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STUDIES OF THE NUCLEAR MATRIX AND CYTOSKELETON OF XENOPUS LAEVIS ERYTHROCYTES AND SPERMATOGENIC CELLS

by

JOHN GAMBINO

A dissertation submitted to the Graduate Faculty in Biology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York.

1983

This manuscript has been read and accepted for the Executive Committee in Biology in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

September 10, 1982 Date	Ronald A. Eckhardt Chairman of Examining Committee Ronald A. Eckhardt, Brooklyn College
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	Institution

The City University of New York

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CHAPTER ONE

BACKGROUND AND INTENT

BACKGROUND

One feature of the eukaryotic genetic apparatus which has intrigued biologists for some time concerns the mechanism by which enormous quantities of DNA can be packaged, organized and selectively expressed within the nucleus of each cell of an organism. Prior to 1970, it was known that DNA in eukaryotic nuclei were tightly associated with a group of evolutionarily conserved, highly basic proteins called the histones. These proteins are the predominant class of nuclear proteins being found in weight ratios of approximately 1:1 with that of nuclear DNA. Five major histone types have been identified (H1, H2A, H2B, H3 and H4) with molecular weights ranging from appoximately 11,000 to 23,000 (Elgin and Weintraub, 1975).

The strong positive charge of these proteins at physiological pH, along with the known highly negative phosphate backbone of DNA, led to early models of chromatin structure where the histones were depicted as being evenly distributed along the DNA helix. Such views were quickly discarded when chromatin research was revolutionized in the early 1970's with the discovery that a basic feature of chromatin structure was a repeating subunit subsequently termed the nucleosome (Oudet et al., 1975; Kornberg, 1977).

Chromatin is now known to possess several levels of organization, the most basic fiber being the 100 Å diameter nucleofilament (Kornberg, 1974). Nucleosomes lie in close aposition in this fiber to form a flexibly jointed chain capable of being organized into higher order structures. Each nucleosome contains a well defined length of DNA which is partially wound around an octomeric aggregate of pairs of the histones H2A, H2B, H3 and H4 (Finch et al., 1977). Nucleosomes of

virtually all eukaryotes presently examined contain a core particle with 146 base pairs of DNA which are relatively resistant to the limited digestive activity of certain nucleases (McGhee and Felsenfeld, 1980).

Early experiments which first demonstrate the presence of this core region of nucleosomal DNA determined its length to be 140 base pairs (Axel, 1975; Sollner-Webb and Felsenfeld, 1975). However, more precise measurements have since shown the correct value to be 146 base pairs (Lutter, 1979). This reevaluation has not significantly altered the current picture of the nucleosome which was developed principally between 1975 and 1978.

Physical and biochemical studies have led to a fairly detailed understanding of the nucleosome. The core particle is roughly wedge-shaped with an approximate diameter of 110 Å and a height of 55 Å (for reviews see, Felsenfeld, 1978; Kornberg and Klug, 1980). DNA is wound around the central histone aggregate in ≃2 negative left handed toroidal supercoils. Moreover, nuclease digestion and x-ray crystallographic analysis of core particles indicate that in all likelihood the particle has a dyad axis of symmetry and 80 base pairs of DNA per supercoil (Sollner-Webb et al., 1978; Finch and Klug, 1978).

Depending on the source (tissue and species) of chromatin studied, the basic nucleosome repeat length can vary from 154 to 241 base pairs (Lewin, 1980). Because the value of nucleosomal core DNA is invariant, these differences in nucleosome repeat length have been attributed to variations in the length of the linker DNA which connects adjacent core particles. Within a given organism, not only do nucleosome repeat lengths differ for various tissues (Thomas and Thompson, 1977), but they can also change during the differentiation of a particular cell type.

This was demonstrated by Weintraub (1978) who showed that during avian erythropoiesis the repeat length increases from 190 to 212 base pairs. Moreover, in a given cell type, the repeat length of nucleosomes of a particular gene can also differ from the value of bulk DNA. For instance, the 5S ribosomal gene of Xenopus erythrocytes has a repeat length of 175 base pairs while the repeat unit for bulk chromatin of that cell is 189 base pairs (Humphries, 1979).

The reasons for the observed variations in nucleosome repeat lengths between different tissues or in specific sequences of a given cell type is not yet understood. The speculation that higher levels of gene activity are correlated with shorter repeat lengths (Morris, 1976; Spadafora et al., 1976) still remains to be firmly substantiated.

The lysine rich histone H1, and H5 in certain nucleated erythrocytes, is also associated with nucleosomes. However, this histone is primarily associated with linker DNA and is believed to play a crucial role in the higher order arrangement of the fundamental nucleofilament into the 200-300 Å (thick) fiber seen in interphase and mitotic nuclei (Thoma and Koller, 1977; Noll, 1977; Worcel and Benyavati, 1977). One molecule of H1 is associated with 10 base pairs of DNA entering and exiting a specific nucleosome core particle. Thus, H1 seals two supercoils of nucleosomal DNA (Kornberg and Klug, 1981). This 166 base pair particle has been termed a chromatosome (Simpson, 1978). In contrast with the nucleosomal histones, the H1 histone shows a much greater heterogeneity in the number of molecular species and tissue subtypes characterized (Isenberg, 1979). The biological significance of these H1 subfractions is not yet clear, although there is some evidence that they may influence chromatin structure and

function. One H1 subfraction, $H1^{\circ}$, has been shown to accumulate in growth inhibited cultured cells (Pehrson and Cole, 1980). Moreover, H1 phosphorylation has been implicated in the initiation of chromosome condensation in mitosis (Matsumoto et al., 1980). In sum, although it is quite clear that H1 is required for the formation of the thick chromatin fiber, the role of this protein in the regulated structural transitions of the basic chromatin fiber is not understood.

Original probes of higher order coiling led to the formation of two different models for the 200-300 Å fiber, the solenoid model (Finch and Klug, 1976; Worcel, 1977) and the superbead model (Hozier et al., 1977). Briefly, the superbead scheme suggests that between 8-12 nucleosomes of the 100 Å diameter fiber are coiled into a "super-supercoil", thus producing a superbead whose diameter will vary between 200-300 Å depending on the nucleosome repeat length (Worcel, 1978). According to the space-filling model of Worcel (1978), superbeads with nucleosome repeat lengths of less than 180 base pairs will be right handed while those above 180 base pairs will be left handed in nature. However, these calculations were done assuming 90 base pairs of DNA per nucleosome supercoil. In view of findings that indicate the number is 80 base pairs (Simpson and Whitlock, 1976; Sollner-Webb et al., 1978; Finch and Klug, 1978), the inflection point between right and left handed superbeads is actually 160 base pairs. Therefore, in most higher eukaryotes, left handed superbeads would prevail.

The solenoid model suggests that the thick fiber is formed as the result of the continuous coiling of the 100 Å nucleofilament so that there are between 6-7 nucleosomes per "super-super coil". It is thought that this fiber when formed in vitro from the primary nucleofilament (as

a result of an increase in ionic strength) does so through the formation of zigzag nucleosome intermediates produced by H1 interactions between adjacent nucleosomes (Thoma, Koller and Klug, 1979). Such a model is consistent with H1 crosslinking data (Kornberg and Klug, 1981). Regardless of the exact nature of the 200 Å chromatin fiber, it is clear that higher levels of chromatin organization are required to account for the packing ratio of DNA within interphase nuclei and mitotic chromosomes.

Although the DNA of individual eukaryotic chromosomes is continuous (Lewin, 1974), experimental evidence from electron microscopy (Laemmli, 1978); Paulsen and Laemmli, 1977), restriction enzyme digestion (Igo-Kemenes and Zachau, 1978) and biophysical studies (Ide et al., 1975; Benyajati and Worcel, 1976; Cook et al., 1976) indicates that DNA in most eukaryotic cell types is constrainted into loops throughout the cell cycle. Supercoiling within the looped domains is at least in part due to released DNA coiling by the nucleosomal histones (Germond et al., 1975). The exact nature of the constraining agent has not been determined. However, evidence does exist that DNA is attached to a predominantly protein skeletal framework in both interphase and metaphase which consists of a group of nonhistone proteins (Laemmli, et al., 1978; Berezney and Coffey, 1977; Comings and Okada, 1970, 1976).

Laemmli and his co-workers have isolated a chromosome "scaffold" from HeLa cell metaphase chromosomes which have been depleted of all their histones as well as most of their non-histone proteins by either polyanion competition or extraction with 2M NaCl. These structures are vulnerable to agents which solubilize or interfere with protein associations, but are resistant to RNase treatment (Adolph et al.,

1977). However, RNA could be protected by proteins and thus still have structural significance. Very recently it has been reported that metalloprotein interactions, most notably with copper, are important stabilizers of the chromosome scaffold (Lewis and Laemmli, 1982). Copper stabilized chromosome scaffolds consist of only two high molecular weight proteins (135,000 and 170,000 daltons) when isolated from chromosomes obtained by methods which prevent cytoskeletal contamination.

Whole mount electron microscopy of spread histone depleted chromosomes shows lateral loops of DNA radiating from the protein scaffold. Loop length has been measured at approximately 50-100 kb and does not appear supercoiled in micrographs. This latter observation is not surprising considering that standard methods of chromosome isolation have been known to introduce many nicks into DNA. Using an alternative chromosome isolation technique, Mullinger and Johnson (1979) appear to have retained the supercoiling within the loops although their preparations are not nearly as well spread as those of Laemmli. Moreover, evidence exists which indicates that a looped arrangement exists in mitotic chromsomes when examined using alternative conditions. For instance, looping of the 200-300 Å diameter chromatin fiber is observable in chromosomes by whole mount electron microscopy (Bahr, 1977) as well as by thin section and scanning electron microscopy of EDTA swollen chromosomes (Marsden and Laemmli, 1979).

The DNA looping phenomenon, as mentioned above, is not restricted to the metaphase chromosome but has been well documented in interphase nuclei. The interphase looped domains are similar in size (50-100 kb) to those measured from metaphase scaffolds and are thought to be

anchored to a primarily proteinaceous residual nuclear structure (Razin et al., 1979; Vogelstein et al; 1980, McCready et al., 1980) which has been termed the nuclear matrix or cage. A more detailed treatment of the possible biological significance of DNA looped domains and their relationship to the nuclear matrix will be presented below.

Until this point, much attention has been given to the significant advances which were made in the last ten years in our understanding of the way in which DNA is compacted and organized in eukaryotic nuclei and chromosomes. During this period, it has also become apparent that after careful removal of the nuclear envelope and chromatin, the "nucleus" contains a dynamic, predominantly proteinaceous, structural network termed the nuclear matrix (for recent reviews see Agutter and Richardson, 1980; Berezney, 1979). Nuclear matrices from a variety of organisms, tissues, and cell lines retain the overall size and shape of in situ nuclei and often consist of: 1. a peripheral residual envelope layer which retains nuclear pores and is frequently referred to as the nuclear pore-lamina complex (NPLC), 2. a nucleolar matrix, and 3. a fibrogranular network which permeates the nuclear space extending to both the NPLC and nucleolar matrix and is termed the intranuclear matrix.

The nuclear envelope consists of an inner and outer nuclear membrane which contain well characterized structures, termed pore complexes, that are involved in nucleocytoplasmic exchange (for a current review see Franke et al., 1981). Ultrastructurally, pore complexes are made up of two annuli (an inner and outer membrane annulus) each of which are composed of eight globular particles symmetrically positioned around the pore periphery as well as eight

radially situated tips that project into the pore lumen. The globular particles are termed traverse fibrils when they have not been collapsed by magnesium. A central granule of variable size is also observed in some nuclear pores. Radiating fibrils which span the pore complex have been observed at the pore margin and may represent traverse fibril projections.

Non-membranous pore complexes, in association with interconnecting material, can be isolated free of nuclear envelopes by the use of non-ionic detergents and high ionic strength conditions (Aaronson and Blobel, 1975). In many cells the nucleoplasmic surface of the inner nuclear membrane contains a continuous layer of non-membranous protein (the fibrous lamina) which is isolated in association with the nuclear pores. The NPLC from rat liver has been well characterized. This structure, which is primarily lamina in content, consists of three major polypeptide components (lamin A, B and C) with molecular weights respectively of 70, 67 and 60 kilodaltons (Gerace et al., 1978). These proteins immunologically localize exclusively at the nuclear periphery and appear to be distinct from the proteins of the intranuclear matrix. Furthermore, the proteins of the NPLC and the intranuclear matrix show functional differences with respect to steroid binding affinity and association with hnRNA (Agutter and Birchall, 1979).

The NPLC is a dynamic structure which is reversibly disassembled during the cell cycle at the time of mitotic apparatus formation and nuclear envelope dissolution (Gerace et al., 1978). During mitosis, lamins A and C are found in a soluble state while lamin B remains associated with membrane fragments within the cytoplasm. The depolymerized state of the lamins is correlated with their higher degree

of phosphorylation (Gerace and Blobel, 1980). It is thought that the reversible phosphorylations of the lamins may mediate the polymerization state of the lamina during the cell cycle.

Fibrous lamina, have been observed *in situ* in a variety of different cell types (Fawcett, 1966) in association with peripherally located chromatin. However, in some cells (Franke *et al.*, 1981) a fibrous lamina is not observed and chromatin directly borders on the inner nuclear membrane. In these cells it is thought that the NPLC consists of a proteinaceous, skeletal meshwork that forms a thin interpore fabric which associates with the nuclear envelope as well as peripheral chromatin.

In contrast with the NPLC, the other nuclear matrix components (intranuclear and nucleolar matrix) are not as well characterized biochemically or ultrastructurally, although in recent years it has become evident that these structures are intimately involved in nuclear metabolism. Research from several laboratories (Berezney and Coffey, 1975; McCready *et al.*, 1980; Pardoll *et al.*, 1980) indicates the nuclear matrix is directly involved in DNA replication. Pulse-chase labelling studies with regenerating rat liver and exponentially growing fibroblast cells show that residual matrix associated DNA is highly enriched in newly synthesized DNA (Pardoll *et al.*, 1980). Electron microscopic autoradiography of these matrix preparations reveals that, as in whole nuclei, the sites of DNA replication are not localized at the nuclear periphery but exist throughout the nuclear matrix. Similar findings were obtained by others (McCready *et al.*; Vogestein *et al.*, 1980) in studies which involved the use of nucleoids. Nucleoids are histone-depleted nuclei in which the intact unnicked looped DNA (as judged by

its superhelicity) remains attached to the nuclear matrix. Pulse labeling of cells with ^{3}H-thymidine for various periods of time followed by autoradio- graphy of isolated nucleoids illustrates that DNA replication occurs at fixed sites at the base of the loops and the newly replicated DNA then moves out to the loop periphery.

Thus, the nuclear matrix is thought to provide fixed sites for the association of replication complexes. DNA, organized into supercoiled looped domains, is reeled through these sites during replication. Several models have been proposed suggesting how this may be accomplished (McCready et al., 1980; Pardoll et al., 1980; Berezney and Buchholtz, 1981).

In addition to its proposed role in replication, evidence has accumulated which suggests that the nuclear matrix may also be involved in nuclear RNA metabolism. Nuclear matrices from a variety of sources have been isolated in association with the bulk of rapidly labelled hnRNA (Miller et al., 1978; Long et al., 1979, Agutter and Birchall, 1979). This hnRNA was shown by radioautography in Hela cells to be associated with internal protein elements (Herman et al., 1978). Recently, an elegant study by van Eekelen and van Venrooij (1981) indicates that association of hnRNA with the nuclear matrix may involve only two specific proteins. These investigators utilized ultraviolet light to crosslink hnRNA to tightly associated proteins in both isolated nuclear matrices and intact cells. Two nuclear proteins (41.5 and 43 kilodaltons) were found crosslinked to hnRNA (irrespective of when cross-linking was performed) which were not released by ribonuclease treatment and therefore represent nuclear matrix proteins. This finding, along with general observations by others that hnRNP isolation

is achieved only by methods which produce nuclear distruction, suggests that in vivo hnRNP are associated with a solid phase nuclear structure. This association might be related to RNA processing and translocation. Ciejek et al. (1981) have presented data which shows that precursors to ovomucoid and ovalbumin mRNA were associated with chick ouiduct nuclear matrices although the mature mRNAs were not. Similar observations were made by these investigators for rRNA precursors.

For some time RNA transcription, like DNA replication, has been thought to occur by mobile polymerases moving along the DNA molecule. Recently, evidence has been presented which suggests this view may need to be re-evaluated. Nucleoids have been isolated and the supercoiled looped DNA digested to various extents in order to determine if the sequences close to the matrix preferentially contained active sequences (Jackson et al., 1981; Robinson et al., 1982). Robinson et al. (1982) observed that the ovalbumin gene is enriched in matrix associated DNA from oviduct cells but not liver cells. Moreover, in contrast the non-transcribed B-globin gene was not preferentially associated with nuclear matrices from oviduct cells. Transcription may then occur by mechanisms similar to those proposed for replication.

Whatever the details of the mechanisms behind eukaryotic replication and transcription turn out to be, it seems likely that the organization of DNA into looped domains will be an important consideration in both processes. Presently, attention is starting to be directed towards the biochemical basis of loop formation as well as the relationships between loops in metaphase and interphase cells. DNA seems to be attached to metaphase scaffolds and nuclear matrices non-randomly. Analysis of the DNA sequences attached to these skeletal

structures indicates that in both instances the associated sequences are a subset of total DNA, specifically intermediately repetitive DNA (Razin et al., 1979). Moreover, comparisons of scaffold and matrix associated DNA (by such criteria as % satellite, buoyant density, renaturation rate, loop size) indicated the DNA to be very similar (Razin et al., 1979). This finding led these investigators to suggest that during the cell cycle the bulk of DNA attachment sites remain the same. More recently, this research group has begun to characterize those proteins which tightly bind to DNA (Razin et al., 1981). They have identified six proteins by molecular weight that tightly bind to DNA and which are constituents of both interphase and metaphase skeletal structures. These proteins were not found in association with skeletal released DNA. In addition, a small molecular weight polypeptide of 18 kilodaltons was identified which tightly bind to both matrix associated DNA as well as DNA scattered throughout the whole loop. These authors speculate that this protein may be able to form associations with certain matrix proteins and therefore represent potential loop attachment sites. These observations are not incongruent with the suggested models for matrix involvement in DNA replication and transcription.

To date, the proteins of the nuclear matrix remain relatively uncharacterized in comparison with those of the NPLC. Peters and Comings (1980) have thoroughly characterized the proteins of rat liver nuclear matrices by two dimensional electrophoresis and noted that the nuclear matrix has a much more diverse polypeptide pattern than originally suggested by one dimensional SDS polyacrylamide gel electrophoresis (Berezney and Coffey, 1977). A nuclear matrix specific

polypeptide has been characterized from amplified nucleoli of Xenopus oocytes (Franke et al., 1981b) and a human specific nuclear matrix protein which associates with the polar region of the mitotic apparatus has also been identified (Lyderson and Pettijohn, 1980). Yet, on the whole, little has been done in the way of two dimensional electrophoretic comparisons between matrices and scaffolds of different tissues and species.

Before leaving the nuclear matrix, it should be noted that I have just touched upon the major areas where this structure appears to play a significant role in nuclear metabolism. In addition however, the matrix has also been implicated in steroid receptor binding (Barrack and Coffey, 1980) and viral coat protein assembly (Hodge et al., 1977; Buckler-White et al., 1980).

During the last ten years, just as major advances were being made in structural-functional relationships within the nucleus, similar advances were made in cytoplasmic research. It was discovered that upon extraction of the plasma membrane with non-ionic detergents, in buffered media having certain ionic parameters, the cytoplasm also contained a highly organized proteinaceous skeletal network made up primarily of microtubules (250 Å in diameter), intermediate filaments (90-120 Å in diameter) and microfilaments (60 Å in diameter).

