H1 Histone Subtypes and Subtype Synthesis Switches in Normal and Delobed Embryos of *Ilyanassa obsoleta*

by

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ABSTRACT

H1 Histone Subtypes and Subtype Synthesis Switches in Normal and Delobed Embyros of *Ilyanassa obsoleta*

The mud snail, *Ilyanassa obsoleta*, provides a way to explore the relationship between histone subtype complexity and major developmental events such as oogenesis, cleavage, gastrulation and organogenesis. Removal of the polar lobe, an anucleate protrusion of segregated egg cytoplasm, results in an embryo which lacks bilateral symmetry, organs, and organ systems, although differentiated cell types, of aberrant structures may be present.

In this study H1 histone subtype complexity and H1 histone subtype synthesis switches were first characterized during the development of normal embryos. The effect of the removal of the polar lobe on the normal H1 pattern of synthesis was then investigated in the delobed embryo to determine if classical polar lobe effects are accompanied by a perturbation of H1 metabolism during development.

SDS gel electrophoresis and fluorography of radiolabelled 5% perchloric acid soluble nuclear extracts resolved six H1 proteins in the *Ilyanassa* embryo. These are designated bands 1 - 6. Bands 1 - 5 migrate as a cluster with similar molecular weights. Band 6 has a substantially higher molecular weight.

The synthesis of band 6 is predominant during the first six hours post-trefoil. During cleavage and gastrulation bands 1 and 2 are predominant while bands 3, 4, and 5 become predominant during organogenesis. Developmental synthesis patterns and electrophoretic mobilities indicate that band 6, band 1, band 2, and the group of bands 3, 4 and 5 may be different subtypes. In addition, it has been found that removal of the polar lobe delays the off-switch of the early bands 6, 1, and 2 and the on-switch of the late bands 3, 4, and 5. Cell number data of normal and delobed embryos reveal that the delay in subtype synthesis switching is not caused by an overall delay of cell division in the delobed embryo.

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RÉSUMÉ

Sous-types d'histone H1 et déterminants de la synthèse des sous-types: Étude réalisée chez Ilyanassa obsoleta (embryons normaux et embryons privées de globules polaires)

L'escargot Ilyanassa obsoleta offre un mode d'exploration du rapport entre la complexité du sous-type d'histone et les principaux événements du développement tels que l'ovogénèse, la segmentation, la gastrulation et l'organogénèse. L'ablation du globule polaire, une protrusion anuclée du cytoplasme de l'oeuf, donne un embryon dépourvu de symétrie bilatérale, de certains organes et de systèmes d'organes, quoique présentant parfois certains types de cellules différenciées de structure aberrante.

Au cours de la présente étude, la complexité des sous-types d'histone H1 et les déterminants de la synthèse des sous-types d'histone H1 ont d'abord été caractérisés au cours du développement d'embryons normaux. Les effets de l'ablation du globule polaire sur le schème normal de synthèse de l'histone H1 a ensuite fait l'objet d'études chez l'embryon privé de globule afin de déterminer s'ils s'accompagnent d'une perturbation du métabolisme de l'H1 au cours du développement.

L'électrophorèse sur gel avec SDS et la fluorographie sur la fraction soluble, dans l'acide perchlorique à 5%, d'extraits

nucléaires radiomarqués, ont permis de distinguer six protéines H1 chez l'embryon d' Ilyanassa. Celles-ci sont désignées les bandes 1 à 6. Les bandes 1 à 5 sont très rapprochées, ayant un poids moléculaire similaire. La bande 6 présente un poids moléculaire nettement plus élevé. La synthèse de la bande 6 est prédominante au cours des six premières heures qui suivent la première segmentation. Au cours de la segmentation et de la gastrulation, les bandes 1 et 2 sont prédominantes tandis que les bandes 3, 4 et 5 deviennent prédominantes au cours de l'organogénèse. Les schèmes de synthèse et de mobilité à l'électrophorèse au cours du développement révèlent que la bande 6, la band 1, la band 2 et le groupe de bandes 3, 4 et 5 peuvent représenter différents sous-types. En outre, on a observé que l'ablation du globule polaire retarde l'interruption de la synthèse des premières bandes 6, 1 et 2 et le déclenchement de la synthèse des bandes tardives 3, 4 et 5. Les données relatives au nombre des cellules tant chez les embryons normaux que chez les embryons privés de globules polaires révèlent que le retard du déclenchement de la synthèse des sous-types n'est pas causé par un retard général de la division cellulaire chez l'embryon privé de globules polaires.

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INTRODUCTION

Early Studies

Investigation of the basic proteins of the cell nucleus began in 1868. Johann Friedrich Miescher, a student with Ernst Hoppe-Seyler in Tubingen, Germany (Luck, 1964) isolated nuclei from human pus cells. With alkaline extraction and acid precipitation, he obtained what he termed soluble and insoluble nuclein. The soluble nuclein was a crude preparation of DNA (Luck, 1964). Later, in 1874, while working on salmon sperm, Miesher isolated a combination of an acidic substance, later called nucleic acid, and an organic base which he named protamine (Johns, 1971). Protamines are now known to be the histone-like basic proteins associated with sperm DNA. Albrecht Kossel, also a student of Hoppe-Seyler, continued Miescher's work in 1877 (Luck, 1964). Using goose erythrocytes, Kossel isolated the basic material associated with the nucleic acid by extraction with dilute hydrochloric acid and named it histone (Johns, 1971).

Since this first isolation of histones attempts have been made to characterize and define them and postulate their function in the cell. Early chromatographic studies showed that histone preparations were not a homogeneous group of proteins. These descriptions of histone fractions utilized the ratio of their arginine:lysine content to distinguish subfractions which were lysine-rich, slightly or moderately

lysine-rich, and arginine rich (reviewed by Murray, 1964a). Analysis of these subfractions by ion-exchange chromatography, starch gel electrophoresis and tryptic digests indicated a great heterogeneity of histones (see Murray, 1964b). However the early characterization of histones was plagued with many technical problems (see Johns, 1971). The analysis of histones solely by total acid amino analysis was not adequate since many proteins with similar molecular weight, also have a high content of basic amino acids, and co-purify with the histones. Also, under the conditions of electrophoresis first used for histones, many histones aggregated producing multiple bands and false heterogeneity. In addition, the histone extraction procedures were not adequate. Preparations contained cytoplasmic and nuclear non-histone contaminants and, in some cases, enzymatic degradation produced artifactual heterogeneity. These problems were resolved with improved extraction techniques, including proteolytic inhibitors, and improved isolation and analytical techniques.

The CIBA Foundation symposium (1974) proposed a universal nomenclature replace the nomenclature which was based upon diverse fractionation protocols. It was decided that the lysine-rich histones (F1, Ia) would be named H1, the slightly lysine-rich histones (F2a2, IIb1 and F2b, IIb2) were named H2A and H2B respectively and the arginine rich histones (F3, III and F2a1, IV) were named H3 and H4 respectively (Bradbury, 1975). The CIBA nomenclature is used throughout this thesis.

Through the work of many laboratories combining physical and biochemical analyses, the existence of a regular repeating pattern of chromatin was elucidated. This repeat structure is the nucleosome or core particle. It is an octomeric complex of two proteins of each of the core histones H2A, H2B, H3, and H4 around which is wrapped 146 base pairs of DNA. Histone H1 exists in half the stochiometric amount of the core histones and is believed to be localized at the point where DNA enters and exits from the core particle and forms a link between two core particles (McGhee and Felsenfeld, 1980; Igo-Kemenes et al., 1982).

Core Histones

In chromatin, core histones are organized with DNA to form the nucleosome (Kornberg, 1977; McGhee and Felsenfeld, 1980; Igo-Kemenes et al., 1982). Core histones H2A and H2B exhibit evolutionary divergence although it is not nearly as extensive as that of H1. H3 and H4 show much less divergence than the other classes of histone, with H3 showing more divergence than H4 (Isenberg, 1979). Histone classes H2A, H2B and H3 contain several different proteins which differ in amino acid sequence (Isenberg, 1979). This heterogeneity has come to be termed "subtypes" or "variant" forms within a class. Additional histone heterogeneity is created by covalent modifications of of amino acids. These modifications are acetylation, phosphorylation, methylation, ADP-ribosylation and, in the case

of H2A, linkage to the protein ubiquitin (see Dixon et al., 1975; Isenberg, 1979). Functional modifications could arise by either directly affecting the histone according to the nature of the amino acid substitutions among subtypes or by providing for an altered ability to undergo amino acid side chain modifications. These alterations in core histones are believed to alter histone:histone and histone:DNA interactions and thus affect chromatin structure (Dixon et al., 1975; McGhee and Felsenfeld, 1980).

The observation of core histone subtypes raised the possibility that within a class of histones there might be subtypes with distinct and similar functions. The discovery that the subtypes are stage specific in their synthesis during development raised the possibility that these subtypes may be functionally significant during embryogenesis (Cohen et al., 1975; Newrock et al., 1978). Since the early discoveries of histone subtypes and histone switching patterns in development, this phenomenon has been found to occur in many species although different species show a large amount of individual variation (see Maxson et al., 1983b).

This brief presentation of the histones is to orient the reader to the histones and their role in chromatin structure. Detailed discussion of the core histones can be found in reviews by McGhee and Felsenfeld (1980) and Igo-Kemenes et al. (1982). The work presented in this thesis is primarily concerned with histone H1. Thus, the remainder of this

introduction deals with H1 with an emphasis on the developmental aspects of this protein.

Function of H1

H1 has been implicated in maintaining and controlling higher order chromatin structures above the level of the nucleosome (Finch and Klug, 1976; see Igo-Kemenes et al., 1982). Little is known about how histone H1 interacts in chromatin to form such higher order structures although it is known that its removal destroys them (Thoma et al., 1979). Analysis of the secondary and tertiary structure of H1 reveals that it consists of three distinct domains: a flexible basic N-terminal tail; an apolar globular central region; and a long very basic flexible C-terminal tail (Thoma et al., 1983). The globular central domain, corresponding to the segment of highest sequence conservation (Isenberg, 1979) is able, alone, to close two full turns of DNA in the nucleosome and thus locates H1 in chromatin (Allan et al., 1980). The globular domain cannot, however, achieve total condensation of the nucleofilament without the C-terminal domain. The very basic, hydrophilic sequence of the C-terminal domain are suitable properties for extended ionic interactions with the DNA (Allan et al., 1980). The N-terminal domain has considerable sequence and length variability (Allan et al., 1980). As such, a fundamental structural role is not likely for this domain although it is believed to be involved in the formation of

higher order structures (Thoma et al., 1979). The very nature of the N-terminal variability suggests it as a region to provide a functional distinction between different H1 species (Allan et al., 1980) H1-DNA complexing studies indicate that different H1 subtypes do, in fact, differ in their interactions with DNA in isolated dinucleosomes (Liao and Cole, 1981).

Additional heterogeneity of histones, over and above amino acid sequence variations, is created by post-translational modifications such as methylation, acetylation, phosphorylation, and ADP-ribosylation (see Dixon et al., 1975; see Isenberg, 1979; Hohmann, 1983). It is possible that these modifications affect histone-histone and histone-DNA interactions and thus affect chromatin structure (Isenberg, 1979). The available modifications to an H1 molecule would be amino acid sequence dependent. Thus, secondary modifications and sequence modifications could work together to create diversity of function.

Removal of H1 from chromatin results in a greater susceptibility of the DNA to endonuclease digestion both in the linker region and in the DNA wrapped around the core histones (Noll and Kornberg, 1977). Additional studies on the nuclease sensitivity of chromatin have shown that active genes are preferentially sensitive to micrococcal nuclease (Weintraub and Groudine, 1976; Wu et al., 1979). DNAase I hypersensitive sites exist near active genes and genes which have been previously expressed (Wu, 1980; Wu and Gilbert, 1981; Burch and Weintraub, 1983). These studies implicate a relationship

between chromatin structure and the regulation of chromatin function.

H1 Subtype Multiplicity

Histone H1 is the histone with the most subtype variability (see Isenberg, 1979). H1 variability was first clearly demonstrated by Kinkade and Cole (1966a) who found that four H1 histone subfractions could be resolved from a preparation of calf thymus H1. Amino acid analysis confirmed that the four lysine-rich histones were distinct molecular species (Kinkade and Cole, 1966b). Further chromatographic, electrophoretic, and amino acid analyses of H1 proteins from a variety of vertebrate, avian, amphibian, and other species verified this multiplicity and showed that the H1 histones are qualitatively different from species to species (Bustin and Cole, 1968; Kinkade, 1969; Panyim et al., 1971; Alfageme et al., 1974). The multiplicity of H1 molecules has also been found in cloned tissue culture cells indicating that several subtypes can indeed be present in one cell type (Hohmann, 1980).

