H1d				H1e				H1f				H1g				H1h				H1i				H1j				H1k				H1l				H1m				H1n				H1o				H1p				H1q				H1r				H1s				H1t				H1u				H1v				H1w				H1x				H1y				H1z				H1aa				H1ab				H1ac				H1ad				H1ae				H1af				H1ag				H1ah				H1ai				H1aj				H1ak				H1al				H1am				H1an				H1ao				H1ap				H1aq				H1ar				H1as				H1at				H1au				H1av				H1aw				H1ax				H1ay				H1az				H1ba				H1bb				H1bc				H1bd				H1be				H1bf				H1bg				H1bh				H1bi				H1bj				H1bk				H1bl				H1bm				H1bn				H1bo				H1bp				H1bq				H1br				H1bs				H1bt				H1bu				H1bv				H1bw				H1bx				H1by				H1bz				H1ca				H1cb				H1cc				H1cd				H1ce				H1cf				H1cg				H1ch				H1ci				H1cj				H1ck				H1cl				H1cm				H1cn				H1co				H1cp				H1cq				H1cr				H1cs				H1ct				H1cu				H1cv				H1cw				H1cx				H1cy				H1cz				H1da				H1db				H1dc				H1dd				H1de				H1df				H1dg				H1dh				H1di				H1dj				H1dk				H1dl				H1dm				H1dn				H1do				H1dp				H1dq				H1dr				H1ds				H1dt				H1du				H1dv				H1dw				H1dx				H1dy				H1dz				H1ea				H1eb				H1ec				H1ed				H1ee				H1ef				H1eg				H1eh				H1ei				H1ej				H1ek				H1el				H1em				H1en				H1eo				H1ep				H1eq				H1er				H1es				H1et				H1eu				H1ev				H1ew				H1ex				H1ey				H1ez				H1fa				H1fb				H1fc				H1fd				H1fe				H1ff				H1fg				H1fh				H1fi				H1fj				H1fk				H1fl				H1fm				H1fn				H1fo				H1fp				H1fq				H1fr				H1fs				H1ft				H1fu				H1fv				H1fw				H1fx				H1fy				H1fz				H1ga				H1gb				H1gc				H1gd				H1ge				H1gf				H1gg				H1gh				H1gi				H1gj				H1gk				H1gl				H1gm				H1gn				H1go				H1gp				H1gq				H1gr				H1gs				H1gt				H1gu				H1gv				H1gw				H1gx				H1gy				H1gz				H1ha				H1hb				H1hc				H1hd				H1he				H1hf				H1hg				H1hh				H1hi				H1hj				H1hk				H1hl				H1hm				H1hn				H1ho				H1hp				H1hq				H1hr				H1hs				H1ht				H1hu				H1hv				H1hw				H1hx				H1hy				H1hz				H1ia				H1ib				H1ic				H1id				H1ie				H1if				H1ig				H1ih				H1ii				H1ij				H1ik				H1il				H1im				H1in				H1io				H1ip				H1iq				H1ir				H1is				H1it				H1iu				H1iv				H1iw				H1ix				H1iy				H1iz				H1ja				H1jb				H1jc				H1jd				H1je				H1jf				H1jg				H1jh				H1ji				H1jj				H1jk				H1jl				H1jm				H1jn				H1jo				H1jp				H1jq				H1jr				H1js				H1jt				H1ju				H1jv				H1jw				H1jx				H1jy				H1jz				H1ka				H1kb				H1kc				H1kd				H1ke				H1kf				H1kg				H1kh				H1ki				H1kj				H1kk				H1kl				H1km				H1kn				H1ko				H1kp				H1kq				H1kr				H1ks				H1kt				H1ku				H1kv				H1kw				H1kx				H1ky				H1kz				H1la				H1lb				H1lc				H1ld				H1le				H1lf				H1lg				H1lh				H1li				H1lj				H1lk				H1ll				H1lm				H1ln				H1lo				H1lp				H1lq				H1lr				H1ls				H1lt				H1lu				H1lv				H1lw				H1lx				H1ly				H1lz				H1ma				H1mb				H1mc				H1md				H1me				H1mf				H1mg				H1mh				H1mi				H1mj				H1mk				H1ml				H1mm				H1mn				H1mo				H1mp				H1mq				H1mr				H1ms				H1mt				H1mu				H1mv				H1mw				H1mx				H1my				H1mz				H1na				H1nb				H1nc				H1nd				H1ne				H1nf				H1ng				H1nh				H1ni				H1nj				H1nk				H1nl				H1nm				H1nn				H1no				H1np				H1nq				H1nr				H1ns				H1nt				H1nu				H1nv				H1nw				H1nx				H1ny				H1nz				H1oa				H1ob				H1oc				H1od				H1oe				H1of				H1og				H1oh				H1oi				H1oj				H1ok				H1ol				H1om				H1on				H1oo				H1op				H1oq				H1or				H1os				H1ot				H1ou				H1ov				H1ow				H1ox				H1oy				H1oz				H1pa				H1pb				H1pc				H1pd				H1pe				H1pf				H1pg				H1ph				H1pi				H1pj				H1pk				H1pl				H1pm				H1pn				H1po				H1pp				H1pq				H1pr				H1ps				H1pt				H1pu				H1pv				H1pw				H1px				H1py				H1pz				H1qa				H1qb				H1qc				H1qd				H1qe				H1qf				H1qg				H1qh				H1qi				H1qj				H1qk				H1ql				H1qm				H1qn				H1qo				H1qp				H1qq				H1qr				H1qs				H1qt				H1qu				H1qv				H1qw				H1qx				H1qy				H1qz				H1ra				H1rb				H1rc				H1rd				H1re				H1rf				H1rg				H1rh				H1ri				H1rj				H1rk				H1rl				H1rm				H1rn				H1ro				H1rp				H1rq				H1rr				H1rs				H1rt				H1ru				H1rv				H1rw				H1rx				H1ry				H1rz				H1sa				H1sb				H1sc				H1sd				H1se				H1sf				H1sg				H1sh				H1si				H1sj				H1sk				H1sl				H1sm				H1sn				H1so				H1sp				H1sq				H1sr				H1ss				H1st				H1su				H1sv				H1sw				H1sx				H1sy				H1sz				H1ta				H1tb				H1tc				H1td				H1te				H1tf				H1tg				H1th				H1ti				H1tj				H1tk				H1tl				H1tm				H1tn				H1to				H1tp				H1tq				H1tr				H1ts				H1tt				H1tu				H1tv				H1tw				H1tx				H1ty				H1tz				H1ua				H1ub				H1uc				H1ud				H1ue				H1uf				H1ug				H1uh				H1ui				H1uj				H1uk				H1ul				H1um				H1un				H1uo				H1up				H1uq				H1ur				H1us				H1ut				H1uu				H1uv				H1uw				H1ux				H1uy				H1uz				H1va				H1vb				H1vc				H1vd				H1ve				H1vf				H1vg				H1vh				H1vi				H1vj				H1vk				H1vl				H1vm				H1vn				H1vo				H1vp				H1vq				H1vr				H1vs				H1vt				H1vu				H1vv				H1vw				H1vx				H1vy				H1vz				H1wa				H1wb				H1wc				H1wd				H1we				H1wf				H1wg				H1wh				H1wi				H1wj				H1wk				H1wl				H1wm				H1wn				H1wo				H1wp				H1wq				H1wr				H1ws				H1wt				H1wu				H1wv				H1ww				H1wx				H1wy				H1wz				H1xa				H1xb				H1xc				H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Appendix II (con't)