In recent years, it has become increasingly evident that the cytoskeleton plays a crucial role in the reproduction of the genetic apparatus and probably can directly influence nuclear activity. The microtubules and microfilaments which respectively are involved in chromosome movement and cyto-kinesis are known not to be synthesized de novo, but to be reassembled from dismantled cytoskeletal components.

Moreover, recent evidence indicates that certain growth factors may initiate DNA synthesis by inducing microtubule disassembly (Crossin and Carney, 1981a, b). Cytoskeletal-nuclear associations and interactions are probably important to a number of vital cellular and developmental processes. This facet of cell biology will undoubtedly receive much attention in the years to come (see Capco et al., 1982).

INTENT

The original intention of this dissertation was to ultrastructurally and biochemically compare nuclear matrices from cell types which differed in nuclear structure, synthetic activity, chromatin structure and chromatin composition. The emphasis of the work was to center on spermatogenic cells and erythrocytes because these cells possess nuclei with certain unique features not found in the somatic cells previously studied by others.

One of the unique values of studying nuclear matrices during spermatogenesis is that in this developmental process the nucleus undergoes temporal changes in nuclear form as well as chromosome arrangement and composition. Spermatogenesis also contains a mitotic and meiotic component. The mitotic component involves the renewal of stem cells and the formation of spermatogonia which will multiply and mature into secondary spermatogonia. Secondary spermatogonia then give rise to spermatocytes which initiate the meiotic phase of the process. Primary spermatocytes undergo a prolonged prophase which involves chromosome condensation, synapsis and crossing over, the last two events being associated with the formation of the synaptonemal complex. The resulting secondary spermatocytes then divide to produce spermatids

which will differentiate toform mature sperm.

Xenopus laevis seemed ideal for such an undertaking because methods had been worked out for the dissociation and fractionation of spermatogenic cells from this organism (Risley and Eckhardt, 1979a). More importantly, these fractionated spermatogenic cells remain viable and continue to differentiate in culture (Risley and Eckhardt, 1979b). Additional advantages for the use of Xenopus were the large amount of nucleated erythrocytes which could be easily obtained from this organism plus the existence of a rapidly growing, non-tumorous, continuous Xenopus cell line, Xl 177.

During the course of this work it became evident, especially from the observations made in studies using spermatocytes and erythrocytes, that cytoskeletal-nuclear interactions were potentially important to the understanding of nuclear functions, position and structure. Thus, a much greater emphasis was placed on isolating nuclear structures which preserved associations with cytoskeletal elements and less emphasis was placed on obtaining nuclei free of "cytoplasmic contamination". Because the work presented in this thesis involves studies of both areas of the cell; the pertinent background information will be given in the introduction of subsequent chapters.

The data presented in the last chapter demonstrates the role calcium plays in the activation of the depolymerization of the Xenopus erythrocyte marginal band microtubules in addition to initiating other changes in the cytoskeleton. The stability of frog marginal bands in intact erythrocytes to cold and colchicine was originally demonstrated by Behnke (1970). His observations are extended in this chapter to include stability when isolated as part of the erythrocyte cytoskeleton

as well as when isolated in medium of relatively low pH. Moreover, evidence is presented which indicates that calcium does not act directly to alter erythrocyte cytoskeletal elements but may activate calmodulin requiring soluble cytoplasmic substances.

CHAPTER TWO

XENOPUS LAEVIS SPERMATOGENIC CELL NUCLEAR MATRICES

INTRODUCTION

Evidence for a non-chromatinic structural framework in eukaryotic nuclei dates back approximately twenty years (Zbarsky et al., 1962). It has only been recently, however, that the functional properties of this structure have begun to be carefully scrutinized. Evidence now exists which suggests that the nuclear matrix may be a multifaceted structure involved in such diverse nuclear processes as DNA replication, RNA metabolism organization of DNA into looped domains as well as steroid hormone receptor binding (for recent reviews see, Berezney, 1979; Agutter and Richardson, 1980).

Given the varied and dynamic nature of the nuclear matrix, we became interested in possible compositional or structural changes in spermatogenic cell nuclear matrices which may reflect the dramatic changes in nuclear structure and function during spermatogenesis. In particular, we were concerned with the changes which might occur during chromosome condensation and synapsis. Comings and Okada (1976) have reported that some nuclear matrices isolated from mouse testes nuclei contain synaptonemal complexes (SCs), and therefore, may help mediate the chromosome interactions of meiotic prophase. Methods employed by Walmsley and Moses (1981), however, failed to yield intact SC containing matrices from nuclei of Chinese hamster testes. Moreover, Stick and Schwartz (1982) using immunochemistry and electron microscopy, were unable to detect the NPLC component of the nuclear matrix in post-spermatogonial cell types from chicken, mouse or the frog, Xenopus laevis.

This chapter demonstrates that nuclear matrices containing structurally integrated SCs can be isolated from Xenopus laevis

spermatocytes. Nuclear matrices were isolated from spermatogonia and spermatocyte nuclei using a method which is relatively gentle and rapid compared to other matrix isolation procedures. The nuclear matrices obtained were similar in their overall size and shape to isolated nuclei. Spermatogonial matrices consisted of a prominent nucleolar matrix connected to a continuous nuclear pore-lamina complex (NPLC) by an extensive fibrogranular network. In contrast to the spermatogonial matrices, the spermatocytes nuclear matrices showed discontinuities in the NPLC, a less dense intranuclear fibrogranular network, and a more stable association with cytoplasmic structures. Many spermatocyte nuclear matrices contained SCs pervading the inner matrix and terminating on the NPLC. Depletion of the fibrogranular network and the discontinuities in the nuclear pore-lamina complex were most pronounced in those matrices containing SCs. Gaps in the NPLC were also observed in isolated nuclei as well as cyto-nuclear skeletons prepared from cells which had been detergent lysed without mechanical disruption.

These observations indicate that the nuclear matrix undergoes substantial changes in meiotic prophase, particularly during the period of chromosome synapsis and movement. Although these observations are probably indicative of important events which occur during meiotic prophase, their biological significance can for the most part only be speculated on. The nuclear matrix remains a collection of diverse unfractionated components which makes designating functional attributes to specific elements impossible at this time.

MATERIALS AND METHODS

Adult male Xenopus laevis laevis were obtained from the South African Snake Farm, Fish Hoek, Cape Province, South Africa. These animals were maintained in deionized water at 18-20°C and fed beef liver at least once a week. The frogs were injected with 200 units of human chorionic gonadotropin (Sigma Chemical Co., St. Louis, MO) two to four weeks prior to use in order to stimulate spermatogenesis.

Cell Fractionation

Cell populations enriched for primary spermatocytes, but containing spermatogenic cells ranging from spermatogonial to pre-elongation stage spermatids, were isolated as previously described in detail (Risley and Eckhardt, 1979a) with a few modifications. Testes were removed from decapitated, pithed, adult males and rinsed once in OR2 (Wallace et al., 1973). They were then placed in 0.4% (w/v) collagenase (Type I, Sigma Chemical Co.) prepared in Defined Nutrient Oocyte Medium (modified as described in Risley and Eckhardt, 1979b) and minced into small pieces. Dissociation was produced by alternate swirling and stirring at 30°C for approximately two hours. The dissociated cells were filtered through a 100 μm Nitex filter (Tetko Inc., Elmsford, NY) prior to collection by centrifugation at 1,750 rpm for 10 min in a Sorvall HB-4 Rotor (DuPont Co., Sorvall Biomedical Div., Newton, CT) at 5°C. The cells were then washed once with cold OR2. Cells were suspended in 12% Metrizamide (Accurate Scientific Corp., Hicksville, NY) prepared in OR2 (modified to maintain iso-osmolarity), overlayered with OR2, and centrifuged at 5,000 rpm for 20 min at 5°C in a Sorvall HB-4 rotor. After removal, the cells were diluted with 3 volumes of cold OR2 and pelleted at 2,500 rpm for 10 min in a Sorvall HB-4 rotor at 5°C.

Isolation of Nuclei and Nuclear Matrices

All procedures were performed at 4°C. The fractionated spermatogenic cell pellet was resuspended, triturated, and then gently homogenized with a Dounce type homogenizer (loose fitting pestle) in cell lysis solution (3.0 mM $CaCl_2$, 10 mM Tris-HCl, pH 7.2, 0.19 M sucrose, 0.5% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, PMSF; the PMSF was added just prior to use from a freshly prepared 50 mM stock in 95% ethanol). This reduced, but did not totally eliminate, cytoskeletal nuclear tags without any apparent deleterious effects on the isolated nuclear matrices. Nuclei were collected by centrifugation at 2,000 rpm for 10 min in a Sorvall HB-4 rotor and subsequently washed twice with nuclear wash and digestion solution (NWD: 3.0 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.2, 1 mM PMSF). Nuclei resuspended in NWD at 5 X 10^7 nuclei/ml were digested with 100 ug/ml of DNase I (Sigma Chemical Co., DN-EP grade) for 6-7 min. At the end of the digestion period, an equal volume of 4 M NaCl, 2-3 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.2 was added dropwise with gentle concurrent rotation of the tube. After 15 min of high salt extraction, matrices were pelleted by centrifugation at 2,000 rpm for 15 min in a Sorvall HB-4 rotor. The pellet was then gently resuspended in 2 M NaCl, 2-3 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.2 and recollected as described above.

Low speed centrifugations of the nuclear matrices during the high salt washes did not cause structural disruption but aggregates formed thus making ultrastructural analysis of individual structures difficult. Clumping was largely avoided if, instead of pelleting the material, the matrices were collected from a dense sucrose interface as described as follows. After the 15 min high salt extraction, the matrices were

layered over 2.4 M sucrose, 2-3 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.2, 1 mM PMSF, centrifuged as described above, and collected from the dense sucrose-high salt interface with a wide-bore pipette. The nuclear matrices were diluted with 2 M NaCl, 2-3 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.2, relayered over dense sucrose and recollected. The samples were then removed and added directly to fixative.

Electron Microscopy

The overall form of isolated nuclei and matrices was best maintained if they were fixed in suspension during preparation for electron microscopy. Unless otherwise indicated, all material prepared for electron microscopy was initially fixed in suspension by the addition of an equal volume of 3% glutaraldehyde, 0.1 M Na-cacodylate (pH 7.2). Glutaraldehyde fixation was carried out overnight at 4°C. All subsequent operations were performed at room temperature. Samples were washed with 0.1 M Na-cacodylate buffer before and after a 1 h post-fixation in buffered 1% osmium. Ethanolic dehydration was followed by sequential infiltration with propylene oxide, propylene oxide-Spurr (a 1:1 mixture) and pure Spurr prior to embedding. Silver and gold sections were cut with a diamond knife on a Sorvall MT-2 microtome and stained with uranyl acetate and lead citrate. All observations were made with a Philips EM 300 electron microscope operated at 80 Kv.

Protein Extraction and Analysis

Total nuclear protein was extracted from nuclei by first resuspending them in 8 M urea, 5% 2-mercaptoethanol and then adding 10% Na-dodecyl sulfate (SDS) to a final concentration of 2% followed by intermittent agitation in a vortex mixer. DNA was removed by ultracentrifugation at 40,000 rpm overnight in a Beckman type 50-Ti

rotor at 7°C. The samples were then stored frozen at -20°C until used. After the last high salt wash, nuclear matrices were washed with NWD and the pellet solubilized and stored as described above. Before electrophoresis, the samples were adjusted to 4.8 M urea, 1.5% SDS, 3% 2-mercaptoethanol, 75 mM Tris-HCl, pH 6.8 and analyzed by electrophoresis on 10% SDS-polyacrylamide slab gels according to the method of Laemmli (1970). Molecular weight markers were obtained commercially (BioRad Laboratory, Rockville Centre, NY). Gels were stained in 0.1% Coomassie Blue R-250, 30% methanol, 10% acetic acid and destained in 30% methanol, 10% acetic acid.

RESULTS

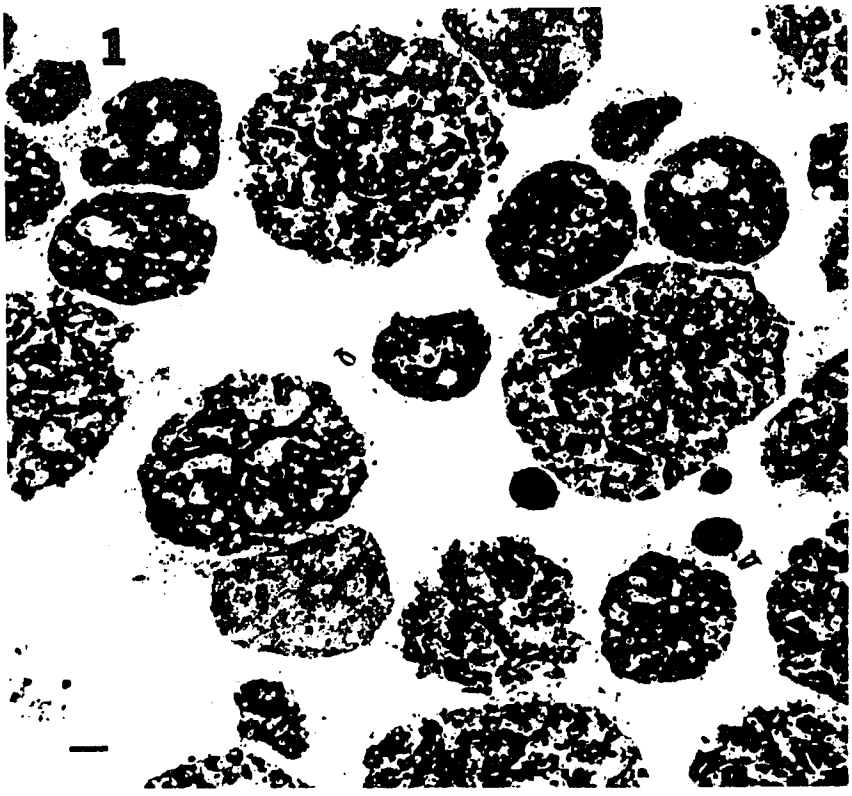
Nuclear Matrix Isolation and Handling

In order to develop a nuclear matrix isolation procedure which minimized morphological distortion, experiments were conducted to study the effects of particular ions and ion concentrations on isolated spermatogonial and spermatocyte nuclear structure. It was determined that, although low divalent cation concentrations (<2mM) have been successfully used in nuclear matrix isolation protocols for many cell types (Berezney, 1979; Agutter and Richardson, 1980), such concentrations adversely influence the stability and morphology of nuclei from spermatogenic cell types (also see, Kalt, 1979).

Spermatogenic cell nuclei isolated by cellular lysis in buffers containing Triton X-100 and divalent cation concentrations (Ca^{2+} or Mg^{2+}) of 2.5 mM or greater were ultrastructurally well preserved and did not aggregate even after repeated low speed centrifugation. These nuclei (fig. 1) clearly lacked nuclear envelopes and had the overall appearance of nuclei from intact cells. Moreover, pachytene and zygotene stage nuclei had well preserved SCs (fig. 2a). Cytoplasmic tags were also observed attached to many nuclei because extensive purification procedures were not used.

Nuclear matrices were isolated without the use of EDTA or low ionic strength buffers. Isolated nuclei (5×10^7/ml) were first digested with 100 ug/ml of electrophoretically purified DNase I at 4°C for 6-7 minutes. The extensively single strand nicked chromatin was then released by the gradual addition, with concurrent mixing, of concentrated 4M NaCl to 2M. Phase microscopy revealed that the resultant structures had the overall size and shape of isolated nuclei

Figure 1. Electron micrograph of nuclei from spermatogonia through pre-elongation spermatid stages. Washed cell pellets were resuspended in cell lysis solution containing 2.5 mM $CaCl_2$ and gently homogenized in a Dounce homogenizer. The nuclei were pelleted and fixed by the direct addition of 1.5% glutaraldehyde, 0.1 M Na-cacodylate, ph 7.4. Bar equals 1 μm (x5000).


1

although there was a noticeable reduction in nuclear material. Thin section ultrastructural observations showed that the nuclear matrices obtained were relatively free of chromatin and were bordered by a NPLC. Zygotene-pachytene spermatocyte matrices contained well preserved SCs and the lateral elements, which were obscured by chromatin in isolated nuclei, could be seen easily (fig. 2).

It is important to note that only spermatogonial and spermatocyte nuclear matrices were obtained by the methods described despite the presence of numerous (approximately 50%) spermatid stage nuclei in the starting nuclear preparations. Approximately 75-80% of the isolated spermatogonial and spermatocyte nuclei were recovered as nuclear matrices based on hemocytometer counts.

Electrophoresis of SDS-urea solubilized proteins of nuclei and nuclear matrices isolated from cells obtained at the 12% Metrizamide/OR2 interface showed the absence of histones providing further evidence for the depletion of chromatin from the matrix preparations (fig. 3). Otherwise, the proteins of isolated nuclei and nuclear matrices had similar electrophoretic profiles. In addition, low molecular weight proteins or polypeptides (<14,300) were not detected in the matrix preparations. These observations suggest that proteolysis was inhibited during the matrix isolation procedure by the protease inhibitor PMSF and by conducting all operations at 4°C. Sodium bisulfite (50 mM), another protease inhibitor, has also been used in the isolation procedure without adverse effects on matrix morphology.

The spermatogenic cell nuclear matrix preparations showed a greater heterogeneity of polypeptides than matrix protein preparations reported from other cell types (Berezney, 1979). This might be attributed to the

Figure 2. Electron micrographs of: (a), an isolated pachytene nucleus containing SCs in both transverse and longitudinal section, and (b), a spermatocyte nuclear matrix containing SCs with lateral elements (arrow) which are now visible as well as the NPLC (arrowhead). Bar equals 1 μm (x20,000).


b
2a

variety of matrix types present as well as the presence of SCs and cytoskeletal remnants. Many procedures often used to obtain nuclei free of cytoplasmic contamination such as vigorous homogenization, agitation on a vortex mixer, and centrifugation at high centrifugal forces through dense sucrose were avoided because they adversely affect spermatogenic cell nuclear matrix integrity (also see, Long et al. 1979).

The two nuclear matrix polypeptides located directly above the 68,000 molecular weight marker (fig. 3) comigrate with two of the major polypeptides of Xenopus erythrocyte cyto-nuclear skeletons (see chapter 3) and probably correspond to the 69,000 and 72,000 molecular weight lamins (LI and LII) found in Xenopus erythrocytes and cultured cells (Krohne et al., 1981).

Ultrastructural Comparisons of Nuclear Matrices from Different Stages

Spermatogonial nuclear matrices closely resembled matrices which have been described previously from somatic tissues (Berezeney, 1979; Agutter and Richarson, 1980). These structures were relatively small and had a prominent nucleolar matrix which was connected to the peripheral NPLC by a labyrinthine, fibrogranular network (fig. 4a). Spermatocyte nuclear matrices could be classified in two groups: 1. those of pre-leptotene/leptotene stages which lacked SCs and contained substantial fibrogranular material (fig. 4b) and 2. zygotene-pachytene stages which were in the process of synapsis and contained unpaired axial cores and SCs but which were depleted of fibrogranular material (fig. 2b, 4c and d). The SCs exhibited a characteristic tripartite structure consisting of paired lateral elements connected to a single central element by numerous transverse filaments (fig. 2b, 5a and b). SCs followed a tortuous route through the matrix as evidenced by the

Figure 3. Coomassie blue stained SDS slab gel. (A) Molecular weight markers from top to bottom 94,000, 68,000, 43,000, 30,000 21,000 and 14,300. (B) Nuclear matrix protein. (C) Total nuclear protein. *= H1 histone. [= core histones.