The different H1 histones of an organism are expressed in varying relative quantities among different tissues (Kinkade, 1969; Hohmann, 1980; Ajiro et al., 1981; Lennox and Cohen, 1983). This finding has increased speculation that they are important in determination of cell type. In addition, the relative amount of H1 subtype synthesis by mammary gland tissue

can be altered by hormone stimulation (Hohmann and Cole, 1969; Hohmann and Cole, 1971). There is some controversy about the significance of these quantitative differences among tissues since many different tissues express the same H1 histone patterns. It has been suggested that the alterations of the H1 composition of chromatin and the differences in total amounts of histone H1 are not tissue-specific but either related to the state of differentiation of the cells (Ajiro et al., 1981; Unger-Ullmann and Modak, 1979) or dependant on whether the cells are in a dividing or non-dividing state (Lennox and Cohen, 1983).

H1 Subtype Synthesis Switches During Development

One way in which cells could come to contain differing amounts of several forms of histone H1 is that they could begin their synthesis at different times during development. It has been found that histone H1, as well as the core histones H2A and H2B, exhibit developmentally related switches of synthesis among several subtypes. Since the first developmental switches of H1 subtype synthesis were reported in the sea urchin, investigations have revealed that this phenomenon occurs in many diverse species. These species are discussed individually below.

Sea Urchin

H1 histone changes in early development were initially characterized in the sea urchin by Seale and Aronson (1973), Ruderman and Gross (1974), and Ruderman, Baglioni, and Gross (1974). There was initially some controversy as to when H1 was first found associated in chromatin. Seale and Aronson (1973) reported that H1 was not present in sea urchin embryos before the 16-cell stage, after which two H1 subtypes were synthesized, H1a at 16-cell stage and H1b at 12 - 16 hours of development. Ruderman and Gross (1974) found, however, that newly synthesized H1 was in fact associated with the chromatin as early as the first cell division in both Arbacia puctulata and Lytechinus pictus. An alteration in the H1 pattern of synthesis occurs but differs in the two species. Arbacia morulae synthesize H1-m (maternal) as early as the first cleavage and, at hatching, incorporation appears in a new lysine-rich histone H1-h. In Lytechinus pictus, a transition of synthesis takes place from H1-m in early cleavage to H1-g at the late blastula-early gastrula stage (Ruderman and Gross, 1974; Poccia and Hinegardner, 1975; Arceci et al., 1976). High resolution SDS slab gels showed that H1-m, H1-h and H1-g each contained at least two resolvable subfractions.

Lytechinus pictus blastomeres isolated at the 16-cell stage synthesize only the early (α) H1 histones. The cultured progeny cells of each blastomere perform the same switch of early (α) to late (β and γ) H1 histone forms as occurs in the intact embryo (Arceci and Gross, 1980). Newrock et al. (1978)

reported further heterogeneity in the histone H1 subtypes of Strongylocentrotus purpuratus. They identified a H1 histone-like protein that is synthesized and incorporated into chromatin during th first S-phase of development. This protein has a considerably higher molecular weight than the already identified H1 proteins. It is the major chromosomal H1 protein during early cleavage and was thus called cleavage stage H1 (CSH1) (Newrock et al.,1978).

In the past decade work from many laboratories has characterized the histone genes and mRNAs which are expressed in the early development of sea urchin embryos. Presented below is a summary of the way in which these progressive changes occur in development.

The unfertilized sea urchin egg contains a large maternal store of histone mRNAs encoding both the e subtype and some CS subtype (Arceci et al., 1976; Newrock et al., 1978; Childs et al., 1979; Weinberg et al., 1983). The egg also contains a store of CS histones which are utilized in pronucleus formation, replacing sperm-specific histones associated with sperm DNA (Poccia et al., 1981).

During the first six hours of development, transcription of the histone genes can be detected but this new transcription cannot alone account for the 40 fold increase in histone protein synthesis which occurs at this time. This increase must therefore be the result of post-transcriptional regulation of stored histone transcripts from the maternal messenger RNP pool (Goustin, 1981). Immediately following fertilization the

CS histone is the major synthesis product but within three hours, synthesis of the ε subtype becomes predominant. After the 16 cell stage, rapid cell division is accompanied by a rapid increase in the rate of ε subtype histone mRNA synthesis (Weinberg et al., 1983). These early mRNAs are transcribed from tandemly organized, highly reiterated early histone genes. In these clusters, H1 and core histones are present in a defined order (5'-H1-H4-H2B-H3-H2A-3') (Maxson et al., 1983a). The rate of early histone mRNA synthesis reaches a peak at the 200 cell stage after which it declines. At this point there is a 50% reduction in the half-life of early transcripts (Mauron et al., 1982; Weinberg et al., 1983). By the hatching blastula stage early subtype synthesis is barely detectable and has been replaced by late subtype synthesis (Childs et al., 1979). The late subtypes are, on the other hand, encoded by a small, dispersed, irregularly arranged class of histone genes (Maxson et al., 1983a). The switch from early to late histone gene expression comes about by the decline of the transcription of early histone genes, a coincident sharp increase in the levels of late gene transcripts, and occurs during the period of the reduction in cell division rates (reviewed by Maxson et al., 1983b).

The incorporation of histone subtypes into the chromatin appears to be controlled by the abundance of synthesis (reviewed by Maxson et al., 1983b). The CS and early subtypes do not appear to be actively removed and replaced by the late histone variants since both CS and ε subtypes are detectable in

chromatin long after their synthesis ceases (Cohen et al., 1975; Newrock et al., 1978). The abundance of the early variants is instead diluted by the increase in synthesis of the late variants.

Urechis caupo

The marine worm, Urechis caupo, undergoes H1 subtype synthesis switches in early development. Germinal vesicles and cleavage-stage nuclei are enriched in H1M (maternal form). During late cleavage a faster migrating H1, H1E (embryonic form), appears. As embryogenesis continues, H1E replaces H1M as the predominant H1 (Franks and Davis, 1983; Das et al., 1982). Nuclei at the gastrula and trocophore larva stages contain predominantly H1E (Franks and Davis, 1983).

H1E synthesis is disproportionately high from midcleavage to midblastula stage and is not balanced with the core histones until the gastrula stage. This observation indicates that there is a non-coordinate regulation of H1 and core histone synthesis during Urechis development (Franks and Davis, 1983). Franks and Davis (1983) suggest several possibilities to explain this: (1) The germinal vesicle pattern is not representative of the histones in mature oocytes as core histones may be accumulated in excess of histone H1 in the cytoplasm; (2) H1E is synthesized in large quantities so that it will replace H1M during subsequent development; (3) H1M turns over more rapidly than the core histones and the increase

of H1E synthesis is necessary to provide sufficient H1 to complement the core histones.

Spisula solidissma

In early embryogenesis of the surf clam, Spisula solidissima, synthesis of two H1 subtypes occurs. H1E is synthesized early in development. Later, at the 32-64 cell stage, a higher-molecular weight H1 histone (designated H1L) is synthesized (Gabrielli and Baglioni, 1975). The trocophore larva synthesizes exclusively histone H1L although the early H1 histone, H1E, is still detectable.

Analysis of the mRNA stored in Spisula eggs indicates that the messages for histone H1 are present in higher amounts than core histone mRNA (Gabrielli and Baglioni, 1977).

Xenopus laevis

There exists some controversy regarding whether or not Xenopus laevis undergoes stage specific switching of H1 histones during development (Flynn and Woodland, 1980; Risley and Eckhardt, 1981). Flynn and Woodland (1980) compared the H1 histones in the oocyte, egg, and gastrula and blastula embryos of Xenopus using 2D gel analysis and found three H1 histones H1A, H1B, and H1C. Results of labelling studies suggest that Xenopus H1 histones do not show a switch in type during development. Risely and Eckhardt (1981) resolved two additional lysine-rich histones H1D and H1E as well as the H1A,

H1B, and H1C previously reported by Flynn and Woodland (1980). After examining the histone composition of embryos (mid-neurula to early tailbud) and various adult tissues (erythrocytes, liver, intestine and testes) it was found that the faster mobility histones H1A, H1B, and H1C were found in both embryonic and adult tissues whereas H1D and H1E were found only in adult tissues. These results suggest that additional H1 heterogeneity is introduced into the Xenopus embryo chromatin during or following metamorphosis (Risley and Eckhardt, 1981).

Since the H1 subtypes H1D and H1E were found only in adult tissues it was suggested that these subtypes may be associated with non-mitotically active or slowly dividing cells of the adult tissues similar to H1° which is enriched in mammalian non-dividing cells (Panyim and Chalkley, 1969). Several observations on the binding capacity of the H1 subtypes suggest that the relative binding strengths are H1A < H1B < H1C < H1D < H1E (Risley and Eckhardt, 1981). This raised the possibility that the H1 histones of adult cells may impose greater restraints on chromatin structure through a greater binding affinity for DNA than the H1 of embryo cells.

Bovine

Valkonen (1980) has examined the post-cleavage stage developmental changes of H1 histones from bovine liver. The H1 histones of young embryo liver (1 - 6 months) has two components, H1a and H1b. At seven months a new component, H1c,

having a lower molecular weight than Hla or Hlb is present. Older embryo liver as well as that of calves and adult cows has four components, the fourth component corresponding to an Hl° subtype (Valkonen, 1980). However the two early Hl proteins, Hla and Hlb, continue to be synthesized in the greatest amounts throughout development. Piha and Valkonen (1979) also observed that the relative amount of Hl histone to core histone in the liver increased two fold during the bovine prenatal period and 3 fold during the whole of ontogeny. They believe that the increase in the amount of the Hl histone during bovine ontogeny may reflect an age related condensation of chromatin and a reduction of RNA synthesis and template activity.

Mouse

Lennox and Cohen (1983) examined the Hl complements of liver, kidney, lung, and thymus tissue of the mouse and the accompanying postnatal developmental changes. In the liver, kidney, and lung two of the major Hl subtypes present 1 week after birth, Hla and Hlb, decline to almost undetectable levels by 8 - 16 weeks. A third Hl subtype, Hld, also diminishes at this time. The amounts of two other Hl subtypes, Hle and Hl°, increase noticeably while Hlc also increases. The disappearance of the two major subtypes, Hla and Hlb, occurs at different times during development. The disappearance of Hla is almost complete by 4 weeks, whereas the decline in Hlb occurs mainly between 4 and 16 weeks, a period when most cells

are non-dividing. Lennox and Cohen (1983) believe that H1b is removed from the chromatin without cell division, that is, its decline is not due to a dilution of H1b by the other H1 proteins.

In the thymus of the mouse, newly formed non-dividing cells have as much H1a and H1b as the dividing cells from which they are derived, but lack H1° even though 90% of the thymus tissue does not divide. The peripheral blood lymphocytes have less H1a and H1b but more H1e than the small thymocytes from which they are derived. Therefore, Lennox and Cohen (1983) believe that H1a and H1b molecules are differentially lost during the transition between small thymocyte and lymphocyte while greater amounts of H1e are incorporated into the chromatin. It is concluded that the H1 subtypes are not tissue specific in somatic tissues but rather the changes in H1 subtypes are related to the formation of non-dividing cells (Lennox and Cohen, 1983).

Lennox and Cohen (1983) characterized each of the H1 subtypes of the mouse using parameters such as synthesis rates, number of phosphorylated forms, maximum number of phosphate groups per molecule, synthesis in dividing and non-dividing cells, presence in non-dividing lymphoid and non-lymphoid cells, and necessity for cell division. They found that each H1 subtype differs from the others in some of the properties examined supporting the idea that individual H1 subtypes differ from each other in some of their functions.

Tetrahymena

Gorovsky and co-workers have studied the histone rearrangements which accompany nuclear differentiation and dedifferentiation in the ciliated protozan Tetrahymena thermophila. There is a clear cut difference in the histone H1 composition between the transcriptionally active somatic macronucleus which supports vegetative growth, yet is eliminated in every sexual generation, and the transcriptionally inactive germinal micronucleus whose cellular function is to provide genetic continuity from generation to generation (Allis and Wiggins, 1984). Gorovsky and Keevert (1975) have shown that macronuclei contain an H1 which is absent in micronuclei. Micronuclei, on the other hand, contain three unique proteins α, β, and γ. These proteins do not have typical H1 solubility properties but are associated with linker regions of micronuclear chromatin (Allis et al, 1979) and may be the equivalent of H1 in some respects although they could have a unique function. During conjugation in Tetrahymena, observations by Allis and Wiggins (1984) demonstrate that the micronuclei dedifferentiate (by losing micronuclear-specific histones) prior to differentiation into new macronuclei. The dedifferentiation process allows new macronuclei, developing from micronuclei, to gain macronuclear-specific H1 histones (Allis and Wiggins, 1984). These results suggest that selective synthesis and deposition of specific histone subtypes plays an important role in the nuclear differentiation process in Tetrahymena (Allis and Wiggins, 1984).