Histones: Gel No.:	HLC			HLC		thl		l	H3			8	n.a.	H3 cores
	1	2&3	4&5	n.a.	cores	cores	cores		2&3	4&5	6&7			
60d	ND	ND	ND	ND	-	3.96	1014	1014	202	108	2414	-	350	0.46
70d	ND	ND	ND	ND	-	6.08	325	325	161	104	1719	295	295	0.44
75d	896	1350	609	899	0.77	3.38	348	348	360	32	3213	3580	502	0.43
79d	ND	ND	ND	ND	-	3.42	-	-	251	156	2006	-	400	0.39
84d	ND	ND	ND	ND	-	4.50	-	-	175	120	1518	1161	299	0.35
90d	ND	ND	ND	ND	-	3.58	-	-	264	201	3177	-	518	0.40
96d	475	656	178	414	1.03	2.62	190	190	109	43	700	767	162	0.41
100d	360	491	160	325	1.25	3.54	134	134	93	42	334	261	106	0.41
105d	ND	ND	ND	ND	-	4.70	-	-	189	95	1618	-	286	0.37
110d	ND	ND	ND	ND	-	4.50	-	-	253	197	3500	1856	517	0.47
114d	ND	ND	ND	ND	-	2.58	393	393	168	21	3409	67	303	0.53
120d	604	1745	836	885	1.06	3.77	123	123	255	187	2429	1117	384	0.46
126d	303	245	122	365	1.81	3.59	130	130	76	26	177	220	84	0.42
130d	ND	ND	ND	ND	-	2.35	-	-	205	172	929	988	344	0.40
140d	ND	ND	ND	ND	-	4.46	36	36	57	10	154	169	45	0.35
146d	ND	ND	ND	ND	-	2.04	245	245	76	36	548	-	154	0.40
150d	ND	ND	ND	ND	-	2.13	-	-	130	87	591	1012	224	0.35
155d	280	312	139	332	1.21	2.78	131	131	114	22	271	-	104	0.38
166d	ND	ND	ND	ND	-	1.36	128	128	37	22	450	1036	130	0.39
178d	ND	ND	ND	ND	-	1.12	91	91	77	6	145	-	60	0.28
181d	ND	ND	ND	ND	-	1.70	-	-	36	7	255	-	46	0.35
186d	362	453	197	583	2.42	5.78	85	85	74	14	349	-	80	0.33
190d	ND	ND	ND	ND	-	2.12	-	-	76	29	310	-	94	0.33
224d	ND	ND	ND	ND	-	1.96	53	53	55	1	166	-	54	0.34
NA	ND	ND	ND	ND	-	-	ND	ND	ND	ND	ND	ND	ND	-

Appendix II (con't)

Histone:		H4					H4	
Gel No.:	1	2&3	4&5	6&7	8	n.a.	cores	
60d	178	254	106	1896	-	154	0.20	
70d	182	105	111	1667	-	128	0.19	
75d	195	344	231	2672	6450	218	0.19	
79d	-	474	280	2511	-	260	0.25	
84d	-	402	243	2007	2281	216	0.26	
90d	-	482	332	2897	-	290	0.23	
96d	90	159	112	694	919	88	0.22	
100d	76	123	96	343	509	63	0.24	
105d	-	306	226	1796	-	187	0.24	
110d	-	393	290	2223	2065	226	0.21	
114d	144	236	15	273	94	85	0.15	
120d	96	179	121	2527	1980	162	0.19	
126d	75	66	21	484	278	43	0.21	
130d	-	289	126	2021	1529	178	0.21	
140d	37	69	12	394	355	32	0.25	
146d	116	92	24	1150	-	76	0.20	
150d	-	226	101	1488	1133	137	0.21	
155d	68	145	27	596	-	62	0.23	
166d	65	64	15	1035	2100	81	0.24	
178d	94	173	3	794	-	64	0.30	
181d	-	61	8	471	-	29	0.22	
186d	52	115	31	659	-	57	0.24	
190d	-	102	30	979	-	64	0.22	
224d	46	65	-	496	-	40	0.25	
NA	ND	ND	ND	ND	ND	ND	-	

Appendix III: The quantity of protein and 14-C-lysine per globin band determined by densitometry of SDS slab gels and accompanying fluorograms. Values were averaged (Av.) and used to calculate specific activities (SA). The molecular weights are from Ducibella (1974a). NA = neonotous adult; * estimated detection limit; (-) not determined

Subtype:		24,500 dalton								19,000/19,500 dalton															
Parameter:		ug/ul				DPM/ul				SA				ug/ul				DPM/ul				SA			
Gel No.:		1&2	3&4	Av.	1&2	3&4	Av.	1&2	3&4	Av.	1&2	3&4	Av.	1&2	3&4	Av.	1&2	3&4	Av.	1&2	3&4	Av.			
60d		0.14	0.07	0.11	0	2	1	9			0.29	0.25	0.27	81	157	119	441								
64d		0.17	0.07	0.12	0	13	7	58			0.53	0.36	0.45	245	520	383	851								
70d		0.31	0.18	0.25	0	7	4	16			0.56	0.49	0.53	490	1008	749	1413								
75d		0.29	0.31	0.30	0	46	23	77			1.20	0.85	1.03	733	1468	1101	1069								
79d		0.54	0.46	0.50	108	5	57	114			1.97	1.08	1.53	384	647	516	337								
84d		0.56	0.43	0.50	153	276	216	432			1.70	0.90	1.30	1413	2210	1812	1394								
90d		0.70	0.38	0.54	172	175	174	322			2.18	0.70	1.44	784	1483	1134	788								
96d		0.75	0.45	0.60	129	54	92	153			2.77	1.46	2.12	360	582	471	222								
100d		0.87	0.53	0.70	104	24	64	91			2.58	1.20	1.89	141	111	126	67								
105d		1.68	0.62	1.15	415	596	506	440			4.11	1.41	2.76	1501	1775	1638	594								
110d		1.23	0.89	1.06	639	651	645	609			4.26	2.08	3.05	1834	2261	2048	672								
114d		0.86	0.24	0.55	206	-	206	375			2.04	0.63	1.34	672	-	672	502								
120d		1.69	0.46	1.08	599	476	538	498			3.40	1.93	2.67	1133	1256	1195	448								
126d		1.95	0.74	1.35	301	0	151	112			3.18	4.27	3.73	442	64	253	68								
130d		0.15	0.43	0.29	37	43	47	161			1.07	1.28	1.18	136	417	276	234								
134d		0.32	0.17	0.25	36	171	103	413			1.53	2.50	2.02	708	755	732	362								
140d		2.34	-	2.34	137	-	137	59			8.33	-	8.33	390	-	390	47								
146d		0.76	1.38	1.07	154	163	192	179			2.74	2.94	2.84	530	626	578	204								
150d		0.83	-	0.83	200	-	200	241			3.12	-	3.12	999	-	999	320								
154d		2.88	-	2.88	330	-	330	115			9.30	-	9.30	1321	-	1321	142								
155d		1.51	1.01	1.26	298	324	311	247			4.41	5.48	4.95	907	732	819	166								
166d		0.37	0.53	0.45	37	34	36	79			1.13	1.82	1.48	480	296	388	262								
178d		2.76	-	2.76	231	-	231	84			9.83	-	9.83	483	-	483	49								
181d		1.64	1.82	1.73	61	49	55	32			3.07	3.53	3.30	183	233	208	63								
186d		2.18	2.67	2.43	112	147	130	54			3.83	6.13	4.98	361	384	372	75								
190d		2.81	-	2.81	210	-	210	75			4.06	-	4.06	416	-	416	103								
224d		4.20	9.72	6.96	137	0	69	10			12.44	4.23	8.34	477	403	440	53								
NA		5.44	11.65	8.55	0	248	83	10			17.28	11.87	14.58	0	232	77	5								

Appendix III (con't)