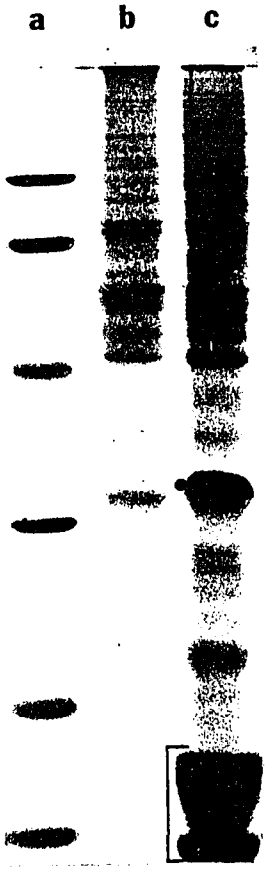
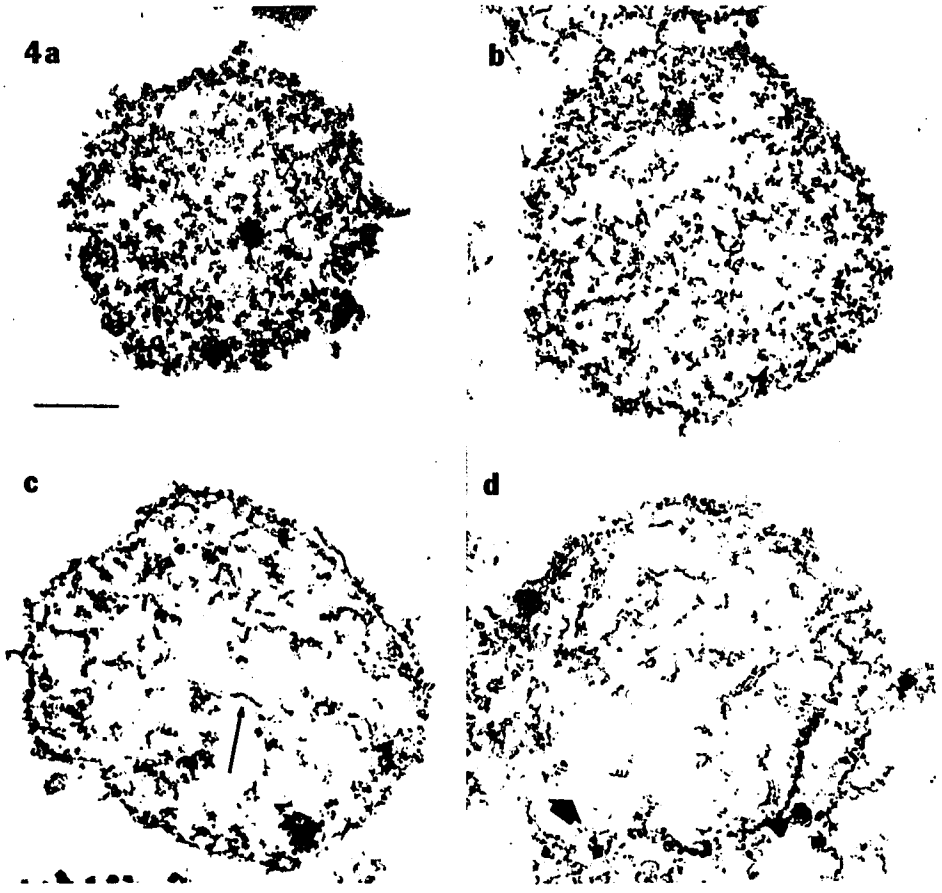

a
b
c

Figure 4. Electron micrographs of nuclear matrices from different spermatogenic stages. Nuclei and nuclear matrices from specific spermatogenic stages were identified using the following criteria: the relative size and abundance of the nuclear structures, nucleolar morphology, and the presence or absence of unpaired axial cores (early meiotic prophase) or SCs (zygotene-pachytene stages). (A) late secondary spermatogonial nuclear matrix. (B) spermatocyte nuclear matrix without distinct axial cores. (C) spermatocyte nuclear matrix with unpaired axial cores (arrow). (D) spermatocyte nuclear matrix containing SCs and a discontinuous NPLC (arrowhead). Bar equals 1 μm (x11,000) in all micrographs.

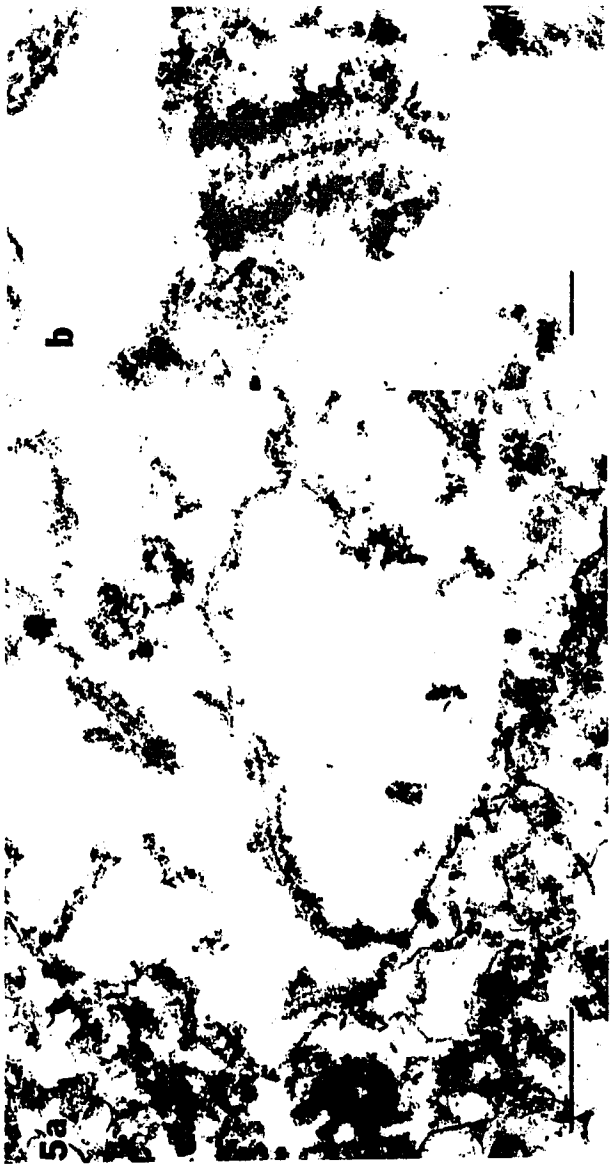

4a
b
c
d

numerous short glancing sections and were anchored at their termini to the NPLC which was often in direct association with residual cytoskeletal elements (see fig. 5a). Comparisons of the dimensions of the central and transverse elements in sections of intact pachytene cells and isolated nuclear matrices suggest that structural rearrangement or extraction of structural components was minimal.

As noted above, spermatocyte nuclear matrices possessed less inner fibrogranular material compared to spermatogonial matrices. The paucity of fibrogranular structures was most pronounced in late zygotene-pachytene matrices where complete SCs were abundant. It was common to observe sections of spermatocyte nuclear matrices where the SC and NPLC were the sole structural constituents. Isolated pachytene stage nuclei (fig. 6a) also have relatively little interchromatinic material between the synapsed homologues. Moreover, a reduction in the intranuclear fibrogranular material during meiotic prophase in Xenopus laevis was previously demonstrated by staining sections of testes with the Bernhard regressive staining technique (Kalt, 1973). These observations and the presence of fibrogranular networks in co-isolated spermatogonial nuclear matrices suggest that during meiotic prophase, the fibrogranular component of the nuclear matrix is either disassembled, incorporated into the SCs, or is altered in such a way that it becomes highly labile during the matrix isolation.

Another consistent difference between isolated nuclei and nuclear matrices containing SCs and those from spermatogonial and early meiotic prophase stages, was in the organization of the NPLC. Isolated zygotene-pachytene nuclei and nuclear matrices regularly were observed to contain gaps in their outer perimeter (fig. 4d and 6a). Such

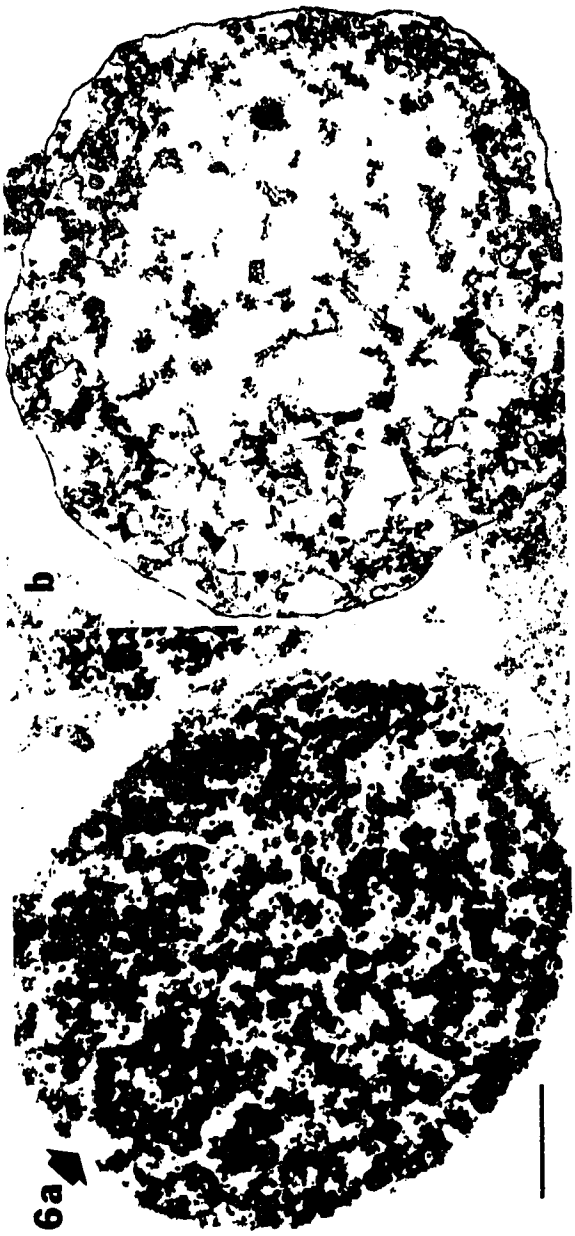
Figure 5. Electron micrographs of synaptonemal complexes. (A) SCs attached to the NPLC. (B) SC terminus showing the characteristic tripartite nature of this structure and the presence of intact transverse filaments. Bar equals 1 μm (x16,500) and 0.1 μm (x70,000) respectively.


b
5a

discontinuities were rarely observed in other spermatogenic stages and have not been seen in other Xenopus somatic cell matrices (see chapter 3).

In order to determine if the gaps in the NPLC were mechanically induced, the nuclear matrix isolation procedure was applied to cells lysed with Triton X-100 without homogenization resulting in matrices containing large cytoplasmic remnants and attachments (cyto-nuclear skeletons). Electron microscopy revealed that the zygotene-pachytene cyto-nuclear skeletons also exhibited characteristic discontinuities in the NPLC (fig. 6b). In addition, the NPLCs in these cyto-nuclear skeletons were often found in direct association with extensive and intricate residual cytoskeletal elements. The identity of the filaments in these cytoskeletal remnants is not certain, but it seems likely that intermediate filaments would be major constituents since the isolation conditions used (cold, DNase I, Ca^{2+}, 2 M NaCl) generally disrupt microfilaments and microtubular arrays. The outer boundary of these cyto-nuclear skeletons often contained a continuous surface layer which resembles the surface lamina previously observed in flat monolayer and spheroidal cell cytoskeletons (Ben-Ze'ev et al., 1979). This structure covers the cytoskeletal network and appears to be derived from the cell surface and plasma membrane proteins (Ben-Ze'ev, et al., 1979).

Figure 6. Electron micrographs of: (a) pachytene stage isolated nucleus with synapsed homologs viewed in cross section. Gaps in the peripheral lamina can be discerned (arrowhead). (B) cyto-nuclear skeleton containing SCs isolated from cells receiving no homogenization after lysis. The discontinuous nature of the NPLC makes it difficult, in certain regions, to distinguish easily the boundaries of the nuclear matrix. Bar equals 1 μm (x15,000).


b
6a

DISCUSSION

Ultrastructural examination of nuclear matrices isolated using rapid and gentle procedures from spermatogenic cells of Xenopus laevis showed that the matrix is structurally altered during the differentiation of spermatogonia into pachytene spermatocytes. The matrix contains less fibrogranular inner matrix components, SCs form and become attached to the NPLC and the nuclear pore-lamina develops structural discontinuities as well as increasingly stable associations with certain cytoskeletal elements. Further structural changes in the nuclear matrix probably also occur following pachytene since matrices were not recovered from spermatids although 50% of the isolated nuclei used for matrix isolations were from spermatids.

Several observations indicate that these changes in the nuclear matrix during meiotic prophase are not due simply to artifactual differences arising from the isolation procedures. First, spermatogenic cells were dissociated and fractionated by methods which allow the cells to live and continue to differentiate when placed in culture (Risley and Eckhardt, 1979b). Second, all spermatogenic cell nuclear matrices were co-isolated from a population of nuclei enriched for spermatogonia, primary spermatocytes and spermatids. Third, relative to methods used to prepare nuclear matrices from other cell types (Berezney, 1979; Agutter and Richardson, 1980), the methods used were both relatively rapid (approx. 75 min.) and gentle.

The structural integration between SCs and the NPLC complex was not unexpected since it had been suggested by whole mount electron microscopy that SCs could be co-isolated with mouse spermatocyte nuclear matrices (Comings and Okada, 1976). The inability of Walmsley and Moses

(1981) to isolate SC containing nuclear matrices from Chinese hamster spermatocytes could have resulted from the use of low $MgCl_2$ concentrations (which caused disruption of Xenopus spermatocyte nuclei), mechanical fragmentation of the fragile spermatocyte NPLC or proteolytic disruption of the matrices resulting from the absence of protease inhibitors in the isolation solutions. Stick and Schwartz (1982) also failed to use proteolytic inhibitors. Therefore, proteolysis may have caused the disintegration of the NPLC in the isolated spermatocyte nuclei they examined. In this chapter, two abundantant proteins are shown which are present in isolated nuclei and nuclear matrices which I have observed co-migrating with the nuclear lamins (LI and LII) of Xenopus erythrocyte on SDS gels. Failure of immunofluorescent procedures to detect nuclear lamins in post-spermatogonial stages might be due to a specific masking of the antigenic determinants in spermatocytes and spermatids.

Although the differences between spermatogonia and spermatocyte intranuclear matrix morphology, as well as the inability to isolate matrices from spermatids, have doubtless functional significance; it is not possible at this time to make any firm biological interpretations. These observed changes in the intranuclear matrix may reflect reorganization and/or disassembly of matrix components but alternatively, they might also reflect biologically induced alterations in matrix elements which make them susceptable to extraction in ways they are not in spermatogonia and in the cell types studied by others.

The significance to spermatogenesis of the depletion of the inner fibrogranular matrix during meiotic prophase is also unclear because the role of this matrix constituent has not been precisely defined. The

inner fibrogranular matrix has been implicated in the processing of hnRNA (Agutter and Birchall, 1979; Long et al., 1979). Precursors for ovomucoid and ovalbumin mRNA have recently been found in specific association with the nuclear matrix (Ciejek et al., 1981). Moreover, chicken (La Fond and Woodcock, 1981) and Xenopus (Gambino et al., 1981) erythrocytes inactive in mRNA synthesis yield nuclear matrices completely devoid of an inner fibrogranular matrix. The depletion of the pachytene spermatocyte inner matrix seems contradictory to its proposed hnRNA processing role since these cells are active in mRNA synthesis (Geremia et al., 1978; Kalt, 1979). Pachytene spermatocytes however, differ from most other cell types in that much of the RNA synthesized by these cells is packaged in a non-translatable storage form for translation during spermatid stages days to weeks after pachytene (Geremia et al., 1977; Iatrou et al. 1978). Thus, one might speculate that depletion of the inner matrix in these cells may be the result, or cause, of a change in the usual sequence of hnRNA processing. A change which could be related to the mechanism of mRNA storage.

In contrast to the observations made on the fibrogranular matrix material, the changes in the NPLC are less ambiguous. This structure has been morphologically well characterized (for a recent review see, Franke et al., 1981). Moreover, the changes reported here are in keeping with those noted by others in whole nuclei.

The structural basis of the increased discontinuity and fragility of the spermatocyte NPLC is not clear. Nuclear matrices isolated from many cell types usually contain continuous, stable NPLCs (Berezney, 1979; Agutter and Richardson, 1980). However, a fibrous lamina situated on the nucleoplasmic surface of the inner nuclear membrane has not been

observed in some cells, including spermatocytes (Franke et al., 1981). The NPLC of spermatocyte nuclear matrices may consist of a delicate interpore network made up of residual membrane proteins. A simillar origin has been proposed for the interpore network associated with the isolated NPLC of Xenopus oocytes (Scheer et al., 1976).

It seems likely that since SCs and the NPLC are structurally integrated, they may also be functionally integrated. The fragile and discontinuous nature of the spermatocyte NPLC may reflect a specific organization of this residual membrane structure which is necessary for membrane mediated movements of the meiotic chromosomes and changes in the distribution of the nuclear pores during meiotic prophase of spermatogenesis. Early in meiotic prophase, SC and chromosome attachment sites are known to cluster on one side of the inner nuclear membrane resulting in the formation, during syapsis, of what has been termed the chromosome bouquet (Moens, 1972, 1973; Gillies, 1975). During pachytene, the attachment sites again move in the plane of the nuclear membrane and become more evenly distributed. Nuclear pore distribution also changes from a relatively dispersed arrangement in spermatogonia to clustered arrangements in spermatocytes. Spermatocyte nuclear pores cluster on the side of the nucleus which contains the SC attachment sites resulting in membrane regions which are devoid of nuclear pores (La Cour and Wells, 1972; Church, 1976, 1977; Fawcett and Chemes, 1979). Since the SCs and the nuclear pores are both attached to the NPLC, the NPLC must exhibit some capacity of being reorganized during the meiotic prophase of spermatogenesis (Fawcett, 1966).

The data presented in this chapter demonstrates that components of the cytoskeleton remain attached to the NPLCs of isolated spermatocyte

cyto-nuclear skeletons. Interactions between the cytoskeleton and the NPLC might directly mediate movements of SC attachment sites of meiotic chromosomes. Cytoskeletal elements could, potentially, generate the forces required for chromosome-SC movements annd specifically direct these movements through a cyto-nuclear skeleton consisting of the SCs, the NPLC, and a cytoskeleton possibly organized by centrioles. In this regard, it is noteworthy that the attachment sites are known to cluster on the side of the nuclear membrane which is adjacent to the centrioles in the cytoplasm (Moens,1969, 1972; Church, 1976; Rasmussen, 1976).

CHAPTER THREE

NUCLEAR STRUCTURE AND CYTOSKELETAL-NUCLEAR ASSOCIATIONS IN XENOPUS ERYTHROCYTES

INTRODUCTION

Xenopus laevis erythrocytes are biconvex, elliptical cells which contain cytoplasmic morphological features shared with other non-mammalian vertebrate erythrocytes. These features include the following: 1. a peripheral bundle of cytoplasmic microtubules (the marginal band) that lie in the plane of cellular flattening (Fawcett, 1959) and which are thought to be involved in the formation, rather than the maintenance, of cell form, and 2. a submembranous cytoskeletal network made up in part of actin and spectrin (or spectrin-like) proteins (Bartelt et al., 1982). In addition, the Xenopus erythrocyte cytoskeleton, as shown below, contains fibrogranular and intermediate filament components, as well as centrioles, within the region between the nucleus and the subsurface cytoskeletal shell. Intermediate filaments have also been observed in avian erythrocytes (Woodcock, 1980; Granger and Lazerides, 1982), however they were not reported in a study of the dogfish erythrocyte cytoskeleton (Cohen et al., 1982). These structural elements may serve an important function in the determination of nuclear shape and position.

Observations presented here, that chromatin depleted nuclei lack any internal nuclear matrix elements, raise the possibility that maintenance of characteristic nuclear morphology and position within these erythrocytes might result from constraints placed on the nuclear lamina by cytoskeletal elements. The nuclear lamina in these preparations is maintained in the overall shape of intact nuclei and remains in association with centrioles, intermediate filaments and the subsurface cytoskeletal shell.