Chick Erythropoiesis

The avian erythrocyte undergoes a series of molecular alterations, including a genetic shut down, as part of its differentiation (Moss et al., 1973). Erythrocytes contain cell-specific histone H5 (Neelin et al., 1964) which has been implicated in both genetic repression (Neelin et al., 1964) and chromatin condensation (Brasch et al., 1972; Brasch, 1976) during erythroid maturation. This histone is detectable in low amounts in the day 4 chick embryo, increases to 13.5% of all histones by day 19 of incubation and finally reaches a level of 21% of all histones in adult hens (Moss et al., 1973). The increase of histone 5 corresponds in time to the transition from primitive to definitive erythroid cell lines in the chick embryo and also to the terminal division of the primitive erythroid cells (Moss et al., 1973; Urban et al., 1980). Histone H5 is located in the linker region of chromatin and has three structural domains, similar to that of other histone H1 molecules (Cary et al., 1981).

Relation of H1 to Cell Cycle

A specialized H1 histone, H1°, has been postulated to be involved in cell cycle regulation. Recent data by D'Anna et al. (1982) suggests a dynamic picture of chromatin in which newly synthesized histone H1° is deposited onto chromatin of Chinese hamster ovary cells during G1 and S. Histone H1, on

the other hand, is possibly lost or becomes dissociated from chromatin during the early stages of DNA replication.

Studies comparing the rate of H1° incorporation into chromatin, relative to the core histones, indicate that H1° incorporation is greater in G1 arrested cells and in G1 traversing cells than in exponentially growing cultures. H1° has also been found in large amounts in cells that have slowed down or stopped DNA synthesis (Panyim and Chalkley, 1969). This led to the generalization that the cellular content of H1° is inversely proportional to the rate of DNA synthesis (D'anna et al., 1982).

Phosphorylation of H1° as well as other H1 subtypes is also believed to occur in a cell-cycle dependent manner. As cells are released from G1 arrest a portion of the H1 molecules become phosphorylated at the C-terminal part of the molecule. As the cells enter S phase, H1 becomes additionally phosphorylated in the C-terminal tails so that by late interphase 55-60% of H1 molecules are phosphorylated. Finally, during mitosis, all H1 molecules become phosphorylated in both their N-terminal and C-terminal tails (reviewed by D'anna et al., 1981). The temporal correlation of H1 phosphorylation with the cell cycle suggest that H1 phosphorylation may take part in the initiation of cell proliferation, DNA synthesis, separation of newly synthesized daughter chromosomes during cell cycle and chromosomal condensation (see D'anna et al., 1981). The mechanisms suggested by which H1 phosphorylation might alter chromatin structure are as follows: (1) H1

phosphorylation may induce changes in H1 conformation which could alter the orientation of nucleosomes and higher orders of chromatin structure; (2) phosphorylation may induce H1:H1 interactions, which could affect chromosomal condensation; (3) H1 phosphorylation may alter H1 interaction with other chromosomal proteins (see D'anna et al., 1981).

Studies on Ilyanassa obsoleta

Although histone synthesis switching has been found in a number of organisms the reason for the observed patterns has remained elusive. It is not clear if the switching reflects other underlying transcriptional changes, or, if the histone subtypes are themselves separately functional. In this study an organism has been utilized in which the switching patterns of histone H1 subtypes can be related to developmental events, in particular, oogenesis, cleavage, and organogenesis. This study, thus, cannot define separate functions for individual subtypes but it can pinpoint where in embryogenesis to look for such functions.

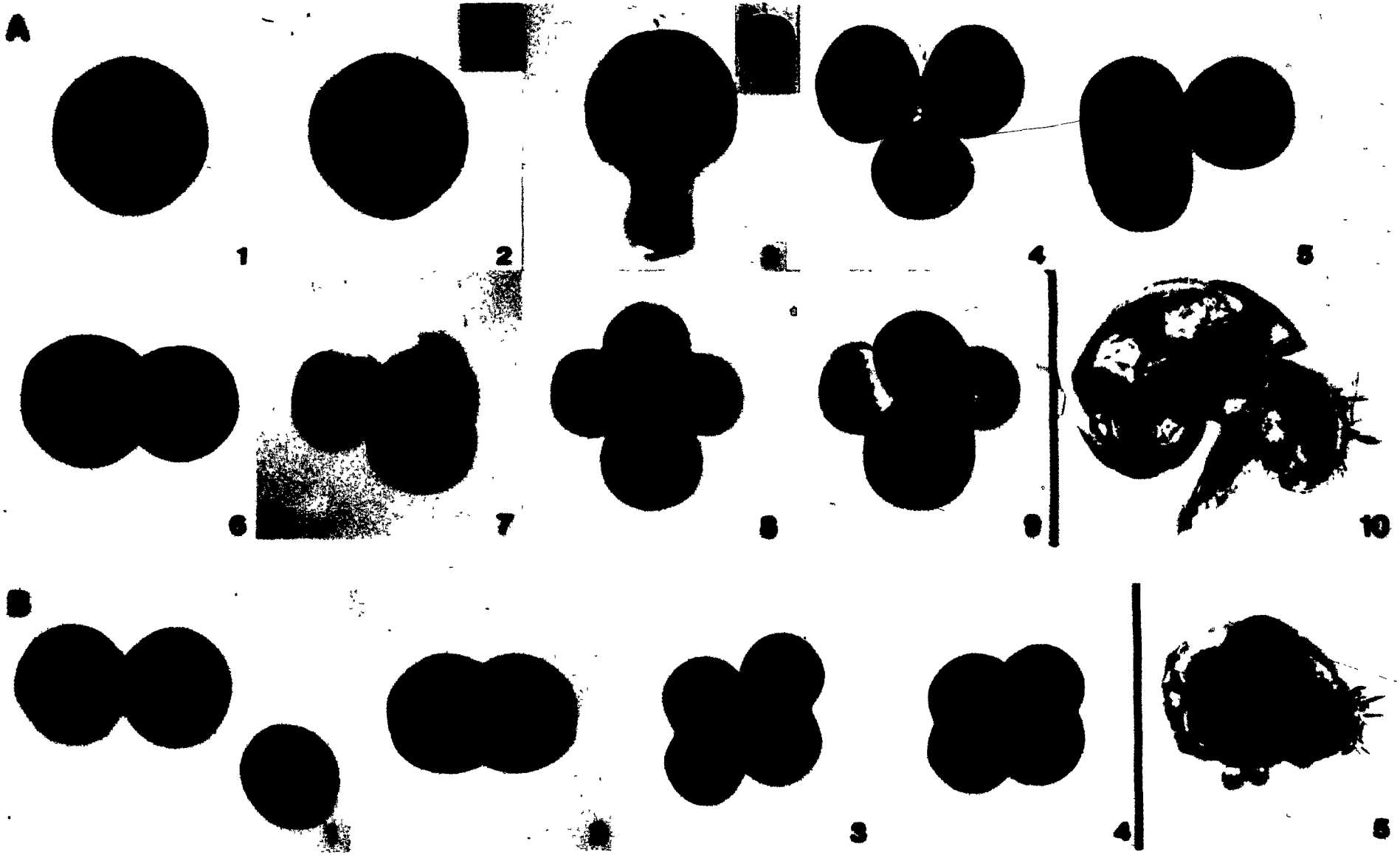
The organism chosen for this study is the marine mud snail Ilyanassa obsoleta. This molluscan embryo undergoes mosaic development (determinate cleavage) in which there is a segregation of egg cytoplasm into the early blastomeres. This cytoplasmic segregation comes about by the extrusion of a series of anucleate cytoplasmic protrusions called polar lobes. The first and second polar lobes are extruded during maturation

of the egg and the third and fourth polar lobes are formed at the first and second cleavage divisions (Crampton, 1896; Clement, 1952). Polar lobe formation in Ilyanassa embryos is illustrated in Figure 1. The third polar lobe, formed during the first cleavage division, called the trefoil stage, is segregated into the "CD" blastomere and finally into the "D" blastomere. (Each of the first four blastomeres, or macromeres, is labelled with a capital letter. Small cells formed from these cells are called micromeres and are indicated by a small letter.) At the trefoil stage the polar lobe can be easily mechanically removed from the embryo resulting in a "delobed" or "lobeless" embryo. The delobed embryo develops lacking mesodermally derived and induced structures such as heart, eyes, intestine, and foot (Clement, 1952). Such embryos are viable for a period, but since they have no organized gut, and thus, cannot feed, they die when they have exhausted their supply of yolk. A detailed histological study by Atkinson (1971) revealed that some cell types of the missing organs are present but exist in disorganized masses. Polar lobe removal, the subsequent lack of a fourth polar lobe, and the larva which develops in the absence of the contents of the polar lobe is shown in Figure 1B.

The polar lobe contains "determinative factors" of cell fate such that cell lineages which come to contain them develop into specific cells and organs. Clement (1968) found that these "determinants" are attached to the cortex in the polar lobe area of the vegetal pole of the egg since centrifugation

Figure 1. Polar lobe formation during Ilyanassa obsoleta embryogenesis. (A) Illustrated is the formation of the four polar lobes. These form during the two meiotic divisions and the first two mitotic cleavage divisions. The normal veliger larva is also shown. (B) Illustrated is development during the corresponding period of (A 4-9) after the removal of the third polar lobe. Also shown is the abnormal veliger larva.

~~(A)~~ (1) The fertilized egg. (2) The first polar lobe. This lobe is barely visible. The insert shows the rounded egg after the first polar lobe has been resorbed. The first polar body is seen as a small bleb at the animal pole. (3) The second polar lobe. The insert shows the second polar lobe in the process of being resorbed and two polar bodies at the animal pole. (4) The third polar lobe (trefoil stage). (5) The third polar lobe in the process of resorption into what will become the AB blastomere. (6) The two cell stage. (7) The fourth polar lobe. (8) The third cleavage division. (9) The four cell stage. The large blastomere at the bottom is the D blastomere which has received the contents of the polar lobe. (10) The normal veliger larva. (B) (1) The third polar lobe (lower right) removed at the trefoil stage. (2) The two cell stage. (3) The third cleavage division. No polar lobe forms. (4) Four cell stage. (5) The abnormal veliger larva formed after the third polar lobe is removed. Egg and cleavage stages are oriented with the animal pole at the top, and the vegetal pole at the bottom, of the photograph. Embryos were photographed under brightfield Nomarski optics. X 175.


A
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of the egg does not dislodge them. In addition, if the egg is separated into the animal and vegetal halves, and if the vegetal half contains a nucleus, the partial egg can form a recognizable, although smaller veliger larva (Clement, 1968).

These studies demonstrate clearly that the Ilyanassa egg contains in its vegetal pole information important to early development. This information is normally segregated into the D blastomere during cleavage via the polar lobe, and can be removed with the lobe. Ilyanassa is not unique in this regard as many molluscs and annelids contain polar lobes (see Davidson, 1976).

Clement (1962) performed a detailed study of the development of Ilyanassa embryos following removal of the "D" macromere (which receives the contents of the polar lobe) at successive stages. This study revealed that the morphogenetic influence of the polar lobe region is exerted during the period of formation of the first four quartets of micromeres (3rd, 4th, 5th, and 6th cell divisions). After this time, the 4D macromere is nutritive but is of no morphogenetic importance.

The lobe-dependent larval features are not all determined at the same time. The normal structural pattern of eyes, foot, shell, and velum is determined at the time of the formation of the third quartet of micromeres. Heart and intestine are determined at the time of formation of the 4d micromere, and are probably derived in part from this cell (Clement, 1962). A process of cytoplasmic segregation appears adequate to account for the determining influence of the polar lobe region in the

development of heart and intestine. In the case of the eyes, foot, shell, and velum a mechanism of induction is probably involved since cell lineage studies indicate that it is highly improbable that all of these structures arise from the 3d micromere (Clement, 1962). This idea is supported by studies on shell gland formation. Cather (1967) has observed an inductive inhibitive interaction between cells of the D blastomere and those cells arising from the A, B, and C blastomeres. Cells containing polar lobe material form the shell gland (2d micromere) however, if the polar lobe is removed, several internal shell masses may be formed. Therefore, Cather (1967) reasoned that the polar lobe exerts an inhibitory influence on the cells arising from the other blastomeres which prevents differentiation leading to shell excretion in the intact embryo.

A number of studies have addressed the biochemistry of Ilyanassa development. Investigations of nucleic acid metabolism indicated that removal of the polar lobe at first cleavage had no effect on the time that rRNA synthesis could first be detected (Newrock and Raff, 1975). Koser and Collier (1976) found no detectable effect on the pattern of total RNA synthesis, as visualized by polyacrylamide gel electrophoresis, during the first day of delobed development (Koser and Collier, 1976). There is, however, a size difference distribution of RNA made by the post-gastrular normal and delobed embryos. The normal embryo accumulates more radioactivity than the delobed embryo in the high molecular weight (34S) fraction of RNA

(suggested to be transcribed hnRNA) while the delobed embryo accumulates proportionately more radioactivity in the low molecular weight range (16S and 12S) of RNA which possibly arises from the accumulation of messenger RNAs that were not translated (Koser and Collier, 1976). A reduction of the overall rate of RNA synthesis in the gastrular (Collier, 1976) and post-gastrular (Davidson et al., 1965) delobed embryo has also been reported. Collier (1975) also reports a decrease in the rate of DNA synthesis in the delobed embryos after the fourth day of development.