Subtype:		15,000 dalton										13,800 dalton										SA	
Parameter:		ug/ul					DPM/ul					ug/ul					DPM/ul					SA	
Gel No.:		1&2	3&4	Av.	1&2	3&4	Av.	1&2	3&4	Av.	1&2	3&4	Av.	1&2	3&4	Av.	1&2	3&4	Av.	1&2	3&4	SA	
60d		0.00	0.16	0.08	0	6	3	38	0.37	0.41	0.39	67	164	116	296		0.39	67	164	116	296		
64d		0.00	0.09	0.05	0	11	6	120	0.84	0.52	0.68	190	396	293	431		0.68	190	396	293	431		
70d		0.00	0.17	0.09	0	44	22	244	1.40	0.75	1.08	399	896	648	560		1.08	399	896	648	560		
75d		0.00	0.30	0.15	0	0	0	0	1.79	1.40	1.60	670	1408	1039	649		1.60	670	1408	1039	649		
79d		0.1*	0.47	0.29	118	44	81	279	2.98	1.56	2.27	254	423	339	149		2.27	254	423	339	149		
84d		0.1*	0.52	0.31	0	101	52	168	2.59	1.25	1.92	1300	1915	1608	837		1.92	1300	1915	1608	837		
90d		0.1*	0.48	0.29	163	133	148	510	3.07	1.92	2.50	469	885	677	271		2.50	469	885	677	271		
96d		0.1*	0.55	0.33	136	77	107	324	3.64	2.55	3.10	248	385	317	102		3.10	248	385	317	102		
100d		0.1*	0.42	0.26	107	24	66	254	3.15	2.34	2.75	108	20	64	23		2.75	108	20	64	23		
105d		0.1*	0.45	0.28	0	357	179	639	5.10	2.98	4.04	748	1225	987	244		4.04	748	1225	987	244		
110d		0.1*	0.41	0.26	523	911	717	2758	5.58	4.16	4.87	450	771	611	125		4.87	450	771	611	125		
114d		0.1*	0.21	0.16	0	0	0	0	2.39	1.29	1.84	360	196	360	196		1.84	360	196	360	196		
120d		1.53	0.56	1.05	507	973	740	705	3.61	3.30	3.46	589	1350	970	280		3.46	589	1350	970	280		
126d		1.57	1.69	1.63	296	101	199	122	2.71	4.86	3.79	262	124	193	51		3.79	262	124	193	51		
130d		0.1*	0.31	0.21	26	91	58	277	0.93	1.51	1.22	10	6	8	7		1.22	10	6	8	7		
134d		0.1*	0.22	0.16	0	247	124	772	2.28	3.68	2.98	256	514	385	129		2.98	256	514	385	129		
140d		3.60	-	3.60	332	-	332	92	7.92	-	7.92	113	-	113	14		7.92	113	-	113	14		
146d		0.1*	1.30	0.70	176	306	241	344	6.33	6.01	6.17	195	581	388	63		6.17	195	581	388	63		
150d		0.1*	-	0.1*	239	-	239	2390	6.44	-	6.44	617	-	617	96		6.44	617	-	617	96		
154d		0.1*	-	0.1*	463	-	463	4630	11.08	-	11.08	241	-	241	22		11.08	241	-	241	22		
155d		1.33	2.14	1.74	336	333	335	192	5.58	6.95	6.27	238	345	292	47		6.27	238	345	292	47		
166d		0.47	0.45	0.46	74	56	65	141	1.17	3.11	2.14	164	235	200	93		2.14	164	235	200	93		
178d		5.17	-	5.17	224	-	224	43	8.58	-	8.58	80	-	80	9		8.58	80	-	80	9		
181d		0.74	2.49	1.62	48	100	73	45	2.42	3.63	3.03	25	94	60	20		3.03	25	94	60	20		
186d		1.09	3.93	2.51	71	202	137	54	4.04	6.15	5.10	34	65	49	10		5.10	34	65	49	10		
190d		2.95	-	2.95	233	-	233	79	5.14	-	5.14	108	-	108	21		5.14	108	-	108	21		
224d		4.78	22.97	13.88	290	187	238	17	4.49	11.97	8.23	0	297	149	18		8.23	0	297	149	18		
NA		6.85	37.01	21.93	0	0	0	0	4.77	19.65	12.21	0	0	0	0		12.21	0	0	0	0		
																						186	

Appendix IV:

In order to test for an excess of labelled precursor under normal incubation conditions, a sample of erythrocytes from eight 100d animals was split into two 1-ml fractions each containing 2×10^8 cells. One aliquot was incubated with the usual amount of 14-C-lysine (20 uCi/ml medium) while the other was incubated with twice this amount of labelled precursor. Both suspensions contained 20 uCi 3-H-thymidine. The amount of label in each aliquot, in samples taken at 10-minute intervals during the incubation and, in samples of cells and cellular fractions, was determined by liquid scintillation counting of TCA precipitates (see MATERIALS AND METHODS).

Samples taken at 10-minute intervals during the incubation period consistently demonstrated approximately 1.7-fold higher levels of lysine incorporation when incubated with twice the usual amount of this precursor (Table 9). The incorporation of 3-H-thymidine was slightly higher (1.2-fold) for the aliquot containing the higher levels of labelled lysine (Table 9). The lysine-incorporation distinction between the two aliquotes was not apparent in samples taken of cells and cellular fractions which had been washed

extensively in saline following the incubation period (Table 9). That is, the enhanced 14-C-lysine incorporation into cells and cellular fractions by the population containing the higher level of this precursor was equal to their slightly higher level of 3-H-thymidine incorporation (Table 9). This suggested that the marginally elevated level of lysine incorporation by this population was due to slightly increased metabolic activity of this population and not the higher 14-C-lysine precursor level.

These results show that TCA precipitation alone does not effectively remove unincorporated precursor from inter- and intra-cellular spaces and that successive washes in saline are necessary to eliminate the influences of high precursor levels during in vitro labelling. The relatively constant level of precursor incorporation into cells and cellular fractions, independent of the amount of 14-C-lysine available in the incubation medium, suggests that incubation with 20 μ Ci 14-C-lysine per-ml incubation suspension is sufficient to overcome the influences of endogenous intracellular amino acid pools.

Table 9: Incorporation of ^{14}C -lysine and ^3H -thymidine into two erythrocyte populations pooled from 8 axolotls 100 days after spawning. One population was incubated with 20 $\mu\text{Ci}/\text{ml}$ of both precursors while the other contained 20 $\mu\text{Ci}/\text{ml}$ ^3H -thymidine and 40 $\mu\text{Ci}/\text{ml}$ ^{14}C -lysine. Total isotope present was determined by liquid scintillation counting of air-dried, 20- μl samples of the suspensions after hydrolysis in PCA and hydrogen peroxide (see MATERIALS AND METHODS). Samples taken during the incubation (t10-t60) were precipitated in 20% TCA, washed in 5% TCA (3X) air-dried and counted. After labelling, suspensions were washed (3X) in 1.5 ml of cold saline and cells and cell fractions were isolated as described in MATERIALS AND METHODS. The ratios of ^{14}C -lysine and ^3H -thymidine incorporated into the two populations, 40 μCi lys (40 μCi ^{14}C -lysine and 20 μCi ^3H -thymidine) and 20 μCi lys (20 μCi ^{14}C -lysine and 20 μCi ^3H -thymidine) are presented.

		Total Label	t 10	t 20	t 30	t 40	t 50	t 60
CPM	40 μCi ^{14}C -lys:	190246	1905	2176	2297	2654	3165	3286
10^4 cells	20 μCi ^3H -thy:	136860	848	1279	1930	2973	4127	5109
CPM	20 μCi ^{14}C -lys:	104092	1118	1164	1224	1495	1863	2167
10^4 cells	20 μCi ^3H -thy:	129751	821	1094	1639	2526	3535	4722
Lysine Incorp.	40 μCi lys:	1.8	1.7	1.9	1.9	1.8	1.7	1.5
Ratio	20 μCi lys							
Thy. Incorp.	40 μCi lys:	1.1	1.0	1.2	1.2	1.2	1.2	1.1
Ratio	20 μCi lys							

		Cells	Sap. Super.	Nuclei	Lysine Rich	Core Hist.	Resid. Pellet
CPM	40 μCi ^{14}C -lys:	1490	634	568	80	147	289
10^4 cells	20 μCi ^3H -thy:	4175	149	2967	12	37	3833
CPM	20 μCi ^{14}C -lys:	1259	486	740	66	138	364
10^4 cells	20 μCi ^3H -thy:	4076	112	4218	10	33	3736
Lysine Incorp.	40 μCi lys:	1.2	1.3	0.8	1.2	1.1	0.8
Ratio	20 μCi lys						
Thy. Incorp.	40 μCi lys:	1.0	1.3	0.7	1.2	1.1	1.0
Ratio	20 μCi lys						

Sap. Super. = saponin supernatant; Lysine-Rich = lysine rich histones; Core Hist. = core histones; Resid. Pellet = pellet after histone extraction

VI. REFERENCES

- Ajiro, K., T.W. Borun, and D. Stolter. 1981. Quantitative changes in the expression of histone H1 and H2B subtypes and their relationship to the differentiation of mouse embryonal carcinoma cells. *Dev. Biol.* 86, 206.
- Alder, D. and M.A. Gorovsky. 1975. Electrophoretic analysis of liver and testes histones of the frog Rana pipiens. *J. Cell Biol.* 64, 389.
- Allan, J., P.G. Hartman, C. Crane-Robinson and F.X. Aviles. 1980. The structure of histone H1 and its location in chromatin. *Nat.* 288, 675-679.
- 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., 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, 47-59.
- Appels, R., C. Bolund and N.R. Ringertz. 1974. Biochemical analysis of reactivated chick erythrocyte nuclei isolated from chick/HeLa heterokaryons. *J. Mol. Biol.* 87, 339-355.
- Appels, R. and N.R. Ringertz. 1974. Metabolism of F1 Histone in G1 and G0 Cells. *Cell Diff.* 3, 1-7.
- Appels, R. and J.R.E. Wells. 1972. Synthesis and Turn-over of DNA-Bound Histone during Maturation of Avian Red Blood Cells. *J. Mol. Biol.* 70, 425-434.
- Arceci, R.J. and P.R. Gross. 1980. Histone Variants and Chromatin Structure during Sea Urchin Development. *Dev. Biol.* 80, 186-209.