Furthermore, the demonstrated lack of an intranuclear fibrogranular

matrix component in these nuclei, which are inert in terms of replication and transcription, supports the view that in more synthetically active nuclear types these elements are dynamic structures which participate in, or are the result of, nuclear metabolism (for reviews see Berezney, 1979; Agutter and Richardson, 1980). Absence of this matrix component also implies that unlike most eukaryotic cells studied, the DNA of these erythrocytes is not organized into supercoiled looped domains. This would be consistent with biophysical data which indicates that mature hen erythrocyte DNA is not organized in this manner (Cook and Brazell, 1976).

Furthermore, work is presented which indicates that maintenance of characteristic nuclear position and morphology in Xenopus erythrocytes may be disrupted by an increase in intracellular calcium. Experimentally induced changes in nuclear and cell shape were detected by exposing erythrocytes, in the presence of calcium, to the divalent cation ionophore A23187 (Reed and Lardy; 1972) for varying periods of time prior to fixation. Light and electron microscopic examination of these samples indicated that this treatment produced both nuclear and cellular rounding. In addition, nuclei were often dislodged from their central position within the cell. The observation that some cells fixed shortly after the addition of ionophore retain a flattened shape, but have spherical nuclei, suggest that the noted changes in cellular and nuclear morphology need not be coordinated. The divalent cation ionophore A23187 appears to be a potentially promising tool which can be used in the detection of the biochemical and ultrastructural changes that occur in the Xenopus erythrocyte nuclear lamina and/or cytoskeleton which result in the loss of characteristic erythrocyte features.

MATERIALS AND METHODS

Adult male Xenopus were purchased from the South African Snake Farm, Fish Hoek, Cape Province, South Africa and maintained in deionized water at 18-20° C. The frogs were fed beef liver at least once a week. The ionophore A 23187 was bought from Calbiochem-Behring Corp.(La Jolla, CA) and electrophoretic grade DNase I from Sigma Chemical Co.(St. Louis, MO). All reagents required for electrophoresis were obtained from Bio Rad Laboratory (Rockville Centre, NY).

Isolation of Cytoskeletons and Cyto-nuclear Skeletons

Decapitated, pithed, adult male Xenopus were bled into Microtubule Stabilization Wash Solution (150 mM NaCl, 1 mM $MgSO_4$, 5 mM EGTA, 20 mM PIPES, pH 6.9) as described by Sloboda and Dickerson (1980), and pelleted at 365 g for 5 min. All centrifugations were carried out in a Sorvall HB-4 Rotor. The supernatant, plus the top layer of leukocytes, were carefully pipetted off and the erythrocytes washed again using the same regimen.

All operations after this point were conducted in an ice bath or in a centrifuge at 0°C. Cytoskeletons were obtained by gently resuspending the cell pellet in a large volume (25 ml/ 1 ml packed erythrocytes) of ice cold lysis solution (0.19 M sucrose, 100 mM KCl, 2.5 mM $MgCl_2$, 10 mM PIPES pH 6.9, 0.3% Triton X-100 and 1 mM Phenylmethylsulfonyl fluoride, PMSF). PMSF was added just prior to use from a 50 mM stock in 95% ethanol. Cytoskeletons were collected by centrifugation at 600 g for 8 to 10 min. and subsequently washed once in wash solution (lysis solution without Triton X-100). After resuspension in Cytoskeletal Digestion Solution (CDS; 2.5 mM $MgCl_2$, 10 mM PIPES pH 6.9, 1 mM PMSF) at a

concentration of 10^8/ml, cytoskeletons were digested with 100 ug/ml of DNase I for 15 min.. Chromatin was then extracted by the gradual addition of an equal volume of 4 M NaCl, 2.5 mM $MgCl_2$, 10 mM PIPES pH 6.9. After 15-20 min. of high salt extraction, the sample was diluted with two volumes of CDS and the cyto-nuclear skeletons were pelleted at 650 g for 15 min.. Next the pellet was re-extracted with 2M NaCl, 2.5 mM $MgCl_2$, 10 mM PIPES pH 6.9, diluted with CDS and recollected. The cyto-nuclear skeletons were washed once with only CDS prior to solubilization with SDS and urea.

Partially chromatin-depleted nuclei were produced without exposure to high salt by the following method. Cytoskeletons were treated with DNase I as described above, with the exception that digestion was extended to 30 min.. At the end of this period an equal volume of 0.1 M EDTA, pH 7.0 was added and the sample allowed to stand for 20 min. The specimens were then fixed in suspension by the addition of an equal volume of 3% glutaraldehyde, 0.1 M Na cacodylate, pH 7.2.

Ionophore A23187 Studies

Frogs were bled into a solution consisting of 80 mM NaCl, 10 mM KCl, 1.35 mM $CaCl_2$ and 2 mM $NaHCO_3$. The erythrocytes were then collected and resuspended in the same solution. Phase contrast and Nomarski interference microscopy indicated that characteristic erythrocyte morphology was maintained in this solution. To experimental samples, divalent cation ionophore A23187 was added to a final concentration of 3 uM. Aliquots were taken at the times indicated, mixed with an equal volume of fixative, and then processed for light and electron microscopy as described below. To assay for the presence of marginal bands, cells were pelleted in a clinical centrifuge, the

supernatant removed and the cells resuspended in Microtubule Stabilization Wash Solution. Cell lysis was produced by the addition of a few drops of 20% Triton X-100. As previously described by Cohen (1978), this procedure allows detection of the marginal band by phase contrast microscopy.

Protein Extraction and Analysis

Cytoskeletal protein was obtained by first resuspending cytoskeletons in 8 M urea, 5% 2-mercaptoethanol (stored in aliquots at -20°C) and then adding 10% SDS to a final concentration of 2%. Samples were then kept refrigerated at 5°C for approximately 2 h. with intermittant agitation in a vortex. DNA was removed by ultracentrifugation at 60,000 g for 14 h. in a Beckman type 50-Ti Rotor at 5 C. The protein samples were stored frozen at -20 C until they were to be characterized. Cyto-nuclear skeletons were washed once with CDS after the second high salt treatment and then solubilized as described above. Prior to electrophoresis, samples were adjusted to 4.8 M urea, 1.5% SDS, 3% 2-mercaptoethanol, 75 mM Tris-HCl, pH 6.8 and analyzed by SDS-polyacrylamide slab gels according to the method of Laemmli(1970). Molecular weight markers were purchased commercially (Bio Rad Laboratories). Chick muscle actin was obtained from Sigma Chemical Co.. Gels were stained overnight in 0.1% Coomassie blue R-250, 25% methanol and 10% acetic acid and destained in 25% methanol, 10% acetic acid.

Microscopy

All material for thick and thin section microscopy was fixed in suspension by the addition of an equal volume of 3% glutaraldehyde, 0.1 M Na cacodylate, pH 7.2. Glutaraldehyde fixation was carried out at room temperature for 1.5 h.. Samples were washed with 0.1 M Na

cacodylate buffer before and after a 1 h. post-fixation in buffered 1% osmium performed at 4 C. Cytoskeletons and cyto-nuclear skeletons, but not erythrocytes, were then washed twice in distilled water and stained en bloc with 1% aqueous uranyl acetate for 1 h. These samples were then washed twice, again with distilled water. All samples were dehydrated in ethanol followed by sequential infiltration with propylene oxide, propylene oxide-Spurr resin (a 1:1 mixture) and pure Spurr prior to embedding. Thick sections (1-2 um) were cut with glass knives on a Sorvall MT-2 microtome and stained with 1% toluidine blue, 0.5% Na borate. Silver and gold sections were cut with a diamond knife and stained with uranyl acetate and lead citrate. Phase contrast observations and light photomicrographs were obtained using a Zeiss Photomicroscope II with Kodak Plus X film. Ultrastructural observations were made with a Philips EM 300 electron microscope at 80 kv.

RESULTS

Erythrocyte and Erythrocyte Cytoskeleton Ultrastructure

Xenopus erythrocytes are elliptical cells which possess a centrally located, distinctly oval nucleus with highly condensed chromatin and a paucity of nuclear-pore complexes (figure 7A). The presence of hemoglobin obscures much of the cytoskeleton of these cells when viewed by electron microscopy. However, if erythrocytes are gently lysed in buffers containing Triton X-100, the cytoskeleton (figure 7B and 8) contained the following structures: 1. a subplasma membrane cytoskeletal network (SPMCN), 2. a peripheral bundle of cytoplasmic microtubules which encircle the cell in the plane of flattening and which are termed the marginal band (Fawcett, 1959) plus, 3. fibrogranular material as well as intermediate filaments which pervade the space between the nucleus and the SPMCN and which, in some instances, can be observed connecting these structures. In addition, a single or pair of centrioles are frequently observed in thin sections of cytoskeletons. In contrast with blood cells of molluscan invertebrates (Nemhauser and Cohen, 1980), these organelles are perinuclear in location and are not associated with the marginal band.

Cyto-nuclear Skeleton Ultrastructure

Nuclear matrices were isolated from cytoskeletons by procedures designed to preserve their association with cytoskeletal components and to minimize morphological disruption. We have termed these composite structures of the nuclear matrix and residual cytoskeleton, cyto-nuclear skeletons. The specific cytoskeletal elements found depended on the method used to deplete chromatin from the nuclei. Chromatin could be extracted from nuclei by limited DNase I digestion followed by treatment

Figure 7. Thin section electron micrographs. A) A portion of a whole erythrocyte illustrating the characteristically oval nucleus with highly condensed chromatin found in this cell. The nuclear envelope appears as a white border around the nucleus and three nuclear pores can be observed within this structure in this section. Hemoglobin obscures the cytoskeleton. (x23,000). B) Low magnification of erythrocyte cytoskeletons showing the general preservation of the oval nuclear shape. (x9,500).

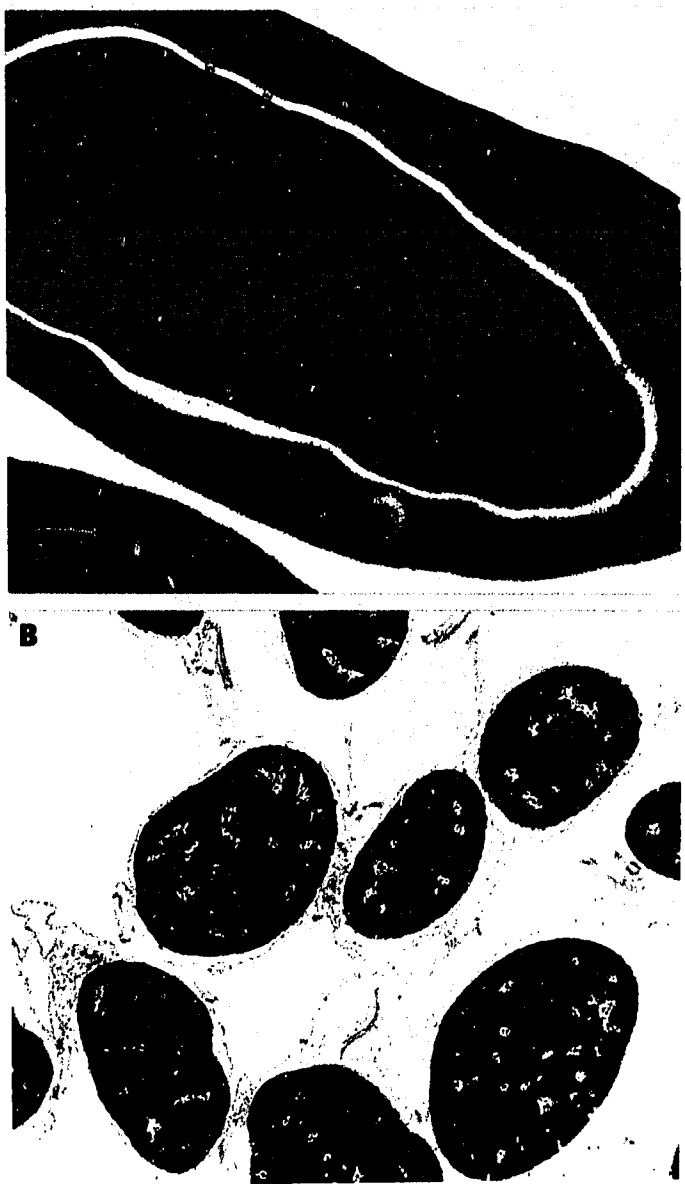
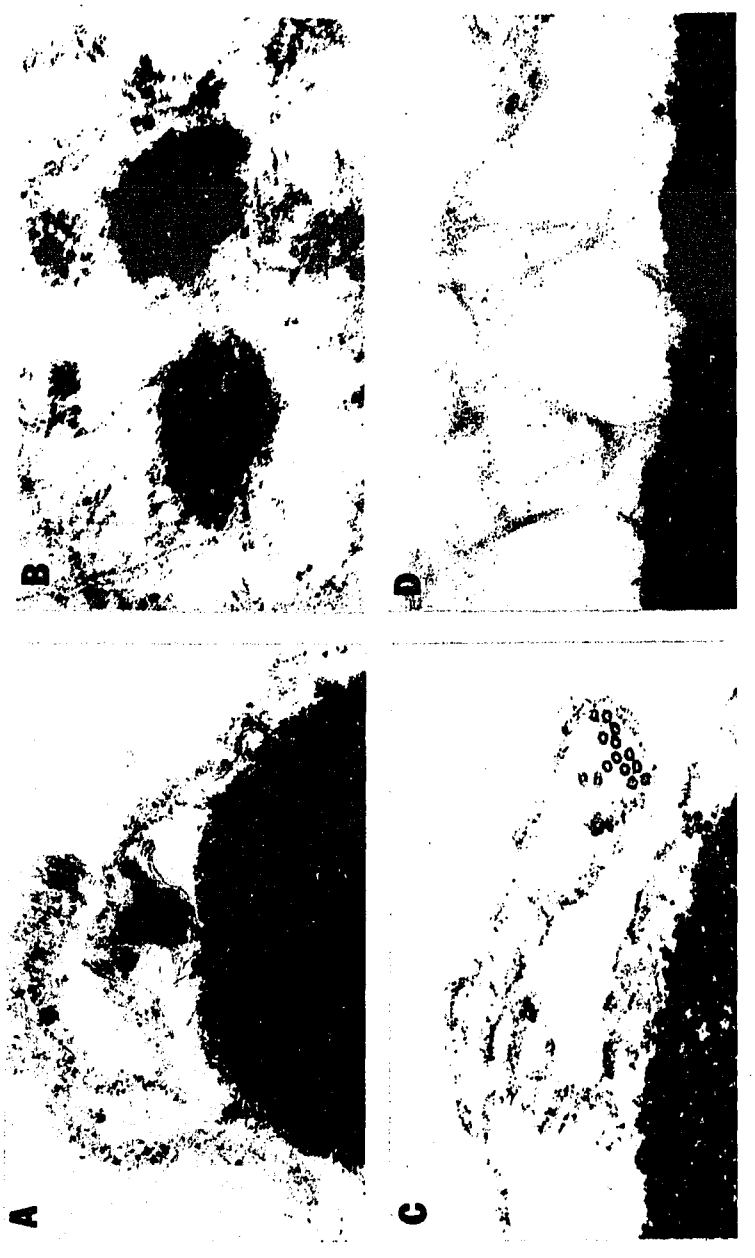

B

Figure 8. High magnification electron micrographs of erythrocytes cytoskeletal elements. (A) A perinuclear centriole, (B) a pair of centrioles surrounded by intermediate filaments and fibrogranular material, (C) cross-section through marginal band microtubules and (D) intermediate filaments extending from the nucleus to the subplasma membrane cytoskeletal network. Magnifications (x46,666; x89,000; 49,000; 94,300 respectively).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with either high salt or EDTA containing solutions.

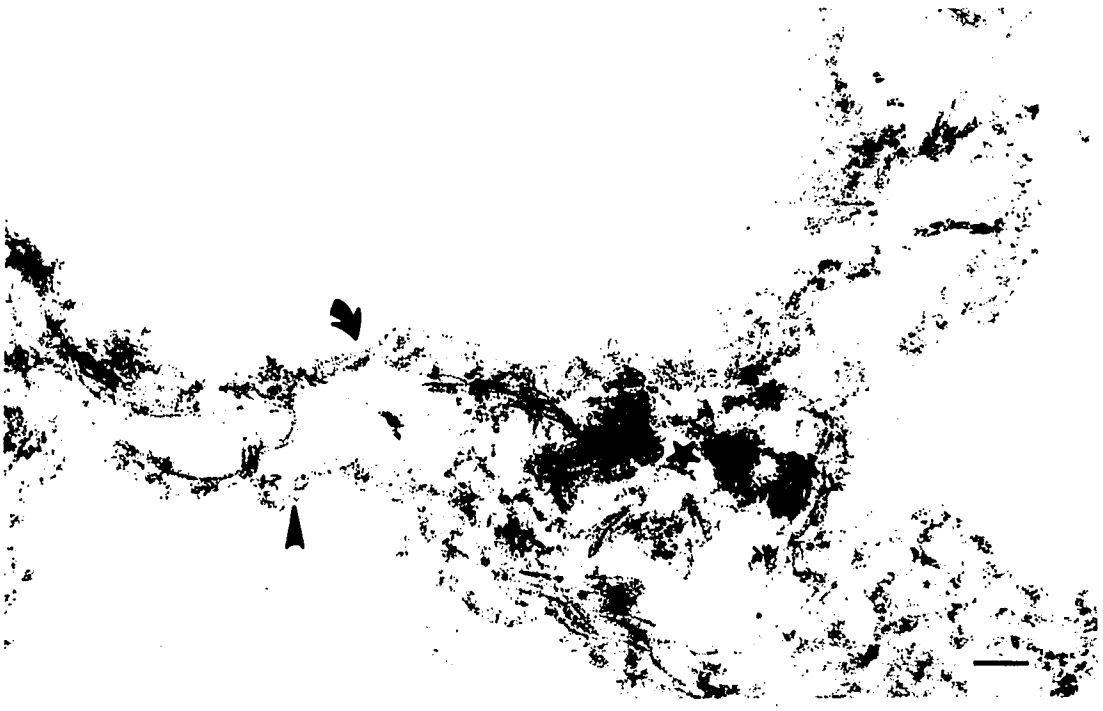
High salt extracted erythrocyte cyto-nuclear skeletons were obtained by digesting cytoskeletons (10^8/ml) on ice with 100 ug/ml of electrophoretic grade DNase I for 15 min. at $0^{\circ}C$. This digestion period is sufficient to render approximately 20% of the DNA acid soluble (data not shown), in addition to producing extensive single strand nicks in the remaining acid insoluble fraction. The gradual addition of NaCl to a concentration of 2M resulted in the removal of chromatin. Phase contrast microscopy of the cyto-nuclear skeletons showed them to contain centrally located nuclei which appeared devoid of nuclear material (figure 9A). The marginal band, which could be discerned in the starting cytoskeleton preparations by phase microscopy, was no longer apparent. Routinely, greater than 80% of the starting cytoskeletons were recovered as cyto-nuclear skeletons as judged by hemocytometer counts of the structures just subsequent to the first high salt extraction. Quantitation by this method becomes unreliable after the first centrifugation because the cyto-nuclear skeletons develop a greater tendency to form aggregates although this is not severe.

Ultrastructural examination of thin sections of this material (figure 9B and 10) showed these cyto-nuclear skeletons to contain roughly oval shaped nuclear matrices, clearly devoid of intranuclear matrix components. Thus nuclear matrices from this cell type consist essentially of a characteristically pore-complex scarce, nuclear lamina. This is in contrast to nuclear matrices from more metabolically active cells which contain extensive intranuclear fibrogranular networks as well as a prominent nucleolar matrix (Berexney, 1979; Agutter and Richarson, 1980). The absence of intranuclear matrix elements in

Figure 9. (A) Phase contrast micrograph of high salt treated cyto-nuclear skeletons. The residual nuclei can be observed within their typical central position. Bar equals 10 μm (x750). (B) Electron micrograph of high salt treated cyto-nuclear skeletons. The nuclear lamina is maintained in the overall form of intact nuclei and is shown to be the only nuclear matrix component present. Bar equals 1 μm (x6000).