Investigations of the electrophoretic patterns of protein synthesis of normal and delobed embryos have been done in order to search for proteins whose synthesis is lobe-dependent. Donohoo and Kafatos (1973) dissociated Ilyanassa embryos into their AB and CD blastomeres and using a double radioactive isotope labelling procedure, detected protein differences between the early cleavage progeny of these two blastomeres. Newrock and Raff (1975) reported differences between normal and delobed embryos at 24 hours, also utilizing double isotope labelling procedures. These studies could not distinguish quantitative from qualitative differences since they employed a one-dimensional SDS gel analytical technique in which groups of proteins from both embryo types were included in common gel slices. Brandhorst and Newrock (1981) and Collier (1981a) analysed the protein synthesis pattern of normal and delobed embryos and isolated polar lobes using two-dimensional (isoelectric focus and SDS) gel electrophoresis to circumvent

some of these problems. There were no unique proteins found in the normal embryo, delobed embryo or isolated polar lobe during early cleavage (2-12 cell stage). These studies did not analyse very basic or acidic proteins, high molecular weight proteins or any proteins which failed to focus in the first dimension. The synthesis of polypeptides in isolated polar lobes demonstrates that concomitant transcription of the genome is not required for their synthesis. Therefore, the mRNAs that are being transcribed must be of oogenic origin. The predominantly oogenic origin of these mRNAs was confirmed by the failure of Actinomycin D (an inhibitor of transcription) to alter the rate of accumulation of any of the polypeptides synthesized during early cleavage (Collier and McCarthy, 1981). These studies indicate that most major proteins are not involved in the etiology of the polar lobe effect.

Analysis of the polypeptides synthesized by the 24 hour normal and delobed Ilyanassa embryo, as compared to those of early cleavage, show stage-specific proteins (Brandhorst and Newrock, 1981; Collier and McCarthy, 1981). The effect of the removal of the polar lobe is restricted to quantitative changes in both proteins which show stage specific synthesis and those which do not (Collier and McCarthy, 1981). Collier and McCarthy (1981) report that these proteins which are synthesized in different quantities in the delobed compared to the normal embryo are all translated from mRNAs transcribed during oogenesis. In addition, a subset of the developmental changes in protein synthesis which occur between the 4 hour and

24 hour embryo occurs in isolated polar lobes (Brandhorst and Newrock, 1981; Collier and McCarthy, 1981). Proteins synthesized by the polar lobe showed both a decrease in the rate of accumulation of some proteins and an increase of some others. This regulation must occur by transcriptionally independent events and may involve the selective regulation of the translation of mRNA stored in the egg (Brandhorst and Newrock, 1981; Collier and McCarthy, 1981). No proteins were detected which were synthesized exclusively in the polar lobes.

Collier (1983) compared proteins synthesized by normal and delobed embryos during late organogenesis. Two-dimensional gel electrophoresis resolved the same set of proteins in the two embryo types. This may relate to the finding of Atkinson (1971) that many cell types of disorganized organs are still present in the delobed embryo.

Attempts to determine why the removal of the lobe results in such a disorganized embryo have been unsuccessful. Analysis of total protein fractions cannot determine the sites of action of the putative determinants which are localized in the polar lobe. It is necessary to examine specific proteins in specific locations which are amenable to a functional interpretation and examine the effect delobing has on these specific proteins. The histones are one such group of proteins as they are specifically located in the nucleus. Thus, this study first elucidates the pattern of histone H1 synthesis in normal Ilyanassa embyros and then focuses on the effect that delobing has on this H1 synthesis pattern.

MATERIALS AND METHODS

Maintaining Snails and Obtaining Embryos

Mud snails, Ilyanassa obsoleta, were obtained from the Marine Biological Laboratories, Woods Hole, Massachusetts. Snail stocks were maintained in hibernation at 4°C in aerated tanks filtered with glass wool and charcoal and contained a gravel layer at the bottom to remove nitrogenous wastes. Breeding snails were maintained at 17°C either in Instant Ocean salts (Aquarium Systems, Inc.) dissolved in deionized water to a density of 1.025 or in natural sea water obtained from the Marine Biological Laboratories. The ten-gallon breeding tanks were well-aerated and filtered with glass wool, charcoal, diatomaceous earth, and gravel. Snails were fed frozen shrimp approximately once a week. Laying snails were kept on a light-dark cycle of 14 hours in light and 10 hours in darkness.

Fertilized Ilyanassa eggs are laid in gelatin-filled capsules which are deposited in strings on the sides of the tanks. Capsules were collected by scraping them from the sides of the tanks with a razor blade, sorted according to embryo stage utilizing a dissecting microscope, and cleaned of adhering algae and other debris with watchmakers forceps. The embryos were allowed to develop up to the point of labelling either within the capsules or removed from the capsules as required by experimental protocol. All experiments were done at 16°C in Millipore-filtered artificial sea water (MFASW; 0.45

M sodium chloride, 0.025 M magnesium chloride, 0.018 M magnesium sulphate, 0.009 M potassium chloride, 0.005 M calcium chloride, 0.002 M sodium bicarbonate)(Cohen et al., 1975) containing 0.1 g/l each of penicillin G sodium and streptomycin sulphate (Sigma). Collier (1981b) has published a useful directive on obtaining and handling Ilyanassa obsoleta.

Polar Lobe Removal

Egg capsules were maintained on a water-cooled cold plate at 9°C to retard development until sorted and cleaned. Single capsules containing 50 to 200 closely synchronous eggs were opened with curved scissors individually in 35 mm X 10 mm polystyrene petri dishes (Corning) and the embryos were released into MFASW with water from a mouth-operated micropipet fashioned from a Pasteur pipet. The brand of petri dish is important since it was found that embryos of some stages adhere to some brands.

In the delobing process embryos were allowed to develop at room temperature (= 22°C) until many embryos of a capsule were at the trefoil stage. The petri dish containing the embryos was then placed on a New Brunswick rotatory shaker and the polar lobes removed by brief rotatory agitation at 250 rpm (Newrock and Raff, 1975). The small diameter of the petri dishes aids the delobing process. Delobed embryos were collected immediately after lobe removal utilizing a mouth-operated micropipet. This is necessary since continued

agitation of delobed embryos can result in the separation of the AB and CD blastomeres. To maintain synchrony between normal and delobed embryos, equal numbers of control embryos (i.e. those embryos from which the polar lobe had not been removed) and delobed embryos were collected from each capsule. Immediately post-trefoil was considered Time 0, and consequently, the first round of DNA synthesis after fertilization was not monitored in experiments reported here. Normal and delobed embryos were then cultured under identical conditions in MFASW until the desired developmental stage. Normal development of agitated control embryos shows that rotatory agitation has no deleterious effects.

Labelling of Embryos

Embryos were labelled with 35 μCi/ml of ^{3}H-Lysine (81.8 Ci/mmole; New England Nuclear) in 3ml of MFASW. Embryos 1 day old and older were labelled for 3 hours as detailed in the figure legends. In order to avoid collecting the fragile nuclei of early embryos, but to still obtain an early time point, embryos were labelled either early (0 - 6 hours) and chased to 24 hours with cold lysine (Sigma; 1 mM in 3 ml MFASW) before nuclear isolation or during the entire first 24 hour period and collected without a chase period.

H1 Histone Isolation

Delobed embryos of Ilyanassa obsoleta are difficult to obtain in large numbers as they must be collected individually after delobing. This material limitation made it necessary to develop a histone isolation technique on a microlevel utilizing the Beckman air-driven ultracentrifuge. With this centrifuge extractions could be accomplished in a total volume of 250 µl.

Normal and delobed embryos (approximately 250 of each) were labelled in petri dishes as described above. Unincorporated label was removed by washing the embryos twice with MFASW by gravity transfer from a wide-mouthed pipet. The seawater was removed by washing embryos with 1 M dextrose-0.1 M NaCl made up in Zweidler's (Z) medium (50 mM glycine; 10 mM potassium-tris-maleate buffer, pH 7.3; 5 mM magnesium chloride; 10 mM β-mercaptoethanol; 0.5% thiodiglycol; 0.1 mM L-1-tosylamide-2-phenyl-ethylchloromethyl ketone, Sigma; 0.1mM phenylmethylsulphonyl fluoride, Boehringer Mannheim) (Cohen et al., 1975). The embryos were transferred in the dextrose-NaCl solution to a 250 µl airfuge tube, allowed to settle and the dextrose-NaCl was removed. The embryos were then homogenized in Z medium with a siliconized pestle made to fit the airfuge tube. The homogenate was then underlayered with 50 µl of 0.1 M sucrose and a 10 µl, 60% sucrose pad (both prepared in Z medium), accelerated to 125,000 g, and immediately allowed to decelerate. The supernatant was removed from above the pellet

which contained the nuclei. Nuclei were then homogenized in the airfuge tube with a buffer containing 0.25% (w/v) Triton X-100 detergent in Z medium. The chromatin was pelleted by acceleration to 125,000 g followed by immediate deceleration, and the supernatant was removed (modified from Cohen et al., 1975). The chromatin pellet was then homogenized in 20 µl of 5% perchloric acid (PCA) and allowed to sit at 4°C for 45 minutes with intermittent stirring (Johns, 1964). The PCA insoluble material was pelleted by acceleration to 125,000 g followed by immediate deceleration and the PCA soluble supernatant was removed to another airfuge tube. The PCA extraction was repeated and the second supernatant pooled with the first. PCA soluble material was then prepared for SDS electrophoresis by adding an equal amount of 2 X SDS sample buffer and adjusting the pH to 7 with sodium hydroxide.

Electrophoresis

SDS polyacrylamide gel electrophoresis was performed using slab gels 14.5 cm x 13 cm x 0.1 cm. A Tris-glycine buffer system was used according to the procedure of Laemmli (1970). The 12 cm separating gel contained 15% acrylamide and 0.4% bisacrylamide (pH 8.8). The 2.5 cm stacking gel contained 6% acrylamide and 0.04% bisacrylamide (pH 6.8) with sample wells 1.5 cm X 0.3 cm X 0.1 cm. Samples of equal volume and approximately equal counts were prepared using 1 X sample buffer (0.625 M Tris-HCl, pH 6.8; 2 % (w/v) sodium dodecyl

sulfate; 10% (v/v) glycerol; 5% (v/v) β- mercaptoethanol; 0.001% (w/v) bromophenol blue)(Laemmli, 1970) to a volume of 20 µl. To these samples approximately 2 µg of unlabelled Ilyanassa obsoleta H1 histone carrier (prepared from embryos ranging in age from egg to late veliger larvae) and 3 µg of unlabelled calf thymus total histone carrier (Worthington) were added.

Gels were run at 100 volts until the tracking dye (bromophenol blue) entered the separating gel. The voltage was then increased to 150 volts and electrophoresis continued for a total of 1000 volt/hours. Gels were stained with 0.4% amido black in 22% methanol and 4.5% acetic acid in distilled water. Gels were then neutralized with a saturated solution of sodium phosphate and fixed in 0.1% glutaraldehyde for 1 hour. After fixation gels were restained using Coomassie blue. Staining with amido black first, then Coomassie blue, is necessary as H1 histones sometimes elute out of gels stained immediately with Coomassie blue (K. M. Newrock and L. H. Cohen, personal communication). Destained gels were photographed, prepared for fluorography using Enhance (New England Nuclear), dried, and exposed to preflashed Kodak XR1 film at -70°C (Laskey and Mills, 1975).

Cleveland Digests of Proteins from Dried Gels

Protein digests were performed using Staphlococcus aureus protease (Sigma; modified from Cleveland et al.,1977). Samples

for digestion were prepared and electrophoresed as described above. Gels were stained as above, but not fixed with glutaraldehyde, prepared for fluorography, dried, and exposed at -70°C. Film and dried gel were notched and marked with ink containing ^{14}C-leucine prior to exposure so that the developed film could be accurately superimposed on the dried gel. The bands corresponding to radioactive H1 proteins were cut from the film and this film was then utilized to cut out the corresponding H1 protein bands from the dried gel. The dried protein bands were soaked for at least 10 minutes in buffer (0.125 M Tris-HCl, pH 6.8; 0.1% (w/v) SDS; 1mM EDTA) which swelled the gel and removed the supporting filter paper from the acrylamide. The bands were then frozen in buffer and stored until needed. A second 15% acrylamide : 0.4% bisacrylamide gel was prepared and the imbibed protein bands were placed at the bottom of the 1.5 cm X 0.6 cm X 0.1 cm sample wells. These bands were then overlayed with the above buffer solution containing 20% (v/v) glycerol and then with 1 µg of S. aureus protease in the same buffer but containing 10% (v/v) glycerol. Samples were electrophoresed at 100 volts until the bromophenol blue dye front reached the end of the stacking gel. Electrophoresis was then halted for 60 minutes, to facilitate protease digestion of the protein, and continued at 150 volts for a total of 800 volt/hours. The gel was then prepared for fluorography, and exposed. A preliminary test comparing calf thymus histone (Worthington) bands cut from a

wet gel to those cut from an Enhanced, dried gel indicated no differences in digestion.