- 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, 363-371.
- Aviles, F.J., S.E. Danby, G.E. Chapman, C. Crane-Robinson and E.M. Bradbury. 1979. The Conformation of Histone H5 Bound to DNA. Maintenance of the Globular Structure after Binding. Biochem. Biophys. Acta. 578, 290-296.
- Balhorn, R., R. Chalkley and D. Granner. 1972a. Lysine-Rich Histone Phosphorylation. A Positive Correlation with Cell Replication. Biochem. 11, 1094-1098.
- Balhorn, R., M. Balhorn and R. Chalkley. 1972b. Lysine-Rich Histone Phosphorylation and Hyperplasia in the Developing Rat. Dev. Biol. 29, 199-203.
- Bates, D.L. and J.O. Thomas. 1981. Histones H1 and H5: One or two molecules per nucleosome? Nuc. Acid Res. 9, 5883-5894.
- Benbassat, J. 1974. The Transition From Tadpole to Frog Hemoglobin during Natural Amphibian Metamorphosis. J. Cell. Sci. 16, 143-156.
- Belyavsky, A.V., S.G. Bavykin, E.G. Gogvadze and A.D. Mirzabekov. 1980. Primary Organization of Nucleosomes Containing all Five Histones and DNA 175 and 165 Base-pairs Long. J. Mol. Biol. 139, 519-536.
- Benjamin, W. 1971. Selective in vitro Methylation of Rat Chromatin Associated Histone After Partial Hepandectomy. Nature (New Biol.) 234, 18-20.
- Benz, E.J.Jr. 1981. Hemoglobin Switching in Animals. Texas Reports on Biology and Medicine 40, 111-123.
- Billet, M.A. and J. Hindley. 1972. A Study of the Quantitative Variation of Histones, and Their Relationship to RNA Synthesis, during Erythropoiesis in the Adult Chicken. Eur. J. Biochem. 28, 451-462.
- Billings, P.C., J.W. Orf, D.K. Palmer, D.A. Talmage, C.G. Pan and M. Blumenfeld. 1979. Anomalous electrophoretic mobility of Drosophila

phosphorylated H1 histone: its relation to the compaction of satellite DNA into heterochromatin. Nuc. Acid Res. 6, 2151-2164.

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.

Boulikas, T., J.M. Wiseman and W.T. Garrard. 1980. Points of contact between histone H1 and the histone octamer. Proc. Natl. Acad. Sci. USA 77, 127-131.

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.

Bradford, M.M. 1976. A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding. Anal. Biochem. 72, 248-254.

Brasch, K., G. Setterfield and J.M. Neelin. 1972. Effects of Sequential Extraction of Histone Proteins on Structural Organization of Avian Erythrocytes and Liver Nuclei. Exp. Cell Res. 74, 27-41.

Brasch, K., G. 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, 659-677.

Brasch, K., G. Adams and J.M. Neelin. 1977. The Morphology of Erythroid Cells Separated by Density Gradient Centrifugation through Ficoll. Cell Tiss. Res. 176, 373-387.

Briand, G., D. Kmiecik, P. Sautiere, D. Wouters, O. Borie-Loy, G. Biserte, A. Mazen and M. Champagne. 1980. Chicken Erythrocyte Histone H5. IV. Sequence of the carboxy-terminal half of the molecule (96 residues) and complete sequence. FEBS Lett. 112, 147-151.

Brown, G.L., R.G. Rutledge and J.M. Neelin. 1981. Anuran erythrocytes and liver both contain satellite histone H1^S. Life Sci. 28, 2993.

- Bustin, M. and R.D. Cole, 1968. Species and Organ Specificity in Very Lysine-Rich Histones. J. Biol. Chem. 243, 4500-4505.
- Bustin, M. and Cole, R.D. 1969. A Study of the Multiplicity of Lysine-Rich Histones. J. Biol. Chem. 244, 5286-5290.
- Bustin, M. and B.D. Stollar. 1972. Immunochemical Specificity in Lysine-Rich Histone Subfractions. J. Biol. Chem. 247, 5716-5721.
- Campbell, G., H. Weintraub, B. Mayalland and H. Holtzer. 1971. Primitive Erythropoiesis in Early Chick Embryogenesis II. Correlation Between Hemoglobin Synthesis and Mitotic History. J. Cell Biol. 50, 669-681.
- Cary, P.D., M.L. Hines, E.M. Bradbury, B.J. Smith and E.W. Johns. 1981. Conformational Studies of Histone H1o in Comparison with Histones H1 and H5. Eur. J. Biochem. 120, 371-377.
- Casale, G.P., E.A. Khairallah and J.A. Grasso. 1980. An Analysis of Hemoglobin Synthesis in Erythropoietic Cells. Dev. Biol. 80, 107-119.
- Chapman, B.S. and A.J. Tobin. 1979. Distribution of Developmentally Regulated Hemoglobins in Embryonic Erythroid Populations. Dev. Biol. 69, 375-387.
- Chegini, N., V. Alepou, G. Bell, V.A. Hilder and N. Maclean. 1979. Production and Fate of Erythroid Cells in Anemic Xenopus laevis. J. Cell Sci. 35, 403-415.
- Childs, G., R. Maxson and L. Kedes. 1979. Histone Gene Expression during Sea Urchin Embryogenesis: Isolation and Characterization of Early and Late mRNAs of Strongylocentrotus purpuratus by Gene-Specific Hybridization and Template Activity. Dev. Biol. 73, 153-173.
- Clissold, P.M., H.R.V. Arnstein and C.J. Chesterton. 1977. Quantitation of globin mRNA levels during erythroid development in the rabbit and the discovery of a new B-related species in immature erythroblasts. Cell 11, 353-361.
- Cohen, L.H., K.M. Newrock, and A. Zweidler. 1975. Stage-specific switches in histone synthesis during embryogenesis of the sea urchin. Science 190, 994-997.

- Cole, R.D. 1977. Special Features of the Structures of H1 Histones. In "The Molecular Biology of the Mammalian Genetic Apparatus", (P. Ts'o, ed.), Elsevier North-Holland Biomedical Press. pp.93-104.
- Commer, P., C. Schwartz, S. Tracy, T. Tamaoki and J-F. Chiu. 1979. Dexamethasone Inhibits Alpha-Fetoprotein Gene Expression in Developing Mouse Liver. Biochem. Biophys. Res. Comm. 89, 1294-1299.
- Cowman, M.K. and G.D. Fasman. 1980. Dependence of Mononucleosome Deoxyribonucleic Acid Conformation on the Deoxyribonucleic Acid Length and H1/H5 Content. Biochem 19, 532-541.
- Cornish-Bowden, A. 1980. Critical Values for Testing the Significance of Amino Acid Composition Indexes. Anal. Biochem. 105, 233-238.
- Crane-Robinson, C., L. Bohm, P. Puigdomenech, P. D. Cary, P.G. Hartman and E.M. Bradbury. 1980. Structural Domains in Histones. In "DNA-Recombination. Interactions and Repair", (S. Zadrazil and J. Sponar, ed.) Pergamon Press, N.Y., pp. 293-300.
- Croft, L.R. 1980. Introduction to Protein Sequence Analysis. John Wiley and Sons, Ltd., U.K. pp. 19-23.
- 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 phosphorylation and induction of an H1-like protein. Biochem. 19, 2656-2671.
- D'Anna, J.A., L.R. Gurley, R.R. Becher, S.S. Barham, R.A. Tobey and R.A. Walters. 1980b. Amino Acid Analysis and Cell Cycle Dependent Phosphorylation of an H1-like, Butyrate-Enhanced Protein (BEP, H1o, IP25) from Chinese Hamster Cells. Biochem. 19, 4331-4341.
- D'Anna, J.A., L.R. Gurley and R.R. Becher. 1981. Histones H1o and H1ob are the same as CHO histones H1(III) and H1(IV): New features of H1o phosphorylation during the cell cycle. Biochem. 20, 4501.