A
B

Figure 10. A high magnification electron micrograph of a portion of a high salt treated cyto-nuclear skeleton. Intermediate filaments are seen within the region between the nuclear lamina (arrow) and the SPMCN (arrowhead). Centrioles (star), surrounded by a network of intermediate filaments, are retained in their perinuclear position. Bar equals 0.1 μm (x55,000).



Xenopus erythrocyte nuclei appears not to be a result of the matrix isolation procedure employed. When basicially the same isolation procedure was used on Xenopus spermatogonia and spermatocytes, intranuclear matrix components, including synaptonemal complexes in zygotene-pachytene stage spermatocytes, were preserved (see chapter one).

In addition to the nuclear lamina, the cyto-nuclear skeletons contained continuous SPMCNs, centrioles in their characteristic perinuclear position, as well as fibrogranular and filamentous structures extending from the nucleus to the cytoskeletal shell. Based on their ultrastructure (approx. 90-110 Å) in diameter), high salt stability and sensitivity to low ionic strength conditions, we have designated the filamentous structures as intermediate filaments. Recently work on avian erythrocyte cytoskeletons, using a variety of approaches, has demonstrated the association of intermediate filaments with both the nucleus (Woodcock, 1980) and the SPMCN (Granger et al., 1982). The only cytoskeletal elements conspicuously absent from Xenopus erythrocyte cyto-nuclear skeletons were the peripheral bundle of cytoplasmic microtubules.

Given the absence of internal matrix elements in cyto-nuclear skeletons produced by high salt extraction, we became interested in determining whether the architectural framework of these nuclei might consist of structural elements which were high salt labile. In order to determine this, another tack was taken to remove chromatin from partially digested nuclei.

Cytoskeletons were once again digested in the cold with DNase I but for twice the time used to produce the high salt extracted cyto-nuclear

Figure 11. Thin section electron micrograph of a partially chromatin depleted, EDTA treated, erythrocyte cyto-nuclearskeleton. The arrow points to a cross-section of marginal band microtubules. Bar equals 0.5 um (x28,000).

skeletons. This was followed by the addition of an equal volume 0.1 M EDTA, pH 7.0. After a 15 min. extraction period the preparations were fixed by the addition of buffered glutaraldehyde and then processed for electron microscopy. The sequestering of divalent cations by EDTA destabilizes higher order levels of chromatin organization (Thoma et al., 1979) allowing the unraveling and release of large amounts of partially digested chromatin. Phase contrast microscopy of these structures suggested that they had been a least partially depleted of chromatin. Moreover, in many instances, the marginal band could still be observed. Ultrastructural observations revealed that substantial, but not complete chromatin extraction was achieved by this procedure (figure 11). However, in the large chromatin free areas found within the nuclei of these structures no internal matrix elements could be detected (figure 11).

While nuclease digestion followed by EDTA extraction has been reported to produce complete chromatin depletion in nuclei from tissue culture cells (Long, 1981); we have not been able to obtain this with Xenopus erythrocytes. Additional DNase digestion and EDTA wash steps lead to a complete decondensation of the remaining chromatin, thus obscuring the intranuclear space. In any event, this approach has allowed us to confirm the findings obtained using high salt. It is also noteworthy that microtubules were ultrastructurally detectable in the EDTA treated samples while the filamentous structures found in cytoskeletons and high salt treated cyto-nuclear skeletons in the region between the nuclear lamina and SPMCN were no longer clearly observable. Interestingly, fibrogranular structures were retained in this region. The former observation is consistant with the known sensitivity of

intermediate filaments to low ionic strength conditions.

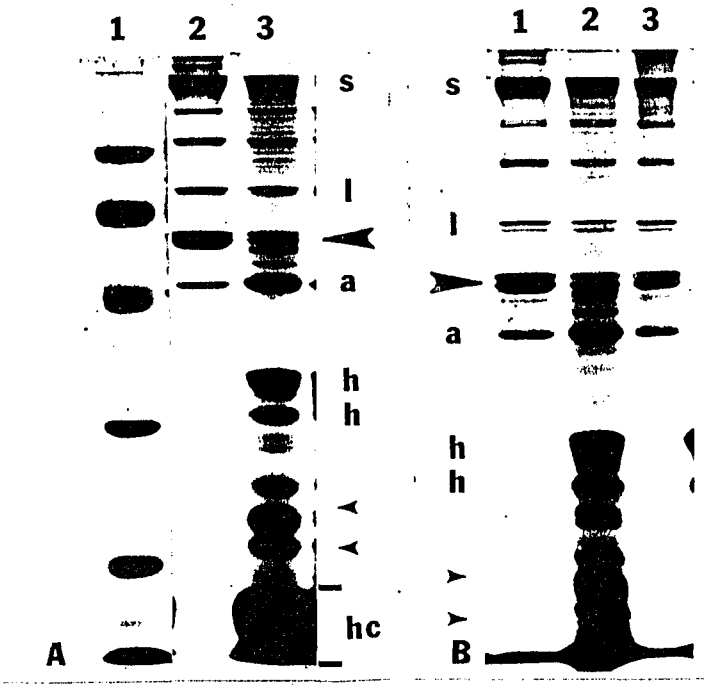
SDS Polyacrylamide Gel Electrophoresis

Electrophoretic characteriztion of SDS-urea solubilized proteins of high salt extracted cyto-nuclear skeletons (figure 12) showed, as expected from the ultrastructural observations, that these structures were totally depleted of the histones. Moreover, polypeptides which might be expected to be found in the SPMCN, based on studies done in other vertebrates (Branton et al. 1981; Bartelt et al., 1982), were also noted. Specifically, a 43,000 molecular weight polypepetide which co-migrates with chick muscle actin and a group of (>150,000) polypeptides with molecular weights similar to spectrin and the spectrin associated polypeptides of human erythrocytes. Both DNase I and high salt are known to solubilize F-actin (Clarke and Spudich, 1977). This might explain the apparent decrease of actin in the electrophoretic profile of the cyto-nuclear skeletons compared to that of the cytoskeletons. This difference has no obvious effect on the integrity of the SPCN.

Two proteins with molecular weights of 69,000 and 72,000 respectively, which have been identified as the major polypeptides of Xenopus erythrocyte and cultured cell nuclear laminae (Krohne et al., 1981), were also observed in the electrophoretic profiles of cytoskeletons and cyto-nuclear skeletons. Consistant with the findings of Krohne et. al. (1981), the higher molecular weight component is the most abundant.

One unexpected finding in the electrophoretic characterization of the high salt extracted cyto-nuclear skeletons was the presence of two polypeptides with the characteristics of the tubulins. This observation was especially surprising since ultrastructural examination of these

Figure 12. (A) 11% SDS polyacrylamide slab gel electrophoresis. Lane 1, molecular weight markers from top to bottom, 94,000, 68,000, 43,000, 30,000 21,000 and 14,000. Lane 2, high salt treated cyto-nuclear skeleton protein. Lane 3, cytoskeletal protein. With the exception of the histones, the electrophoretic profiles of the cytoskeletons and cyto-nuclear skeletons look similar. Symbols: star (SPMCN proteins), l (nuclear lamina proteins), arrowhead (the tubulins), a (actin), h (H1 histones), hc (nucleosomal core histones), small arrowheads (probable H1 degradation products). (B) 9.5% SDS polyacrylamide slab gel electrophoresis. Lane 1 and 3, cyto-nuclear skeleton protein from separate isolations. The nuclear lamina proteins and the tubulins are better resolved than in the 11% gel. Lane 2, cytoskeletal protein, the core histones now run with the tracking dye.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cyto-nuclear skeletons gave no morphological evidence for the presence of cytoplasmic microtubules. These proteins were identified as the tubulins based on the following criteria: 1. molecular weight, 2. co-migration on SDS polyacrylamide gels with tubulin from Tetrahymena axonemes (see chapter four) and 3. their specific absence from the polypeptide profiles of cells lysed under conditions which cause the depolymerization of the marginal band microtubules as judged by phase contrast, electron microscopy and indirect immunofluorescence with tubulin anti-serum (see chapter four). The intermediate filament protein vimentin has been tentatively identified as the polypeptide migrating just ahead of the tubulins on 9% SDS polyacrylamide gels.

Experimentally Induced Changes in Erythrocyte Morphology

Given the lack of internal matrix elements in the nucleus of Xenopus erythrocytes, we became interested in the forces responsible for maintenance of nuclear shape and position in this cell. Because of the exsistence of cytoskeletal elements extending from the nuclear lamina to the SPMCN, we were particularly intrigued with any possible connection between cell shape and nuclear morphology or location. The flattened shape of blood platelets from certain vertebrate groups can be transformed into irregular spherical configurations by exposure to agents (such as cold, colchicine, vinblastine sulfate) known to depolymerize steady-state microtubules (Dustin, 1978). The insensitivity of Xenopus erythrocyte marginal bands to these agents, but their lability when lysed in the presence of calcium, led us to the use of the divalent cation ionophore A23187 (Reed and Lardy, 1972) in an effort to manipulate cell, and possibly nuclear shape.

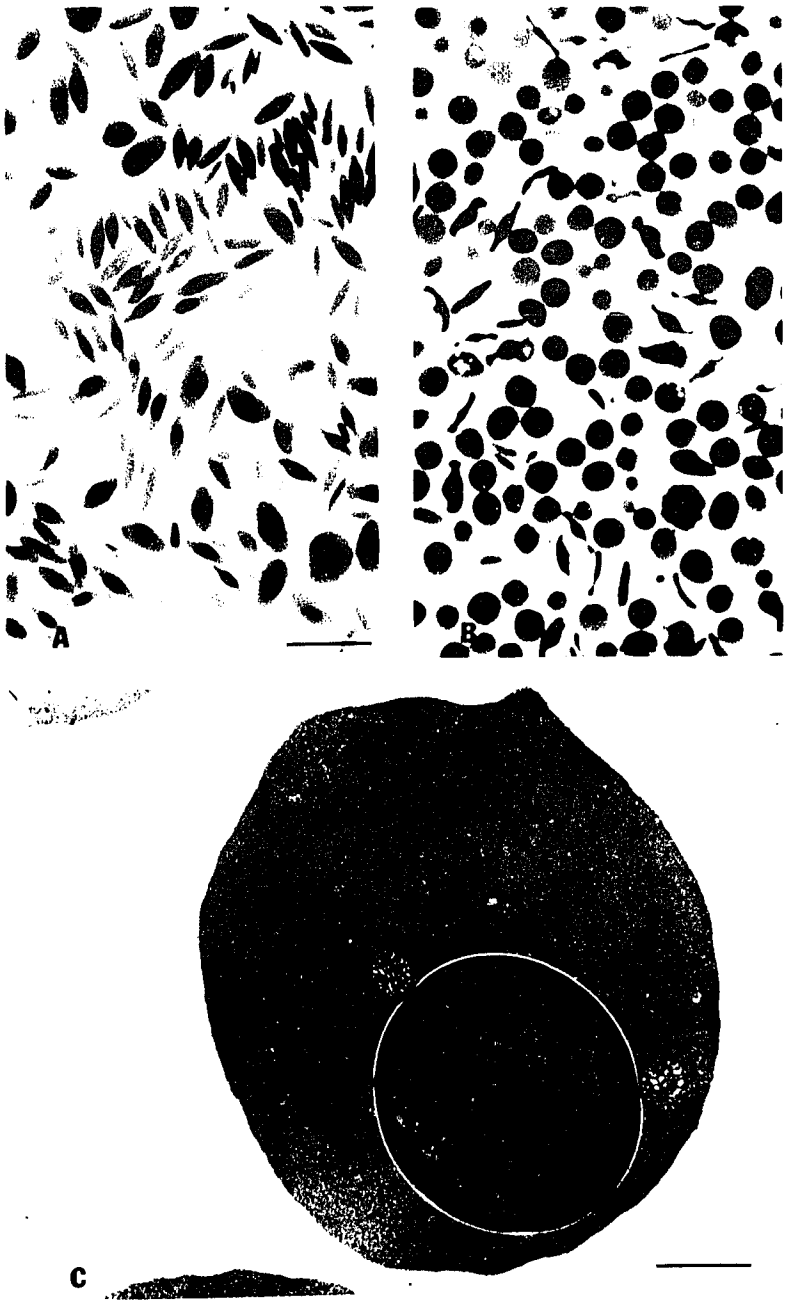
When erythrocytes were incubated in an isoosmotic salt solution

containing 1.35 mM $CaCl_2$ in the presence of ionophore, within 25 to 30 min. there was almost complete cell rounding as judged by combined phase contrast and thick section analysis. Whereas control cells remained flattened (figure 13A and B). Phase contrast microscopy of cells lysed in EGTA containing microtubule stabilization buffers (Sloboda and Dickerson, 1980) revealed that cytoskeletons from the ionophore treated cells contained distinctly spherical nuclei but lacked marginal bands. In contrast, cytoskeletons from control cells possessed roughly oval-shaped nuclei and intact marginal bands (Data not shown).

Light and electron microscopy indicated that treatment of erythrocytes with ionophore for periods sufficient to produce maximal cell rounding altered cell and nuclear shape, but in many instances caused nuclear dislodgement from the cell center (figure 13B and C). Superficially, these findings are consistant with the interpretation that microtubules, and/or other calcium labile structures, determine cell morphology and thereby determine nuclear shape and position, presumably mediated via structural elements which extend from the nuclear lamina to the peripheral cytoskeletal network. It is noteworthy that in these preparations a spherical cell has yet to be observed which possesses an elongate nucleus. This indicates that the abundantly noted circular nuclei within circular cells seen in sections of ionophore treated cells are likely to be spherical and not merely spherical appearing due to the plane of section.

On the other hand, it also needs to be considered that if cells were exposed to ionophore for only short periods of time prior to fixation, nuclei were observed undergoing morphological changes in cells that were still flattened (figure 14). After a one minute exposure to

Figure 13. Thick section light micrographs of erythrocytes kept in calcium salt solution for 30 min. (A) without ionophore. (B) with 3 uM ionophore. Bar equals 10 um (x1,100). C) Thin section electron micrograph of an erythrocyte exposed to ionophore for 30 min. showing nuclear rounding as well as displacement from the cell center. Bar equals 1 μm (x7,500).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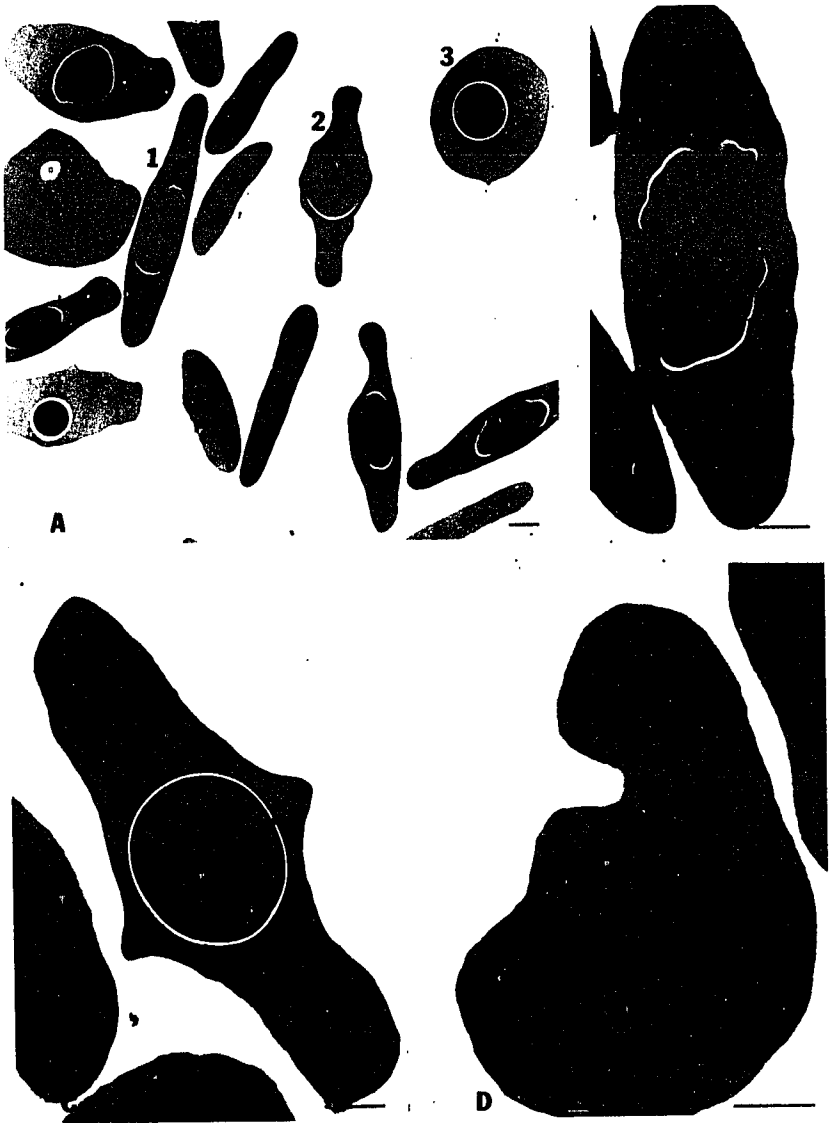
B
C

ionophore there was little evidence of cell shape change by phase contrast microscopy. Electron microscopy of this sample however, indicated that in contrast with controls, some nuclei began to show contorted configurations (figure 14A). In aliquots fixed after a 5 min. treatment, a more diverse combination of cellular and nuclear forms were evident (figure 14B,C and D). These configurations were not observed at latter time points (i.e. 20 min.) when the vast majority of cells had circular nuclear and cell morphology when viewed in sections. Cells could be detected in which nuclei had rounded although the cell remained flattened. This observation does not necessarily demonstrate that nuclear morphology is maintained independently of cell shape. It may only indicate that the two phenomena can be uncoupled.

Figure 14. Thin section electron micrographs of ionophore treated cells. (A) Cells exposed to ionophore for 5 min. prior to fixation. (1) A cell which resembles those found in controls containing an elongate, centrally located, oval nucleus. (2) A cell which is flattened but in which the nucleus has rounded. (3) An erythrocyte in which both nuclear and cellular rounding has occurred. (B) Nuclear disfiguration found within a cell exposed to ionophore for 1 min. (C) An erythrocyte exposed to ionophore for 5 min, the nucleus appears to have rounded while the cell has just begun to lose its flattened shape. (D) A cell exposed to ionophore for 5 min. which is in a more advanced stage of rounding than that illustrated in C. Bar equals 1 um in all micrographs. Magnifications (x3,500; x7,000; x7,000; x10,000 respectively).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DISCUSSION

Ultrastructural evidence is presented that nuclear matrices from Xenopus erythrocytes lack intranuclear matrix elements and therefore consist solely of the nuclear lamina. This structure, in association with the residual cell skeleton has been termed a cyto-nuclear skeleton. Within this structure the nuclear lamina is maintained in the overall nuclear form and position found in intact erythrocytes and cytoskeletons.

Several precautions were taken to reduce the possibility that the failure to observe internal nuclear matrix components was not the result of procedurally induced artifacts. To eliminate the possibility that these components were being fragmented by mechanical treatment and then lost upon the extraction of chromatin, cytoskeletons rather than purified nuclei were used in the matrix isolation procedure. With this in mind, it might be remarked that Krohne et al. (1981) commented on the absence of intranuclear matrix structures in their high salt, DNase treated, Xenopus erythrocyte nuclear lamina material. Although, in order to obtain lamina preparations significantly free of cytoplasmic attachments, these investigators utilized severe homogenization protocols as well as centrifugation through dense sucrose at high centrifugal forces. It has been our experience with nuclear matrices from spermatogenic cells that these manipulations may adversely influence the integrity of the nuclear matrix. It should also be pointed out that in the cytoskeletally free nuclear lamina preparations of Krohne and coworkers (1981), the lamina were collapsed and did not resemble the nucleus in overall form.