Embryo Cell Number Determination

Normal and delobed embryos were collected as described above and washed in 1 M dextrose-0.1 M NaCl to remove sea water. Individual embryos were placed in a drop of Mithramycin (125 µg/ml, Sigma)(Coleman et al., 1981) prepared in Z medium and squashed with a glass coverslip on slides coated with a mixture of egg white and glycerol (1:1). Nuclear fluorescence was visualized using a Leitz Orthoplan microscope equipped with incident illumination fluorescence. Squashes were photographed and fluorescent nuclei later counted on the negatives or on enlarged projections. Nuclei lying outside the field of the photographs were counted without the aid of photography and later added to the count. Typical squashes of normal and delobed embryos of early and late stages are shown in Figures 5 and 6. Values obtained for the comparison of cell numbers of normal and delobed embryos were then analyzed using Student's t test (SAS Institute Inc., Raleigh, N.C.). The percent differences in cell number of normal and delobed embryos were calculated from the means.

Molecular Weight Determination

Molecular weights of the Ilyanassa H1 proteins were determined by SDS polyacrylamide gel electrophoresis utilizing

previously reported histone molecular weights as markers. The markers used were calf thymus H4 (11,282; Delange et al., 1969), thymus H3 (15,324; Delange et al., 1972) and sea urchin H1e (Levy et al., 1982) (calculated molecular weight of 25,824). Ilyanassa H3 and H4, as expected, have the same mobility in SDS polyacrylamide gels as the calf thymus histones and Ilyanassa H1 band 3 has the same mobility as sea urchin histone H1e (Mackay and Newrock, 1982).

RESULTS

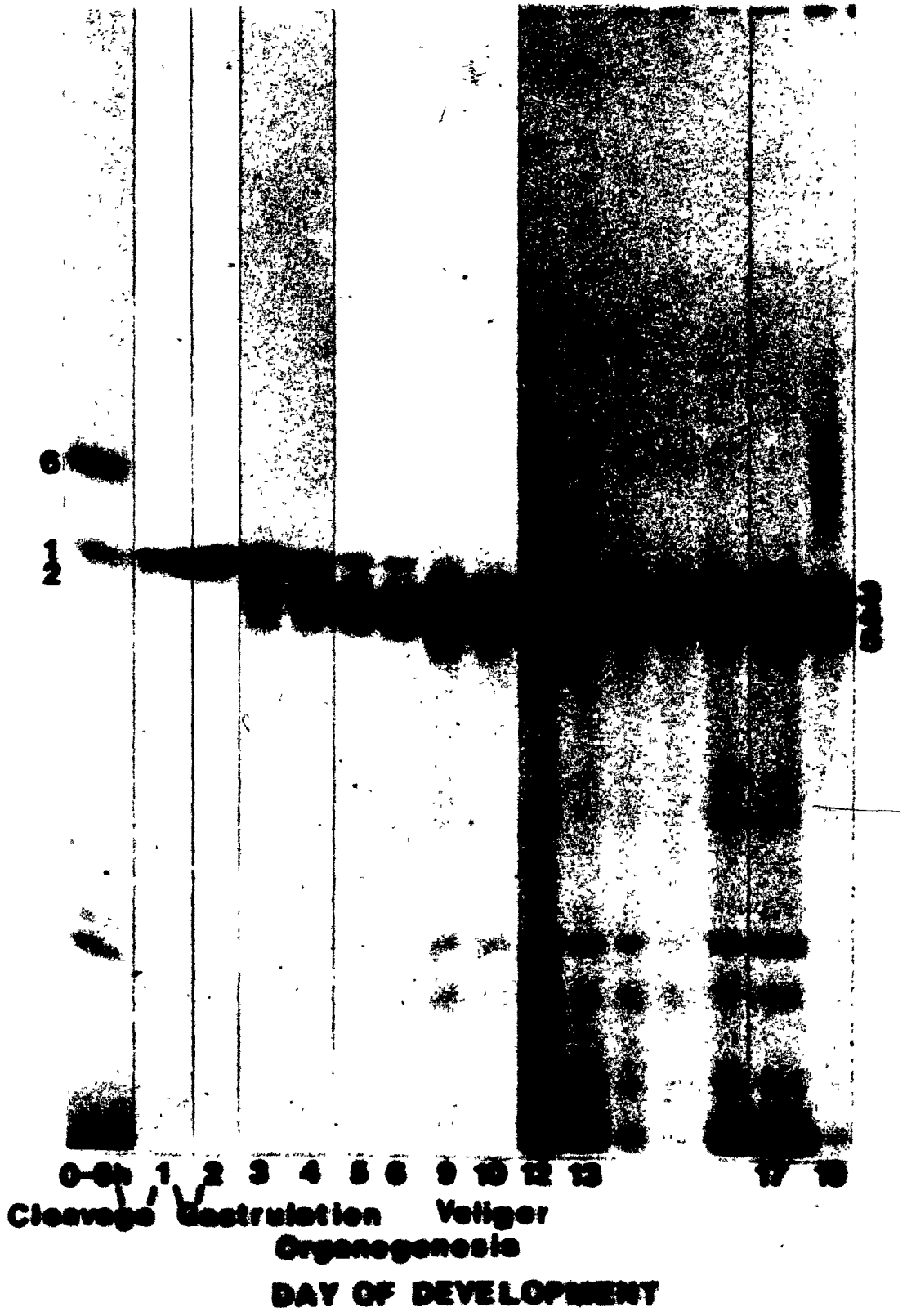
Hl Histone Synthesis During Normal Development

The synthesis of Hl proteins was first studied during normal development, after which the normal pattern was compared to that of delobed embryonic development. Radioactive Hl proteins were isolated as 5% perchloric acid soluble nuclear proteins, displayed by electrophoresis and visualized by fluorography as described in Methods. Exposures were adjusted for both accurate depiction of relative band intensities and band clarity. For all but the earliest time point, for which there was a material limitation, extended exposures were done to determine trace amounts of incorporation. Such exposures are depicted in diagrammatic form only (Figure 7).

During Ilyanassa development six radioactive bands can be detected which are Hl proteins according to the above criteria. These bands are designated "1 - 6" in Figure 2. Bands 1 - 5 are clustered and have molecular weights in the range of 26,800 - 24,700 whereas band 6 has a substantially higher molecular weight of approximately 29,500. Three of these bands (3,4,5) have been previously observed (Mackay and Newrock, 1982). Band 3 has a molecular weight of approximately 25,800. It has the same mobility in SDS gel electrophoresis as sea urchin histone Hl $_{e}$ (Mackay and Newrock, 1982).

During early cleavage (trefoil until approximately the 32 - 64 cell stage) the predominant Hl band is band 6.[1] During

Figure 2. H1 histones of normal Ilyanassa embryos. Histones were prepared, resolved and displayed by SDS gel electrophoresis and visualized by fluorography as described in Methods. 0 - 6 hour embryos were labelled with ^{3}H-lysine for 6 hours beginning at the trefoil stage and chased with cold lysine to 24 hours. A 3 hour labelling period beginning at 24 hour intervals was used for all other stages. 1 - 6 indicate H1 histone bands. The day of development for each lane is indicated.


6
1
2
0-2
1
2
3
4
5
6
9
10
12
13
17
18
Cleavage
Gastrulation
Veliger
Organogenesis
DAY OF DEVELOPMENT

early cleavage, incorporation into band 6 is greater than into either bands 1 or 2 (Figure 2, 0-6 hrs). By twenty-four hours of development (gastrulation), only a trace amount of radioactivity into band 6 can be seen and only in extended fluorographic exposures. By day two bands 1 and 2 are predominant and show incorporation in nearly equal amounts (Figure 2, day 1). At two days of development band 2 shows a decrease in relative intensity and by day five is detectable only in a minor amount (Figure 2). After this time it is detectable only with very long exposure times when other proteins are overexposed. Incorporation into band 1 decreases and becomes a trace with a different schedule than band 2. Band 1 is still seen as a relatively intense band at day six and is seen in a minor amount by day nine, the next time point (Figure 2).

Bands of faster mobility, bands 3, 4, and 5, are first present in relatively large amounts at day three of development (during early organogenesis)(Figure 2, day 3). They are present as early as day one but only in trace amounts. At day four, bands 3, 4, and 5 show the same amount of incorporation as bands 1 and 2. By day five, bands 3, 4, and 5 become the predominant H1 histone bands and continue to be so throughout the remainder of development (Figure 2). Of these three bands, band 3 is the predominant band. Band 4 and band 5 appear in lower and approximately equal amounts to one another. Occasionally, another band of faster mobility than band 5 is resolved (e.g. Figure 2, day 13). A summary of the H1 histone

synthesis during normal development is presented diagrammatically in Figure 7.

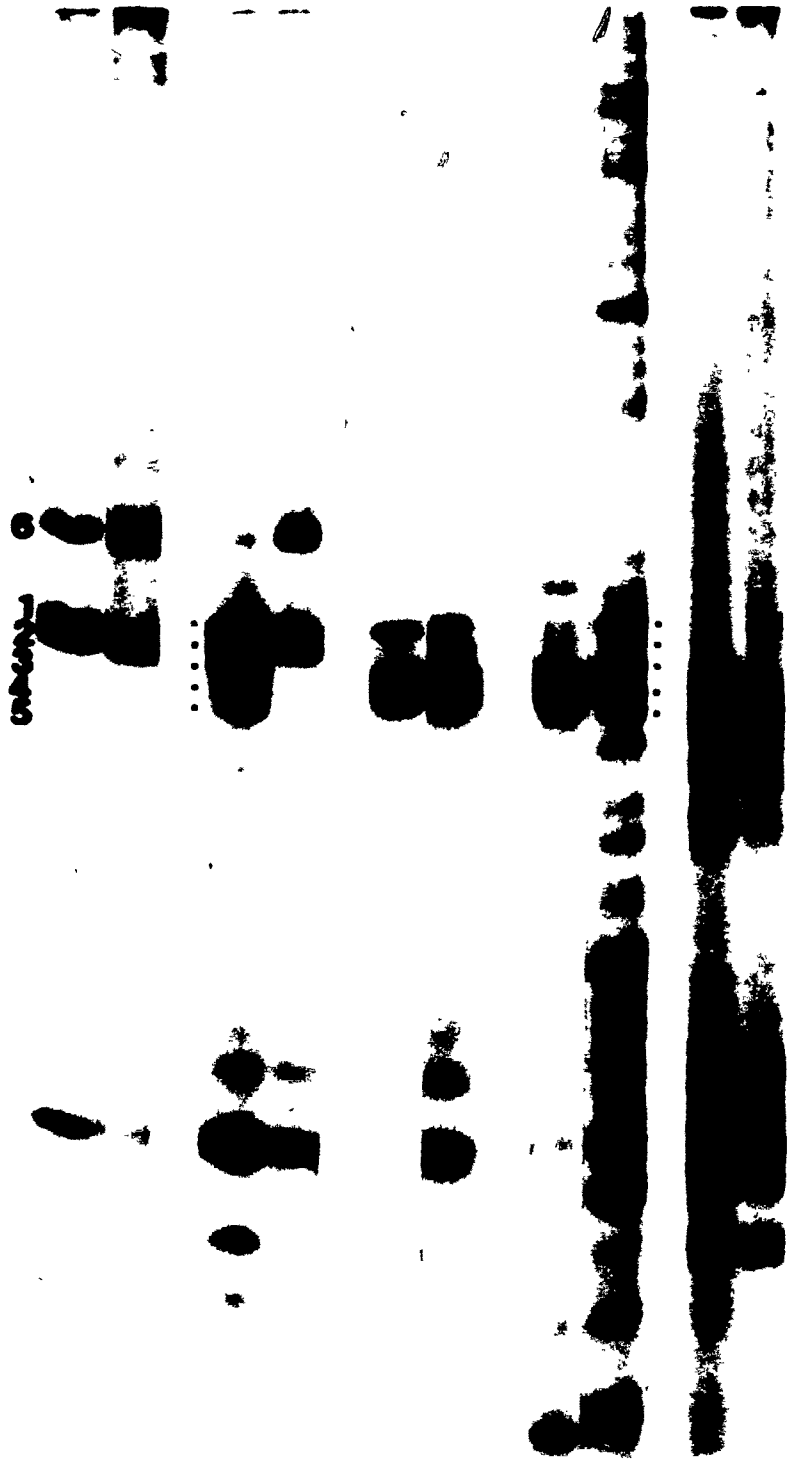
[1]During early spiralian development cleavage does not necessarily progress geometrically. However, in the absence of a detailed study of these early stages in this report average cell numbers are used.