- Dardick, I. and G. Setterfield. 1976. Volume of Condensed Chromatin in Developing Primitive-Line Erythrocytes of Chick. *Exp. Cell Res.* 100, 159-171.
- Darzynkiewicz, Z., T. Sharpless, L. Staiano-Coico and M.R. Malamed. 1980. Subcomponents of the G1 phase of the cell cycle detected by flow cytometry. *Proc. Natl. Acad. Sci. USA* 77, 6696-6600.
- Darzynkiewicz, Z., F. Traganos, S-B. Xue and M.R. Malamed. 1981. Effect of N-Butyrate on Cell Cycle Progression and in situ Chromatin Structure of L1210 Cells. *Exp. Cell Res.* 136, 279-293.
- Delegeane, A.M. and A.S. Lee. 1982. Coupling of Histone and DNA Synthesis in the Somatic Cell Cycle. *Science* 215, 79-81.
- 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. *Biochem. Biophys. Acta.* 577, 61-70.
- DeWitt, W., R.P. Price, G.L. Vankin and E.M. Brandt. 1972. Production of Erythroid Cells in Phenylhydrazine-treated Rana catesbeiana Tadpoles. *Dev. Biol.* 27, 27-37.
- Ducibella, T. 1974a. The Occurrence of Biochemical Metamorphic Events Without Anatomical Metamorphosis in the Axolotl. *Dev. Biol.* 38, 175-186.
- Ducibella, T. 1974b. The Influence of L-thyroxine on the Changes in Red Blood Cell Type in the Axolotl. *Dev. Biol.* 38, 187-194.
- Edwards, L.J., and L.S. Hnilica. 1968. The Specificity of Histones in Nucleated Erythrocytes. *Experientia* 24, 228-229.
- Edwards, J.A. and J.T. Justus. 1969. Hemoglobins of Two Urodeles: Changes with Metamorphosis. *Proc. Soc. Exp. Biol. Med.* 132, 524-526.
- Elgin, S. and H. Weintraub. 1975. Chromosomal Proteins and Chromatin Structure. *Ann. Rev. Biochem.* 44, 725-775.
- Felsenfeld, G. 1978. Chromatin. *Nature* 271, 115.

- Flavin, M., Y. Blouquit and J. Rosa. 1978a. Biochemical Studies of the Hemoglobin Switch During Metamorphosis in the Salamander Pleurodeles waltlii. I. Partial Characterization of the Adult Hemoglobin. Comp. Biochem. Physiol. 61B, 533-537.
- Flavin, M., Y. Blouquit, A.M. Duprat and J. Rosa. 1978b. Biochemical Studies of the Hemoglobin Switch During Metamorphosis in the Salamander Pleurodeles waltlii. II. Comparative Studies of Larval and Adult Hemoglobins. Comp. Biochem. Physiol 61B, 539-544.
- Flavin, M., A.M. Duprat and J. Rosa. 1979. Ontogenic changes in the Hemoglobins of the Salamander Pleurodeles waltlii. Cell Diff. 8, 405-410.
- Flavin, M., A.M. Duprat and J. Rosa. 1982. Effect of Thyroid Hormones on the Switch from Laval to Adult Hemoglobin Synthesis in the Salamander Pleurodeles waltlii. Cell Diff. 11, 27-33.
- Gallwitz, D. 1975. Kinetics of inactivation of histone mRNA in the cytoplasm after inhibition of DNA replication in synchronized HeLa cells. Nature 257, 247-248.
- Garrard, W.T. and J. Bonner. 1974. Changes in the chromatin proteins during liver regeneration. J. Biol. Chem. 249, 5570.
- Gjerset, 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, 349-357.
- Glotov, B.O., L.G. Nikolaev, S.N. Kurochkin and S.E. Severin. 1977. Histone H1-DNA Interaction. Influence of phosphorylation on the interaction of histone H1 with linear fragmented DNA. Nucl. Acid Res. 4, 1065-1082.
- Glotov, B.O., L.G. Nikolaev and S.E. Severin. 1978. Histone H1-DNA Interaction. On the mechanism of DNA strands crosslinking by histone H1. Nucl. Acid Res. 2587-2605.
- Gooderham, K. and P.M. Naha. 1981. Biochemical Controls of the G1 phase of a Mammalian Cell Cycle. II Synthesis of H1 Histones in Temperature-Sensitive Mouse Cells, Arrested in G1 Phase. Cell Biol. Intl. Rep. 5, 1105-1114.

- Gorka, C. and J.J. Lawrence. 1979. The distribution of H1 subfractions in chromatin subunits. Nucl. Acid Res. 7, 347-359.
- Grasso, J.A. and J.W. Woodard. 1967. DNA Synthesis and Mitosis in Erythropoietic Cells. J. Cell Biol. 33, 645-655.
- 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, 206-222.
- Groudine, M. and H. Weintraub. 1981. Activation of Globin Genes during Chicken Development. Cell 24, 393-401.
- Gurley, L., J.A. D'Anna, S.S. Barham, L.L. Deaven and R.A. Tobey. 1978. Histone Phosphorylation and Chromatin Structure during Mitosis in Chinese Hamster Cells. Eur. J. Biochem. 84, 1-15.
- Hagiwara, H., K. Miyazaki, Y. Matuo, J. Yamashita and T. Horio. 1980. Novel Protease Bound with Chromatins in Normal and Tumorous Tissues of Rats. Biochem. Biophys. Res. Comm. 94, 988-995.
- Hentschel, C.C. and M.L. Birnsteil. 1981. The Organization and Expression of Histone Gene Families. Cell 25, 301.
- 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 the Sequences Derived from the Adult Globin mRNA. Dev. Biol. 72, 350-363.
- Herner, A.E. and E. Friesen. 1961. Biochemical Changes during Anuran Metamorphosis. VIII Changes in the Nature of the Red Cell Proteins. Arch. Biochem. Biophys. 95, 25-35.
- Hieter, P.A., M.B. Hendricks, K. Hemminki and E.S. Weinberg. 1979. Histone Gene Switch in the Sea Urchin Embryo. Identification of the Late Embryonic Messenger Ribonucleic Acids and the Control of Their Synthesis. Biochem. 18, 2707-2716.
- Hnilica, L.S. 1967. Proteins of the Cell Nucleus. Prog. Nuc. Acid Res. 7, 25.

- Hohmann, P. 1978. The H1 class of histone and diversity in chromosomal structures. *Subcell. Biochem.* 5, 87.
- Hugues, B. and H.B. Osbourne. 1981. Dexamethasone inhibits a heme-independent event necessary for the terminal differentiation of murine erythroleukemia cells. *Biochem. Biophys. Res. Comm.* 102, 1342-1349.
- Hwan, J.C., I.M. Leffak, H.J. Li, P.C. Huang and C. Mura. 1975. Studies on the interaction between histone V (f2c) and deoxyribonucleic acids. *Biochem.* 14, 1390-1396.
- Isenberg, I. 1979. Histones. *Ann. Rev. Biochem.* 48, 159.
- Just, J.J., and B.G. Atkinson. 1972. Hemoglobin Transition in the bullfrog, Rana catesbeiana, during Spontaneous and Induced Metamorphosis. *J. Exp. Zool.* 182, 271-280.
- Jurd, R.D. and N. Maclean. 1970. An Immunofluorescent study of the hemoglobins in metamorphosing Xenopus laevis. *J. Embryol. Exp. Morph.* 23, 299-309.
- Johmann, C., R. Eckhardt and M. Gorovsky. 1973. The Histones Associated with Condensed and Extended Chromatin in Mouse Liver. *J. Cell Biol.* 58, 119-125.
- Kano, Y., T. Miyashita, H. Nakamura, K. Kuroki, A. Nagota and F. Imamoto. 1981. In vivo correlation between DNA supercoiling and transcription. *Gene* 13, 173-184.
- Keppel, F., B. Allet and H. Eisen. 1977. Appearance of a chromatin protein during 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 Location of the Chromosomal Protein IP25. *Eur. J. Biochem.* 96, 477-482.
- Kernell, A.M., L. Bolund and N.R. Ringertz. 1971. Chromatin Changes during Erythropoiesis. *Exp. Cell Res.* 65, 1-6.
- Kincade, J.M.Jr. 1969. Quantitative Species Differences and Quantitative Tissue Differences in the Distribution of Lysine-Rich Histones. *J. Biol. Chem.* 244, 3375-3386.