To curtail proteolytic degradation, all procedures after cell lysis

were conducted in the cold and the serine protease inhibitor, PMSF was added to lysis and wash solutions. More importantly, leukocytes were removed from the starting cell pellets. These precautions inhibited widespread general proteolysis. However, on occasion, some degradation products of the H1 histones were observed. No detectable proteolysis takes place during the DNase I digestion step and the subsequent high salt extractions or wash steps because with the exception of the histones, the electrophoretic profiles of the cytoskeletons and the cyto-nuclear skeletons are the same.

The electrophoretic detection of considerable amounts of and tubulin associated with the high salt treated cyto-nuclear skeletons was surprising because these structures no longer possess discernable marginal band microtubules when viewed by electron microscopy. The association of the tubulins with cyto-nuclear skeletons might reflect more than simply a non-specific binding phenomenon. Xenopus erythrocyte marginal band microtubules differs from the cytoplasmic microtubules of many other cell types in two respects: 1. they are organized into a single well defined structural unit and 2. they exhibit stability properties not characteristic of steady-state microtubules. Specifically, they are resistant to cold, mitostatic agents,as well as millimolar concentrations of calcium (see chapter four). The latter if in the absence of soluble cytoplasmic contaminants. In this regard, it is interesting that we have only been able to achieve total tubulin release from these cytoskeletons by calcium lysis (see chapter four). Thus, while high salt treatment might interfere with interactions between tubulin dimers required for microtubule integrity, it may not disrupt interactions, possibly hydrophobic, between tubulin and other

cytoskeletal proteins which play a role in influencing microtubule organization and/or stability. Furthermore, in vitro, tubulin has been known to assemble into a variety of non-tubular structures under different experimental conditions. We are currently exploring the possibility that calmodulin activates a modification of either the tubulins or their associated proteins which would lead to an alteration in microtubule stability (as well as tubulin-cytoskeletal interactions) and the subsequent release of its constituent proteins.

The ability of the divalent cation ionophore A23187 to induce changes in nuclear and cell form as well as nuclear location suggests that this reagent may be a promising tool in the detection of biochemical and ultrastructural changes that occur in the nuclear lamina and the cytoskeleton during the loss of characteristic erythrocyte features. The observation that nuclear and cellular rounding may not be coordinated events cannot be easily explained. Structural elements required for both nuclear and cell shape may both be directly affected by calcium but show kinetic differences in their sensitivities. Alternatively, the possibility that an increase in intracellular calcium causes secondary changes in the cytoplasmic ionic milleu which adversely affect nuclear morphology and position remains open. It is noteworthy that exposure of serum-deprived human fibroblasts to A23187 in the presence of calcium has been demonstrated to dramatically increase sodium influx within these cells in several minutes (Owen and Villereal, 1982). Recently investigators have shown that quantitative and qualitative changes in the ionic environment may significantly influence intermediate filament integrity (Traub and Nelson, 1982; Nelson and Traub, 1982a). Moreover, a calcium activated proteinase specific for

the intermediate filament proteins vimentin and desmin has been identified in cultured cells from a wide variety of vertebrates, including Xenopus (Nelson and Traub; 1982b). It would be valuable to determine if such a protease was present in vertebrate erythrocytes. Intermediate filaments have been implicated in other cells in the anchorage of nuclei within the cytoskeleton (Lehto et al., 1978).

In closing, the Xenopus erythrocyte cytoskeleton appears to be a relatively simple, cold stable, structure that can be manipulated for the study of cytoskeletal and nuclear-cytoskeletal interactions responsible for the maintenance of the unique cell and nuclear form of non-mammalian vertebrate erythrocytes.

CHAPTER FOUR

THE DEPOLYMERIZATION OF NON-STEADY STATE XENOPUS ERYTHROCYTE CYTOSKELETAL MICROTUBULES BY CALCIUM LYSIS

INTRODUCTION

Microtubules are cylindrical structures, with a diameter of 25 nm and a hollow core of 15 nm, that are common to eukaryotic cells. These organelles can be organized into a wide variety of assemblages (the mitotic apparatus, ciliary and flagellar axonemes, cytoplasmic microtubule networks, centrioles, and others) which participate in very diverse cellular processes. Ultrastructurally and biochemically microtubules from various sources show many similarities. They are composed principally of two 55,000 dalton proteins (α and β tubulin) which together are thought to make up the 5 nm subunits found in the wall of these structures. Microtubules however, can also exhibit differences, particularly in terms of their sensitivity to chemical and physical treatments (Behnke and Forer, 1967; Dustin, 1978).

Most cytoplasmic microtubules, like those which have been assembled *in vitro*, are usually rapidly depolymerized by such conditions as cold, colchicine and calcium. This contrasts with the properties of microtubules found in ciliary and flagellar axonemes, or centrioles, which are not depolymerized by such conditions. The factors responsible for differences in microtubule stability, or those involved in the dismantling of the cytoplasmic microtubule network prior to mitosis are still unknown. Within recent years, much attention has focused on the way in which microtubule associated proteins (Sloboda *et al.*, 1975) might be involved in mediating the assembly and stability of microtubules (Sloboda *et al.*, 1976, Deery and Weisenberg, 1981).

Marginal band microtubules are major cytoskeletal elements found in certain blood cells including all non-mammalian vertebrate erythrocytes (Behnke, 1970). Moreover, their existence has also been noted in blood

cells of some invertebrates (Nemhauser et al., 1980; Cohen and Nemhauser,1980). Although structurally similar, marginal bands from different sources do not always have the same stability properties. For instance, those of mammalian blood platelets can be depolymerized by cold or high concentrations of colchicine whereas frog erythrocyte marginal bands are cold stable (Behnke, 1970). The marginal band of dogfish erythrocytes are disassembled after 30 min. at 0°C but are unperturbed after an hour incubation in 1 mM colchicine (Cohen et al. 1982). Similarly, marginal bands of chick erythrocytes are cold sensitive although those of "mature" cells show a slower rate of disappearance than "immature" ones (Barrett and Dawson, 1974). Xenopus laevis erythrocyte cytoskeletons contain a peripheral bundle of cytoplasmic microtubules (the marginal band) which has stability properties unlike those exhibited by most cytoplasmic microtubules. If erythrocytes were lysed in calcium free medium, the marginal band remained intact after long exposures to cold temperatures, high concentrations of the microtubule inhibitors colchicine, vinblastine sulfate and nocodazole as well as relatively low pH (5.5). However, if lysed in solutions containing calcium, the cytoskeletons were devoid of marginal band microtubules as ascertained by phase contrast and electron microscopy. SDS polyacrylamide gel electrophoresis showed that these structures were depleted of the tubulins and also suggested the existence of a calcium activated cytoplasmic factor which produces a new high molecular weight cytoskeletal polypeptide. Lysis and washing of cytoskeletons in calcium free media prior to the addition of calcium renders the marginal band insensitive to depolymerization by this ion. Moreover, the appearance of the new high molecular weight polypeptide

was no longer observed. Addition of calmodulin and/or ATP to the calcium wash did not alter this observation.

It has been reported (Nemhauser and Cohen, 1981) that the marginal band of the blood clam, Noetia, is in direct association with centrioles and that this organelle appears to be part of a microtubule organizing center which functions during the in vivo reassembly of the marginal band. Furthermore, in many interphase cells which have steady-state microtubule networks, the microtubule organizing center is also localized at the centrosomal region (Brinkley et al., 1981). Centrioles were frequently observed within the cytoskeleton of Xenopus erythrocytes. However, they were not in association with the marginal band but were perinuclear in position. This perinuclear location might reflect the non-steady state nature of the Xenopus erythrocyte marginal band cytoplasmic microtubules.

MATERIALS AND METHODS

Adult male Xenopus were purchased from the South African Snake Farm, Fish Hoek, Cape Provine, South Africa; maintained in deionized water at 18-20°C and fed beef liver at least once a week. The ionophore A 23187 was obtained from Calbiochem-Behring Corp. (La Jolla, CA) and kept as a 1 mM stock in 95% ethanol at -5°C. Bovine heart calmodulin, colchicine, vinblastine sulfate, nocodazole, plus Na^{+} and Mg^{++} ATP were bought from Sigma Chemical Co. (St. Louis, MO). Fluoroscein isothiocyanate conjugated goat anti-rabbit IgG was purchased from Miles-Yeda Ltd. (Rehovot, Isreal). The following materials were obtained via generous gifts, homogeneous bovine brain calmodulin (Dr. Donald Wolff, Rutgers Medical School), the Xenopus epithelial cell line, Xl 177 (Dr. Leo Miller, Univ. of Illinois at Chicago Circle), and anti-tubulin serum (Dr. Joanna Olmsted, Univ. of Rochester).

Solutions

Calcium Erythrocyte Collection Solution: 80 mM NaCl, 10 mM KCl, 1.35 mM $CaCl_2$, 2 mM $NaHCO_3$. EGTA Lysis Solution pH 5.5: 150 mM NaCl, 1 mM $MgSO_4$, 5 mM EGTA, 20 mM PIPES pH 6.9, 1 mM TAME (N-p-Tosyl-L-arginine Methyl Ester), 0.4% Triton X-100. EGTA Lysis Solution pH 6.9: 150 mM NaCl, 1 mM $MgSO_4$, 5 mM EGTA, 20 mM PIPES, 1 mM TAME, 0.4% Triton X-100, pH adjusted with 1 N NaOH. Magnesium or Calcium Lysis Solution: 0.19 M sucrose, 3 mM Mg or $CaCl_2$, 75 mM KCl, 10 mM PIPES pH 6.9, 0.4% Triton X-100, 1 mM PMSF (Phenylmethylsulfonyl fluoride, added fresh). Wash Solutions: the same as the lysis solution with the exception that they lacked Triton X-100. Phosphate Buffered Saline (PBS): 145 mM NaCl, 10 mM phosphate buffer, pH 7.4.

Cell Culture

The Xenopus laevis epithelial cell line, Xl 177, was grown at 22-25°C in Leibovitz medium (Flow laboratories, Rockville, MD) containing 10% fetal calf serum (Gibco, Grand Island, NY) and 1% anti-biotics/anti-mycotics (Gibco). Cells were subcultured using 70% of 1X Trypsin-EDTA (Gibco) and washed with 70% Earle's Balanced Salt Solution (BSS) without calcium or magnesium.

Cells to be used for immunofluorescence were planted in petri dishes containing circular coverslips 24 to 48 hours prior to use. Colchicine treatment experiments were done by placing the cell containing coverslips in media to which the drug was added. Cold exposure was accomplished by placing the cells in prechilled petri dishes containing ice cold medium for the times specified. Tetrahymena thermophila were grown at 35°C in medium of 1.5% bactopeptone (Difco, Detroit, MI) and 0.4% yeast extract (Difco).

Collection of Erythrocytes and the Isolation of Cytoskeltons

Decapitated, pithed, adult male Xenopus laevis were bled into EGTA Wash Solution and the erythrocytes were pelleted at 365 g for 5 min.. All centrifugations were carried out in a Sorvall HB-4 rotor. Unless otherwise indicated, leukocytes were removed (to less than 1% contamination) by either repeated removal of the surface layer from erythrocyte pellets of by centrifugation of erythrocytes through 0.5 M sucrose, 3 mM $MgSO_4$. Studies on intact erythrocytes were done on aliquots of these cells resuspended in EGTA Wash Solution with the specified experimental modifications (i.e. colchicne, cold, etc.). Erythrocytes used for experiments involving the effects of increasing intracellular calcium were collected in Calcium Erythrocyte Collection Solution. To experimental samples, the ionophore A23187 was added to 3

uM. Control cells resembled those kept in calcium free solutions as determined by phase contrast and electron microscopy.

Cytoskeletons were isolated by resuspending erythrocyte cell pellets in the particular lysis solution of choice (approx. 0.5 ml of packed erythrocytes per 10 ml of solution). In experiments where it was not desirable to completely wash away cytoplasmic elements, the cells were lysed in only a few ml of solution. On the other hand, in experiments such as the calmodulin reconstitution study where it was beneficial to reduce contaminating cytoplasmic factors the cell pellets were lysed in 20-25 ml of solution. Centrifugation at 600 g for 8-10 min. was sufficient to collect the cytoskeletons. The pellet was then resuspended in the appropriate wash solution. Where indicated, the samples were washed a second time before protein extraction. Cytoskeletons isolated in the cold, were resuspended in ice cold solutions, and centrifuged at 0°C. During the phase contrast observations of sample aliquots routinely made between centrifugations, the cytoskeletons were kept in an ice bath.

Indirect Immunofluorescence

Tissue culture cells grown on coverslips were removed from petri dishes, dipped three times in 70% BSS and fixed in 3.8% paraformaldehyde in BSS, pH 7.0-7.4, for 20 min at room temperature. The coverslips were then rinsed in PBS, post-fixed in acetone for 7 min. at -20°C and then rehydrated in PBS. Staining with anti-tubulin serum (characterized previously Van der Water and Olmsted, 1980), diluted 1:20 with PBS, was done in a moist chamber at 37 C for 60 min.. The coverslips were then washed three times for 20 min. each in PBS. FITC-conjugated goat anti-rabbit IgG (diluted 1:20 with PBS) staining was done in a moist chamber

at 37°C for 30 min.. The coverslips were next washed for at least 90 min. in several changes of PBS prior to mounting on slides for photomicroscopy. Cells not treated with tubulin anti-serum showed no fluorescence. Photomicrographs were taken with a Zeiss Photomicroscope II equipped with epifluorescence optics using Kodak Plus X film at ASA 400 and developed with Diafine developer.

Xenopus erythrocyte cytoskeletons were prepared for indirect immunofluorescence as follows. Erythrocytes collected into 100 mM NaCl, 15 mM Na citrate, 3 mM $MgSO_4$, were allowed to settle onto polylysine coated coverslips in a moist chamber for about 15 min. This had no adverse effect on cell shape. They were then dipped in a particilar cell wash solution, drained and lysed by the addition of a few drops of the corresponding lysis solution. After 2-3 min., they were dipped consecutively in three beakers containing the appropriate wash solution and then fixed for 20 min. in wash solution containing 3.8% formaldehyde. Anti-serum staining and the subsequent wash steps were as described for the Xl 177.

Electron Microscopy

Cytoskeletons suspended in wash solution were fixed by the addition of an equal volume of 3% glutaraldehyde, 0.1 M Na cacodylate, pH 7.2. Glutaraldehyde fixation was at room temperature for 1.5 h. Samples were washed with 0.1 M Na cacodylate buffer before and after a 1 h. post-fixation in buffered 1% osmium tetroxide at 4°C. After an additional buffer wash, the samples were dehydrated in ethanol followed by sequential infiltration with propylene oxide, propylene-oxide-Spurr (1:1) and pure Spurr prior to embedding. Silver and gold sections were cut with a diamond knife and stained with uranyl acetate and lead

citrate prior to viewing on a Philips EM 300 electron microscope operated at 80 kv.

Protein Preparation and Analysis

Cytoskeletal protein was extracted by first resuspending the cytoskeletal pellet in 8 M urea, 5% 2-mercaptoethanol. During the course of this research it was observed that the freshness of this solution noticably influenced the migration of the tubulins in SDS gels, although it had no detectable effect on the electrophoretic mobility of other cytoskeletal polypeptides. To insure consistent migration, this solution was stored in aliquots at -20°C. SDS was then added to a final concentration of 2%. Samples were next refrigerated for approximately 2 h. with intermittant agitation in a vortex. DNA was removed by ultracentrifugation at 60,000 g, for 14 h. in a Beckman type 50-Ti Rotor at 5°C. The protein samples were then stored at -20 C. Protein from supernatants was dialyzed against distilled water in the cold and then lyophilized. Cilia were isolated by the ethanol fixation/calcium release method of Gibbons (1965). Ciliary axonemes were prepared as described by Gavin (1980). Prior to electrophoresis, samples were adjusted to 4.8 M urea, 1.5% SDS, 3% 2-mercaptoethanol, 75 mM Tris-HCl pH 6.8 and analyzed by SDS-polyacrylamide gel electrophoresis according to the method of Laemmli (1970). Protein molecular weight markers were purchased from Bio Rad Laboratories. Gels were stained in 0.1% Coomassie blue R-250, 25% methanol and 10% acetic acid overnight and destained in 25% methanol, 10% acetic acid.

RESULTS

Comparison of the Effects of Microtubules Depolymerizing Agents on Erythrocytes and Tissue Culture Cells

Xenopus laevis erythrocytes are oval, nucleated, elliptical cells which possess typical marginal band microtubules in the plane of cellular flattening. Characteristic cell morphology, as visualized by phase contrast and Nomarski interference optics, is illustrated in figure 15.

Intact erythrocytes were unaffected by agents known to depolymerize cytoplasmic microtubules in other cell types. Incubation of erythrocytes for 30 min. at room temperature in solutions containing relatively high concentrations of the microtubule poisons colchicine or vinblastine sulfate, 5 mM and 420 uM, respectively, had no detectable influence on cell form. Moreover, cytoskeletons obtained from colchicine and vinblastine-treated cells have marginal bands as judged by phase contrast and electron microscopy. In addition to the specified alkaloid insensitivity, Xenopus erythrocytes were also unaffected by low temperatures. Cells kept at $0^{\circ}C$ for 30 min. were indistinguishable from control cells maintained at $20^{\circ}C$. Cellular lysis produced by the addition of a few drops of 20% Triton X 100 indicated that marginal bands were still present within these cells. This was also confirmed by electrophoretic analysis of the cytoskeletons.

Although the low temperature and alkaloid treatments described above had no apparent adverse influence on Xenopus erythrocyte morphology and marginal band integrity, these conditions had devastating effects on the cell form and cytoplasmic microtubule network of the epithelially derived (Miller, 1977), Xenopus cell line , XL177.

Figure 15. Phase contrast (A) and Nomarski interference optics (B) micrographs of red blood cells in Calcium Erythrocyte Collection Solution. Bar equals 10 μm (x800).


B
A

Incubation of XL177 cells for only 15 min. in ice cold medium was sufficient to disassemble much of the intricate cytoplasmic microtubule network normally observed in these cultured cells by indirect immunofluorescence with anti-tubulin serum (figure 16a-d). Slightly longer cold treatment caused the detachment of cells from the coverslips. Exposure of the cells to 1 mM colchicine for 30 min. resulted in cellular distortion as well as the loss of the microtubular cytoskeleton (figure 16e,f). These observations indicate that the microtubules of the XL177 cell cytoplasm have stability properties similar to the cytoplasmic microtubules of most other cells.

Although Xenopus erythrocyte morphology was unchanged by extended cold treatment and exposure to the alkaloids tested, it was very sensitive to an increase in intracellular calcium. If erythrocytes were kept in isoosmotic salt solutions containing 1.35 mM $CaCl_2$, the addition of the divalent cation ionophore A23187 caused almost complete cell rounding within a 30 min. period (figure 17). Control cells kept in the same solution, but without ionophore, maintained the characteristic morphology depicted in figure 15. When control and experimental cells were pelleted, resuspended in EGTA containing wash solution and subsequently lysed by the addition of Triton X-100, marginal bands were scarce in the ionophore treated samples yet abundant in the cytoskeletons obtained from the control cells as judged by phase contrast microscopy.