H1 Histone Synthesis in Embryos Delobed during the First Cleavage

During the first twenty-four hours of normal development, when the labelling protocol is to label for the entire period, bands 1 and 2 show incorporation of isotope in equal amounts while incorporation into band 6 is lower (Figure 3, trefoil - day 1). The situation is different in delobed embryos. In these embryos, incorporation into band 1 is less than band 2 while band 6 is dominant (Figure 3, trefoil - day 1). Such differences in incorporation patterns between normal and delobed embryos are found throughout development. These differences represent a delay in the timing of the histone switches as illustrated by examining subsequent development.

At day four, when incorporation into bands 3, 4, and 5 is significant and incorporation into band 6 is reduced to only a trace in normal embryos, in delobed embryos the incorporation into bands 1, 2, and 6 resembles more closely that found in normal embryos labelled for the first twenty-four hours of

Figure 3. H1 histones of delobed Ilyanassa embryos. The histones of normal embryos are included for comparison. One day embryos were labelled for 24 hours beginning at the trefoil stage. 4, 5, 9, and 12 day embryos were labelled for 3 hours. Histones were prepared, resolved and displayed by SDS polyacrylamide gel electrophoresis and visualized by fluorography as described in Methods. Bands 1 - 6 at the far left indicate the H1 protein bands. The small squares are an aid for easy alignment. Days 1, 4, and 12 are from the same gel. Day 5 and 9 are from a separate gel. N, normal embryo; D, delobed embryo.


N D
Trefoil-1
N D
4
N D
5
N D
9
N D
12
DAY OF DEVELOPMENT

development (Figure 3, trefoil - day 1). Incorporation into bands 2 and 6 is approximately equal while incorporation into band 1 is somewhat less although its relative incorporation has greatly increased compared to that seen in the trefoil-day one labelling period. The onset of synthesis of bands 3, 4, and 5 is also delayed in the delobed embryo. Incorporation into these bands is detectable in the four day delobed embryo only after a very long fluorographic exposure whereas in the four day normal embryo incorporation into bands 3, 4, and 5 is pronounced relative to bands 1 and 2.

At day five of development, normal embryos show incorporation into bands 1, 3, 4, and 5 (Figure 2) although in this gel bands 4 and 5 did not resolve well (Figure 3, day 5). Incorporation into band 2 occurs only in very low amounts (Figure 3, day 5). The five day delobed embryo, on the other hand, shows a substantial amount of incorporation into band 2 relative to bands 1, 3, 4, and 5. Incorporation into bands 3, 4, and 5 has increased greatly (Figure 3, day 5) compared to day four of delobed development. These bands are now present in amounts similar to bands 1 and 2 (Figure 3, day 5).

Delayed switching is again apparent in the nine day delobed embryo. In the nine day normal embryo band 1 can be detected only in low amounts. However, in the day nine delobed embryo band 1 continues to show a relatively high level of incorporation (Figure 3, day 9). The nine day delobed embryo also shows a high level of incorporation into band 2 which is synthesized only in minor amounts after day four in the normal

embryo (Figure 3 and Figure 2). In fact, at day nine of delobed development band 2 has begun to decrease in a manner consistent with the normal pattern such that it shows relatively lower incorporation than band 1. Incorporation into bands 3, 4, and 5 also occurs in the nine day delobed embryo to give a pattern which resembles that seen on day four of normal development (Figure 3, day 9).

By day twelve of development, bands 1 and 2 have ceased to be synthesized in both normal and delobed embryos (Figure 3, day 12). Thus, the off-switch of synthesis of these bands does eventually occur in the delobed embryos, albeit, in a delayed manner. Bands 3, 4, and 5 of this preparation, and in all other day twelve preparations made for this type of comparison, did not resolve well. As such, no comparisons of incorporation among these bands between normal and delobed embryos is possible. A diagrammatic summary of the H1 histone synthesis schedule during delobed development is found in Figure 7.

There is no difference in the timing of the H1 switches in normal embryos cultured free in seawater (Figure 3) as compared to those embryos maintained in their capsules until labelling (Figure 2).

Cell Numbers of Normal and Delobed Embryos

Cell numbers were determined by counting the nuclei of embryos at twenty-four hour intervals throughout development (Table 1; Figures 4, 5, and 6). The data was collected in two

Table 1. Cell numbers of normal and delobed embryos. The data were collected in two data sets as indicated by procedures described in Methods. The cell counts of normal and delobed embryos aged day 1 to day 12 were compared using the Student's t test. Significant differences were calculated at the 1% level of probability.

Table 1

Day	Embryo Type	Number of Embryos	Mean of Cell Numbers ± Standard Deviation	Range of Cell Numbers	T Value	Degrees of Freedom	Probability of > |T|	Significant Difference at 1% Probability Level	% Difference between Means of Cell Numbers
Data Set 1									
1	N	11	36.3± 3.4	28- 40	0.95	16 9	0 36	NO	0 06
1	D	13	38.5± 7.8	27- 48					
2	N	11	82.6± 7.8	69- 93	-2.20	21 0	0 04	NO	0 07
2	D	12	76.9± 4.4	70- 84					
3	N	6	104.2± 9.7	93- 115	1.24	12.0	0 24	NO	0 07
3	D	8	111.6± 12.0	93- 134					
4	N	10	146.3± 15 7	116- 169	-1 62	13 2	0 13	NO	0.06
4	D	8	137.3± 7.2	131- 153					
5	N	7	161.7± 16.6	139- 188	0.26	13 0	0 80	NO	0 02
5	D	8	164.3± 20.4	135- 200					
6	N	8	249.0± 33 4	195- 307	-2 84	15.0	0 01	YES	18 5
6	D	9	202.9± 33.3	160- 249					
7	N	7	325.3± 43.5	241- 371	-3.97	11 0	0.002	YES	25 1
7	D	6	243 8± 26.8	217- 286					
Data Set 2									
7	N	9	697 9± 48.9	644- 791	-4.37	17.0	0 0004	YES	20 7
7	D	10	553.6± 87.5	396- 676					
8	N	10	934.5± 62.6	800-1022	-5 23	18.0	0 0001	YES	21 4
8	D	10	735.0±103.0	637- 996					
9	N	9	1316.5±188.3	979-1691	-3.29	16.0	0.005	YES	21 9
9	D	9	1028.8±182.9	787-1318					
10	N	8	1744.0±127.8	1514-1892	-5.57	12.0	0.0001	YES	25.0
10	D	6	1308.8±165.7	1079-1433					
12	N	8	2434.3± 96.5	2260-2541	-11.52	14 0	0 0001	YES	22.9
12	D	8	1876.4± 98.1	1747-2015					

Figure 4. Graphic representation of cell numbers of normal embryos as compared to delobed embryos. The plotted values are the means obtained from Table 1. ● Normal embryos. O Delobed embryos. Day 1 - 7 , data set 1. Day 7 - 12, data set 2.

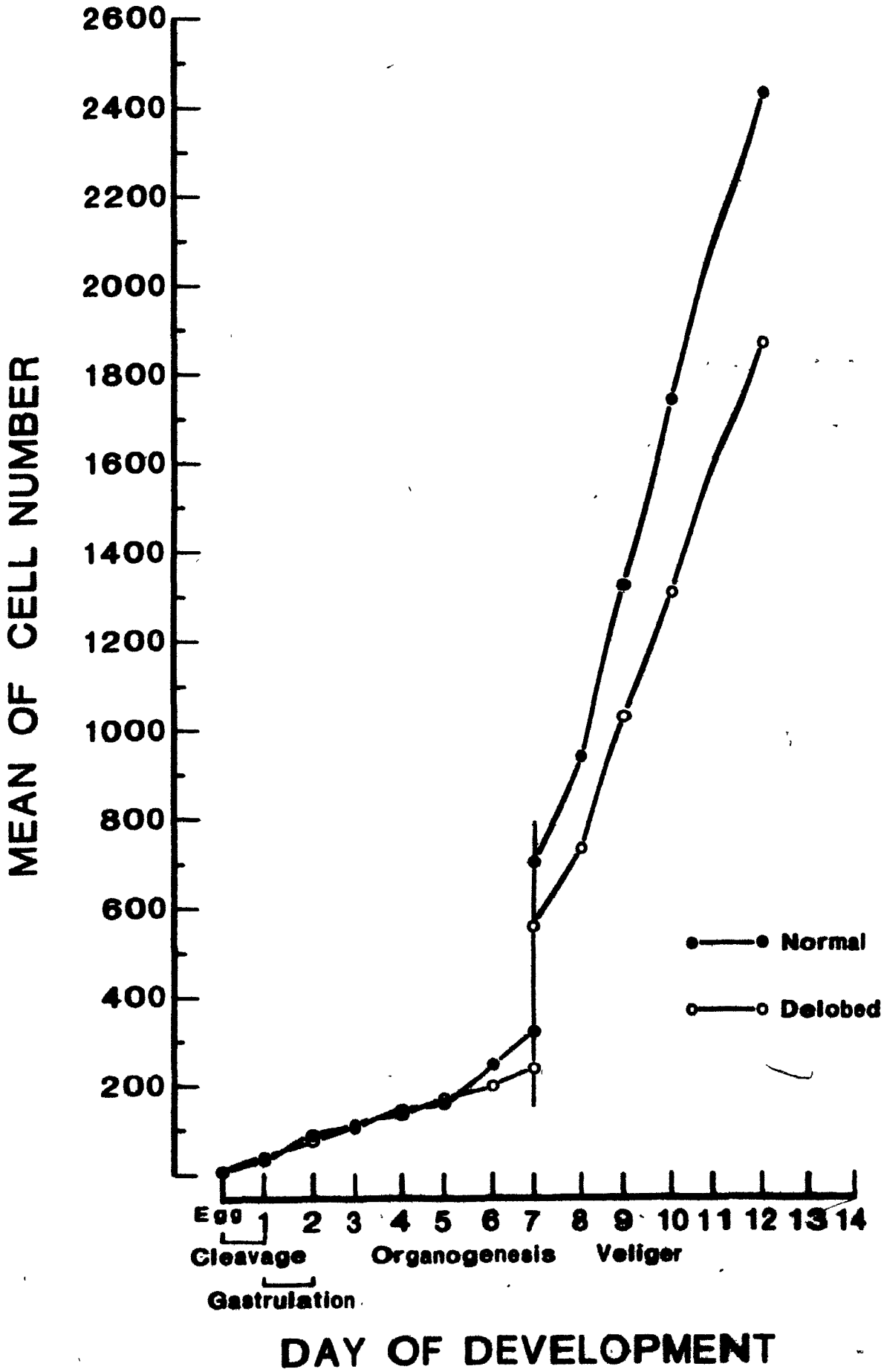

MEAN OF CELL NUMBER
2600
2400
2200
2000
1800
1600
1400
1200
1000
800
600
400
200
Egg
1
2
3
4
5
6
7
8
9
10
11
12
13
14
Cleavage
Gastrulation
Organogenesis
Veliger
Normal
Delobed
DAY OF DEVELOPMENT

Figure 5. Nuclear fluorescence of normal embryos. (A) Day 1. (B) Day 12. Embryos were squashed in mithramycin (125 µg/ml) and fluorescence visualized as described in Methods. The 1 day embryo squash contains 34 brightly fluorescing nuclei plus 2 very dim nuclei. The 12 day embryo squash has 2432 nuclei plus 70 nuclei which lay outside the frame of the montage. X 100