- Kornberg, R.D. 1977. Structure of Chromatin. *Ann. Rev. Biochem.* 46, 931.
- Kumar, N.M. and I.O. Walker. 1980. The Binding of Histones H1 and H5 to Chromatin in Chicken Erythrocyte Nuclei. *Nucl. Acid Res.* 8, 3535-3551.
- Langan, T.A. 1978. Methods for the Assessment of Site-Specific Phosphorylation. In "Methods in Cell Biology" XIX, (G. Stein, J. Stein and L.J. Kleinsmith, ed.) Academic Press, N.Y. pp 127-142.
- Larsen, A. and H. Weintraub. 1982. An Altered DNA Conformation Detected by S1 Nuclease Occurs at Specific Regions in Active Chick Globin Chromatin. *Cell* 29, 609-622.
- Laskey, R.A. and A.D. Mills. 1975. Quantitative Film Detection of 3-H and 14-C in Polyacrylamide Gels by Fluorography. *Eur. J. Biochem.* 56, 335-341.
- 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, 862-867.
- Lewin, B. 1974. Gene Expression 2 Eukaryotic Chromosomes. John Wiley and Sons. Ltd., Toronto. pp. 101-147.
- Liao, L. and R.D. Cole. 1981a. Differences Among H1 Subfractions in Binding to Linear and Superhelical DNA. *J. Biol. Chem.* 256, 11145-11150.
- Liao, L. and R.D. Cole. 1981b. Condensation of Dinucleosomes by Individual Subfractions of H1 Histones. *J. Biol. Chem.* 256, 10124-10128.
- Lucas, A.M. and C. Jamroz. 1961. Atlas of Avian Hematology. Dept. of Agriculture, Washington, DC
- Maclean, N., V.A. Hilder and Y.A. Baynes. 1973. RNA Synthesis in Xenopus Erythrocytes. *Cell Diff.* 2, 261.
- Maclean, N., and R.D. Jurd. 1971. Electrophoretic Analysis of the Hemoglobins of Ambystoma mexicanum. *Comp. Biochem. Physiol.* 40B, 751-755.
- Maniatis, G.M. and V.M. Ingram. 1971a. Erythropoiesis during Amphibian Metamorphosis. II Immunochemical Study of Larval and Adult Hemoglobins of Rana catesbeiana. *J. Cell Biol.* 49, 380-389.

- Maniatis, G.M. and V.M. Ingram. 1971b. Erythropoiesis during Amphibian Metamorphosis. III Immunochemical Detection of Tadpole and Frog Hemoglobins (*Rana catesbeiana*) in Single Erythrocytes. *J. Cell Biol.* 49, 390-397.
- Marashi, F., L. Baumbach, R. Rickles, F. Sierra, J. Stein and G. Stein. 1982. Histone Proteins in HeLa S3 Cells are Synthesized in a Cell Cycle Stage-Specific Manner. *Science* 215, 683-685.
- Marchalonis, J. and J.K. Weltman. 1971. Relatedness among proteins: A new method of estimation and its application to immunoglobulins. *Comp. Biochem. Physiol.* 38B, 609-625.
- Marekov, L., G. Angelov and B. Beltchev. 1978. Studies on the Preferential Binding of Histone H1 to AT-rich DNA. *Biochimie* 60, 1347-1349.
- Marsh, W.H., and P.J. Fitzgerald. 1973. Pancreas Acinar Cell Regeneration. XIII Histone Synthesis and Modification. *Fed. Proc.* 32, 2119-2125.
- Marinage, A., C. Quirin-Stricker, M. Chamagne and P. Sautiere. 1981. Phosphorylated Sites of Chicken Erythrocyte H5 by a cAMP-Independent Protein Kinase from Mouse Plasmocytoma Cells. *FEBS Lett.* 134, 103-106.
- Mathews, H.R. and E.M. Bradbury. 1978. The Role of H1 Phosphorylation in the Cell Cycle. *Exp. Cell Res.* 111, 343-351.
- Mathis, D., P. Oudet and P. Chambon. 1980. Structures of transcribing chromatin. *Prog. Nuc. Acid Res. Mol. Biol.* 24, 1.
- Matsumoto, Y-i., H. Yasuda, S. Mita, T. Marunouchi and M-a. Yamada. 1980. Evidence for the involvement of histone H1 phosphorylation in chromosome condensation. *Nature* 284, 181-183.
- Mazen, A., M. Champagne, M. Wilhelm and F.X. Wilhelm. 1978. The Structure of *Sipunculus nudus* erythrocyte chromatin. *Exp. Cell Res.* 117, 431-438.
- McGee, J.D., D.C. Rass, E. Charney and G. Felsenfeld. 1980. Orientation of the Nucleosome within the Higher Order Structure of Chromatin. *Cell* 22, 87-96.

- Medvedev, Zh. A. and M.N. Medvedeva. 1980a. High H1o/H1 ratios in spontaneous hepatomas in aging mice. IRCS Med. Sci. 8, 431.
- Medvedev, Zh. A. and M.N. Medvedeva. 1980b. A Group of Satellite Acid-Soluble Non-Histone Chromatin Proteins. FEBS Lett. 112, 35-38.
- Medvedev, Zh. A. and M.N. Medvedeva. 1980c. Correlation between the increase of H1o histone and the decrease in H1-2 in different tissues. IRCS Med. Sci. 8, 848.
- Medvedev, Zh. A., M.N. Medvedeva and H.M. Crowne. 1981. Sharp increase in the content of liver-specific satellite chromatin protein in spontaneous mouse hepatomas. IRCS Med.Sci. 9, 860-861.
- Medvedeva, M.N., L.I. Huschtscha and Zh.A. Medvedev. 1975. The methionine-containing subfraction of F1 histone from rat tissues. FEBS Lett. 53, 253-257.
- Meints, R.H. and C. Forehand. 1977. Hemoglobin Switching in the Frog, Rana pipiens and Rana catesbeiana : The Effect of Hemorrhage on the Transition from Adult to Tadpole Hemoglobin Patterns. Comp. Biochem. Physiol. 58A, 265-268.
- Melli, M., G. Spinelli and E. Arnold. 1977. Synthesis of Histone Messenger RNA of HeLa Cells During the Cell Cycle. Cell 12, 167-174.
- 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. 1977. Comparison of histones from fish erythrocytes. Can. J. Biochem. 55, 1220-1227.
- Miki, B.L.A. and J.M. Neelin. 1980. DNA repeat lengths of erythrocyte chromatins differing in contents of H1 and H5. Nucl. Acid. Res. 8, 529-542.
- Miller, G.L., L. Berlowitz and W. Regelson. 1972. Chromatin and Histones in Hen Erythrocyte Nuclei - Morphologic change induced by selective binding of histones with synthetic polyanion. Exp. Cell Res. 71, 409-421.

- Mitsui, Y., H. Sakagami, S-I. Murota and M-A. Yamada. 1980. Age-related Decline in Histone H1 Fraction in Diploid Fibroblast Cultures. *Exp. Cell Res.* 126, 289-298.
- Miura, Y. and F.H. Wilt. 1971. The Effects of 5-Bromodeoxyuridine on Yolk Sac Erythropoiesis in the Chick Embryo. *J. Cell Biol.* 48, 523-532.
- Moreau, J., L. Marcáud, F. Machat, J. Kejzlarova, J-A. Leperant and K. Scherrer. 1982. A and T-rich linkers define functional domains in eukaryotic DNA. *Nature* 295, 260-262.
- Morris, S.M. and R.D. Cole. 1978. Histone Metabolism During Amphibian Metamorphosis. Isolation, Characterization and Biosynthesis. *Dev. Biol.* 62, 52-64.
- Moss, B. and V.M. Ingram. 1965. The repression and induction by thyroxine of hemoglobin synthesis during amphibian metamorphosis. *Proc. Natl. Acad. Sci. USA* 54, 967-974.
- Moss, B., and V.M. Ingram. 1968a. Haemoglobin synthesis during amphibian metamorphosis. I. Chemical studies of haemoglobin from the larval and adult stages of Rana catesbeiana. *J. Molec. Biol.* 32, 481-492.
- Moss, B., and V.M. Ingram. 1968b. Haemoglobin synthesis during amphibian metamorphosis. II. Synthesis of adult haemoglobin following thyroxine administration. *J. Molec. Biol.* 32, 493-504.
- Moss, B.A., W.G. Joyce and V.M. Ingram. 1973. Histones in Chick Embryo Erythropoiesis. *J. Biol Chem.* 248, 1025-1031.
- Mura, C., S.W. Craig and P.C. Huang. 1978. Immunofluorescent and Autoradiographic Analysis of the Relationship Between the Presence of Histone H5 and DNA Synthesis in the Developing Chick Erythroid Cells. *Exp. Cell Res.* 114, 301-305.
- Mura, C. and B.D. Stollar. 1981. Sereological Detection of Homologies of H1o with H5 and H1 Histones. *J. Biol. Chem.* 256, 9767-9769.
- Nazar, R.N., M. Yaguchi, G. Willick, C.F. Rollin and C. Roy. 1979. The 5-S RNABinding Protein from Yeast (Saccharomyces cerevesiae) Ribosomes. Evolution of the Eukaryotic 5-S RNA Binding Protein. *Eur. J. Biochem.* 102, 573-582.