These observations raised the possibility that calcium can act to initiate marginal band disassembly, however, it does not necessarily suggest that maintenance of flattened elliptoid morphology and marginal band integrity are co-requirements. In fact, evidence from other

Figure 16. Phase contrast (A, C, E) and indirect immunofluorescenece (B, D, F) microscopy of Xl 177 cells. A and B are control cells grown at 24°C. Anti-tubulin staining reveals the presence of a complex cytoplasmic microtubule network which extends to the cell periphery. Micrographs C and D are of cells incubated in ice cold medium. Cells in C were kept in the cold for 25 min. prior to fixation. After this time, the cells began to round up and shortly thereafter would start to detach from the coverslips. The cell illustrated in C was kept in the cold for only 15 min. The cytoplasmic microtubule network has already become faint and limited to the region around the nucleus. The effects of a 30 min. exposure to 1 mM colchicine on these cells is illustrated in E and F. The cells become distorted and all evidence of a fibrous cytoplasmic microtubular cytoskeleton has been removed. Bar equals 10 μm.

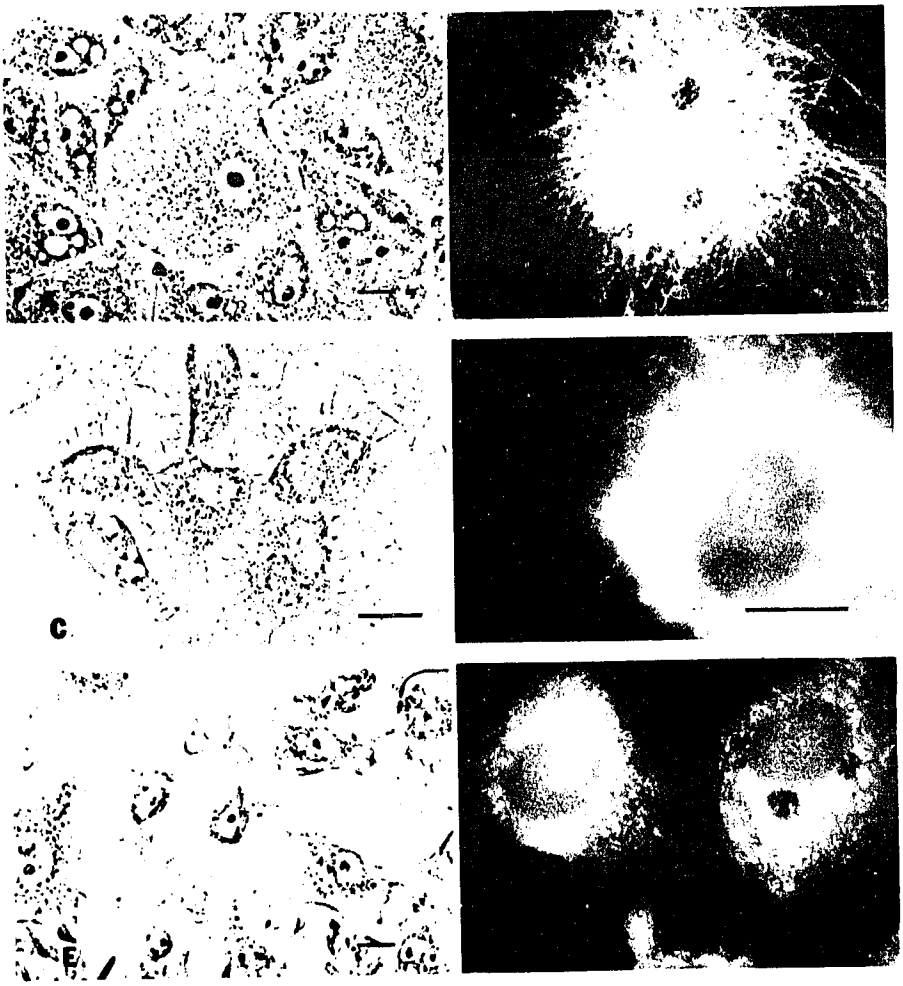
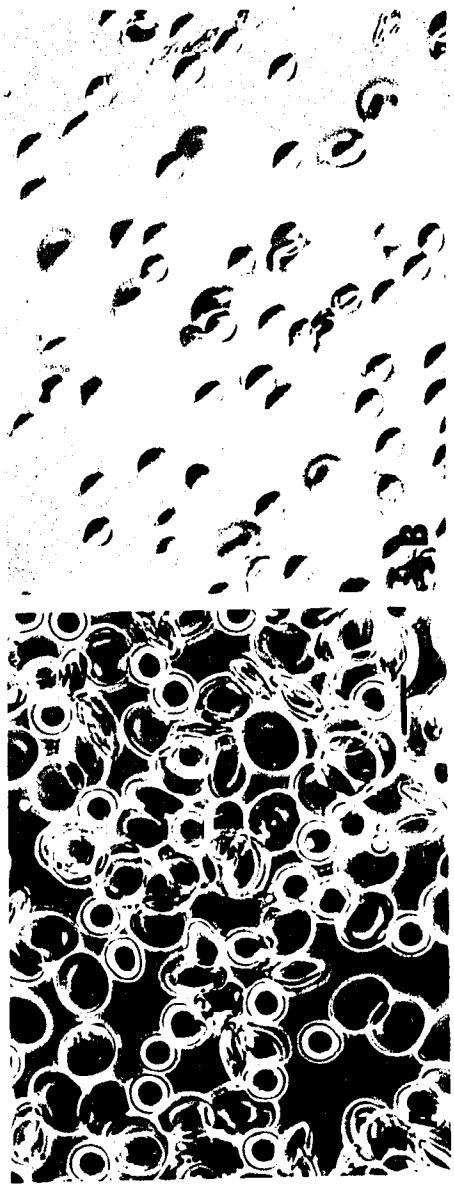

c

Figure 17. Phase contrast (A) and Nomarski interference optics (B) of erythrocytes treated with the ionophore A 23187 in the presence of calcium for 20 min (A) and 25 min (B) prior to fixation with glutaraldehyde. Bar equals 10 μm (x800).



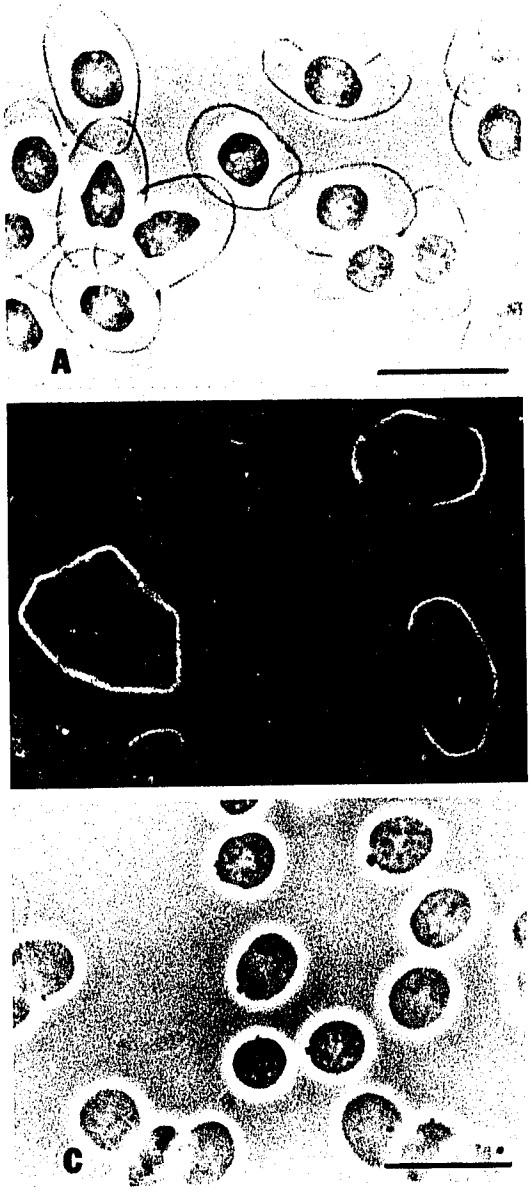
systems (Barrett and Dawson, 1974; Cohen et al.,1982) indicates that in mature erythrocytes there is no such requirement. Furthermore, it needs to be considered that an increase in cellular calcium activates non-speciific proteases so the failure to observe marginal bands in lysed post-ionophore treated cells is due to their proteolytic degradation. In order to circumvent this possibility, marginal band integrity was examined in cells lysed in calcium containing solutions at low temperatures. This allowed the use of protease inhibitors and assesment of the integrity of previously well characterized nuclear and cytoskeletal proteins. It also permitted the detection of changes in other cytoskeletal components as a result of exposure to calcium.

Erythrocyte Cytoskeleton: Isolation and Structure

When Xenopus erythrocytes were cleared of hemoglobin by lysis in calcium free buffers containing non-ionic detergents and examined by phase contrast microscopy, the marginal band was detectable surrounding the nucleus (figure 18a). This structure specifically fluoresced when viewed by indirect immunofluorescence using anti-tubulin serum even when the cells were rinsed, lysed and washed in ice cold solutions prior to formaldehyde fixation (figure 18b). Lysis in calcium containing buffers however, caused the immediate disappearance of the marginal band (figure 18c).

Before long term stability studies on Xenopus erythrocyte cytoskeleton microtubules were conducted, a variety of lysis and wash solutions were tested for their ability to inhibit proteolysis while preserving the integrity of the marginal band. Because Xenopus erythrocyte H1 histones are highly sensitive to minimal protease

Figure 18. Phase contrast (A, C) and indirect immunofluorescence (B) microscopy of Xenopus erythrocyte cytoskeletons. Erythrocytes were adhered to coverslips and lysed by the addition of a few drops of the specified lysis solution. This produced better visualization of the marginal band compared to lysis in suspension because there was less twisting of this structure by this method. (A) Cytoskeletons obtained using EGTA Lysis Solution, pH 5.5 at 20°C. (B) Anti-tubulin indirect immunofluorescence of cytoskeletons obtained from cells which were rinsed, lysed and washed in ice cold EGTA, pH 5.5 solutions prior to formaldehyde fixation. The marginal band remains intact. Where the marginal band is obscured by the nucleus (also see A), there is a gap in the fluorescence. (C) Cytoskeletons produced via cold calcium lysis, note the complete absence of marginal bands. Bar equals 10 um (x1,700).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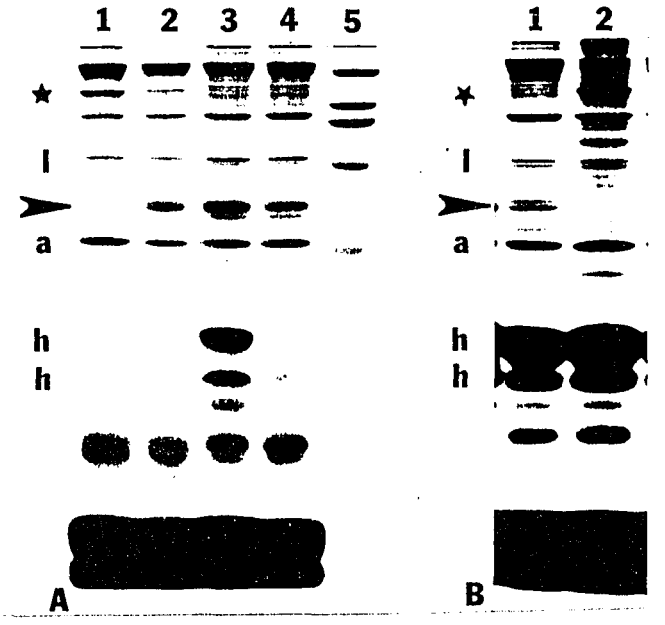
activity (Risley and Eckhardt, 1981), their integrity as evaluated by mobility on SDS polyacrylamide gels was used as the basis for judging the extent of proteolysis in erythrocyte cytoskeletal preparations. Under conditions of maximal protease activity (leukocytes were not removed from the starting erythrocyte pellets), moderately acidic pH (5.5) appeared to be the best inhibitior of general proteolysis when viewed by the preservation of the H1 histones (figure 19). Cytoskeletons isolated at room temperature at pH 5.5 showed beter H1 preservation than those isolated at pH 6.9, with ice cold solutions, containing PMSF. This enhanced protease inhibition was attributed to the difference in pH and not to the inclusion of the protease inhibitor, TAME, in the pH 5.5 lysis and wash solutions. When the pH of this solution was adjusted to 6.9, H1 integrity was not observed under the conditions described above (data not shown).

Removal of leukocytes by repeated removal of the surface layer on the erythrocyte pellet or by centrifugation of erythrocytes through 0.5 M sucrose, as done by Cohen and co-workers (1982), dramatically reduced proteolysis in cytoskeletons isolated at neutral pH in the cold. Although prolonged incubation ($>$ 30 min.) of comparable samples at room temperature often caused chromatin decondensation and cytoskeletal clumping probably as a result of H1 degradation. In contrast, cytoskeletons from leukocyte depleted erythrocyte cell pellets lysed in EGTA Lysis Solution pH 5.5, have on many occasions been kept at room temperature for over an hour without clumping and with remarkable maintenance of the marginal band as judged by phase contrast and electron microscopy. These observations are consistant with those made on dogfish erythrocyte marginal band microtubules which are stable at pH

Figure 19. SDS 10% polyacrylamide gel electrophoresis of erythrocyte cytoskeletal protein. Figure A, erythrocytes were collected in EGTA Wash Solution, pH 5.5, aliquoted and pelleted without removal of contaminating leukocytes. The cells were then lysed, collected, washed, recollected and the protein extracted. Lane (1) Calcium Lysis and Wash Solution at 0°C, (2) Magnesium Lysis and Wash Solution at 0°C, (3) EGTA Lysis and Wash Solution, pH 5.5 at 0°C, (4) EGTA Lysis and Wash Solution, pH 5.5 at 20°C, (5) molecular weight markers, from top to bottom, 200,000, 116,500, 94,000, 68,000, 43,000 daltons. Figure B, erythrocyte pellets from which leukocytes were removed by repeated removal of the surface layer of cells were used to obtain cytoskeletal protein. Lane (1) EGTA Lysis and Wash Solution, pH 5.5 at 0°C, (2) Calcium Lysis and Wash Solution at 0°C. Although the H1 histones are now preserved, tubulin release and CSHMWP formation are still noted in the calcium lysed cytoskeletons. Symbols: star (CSHMWP), l (nuclear lamina proteins), a (actin), arrowhead (tubulin region), h (H1 histones).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4 (Cohen et al., 1982).

It was interesting that even in samples where protease activity was considerable (figure 19), the cytoskeletal polypeptide electrophoretic profiles resembled those of samples in which protease activity was minimized while the H1 histones were severely degraded. The absence of the tubulins and the emergence of a new cytoskeletal high molecular weight polypeptide (CSHMWP) in the calcium lysed samples was not a general phenomenon. When an equal concentration of cells were lysed in media of the same composition, with the exception that 3 mM $MgCl_2$ was substituted for 3 mM $CaCl_2$, H1 degradation was comparable in both samples yet the magnesium lysed cytoskeletons were not tubulin depeleted nor did they show a prominent CSHMWP (figure 19A, lanes 1 and 2). Moreover, if erythrocytes were freed of leukocytes prior to calcium lysis, the H1 histones were preserved, although tubulin was released and the CSHMWP noted (figure 19B).

In addition to the peripheral bundle of marginal band microtubules, Xenopus erythrocyte cytoskeletons (figure 20 and 21) also consist of a subsurface cytoskeletal layer (SPMCN) plus intermediate filaments as well as fibrogranular structures in the region between the nucleus and the outer cytoskeletal shell. A pair of perinuclear centrioles surrounded by a network of intermediate filaments were also frequently observed in thin sections. Ultrastructurally, microtubules were abundant in all calcium free conditions tested (EGTA Lysis and Wash pH 5.5 and 6.9; Magnesium Lysis and Wash). When viewed in cross section figure 21), the average number of microtubules per marginal band was approximately 15, however bundles containing as few as 9, or as many as 23 microtubules have been observed. Cytoskeletons obtained by EGTA

Figure 20. Thin section electron micrograph of an erythrocyte cytoskeleton isolated at 0°C using Magnesium Lysis and Wash Solutions. The SPMCN (star) is a continuous subsurface network found at the periphery of the cytoskeleton. The nucleus remains centrally positioned and intermediate filaments are present within the region between the SPMCN and the nucleus. A centriole (arrow) is also observed in this area near the nucleus. Marginal band microtubules (arrowheads) can be viewed in both cross and longitudinal section. Bar equals 1 um (x29,500).

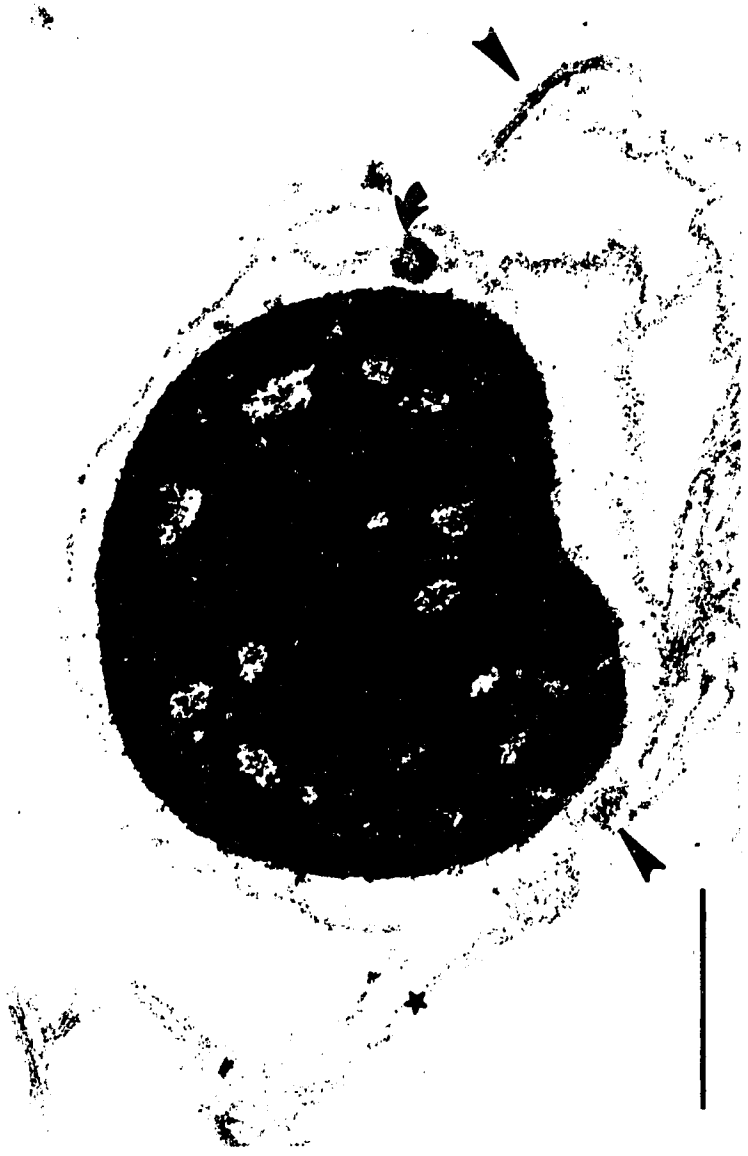
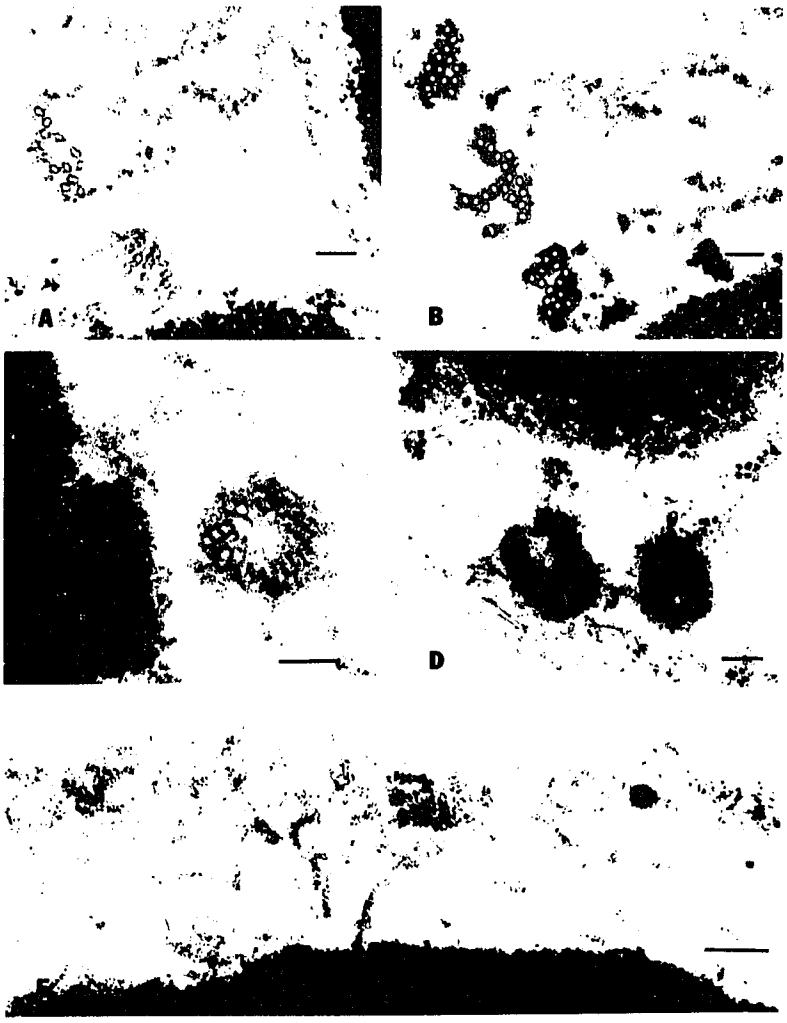