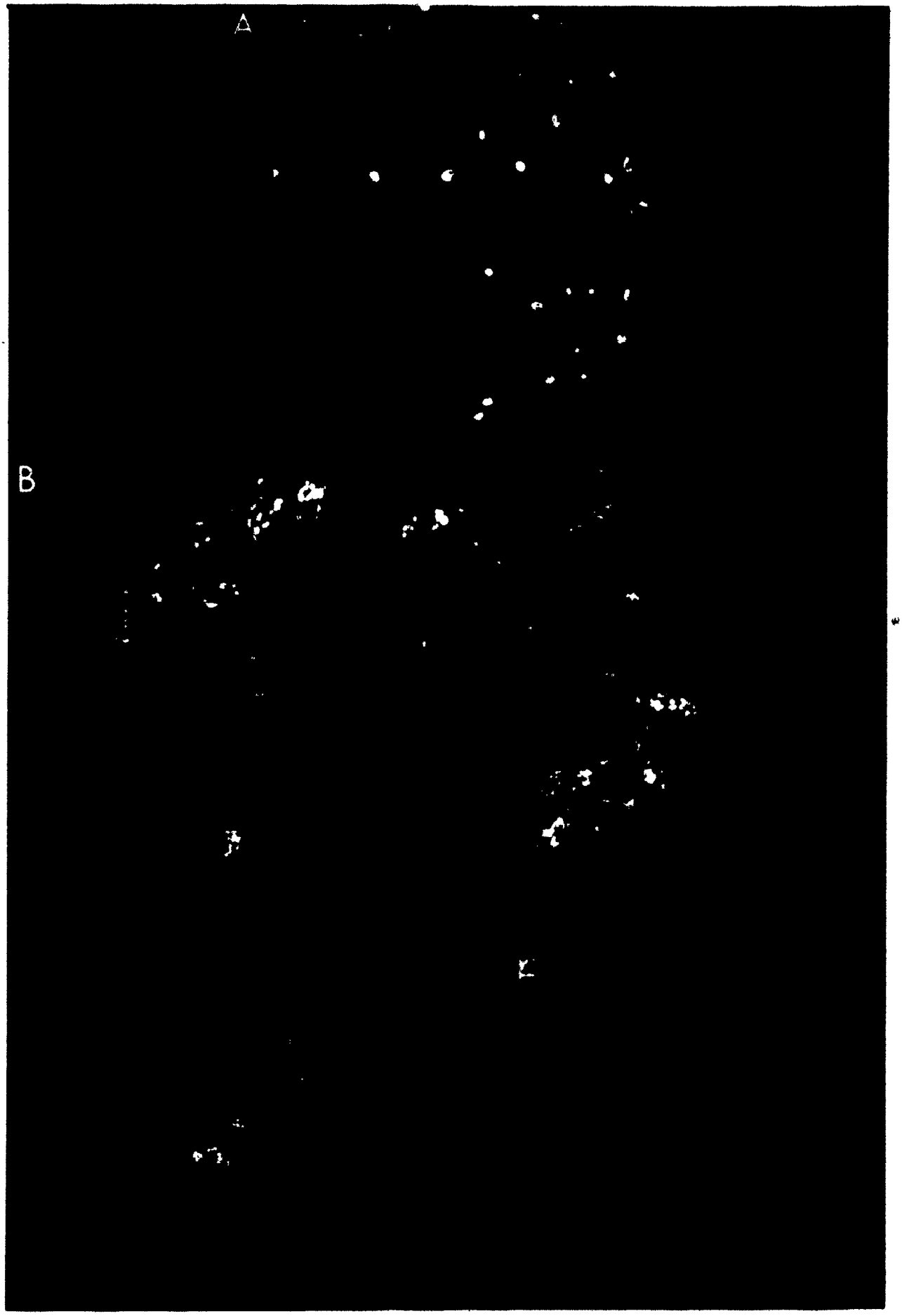
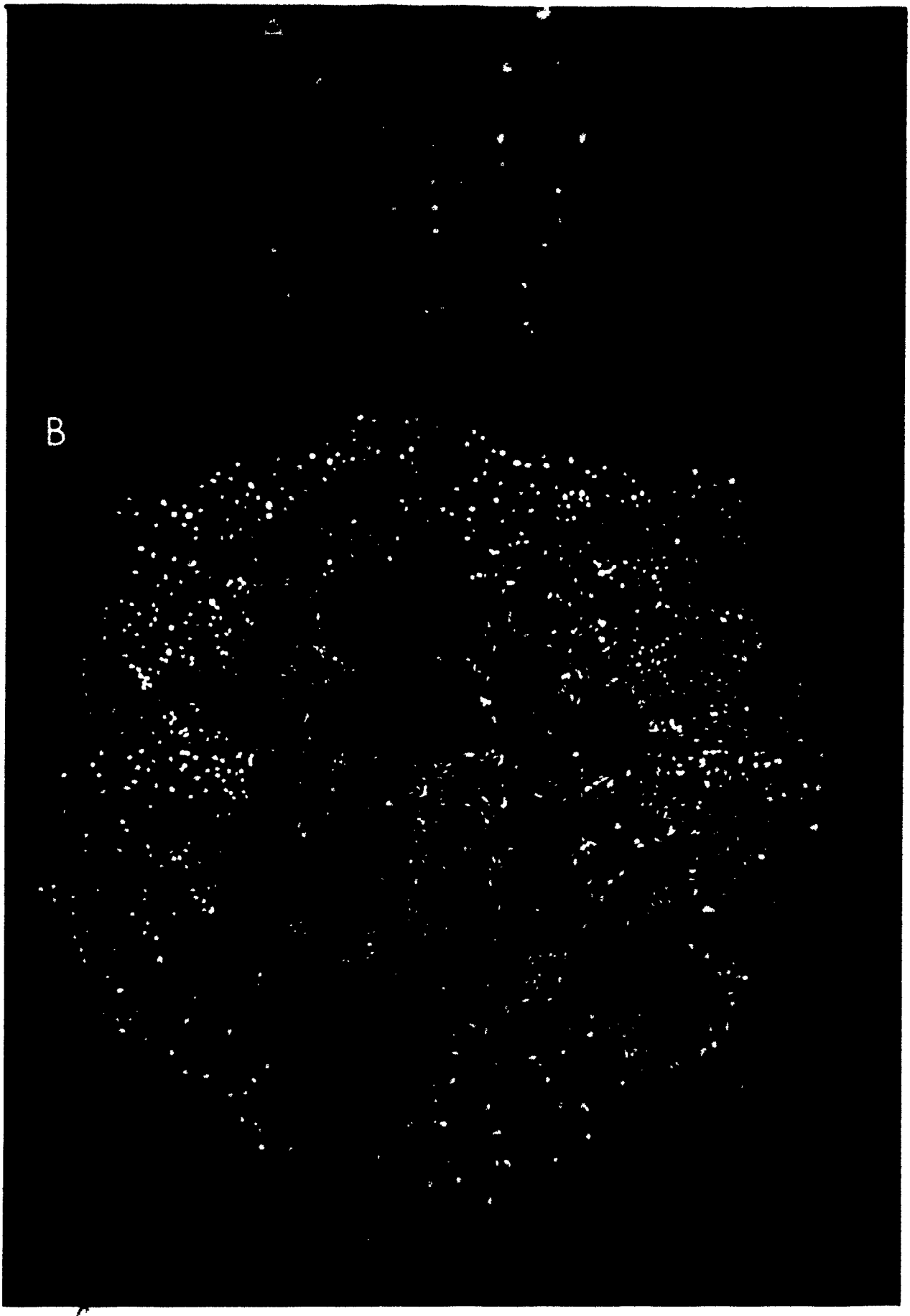

A
B

Figure 6. Nuclear fluorescence of delobed embryos. (A) Day 1. (B) Day 12. The 1 day embryo has 42 brightly fluorescing nuclei and 4 very dim nuclei while the 12 day embryo has 1859 fluorescing nuclei. X 100


B

sets, the first set consisted of embryos one to seven days old and the second set of seven to twelve day old embryos. The number of cells in the day seven embryos of the second data set is approximately twice that in the first data set. This difference represents one round of cell division and could be a result of embryo variation between the two experimental groups. Delobed embryos were cultured to only twelve days. It is not possible to culture such embryos much longer as they deplete their stored nutrient supply and die.

Statistical analysis of the cell numbers indicated that there was no significant difference at the 1% probability level between the cell number means of normal and delobed embryos from day one to day five of development. On the other hand, means of the cell numbers of normal and delobed embryos from day six to day twelve are significantly different at the 1% level of probability (Table 1). The results from day seven of both data sets agree in that there is a significant difference in cell number between normal and delobed embryos.

Between day five and day seven, during mid-organogenesis, the normal embryos undergo cell divisions which do not occur in the delobed embryos (Table 1 and Figure 4). These cell divisions result in an 18% difference in cell number between the normal and delobed embryos. The difference in cell number is maintained at 18-25% for the remainder of the developmental period studied (Table 1). Thus, this phenomenon occurs only once during the observed developmental period. At the end of the period examined, the cells of both normal and delobed

embryos are still dividing at a rapid rate. This rate has changed little since early development (Figure 4).

Nuclear Size in Normal and Delobed Embryos

The nuclei of one day squashes of embryos have an approximately 3 times greater diameter than do the twelve day embryos. This result is the same for both the normal and delobed embryos (Figures 5 and 6). The size limits of the nuclei of normal as compared to delobed embryos are similar at these stages (Figures 5 and 6).

DISCUSSION

H1 Histone Subtype Complexity and Subtype Synthesis Switches: Normal Development

H1 histone synthesis in *Ilyanassa* is similar to that of the sea urchin (Seale and Aronson, 1973; Ruderman and Gross, 1974; Poccia et al., 1975). It is possible that band 6 is the *Ilyanassa* equivalent of the sea urchin CSH1 (Newrock et al., 1978). In a manner similar to the sea urchin CSH1, band 6 has a significantly slower mobility than the other components of the H1 fraction, and, again like the sea urchin CSH1, its synthesis during development is confined to early cleavage. *Ilyanassa* embryos also have early forms (α) (bands 1 and 2) and late forms (β) (bands 3, 4, and 5) similar to the sea urchin. In the sea urchin, the CS and α forms are maintained in chromatin long after their synthesis ceases (Cohen et al., 1975; Newrock et al., 1978). No evidence on this point has been obtained in this study for *Ilyanassa*.

With the available data it is not possible to make a definitive statement as to whether or not each of the six bands described in this study represent subtypes with different primary amino acid sequences, or, instead arise due to post-translational modifications of one or a few subtypes. However, the data indicate that at least four distinct polypeptides are involved. Band 6 is synthesized only during cleavage and is a larger molecule than the other H1 proteins.

Bands 1 and 2 have synthesis patterns distinct from all other bands as well as from one another. In addition, V8 protease peptide maps indicate that these proteins, although related to one another, are different (see Appendix I). However, peptide mapping data are not conclusive since amino acid modifications could affect peptide mobility. Bands 3, 4, and 5 can be distinguished from bands 1, 2, and 6 by their developmental synthesis patterns. It is entirely possible, however, that they differ from one another only by post-translational modifications. The question regarding whether or not each of the six bands represent subtypes can only be unequivocally answered by amino acid or gene sequencing.

In an earlier study of the H1 histones of Ilyanassa Mackay and Newrock (1982) described three H1 protein bands in the veliger larvae. These bands correspond to bands 3, 4, and 5 observed in this study. They found differential incorporation of isotope (3>>4>5) into these bands at the veliger stage, although the three proteins were present in equivalent mass. In this study it has been found that the relative synthesis pattern among these proteins is established early in embryogenesis and is maintained throughout development. How the three proteins come to have equivalent masses, given the non-equivalent incorporation patterns, is not clear, although it may indicate that they are modified forms which can be converted into one another.

Unlike the sea urchin embryo, Ilyanassa embryos undergo a complex organogenesis. Under these experimental conditions

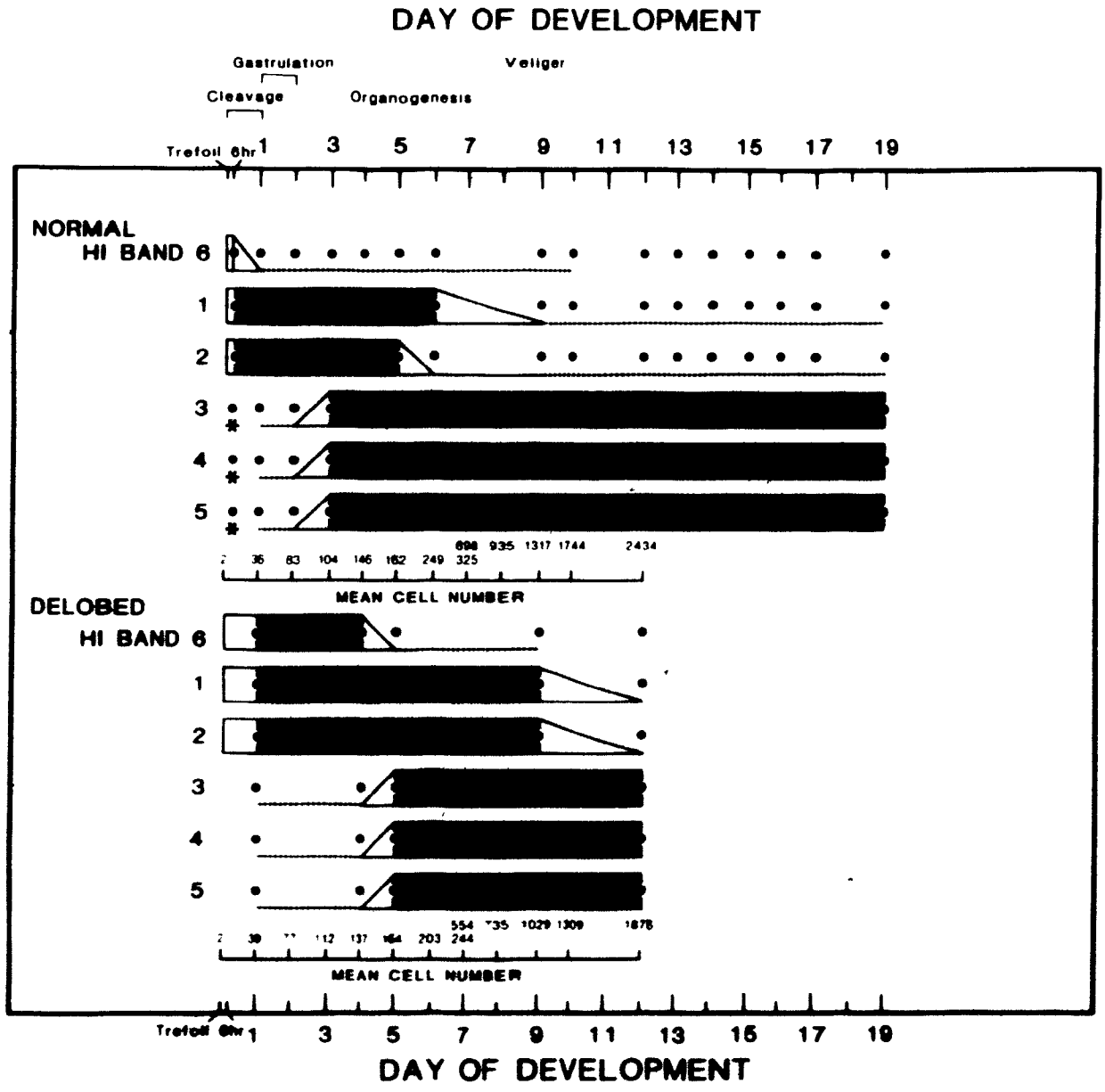
this organogenesis begins between the third and fourth day of development (after the completion of gastrulation), and it continues for several days. At some point during this process, the relative amount of synthesis of bands 1 and 2 decreases rapidly, while bands 3, 4, and 5 increase accordingly (Figure 7). The changes in H1 synthesis described here introduce new H1 proteins into chromatin. If these H1 subtypes are functionally different, then the introduction of a new subtype into chromatin would be developmentally significant. Since several of these switches occur during organogenesis they may be functionally related to this event whether or not each of the subtypes has a distinct function.