- Neelin, J.M., P.X. Callahan, D.C. Lamb and K Murray.
1964. The Histones of Chicken Erythrocyte Nuclei.
Can. J. Biochem. 42, 1220-1227.
- Nelson, P.P., S.C. Albright and W.T. Garrard. 1979.
Nucleosome Arrangement with Regard to the DNA Base
Composition. J. Biol. Chem. 254, 9194-9199.
- Nelson, R.D. and J.J. Yunis. 1969. Species and Tissue
Specificity of Very Lysine-Rich and Serine-Rich
Histones. Exp. Cell Res. 57, 311-318.
- Newrock, K.M., C.R. Alfragme, R.V. Nordix and L.C. Cohen.
1978. Histone changes during chromatin remodeling
in embryogenesis. Cold Spring Harbour Symp.
Quant. Biol. 42, 421-431.
- Panyim, S., D. Bilek and R. Chalkley. 1971. An
Electrophoretic Comparison of Vertebrate Histones.
J. Biol. Chem. 246, 4206-4215.
- Panyim, S. and R. Chalkley. 1969a. A New Histone Found
Only in Mammalian Tissues with Little Cell
Division. Biochem. Biophys. Res. Comm. 37,
1042-1049.
- Panyim, S. and R. Chalkley. 1969b. The Heterogeneity of
Histones. I. A Quantitative Analysis of Calf
Histones in Very Long Polyacrylamide Gels.
Biochem. 8, 3972-3979.
- Pardee, A.B., R. Dubrow, J.L. Hamlin and R.F. Kletzien.
1978. Animal Cell Cycle. Ann. Rev. Biochem. 47,
715-750.
- Pehrson, J. and R.D. Cole. 1980. Histone H1o
accumulation in growth-inhibited cultured cells.
Nature 285, 43-44.
- Pehrson, J. and R.D. Cole. 1981. Bovine H1o Histone
Subfractions Contain an Invariant Sequence Which
Matches H5 Rather than H1. Biochem. 20, 2298-2301.
- Pehrson, J. and R.D. Cole. 1982. Histone H1 Subfractions
and H1o Turnover at Different Rates in Nondividing
Cells. Biochem. 21, 456-460.
- Pieler, C., G.R. Adolf and P. Swetly. 1981.
Accumulation of Histone H1o During Chemically
Induced Differentiation of Murine Neuroblastoma
Cells. Eur. J. Biochem. 115, 329-333.

- Piha, R.S. and K.H. Valkonene. 1979. Changes in Liver Nuclear Proteins During Bovine Ontogeny. FEBS Lett. 108, 326-330.
- Prahlad, K.V. 1968. Induced Metamorphosis: Rectification of a Genetic Disability by Thyroid Hormone in the Mexican Axolotl Siredon mexicanum. Gen. Comp. Endoc. 11, 21-30.
- Prahlad, K.V. and L.E. DeLanney. 1965. A Study of Induced Metamorphosis in the Axolotl. J. Exp. Zool. 160, 137-146.
- Prescott, D. 1976. The Cell Cycle and the Control of Cellular Proliferation. Adv. Gen. 18, 99-177.
- Radford, I.R., R.F. Martin and L.R. Finch. 1982. Effects of Hydroxyurea on DNA Synthesis in Mouse L-Cells. Biochem. Biophys. Acta. 696, 145-153.
- Rattle, H.W., T.A. Langan, S.E. Danby and E.M. Bradbury. 1977. Studies on the Role and Mode of Operation Of the Very-Lysine-Rich Histones in Eukaryotic Chromatin. Effect of A and B Site Phosphorylation on the Conformation and Interaction of Histone H1. Eur. J. Biochem. 81, 499-505.
- Rickles, R., F. Marashi, F. Sierra, S. Clark, J. Wells, J. Stein and G. Stein. 1982. Analysis of histone gene expression during the cell cycle in HeLa cells by using cloned human genes. Proc. Natl. Acad. Sci. USA 79, 749-753.
- Riggs, A. 1951. The metamorphosis of hemoglobin in the bullfrog. J. Gen. Physiol. 35, 23-40.
- Risley, M.S. and R.A. Eckhardt. 1981. H1 Histone Variants in Xenopus laevis. Dev. Biol. 84, 79-87.
- Roberts, R.V., P.J. Weatherall and J.B. Clegg. 1972. The synthesis of human hemoglobin A2 during erythroid maturation. Biochem Biophys. Res. Comm. 47, 81-87.
- Rosenberg, M. 1970. Electrophoretic Analysis of Hemoglobin and Isozymes in Individual Vertebrate Cells. Proc. Natl. Acad. Sci. USA 67, 32-36.
- 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.

- Ruiz-Vazquez, R. and A. Ruiz-Carrillo. 1982. Construction of chimeric plasmids containing histone H5 cDNA from hen erythrocytes. DNA sequence of a fragment derived from the 5' region of H5 mRNA. Nucl. Acid Res. 10, 2093-2108.
- Russanova, V., C. Venkov and R. Tsanev. 1980. A Comparison of Histone Variants in Different Rat Tissues. Cell Diff. 9, 339-350.
- Rutledge, R.G. 1982. Histone Synthesis during Erythropoiesis in Xenopus laevis. M.Sc. Thesis, Carleton University, Ottawa.
- Rutledge, R.G., C. Shay, G.L. Brown and J.M. Neelin. 1981. The similarity of histones from turtle erythrocytes and liver. Can. J. Biochem. 59, 273.
- Sawicka, J., L. Kornacka and J. Malec. 1980. Extraction of mouse DNA by precipitation with F1 histones into fragments differing in their base composition. Experientia 34, 309-310.
- Schlegel, R.A., K.R. Haye, A.H. Litwack and B.M. Phelps. 1980. Nucleosome Repeat Lengths in the Definitive Erythroid Series of the Adult Chicken. Biochem. Biophys. Acta. 606, 316-330.
- Seligy, V.L., G.H. Adams and J.M. Neelin. 1973. The Role of Histones in Avian Erythropoiesis. In "The Biochemistry of Gene Expression in Higher Organisms" (J. Pollack and J.W. Lee, ed.), Australia and New Zealand Book Co. TTY Ltd., pp. 177-190.
- 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. and J.M. Neelin. 1970. Transcription properties of stepwise acid-extracted chicken erythrocyte chromatin. Biochem. Biophys. Acta. 213, 380.
- Senger, D.R., R.J. Arceci and P.R. Gross. 1978. Histones of Sea Urchin Embryos; Transients in Transcription, Translation and the Composition of Chromatin. Dev. Biol. 65, 416-425.
- Seyedin, S.M. and R.D. Cole. 1981. H1 Histones of Trout. J. Biol. Chem 256, 442-444.

- Seyedin, S.M. and W.S. Kistler. 1979. Levels of chromosomal protein high mobility group 2 parallel the proliferative activity of testes, skeletal muscle and other organs. *J. Biol. Chem.* 254, 11264.
- Shaw, L.M.J. and P.C.C. Huang. 1970. A Description of Two Procedures Which Avoid the use of Extreme pH Conditions for the Resolution of Components Isolated from Chromatins Prepared from Pig Cerebellar and Pituitary Nuclei. *Biochem* 9, 4530-4542.
- Shimada, T., Y. Okihama, C. Murata and R. Shukuta. 1981. Occurance of H1o-like Protein and Protein A24 in the Chromatin of Bullfrog Erythrocytes Lacking H5. *J. Biol. Chem.* 256, 10577-10582.
- Singer, D.S. and M.E. Singer. 1976. Studies of the interaction of H1 histone with superhelical DNA: Characterization of the recognition and binding regions of H1 histone. *Nucl. Acid Res.* 3, 2531-2547.
- Singer, D.S. and M.E. Singer. 1978. Characterization of the Complexes of Superhelical and Relaxed Closed Circular DNA with H1 and Phosphorylated H1 Histones. *Biochem.* 17, 2086-2095.
- Smerdon, M.J. and I. Isenberg. 1976. Conformational Changes in the Subfractions of Calf Thymus Histone H1. *Biochem.* 15, 4233-4242.
- Smith, B.J. and E.W. Johns. 1980a. Isolation and Characterization of Subfractions of Nuclear Protein H1o. *FEBS Lett.* 110, 25-29.
- Smith, B.J. and E.W. Johns. 1980b. Histone H1o: Its location in chromatin. *Nucl. Acid Res.* 8, 6069-6079.
- Smith, B.J., J.M. Walker and E.W. Johns. 1980. Structural Homology Between Mammalian H1o Subfraction and Avian Erythrocyte-Specific Histone H5. *FEBS Lett.* 112, 42-44.
- Sponar, J. and Z. Sormova. 1972. Complexes of Histone F1 with DNA in 0.15M NaCl. Selectivity of Interaction with Respect to DNA Composition. *Eur. J. Biochem.* 29, 99-103.