H1 Histone Subtype Complexity and Subtype Synthesis Switches: Delobed Development

The H1 histone subtype synthesis pattern of the delobed embryo differs from that of the normal embryo. The differences are shown in diagrammatic form in Figure 7. In general, in the delobed embryo there is a delay of the switching of early (CS and α) H1 proteins to late (β) H1 proteins. However, the switch from early to late H1 histone proteins does eventually occur in delobed embryos. Therefore, the presence of the polar lobe material is not necessary for the switches to occur but rather for the proper timing of the switches. The complexity of the H1 fraction in the two embryo types is the same.

Figure 7. Diagrammatic summary of the H1 histone synthesis switches during the development of normal and delobed Ilyanassa embryos. The H1 synthesis patterns from the data of Figures 2 and 3 are summarized. The corresponding mean cell number is located beneath the synthesis data as two tiers of numbers, each tier representing one data set.

Black circles represent the data points. Solid bars indicate substantial synthesis of an H1 band. Open bars represent considerable relative synthesis at an undetermined time. Open triangles represent an increase or a decrease in synthesis between the days indicated. A solid line indicates a low amount of synthesis and a dashed line indicates a trace synthesis. The asterisk represents a time point for which there was insufficient exposure to determine if there was a trace amount of synthesis.


DAY OF DEVELOPMENT
Gastrulation
Cleavage
Organogenesis
Veliger
Trefoil 6hr
1 3 5 7 9 11 13 15 17 19
NORMAL
HI BAND 6
1
2
3
4
5
698 935 1317 1744 2434
2 36 83 104 146 162 249 325
MEAN CELL NUMBER
DELOBED
HI BAND 6
1
2
3
4
5
554 735 1029 1309 1878
2 39 112 137 164 203 244
MEAN CELL NUMBER
Trefoil 6hr
1 3 5 7 9 11 13 15 17 19
DAY OF DEVELOPMENT

It seemed possible that the observed delay of H1 synthesis switches could be due to a general delay of development of the entire embryo. To explore this possibility the number of cells comprising normal and delobed embryos were determined for the first twelve days of development. Embryo cell number was then used as a marker for developmental progress. A positive correlation of cell number to the H1 subtype switches would exist if delobed embryos showed a delayed increase of cell number, and if the H1 switches occurred in the delobed embryo when it had the same number of cells as the normal embryo has when the switches occur. This conjecture was not the case. In fact, at the time bands 2 and 6 decrease in relative synthesis in the normal embryo, and when their decrease is first noticeably delayed in the delobed embryo, the two embryo types have the <u>same number</u> of cells (Figure 7). When the off switches of bands 2 and 6, and band 1, finally do occur in the delobed embryo the embryo has many more cells than its normal counterpart does at the same switch. Thus, there is no simple relationship between an H1 histone switch and the number of cells in an embryo. The switching schedule must be related to another phenomenon.

The delobed embryo shows aberrant organogenesis (Crampton,1896; Clement, 1952; Atkinson, 1971). Thus, the polar lobe is necessary for proper organogenesis. Possibly the H1 switches observed here are related to organogenesis since

during normal development bands 1 and 2 show a rapid decline and bands 3, 4, and 5 a rapid rise in relative synthesis during this period.

It is possible that either H1 histone proteins, H1 histone mRNA, or factors which affect the transcription and translation of H1 histone mRNAs are removed with the polar lobe. Removal of any of these molecules could affect the timing of the H1 switches. In the sea urchin the histones and histone mRNAs of the CS and α subtypes are stored in the egg and transcribed and translated during early cleavage (Newrock et al., 1978; Weinberg et al., 1983). If CS and α histones and mRNAs are stored in the Ilyanassa egg, as in the sea urchin egg, and if these molecules are removed and not replaced by new transcription and translation of early histone mRNA, then the expression of the late subtypes might begin earlier. If, on the other hand, new transcription of CS and early subtypes does occur, there would be no effect at all on the H1 protein switches. If there are late histones or late histone mRNAs stored in the egg, and, if these are removed, then the observed phenomenon could result. The presence of late histones and late histone mRNAs in the Ilyanassa egg is not likely since the evidence in sea urchin from inhibitor studies (Newrock et al., 1978) and from in vitro translation of mRNA isolated from eggs (Arceci et al., 1976; Weinberg et al., 1983) and embryos (Newrock et al., 1978; Childs et al., 1979; Arceci et al., 1976) indicate that late histone mRNAs are not stored in the egg but are synthesized later in development. Thus, it is not

likely that removal of histones or histone mRNAs with the polar lobe causes the observed phenomenon.

A Cell-Cell Interaction May Be Part of the Normal Histone Switch Mechanism in Ilyanassa

The data indicate that during normal development cell-cell interactions may be part of an embryo-wide coordinated histone synthesis switch. During early development all cell lineages are dividing, and presumably all are incorporating H1 histones into the genome. The H1 histones made during this period are bands 1 and 2 and 6 (CS and α forms). The synthesis of the early histones then all but ceases in the entire embryo, as evidenced by the absence of newly synthesized material from total embryo chromatin preparations. Removal of the polar lobe contents results in a delay in histone subtype switching in the entire embryo even though the contents of the polar lobe are segregated into only one cell lineage. Thus, in the delobed embryo, the "D" lineage affects the switch of H1 synthesis in the entire embryo. If the entire embryo was not affected, a delay in H1 subtype synthesis switching would not have been seen since the switching would have occurred at the proper time in the A, B, and C cell lineages.

Difference in Cell Number between Normal and Delobed Embryos

In the period from day one to day five normal and delobed embryos have a similar number of cells. Then, at some point

during the sixth day of development, there is a preferential increase in the cell number in the normal embryo such that it ultimately contains 20-25% more cells than the delobed embryo. This increase is complete by the end of day seven. The difference between the two embryo types is then maintained without substantial fluctuation throughout the remainder of development studied. The increase in the normal embryo does not represent a doubling of cell number. Thus, there must be a subpopulation of cells which divide in the normal embryo but which do not divide in the delobed embryo between days five and seven. These divisions occur in the normal embryo during organogenesis, a period of development when polar lobe effects might be expected since the delobed embryo shows aberrant organogenesis. It is possible that the increase in cell number is related to this process. Of course, the delobed embryo later catches up in cell number, but, from this point on there may be a (an additional) qualitive difference between the cells of the two embryo types such that they are irreversibly altered.

At twelve days of development the normal embryo is increasing in cell number as rapidly as at any point in post-cleavage development. As such, the maximum number of cells in the veliger larva has not yet been determined. At the time of death the delobed embryo has 20% fewer cells than the normal embryo, the difference established between days five and seven. However, its cells are increasing in number at this point at the same rate as those of the normal embryo. It seems

possible that if the delobed embryo had not died due to lack of nutrients there would have been a further increase in cell number in these embryos also.

Equivalent rates of early development of normal and delobed embryos agrees with the results of Davidson et al., (1965) who found the cell number of normal and delobed embryos was similar up to sixty- two hours, the time period studied, and with Newrock and Raff, (1975) who found that the appearance of ribosomal RNA synthesis, the appearance of apical cilia, and the formation of velar structures occurred at the same time in normal and delobed Ilyanassa embryos. Results showing later differences in cell number between the two embryo types are also consistant with those of Collier (1975) who measured DNA content of normal and delobed embryos as a function of development.

During early cleavage it has been reported (Clement, 1952) that normal and delobed embryos have slightly different numbers of cells due to an altered rate of division in the "D" lineage. This cannot be the cause of the differences observed in this study since no significant numerical differences are observed by these methods from day one to five of development. This does not mean that differences seen earlier are not developmentally significant, but that such differences are not directly translated into the differences in cell number observed here.

At first glance it may seem possible that there are fewer cells in the delobed embryo because one third of its mass was

removed with the polar lobe. Two points argue against this explanation. First of all, when the polar lobe is removed no nuclei are removed. Thus, no potential centres for cell division were removed. Secondly, during normal development, the "D" macromere, after the micromeres have formed, stops dividing and becomes a source of nutrient (Clement, 1952), not cells. Thus, the presence of this extra mass cannot account for the 18 - 25% additional cells in the normal embryo.

The Influence of Maternally Stored Information

This study shows an effect on H1 subtype synthesis schedules after removal of the polar lobe. The polar lobe presumably contains oogenetically synthesized and stored information and the differences observed between normal and delobed embryos are due to the deletion of these products. Thus, there is likely a maternal influence on chromatin remodeling during development. The precise developmental consequences to the delobed embryo of the perturbation of the H1 switches, which could be one of several perturbations associated with polar lobe removal, will depend upon what, if any, distinct functions the individual H1 subtypes have. Alternatively, it remains possible that the observed effect is indirect and could be the consequence of another aberration which then affects H1 synthesis.

Nuclear Size Changes during Development

The decrease in nuclear size as a function of development may be related to the incorporation of different H1 molecules into the nucleus. To fully explore this possibility it is necessary to determine the full distribution of nuclear sizes in individual embryos at progressive stages of development and compare this distribution to the H1 switching schedule. Alternatively, a procedure to identify individual subtypes in different sized nuclei could be used.

Cell Type Distribution of H1 Subtypes Must be Determined

This study has involved whole embryos. It is important to determine the subtype composition of distinct cell types. In a highly mosaic organism such as Ilyanassa this can be accomplished by isolating A, B, C, and D blastomeres after the second cleavage. As these blastomeres will develop in isolation (Donohoo and Kafatos, 1973) their subtypes could be compared as a function of development. In addition, the complexity of the veliger larva allows for the isolation of tissue and organ types. Analysis of the H1 proteins would determine if there is an association of specific H1 subtypes with specific tissues and organs. A priori it seems more likely that the subtypes will be associated with "early-late" events in the entire embryo and not with specific cell type determinations. This possibility seems likely as there are no completely determined cell types at the CS switch but rather

cells with a great deal of developmental potential. At the later switch organogenesis has begun but there are not as many different H1 subtypes as there are cell types.

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APPENDIX I

V8 Protease Digestion of H1 Histone Bands

Preliminary data from the V8 protease digestions indicates that the six H1 bands have both amino acid sequence homology and differences among them. Band 6 has similar major digestions products as those peptides produced by digestion of bands 1 and 2 (Figure 8). This further confirms its identification as an H1 protein. Band 1 has a digestion product that is missing in band 2 and the relative amounts of several peptides vary in the digestion patterns of the two bands. The two major digestion products of band 3 have similar mobilities to two peptides of band 2. The major digestion product of bands 3, 4 and 5 increases in mobility in the order 5>4>3 (Figure 8). A difference such as this could be due to sequence differences or to amino acid modification. A longer fluorograph exposure is necessary to see the full digestion products of bands 6, 1, 3, 4, and 5. Without this exposure no conclusions can be drawn from this data.

Figure 8. V8 protease digestion of H1 histone bands 1 - 6. Lanes a - f correspond to bands 6, 1, 2, 3, 4, and 5 respectively. The two arrows indicate the two main digestion products of band 6 which correspond in mobility to peptides of bands 1, 2, and 3. Protein digests were displayed by SDS polyacrylamide gel electrophoresis and visualized by fluorography as described in Methods.


a
b
c
d
e
f