- Stalder, J., M. Groudine, J. Dodgson, J. Engel and H. Weintraub. 1980. Hb Switching in Chickens. *Cell* 19, 973-980.
- Stein, G.S., J.L. Stein, W.D. Park, S. Detke, A. Lichtler, E.A. Sheppard, R.L. Jansing and I.R. Phillips. 1978. Regulation of Histone Gene Expression in HeLa S3 Cells. *Cold Spring Harbour Sym. Quant. Biol.* 42, 1107.
- Sung, M.T. 1977. Phosphorylation and dephosphorylation of histone V (H5): Controlled condensation of avian erythrocyte chromatin. *Biochem.* 16, 286.
- Sung, M.T. and E.F. Freeland. 1978. Sites of In Vivo phosphorylation of Histone H5. *Biochem.* 17, 1884-1890.
- Sung, M.T., J. Harford, M. Bundman and G. Vidalakas. 1977. Metabolism of Histones in Avian Erythroid Cells. *Biochem.* 16, 279-285.
- Tarnowka, M.A., C. Baglioni and C. Basilico. 1978. Synthesis of H1 histones by BHK cells in Gl. *Cell* 15, 163-171.
- 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, 709-733.
- Thomas, N. and N. Maclean. 1975. The Erythroid Cells of Anemic Xenopus laevis. I. Studies on Cellular Morphology and Protein and Nucleic Acid Synthesis During Differentiation. *J. Cell Sci.* 509-520.
- 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.
- Tompkins, R. and J.K. Townsend. 1977. Control of Metamorphic Events in the Neotenus Urodele Ambystoma mexicanum. *J. Exp. Zool.* 200, 191-196.
- Tsai, Y.H. and L.S. Hnilica. 1975. Tissue specific histones in the erythrocytes of chicken and turtle. *Exp. Cell Res.* 91, 107.
- Urban, M.K., S.G. Franklin and A. Zweidler. 1979. Isolation and characterization of histone variants in chicken erythrocytes. *Biochem* 18, 3952-3960.

- Urban, M.K., J.M. Neelin and T.W. Betz 1980.
Correlation of chromatin composition with metabolic changes in nuclei of primitive erythroid cells from chicken embryos. *Can. J. Biochem.* 58, 726-31.
- Valkonen, K.H. 1980. Developmental Changes in the H1 Histones from Bovine Liver. *Life Sci.* 27, 1217-1224.
- Varricchio, F. 1977. Postnatal Increase in Histone H1a in the Rat Pancreas. *Arch. Biochem. Biophys.* 179, 716-717.
- Vendrely, R and M. Picaud. 1968. Étude Comparée des Histones D'erythrocytes de Differentes Espèces Animals. *Exp. Cell Res.* 49, 13-24.
- Villeponteaux, B., L. Laskey and I. Harary. 1978. Lysine-Rich Histones and the Selective Digestion of the Globin Gene in Avian Red Blood Cells. *Biochem.* 17, 5532-5536.
- Von Holt, C, W.N. Strickland, W.F. Brandt and M.S. Strickland. 1979. More Histone Structures.. *FEBS Lett.* 100, 201-218.
- Weintraub, H. 1972. A possible role for histone in the synthesis of DNA. *Nature* 240, 449-453.
- Weintraub, H. 1978. The nucleosome repeat length increases during erythropoiesis in the chick. *Nucl. Acid Res.* 5, 1179-1187.
- Weintraub, H., G. 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, 652-668.
- Weintraub, H., G. Campbell and H. Holtzer. 1972. Identification of a Developmental Program Using Bromodeoxyuridine. *J. Mol. Biol.* 70, 337-350.
- Weintraub, H., S.J. Flint, I.M. Leffak, M Groudine and R.M. Grainger. 1978. The generation and propagation of variegated chromosome structures. *Cold Spring Harbour Sym. Quant. Biol.* 42, 401-407.
- Weintraub, H. and M Groudine. 1976. Chromosomal Subunits in Active Genes Have an Altered Conformation. *Science*, 193, 848-856.

- Weintraub, H., A. Larsen and M. Groudine. 1981. Alpha-Globin-Gene Switching during the Development of Chick Embryos: Expression and Chromosome Structure. *Cell* 24, 333-344.
- Welch, S. L. and R.D. Cole. 1980. Differences Among Subfractions of H1 Histones in Retention of Linear and Superhelical DNA on Filters. *J. Biol. Chem.* 255, 4516-4518.
- Wilhelm, M.L., A. Mazen and F.X. Wilhelm. 1977. Comparison of the DNA Repeat Length in H1- and H5-Containing Chromatin. *FEBS Lett.* 79, 404-408.
- Wilkes, R.R., G.B. Birnie and R.W. Old. 1978. Histone Gene Expression during the Cell Cycle Studied by in situ Hybridization. *Exp. Cell Res.* 115, 441-444.
- Worcel, A. 1977. Molecular Architecture of the Chromatin Fiber. *Cold Spring Harbour Sym. Quant. Biol.* 42, 313-323.
- Worcel, A. and c. Benyajati. 1977. Higher Order Coiling of DNA in Chromatin. *Cell* 12, 83-100.
- Wu, R.S. and W.M. Bonner. 1981. Separation of Basal Histone Synthesis from S-phase Histone Synthesis in Dividing Cells. *Cell* 27, 321-330.
- Yaguchi, M., C. Roy, M. Dove and V. Seligy. 1977. Amino Acid Sequence Homologies Between H1 and H5 Histones. *Biochem. Biophys. Res. Comm.* 76, 100-106.
- Yaguchi, M., C. Roy and V. Seligy. 1979. Complete amino acid sequence of goose erythrocyte H5 and the homology between H1 and H5 histones. *Biochem. Biophys. Res. Comm.* 90, 1400.
- Zlatanova, J. 1980. Lack of coupling between DNA and histone synthesis in growth-arrested Friend erythroleukemia cells. *Mol. Cell. Biochem.* 35, 49-53.
- Zlatanova, J., K. Oberhammer and P. Swetly. 1980. Expression of histone H1o during differentiation of erythroleukemic mouse cells. In "In vivo and in vitro Erythropoiesis: The Friend System" (G.B. Rossi, ed), Elsevier/North-Holland Biomedical Press. pp. 297-307.

Zlatnova, J. and P. Swetly. 1978. Uncoupled synthesis of histones and DNA during Friend cell differentiation. *Nature* 286, 276-277.

Zweidler, A. 1976. Complexity and variability of the histone complement. In "Organization and Expression of Chromosomes" (V.G. Allfrey, E.K.F. Bautz, B.J. McCarthy, R.T. Schimke and A. Tisieres, eds.), Life Sciences Research Report #4, Abakon Verlagsgesellschaft, Berlin. pp. 187-196.

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10, 365-388.

R.G. Rutledge, C.S. Shay, G.L.
Brown and J.M. Neelin (1981) The
Similarity of Histones From Turtle
Erythrocytes and Liver. Can. J.
Biochem. 59, 273-279.

G.L. Brown, R.G. Rutledge and J.M.
Neelin (1981) Anuran Erythrocytes
and Liver Both Contain Satellite
Histone H1 . Life Sciences 28,
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ABSTRACTS PUBLISHED

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G.L. Brown and M. Locke (1977)
Ultrastructural Localization of
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G.L. Brown (1978) Ultrastructural
Localization of Specific Nucleo-
protein Fractions. Ninth Intl. Con.
on Electron Microscopy, Toronto,
Ontario. Vol. II, 204-205.

G.L. Brown and J.M. Neelin (1981)
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