Figure 21. High magnification thin section electron micrographs of Xenopus erythrocyte cytoskeletal elements. (A) cross-section of marginal band microtubules found in cytoskeletons obtained using Magnesium Lysis and Wash Solution at 0°C. Much of the granular material often observed surrounding the marginal band microtubules of cytoskeletons produced using EGTA Lysis and Wash Solutions is not present. Extraction of this material has no obvious effect on marginal band stability. (B) cross-section of marginal band microtubules from cytoskeletons produced using EGTA Lysis and Wash, pH 5.5 containing 5 mM colchicine at 20°C. (C) and (D) centrioles from cytoskeletons isolated using cold Magnesium Lysis Solution in their characteristic perinuclear position. The centrioles are surrounded by a network of fibrogranular and filamentous cytoskeletal elements. Centriolar microtubules are preserved in cytoskeletons lysed in calcium containing solutions. (E) intermediate filaments found within the region between the SPMCN and the nucleus. These filaments are conspicuously absent in cytoskeletons produced using EGTA Lysis and Wash Solutions. Bar equals 0.1 um (magnifications: x50,000; x50,000; x80,000; x50,000 and x80,000 respectively).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Lysis Solution (pH 5.5 or 6.9) differed from those produced with Magnesium or Calcium Lysis Solution in that they were notably devoid of intermediate filaments. This difference might be attributed to the lower divalent cation concentration (1 mM vs 3 mM) and the absence of sucrose from the EGTA Lysis and Wash Solutions. Cells lysed in Magnesium or Calcium Lysis Solution were identical ultrastructurally with the exception that marginal band microtubules were never observed in the cytoskeletons produced using calcium lysis.

In the microtubule stability studies to be presented below, EGTA Lysis Solution pH 5.5, was used because of the enhanced inhibition against proteolysis offered by this medium as well as the observed marginal band integrity at this pH. It should be mentioned however, that most of these experiments were also conducted using Magnesium Lysis or EGTA Lysis pH 6.9 with the same results.

Xenopus Erythrocyte Cytoskeletal Microtubule Stability Properties

Lysis of erythrocytes in calcium caused the complete depolymerization of Xenopus erythrocyte marginal band microtubules as well as the disappearance of the tubulins from cytoskeletal electrophoretic profiles. Therefore, all microtubule stability studies were done on cells lysed at concentrations which produced microtubule disassembly in companion samples lysed in solutions containing calcium. This insured that all the cytoplasmic requirements for microtubule depolymerization (i.e. ATP) were present at concentrations capable of causing the solublization of the cytoplasmic microtubules. Bershadsky and Gelfand (1981) have shown that the depeletion of cellular ATP results in resistence to microtubule disassembly by colcemid and vinblastine.

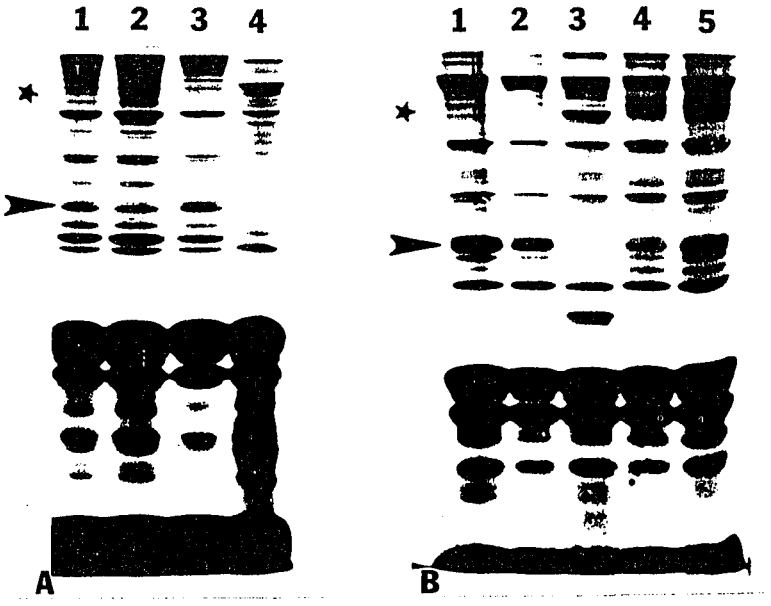
To insure that impermeability of the microtubule inhibitors tested to the Xenopus erythrocyte plasma membrane was not responsible for the inabiltiy of these drugs to produce disassembly, cells were lysed in solutions containing these inhibitiors. Addition of 2.5 mM colchicine, 210 uM vinblastine sulfate or 100 uM nocodazole to the EGTA lysis medium at room temperature had no deleterious effects on the marginal band as judged by phase contrast microscopy and SDS polyacrylamide gel electrophoresis (figure 22A). Moreover, cytoskeletons have been kept in EGTA Lysis Solution (pH 5.5 or 6.9) containing 5 mM colchicine for an hour. Electron microscopy of these samples and controls showed no differences. Marginal band microtubules were abundant in both preparations.

Cytoskeletons lysed in ice cold, calcium free media possessed marginal band microtubules and retain tubulin after incubation in these media at $0^{o}C$ for at least 60 min. (figure 22B). In contrast, lysis in ice cold, calcium containing medium caused immediate solubilization of the tubulins as well as production of the CSHMWP. In some gels (figure 21B, lane 3), an additional new component was also seen migrating just below actin.

Calcium Activated Cytoplasmic Factors

The emergence of a new high molecular weight cytoskeletal polypeptide by calcium lysis was observed to be mediated by cytoplasmic agents (figure 23). Resuspension of cytoskeletons in calcium wash solution after previously lysed and washed in calcium free media did not result in the formation of a CSHMWP. Resuspension of washed cytosketons in the calcium lysis supernatant from another cytoskeletal pellet, however, did produce microtubule depolymerization and formation of a

Figure 22. SDS 10% polyacrylamide gel electrophoresis of erythrocyte cytoskeletal protein. Figure A, microtubule inhibitor study. Cells were lysed at room temperature in EGTA Lysis Solution, pH 5.5 (lanes 1-3) containing the specified inhibitors or Calcium Lysis Solution (lane 4). The cytoskeletons were then washed and the proteins extracted. Lane (1) 2.5 mM colchicine, (2) 210 uM vinblastine sulfate, (3) 100 uM nocodazole, (4) Calcium Lysis Solution. Figure B, long term cold stability study. Where indicated, erythrocytes were resuspended in ice cold lysis solution and either processed immediately (lanes 1,2,3) or kept at 0°C for the times indicated. The minimum processing time for lysis, collection, wash and recollection was about 35 min. Lane (1) EGTA Lysis and Wash Solution, pH 5.5 at 0°C, (2) EGTA Lysis and Wash Solution, pH 5.5 at 20°C, (3) Calcium Lysis and Wash Solution at 0°C, (4) EGTA Lysis and Wash Solution, pH 5.5, incubated for 30 min. at 0°C after lysis prior to processing, (5) EGTA Lysis and Wash Solution, pH 5.5, incubated at 0°C for 45 min. after lysis prior to processing. Thus, samples in lanes 4 and 5 were exposed to cold for over an hour prior to protein extraction.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CSHMWP as determined by electrophoretic characterization of the sample. Addition of ATP (Na^+ or Mg^{++} salt) to the calcium wash had no effect (see below) on the high molecular weight polypeptides. These polypeptides are probably constituents of the SPMCN since they are not nuclear proteins (they are present in cyto-nuclear skeletons, see chapter 3, figure 12) and their molecular weights (approximately 200,000) are in keeping with the non-actin polypeptides of other vertebrate erythrocyte subsurface cytoskeletal layers (Branton, 1981; Bartelt, 1982). In some of my better gels, emergence of the CSHMWP coincides with the absence of a component among the 200,000 dalton polypeptides (see figure 23, lane 2). Thus, formation of the CSHMWP may be the result of the partial cleavage of this component.

Microtubules, such as those which are assembled and disassembled in vitro (Weisenberg, 1972; Borisy and Olmsted, 1972), are depolymerized by calcium (Olmsted and Borisy, 1973, 1975). In contrast, Xenopus erythrocyte marginal band microtubules were found only to be sensitive to calcium depolymerization if in the presence of soluble cytoplasmic elements. When cytoskeletons obtained by EGTA Lysis Solution pH 5.5, were pelleted and resuspended in Calcium Wash Solution, undegraded tubulins as well as some minor high molecular weight polypeptides (120,000), were the predominant polypeptides found in the supernatants (figure 24A). Hemoglobin and trace amounts of actin were also noted, however, these proteins were also observed in EGTA Wash Solution supernatants. Phase contrast examination of the calcium washed cytoskeletons suggested that marginal bands were still present. This was confirmed by electron microscopy (figure 24B). Moreover, electrophoretic characterizations of calcium washed cytoskeletons showed

Figure 23. SDS 9.5% polyacylamide gel electrophoresis of erythrocyte cytoskeletal protein. Formation of a calcium lysis specific high molecular weight cytoskeletal polypeptide. All isolations were done at 0°C. Lane (1) molecular weight markers from top to bottom 200,000, 116,500 and 94,000 daltons. Lane (2) calcium lysed and twice washed cytoskeletal protein. Lane (3) EGTA, pH 5.5 lysis and twice washed cytoskeletal protein. Lane (4) cytoskeletons EGTA, pH 5.5 lysed and washed followed by a calcium wash. Lane (5) cytoskeletons were EGTA, pH 5.5 lysed and washed followed by resuspension in the calcium lysis supernatant from the cytoskeletons characterized in lane 1.

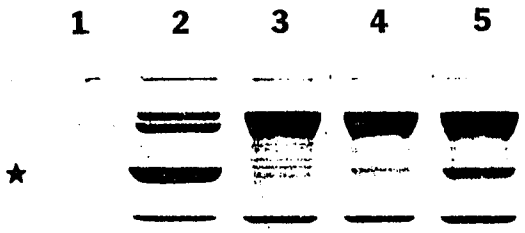
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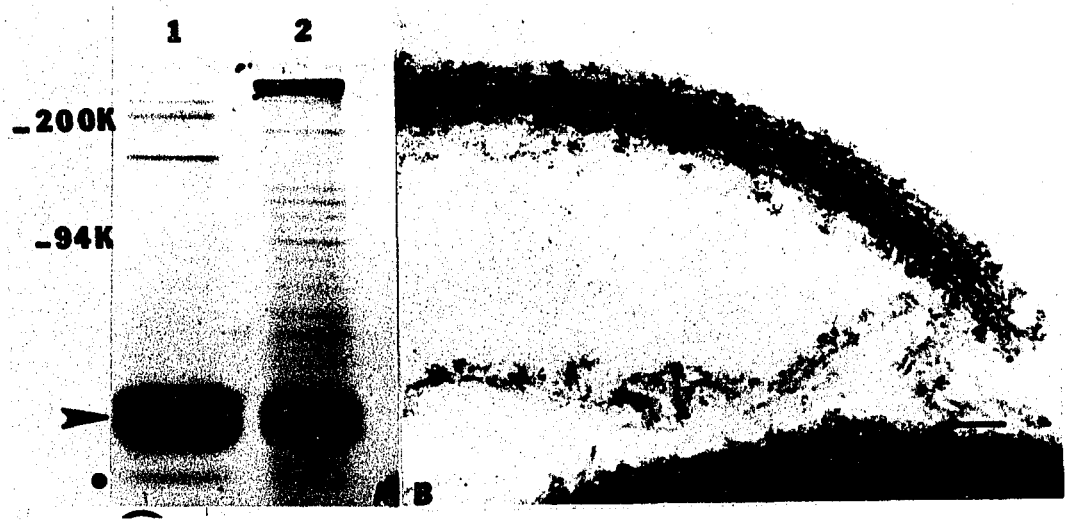
Figure 24. (A) SDS 9% polyacrylamide gel electrophoresis. All procedures were done at 0°C. Lane 1, 1.5 ml of packed erythrocytes was resuspended in EGTA Lysis Solution, pH 5.5, collected and then washed in a few ml of Calcium Wash Solution. The cytoskeletons were collected and the supernatant removed. The supernatant was next recentrifuged to remove any cytoskeletal contamination followed by dialysis against distilled water prior to lyophilization. Lane 2, Tetrahymena axoneme tubulin standard. (B) thin section electron micrograph showing the presence of marginal band microtubules in calcium washed cytoskeletons. Bar equals 0.1 μm (x86,000).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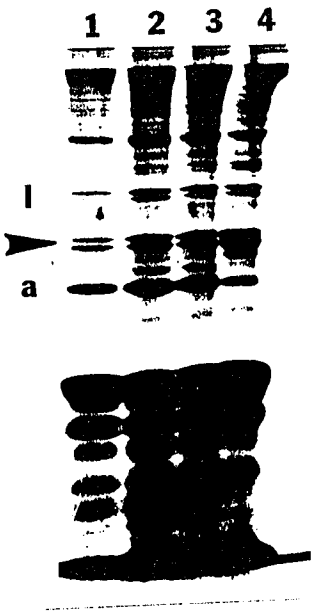
significant amounts of the tubulins. If cytoskeletons were lysed and washed with copious amounts of calcium free solutions prior to resuspension in Calcium Wash Solution, the cytoskeletal profiles could not be distinguished from those obtained without calcium treatment. Furthermore, analysis of the calcium supernatants showed, at most, only traces of the tubulins.

Recently, cold stable microtubules from rat brain have been shown to be disassembled by calcium in the presence of calmodulin (Job et al., 1981). Moreover, calmodulin binding proteins have been reported to be components of the erythrocyte cytoskeleton of certain vertebrates (Sobue et al., 1981; Bartelt et al., 1982). Addition of calmodulin and/or ATP to the calcium wash solution however, did not result in microtubule disassembly (figure 25). Thus, if calmodulin does mediate the change in microtubule stability, it might do so through cytoplasmic calmodulin receptor proteins. Preliminary reconstitution experiments using Calcium Lysis Solution supernatants have indicated that marginal band disassembly can be prevented by the calmodulin inhibitor trifluoperazine.

Figure 25. SDS 10% polyacrylamide gel electrophoresis of erythrocyte cytoskeletal protein from a calmodulin reconstitution study. Cytoskeletons were lysed and washed in EGTA, pH 5.5 solutions followed by a third wash in the following: lane 1- EGTA Wash Solution, pH 5.5, lane 2- Calcium Wash Solution, lane 3- Calcium Wash Solution containing 20 mM Na_2ATP, lane 4- Calcium Wash Solution containing 20 mM Na_2ATP, 100 ug/ml beef heart calmodulin. The same results were obtained in experiments using 20 mM $Mg^{++}ATP$ and 100 ug/ml of homogeneous bovine brain calmodulin.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DISCUSSION

Xenopus erythrocyte marginal band microtubules exhibit many stability properties which are characteristic of microtubules that are found in ciliary and flagellar axonemes or centrioles. These features include insensitivity to cold and mitostatic agents in addition to the direct action of calcium. They differ however, in that they can be rapidly depolymerized by calcium if in the presence of soluble cytoplasmic factors. Thus, calcium appears to indirectly activate either the modification of the tubulins or their associated proteins so that the microtubules become highly labile.

Microtubule associated proteins (MAPs), were originally identified as those proteins which associate with microtubules which have undergone several cycles of in vito assembly and disassembly (Sloboda et al., 1975, Berkowitz et al., 1977). Certain such high molecular weight MAPs have been shown to enhance the assembly as well as stability of in vitro assembled microtubules (Sloboda et al., 1976; Deery and Weisenberg, 1981). Recently however, Solomon and co-workers have called attention to the limitations of such a narrow definition of MAPs and have suggested that the proteins which are co-released with tubulin when cytoskeletal microtubules are depolymerized by specific agents also be included within this category (Solomon et al., 1979; Duerr et al., 1981).

Whether MAPs are responsible for the unusual stability of Xenopus erythrocyte cytoskeletal microtubules remains an exciting, yet unanswered question. A preliminary report on rat brain cold stable microtubules has suggested that the phosphorylation and dephosphorylation of a 64 kilodalton MAP may determine the cold

stability or lability of these proteins (Margolis and Rauch, 1981).

Certain high molecular weight polypeptides were often observed in our calcium wash supernatants that contain significant amounts of tubulin. Whether all or some of these polypeptides represent authentic or partially degraded MAPs (as considered by Solomon) is uncertain at this time. Although these polypeptides are co-released with tubulin, they are not done so by an agent which specifically acts on microtubules. Calcium is known to influence cytoskeletal elements other than microtubules (Phillips and Jakabova, 1977; Wang and Bryan, 1981). Moreover, even in this system, calcium acts to modify high molecular weight cytoskeletal components which are most probably associated with the SPMCN.

The existence of MAPs in marginal band systems remains to be more fully explored. A protein which is antigenically similar to MAP 2 from chick brain has been identified in marginal bands of toad erythrocytes (Sloboda and Dickerson, 1980). However, high molecular weight components were not observed in purified marginal band preparations from dogfish erythrocytes (Cohen et al., 1981). We hope two dimensional electrophoretic analysis of Xenopus erythrocyte cytoskeletal proteins will aid in the detection of proteins which are co-released with tubulin. Pretreatment of cells with taxol should make the marginal band insensitive to calcium (Parness and Horwitz, 1981) and thus allow us to distinguish which polypeptides are released from cytoskeletons as a result of the non-microtubular ramifications of calcium lysis. Zieve and Solomon (1982) have used such a similar approach to identify a specific MAP associated with the mitotic spindle.

Alternatively, the possibility that the unusual microtubule

stability of Xenopus erythrocyte cytoplasmic microtubules is due to unique forms or modified tubulin remains open. Tubulin has been shown in several diverse organisms to exist in more than one form (Sheir-Neiss et al., 1978; Mc Keithan and Rosenbaum, 1981). The in vivo significance of these different tubulins however, is not yet known. The two dimensional electrophoretic characterization of tubulin and MAPs from Xl 177 cells, which exhibit stability properties of steady-state microtubules, is already in progress and it will be significant to see if these proteins show any major differences from those identified in erythrocytes.

The mechanism by which calcium acts to initiate microtubule disassembly as well as the emergence of a new high molecular weight cytoskeletal polypeptide is under investigation. The possibility that calmodulin is involved in these processes appears likely. Evidence exists which suggests that this protein may be involved in the calcium dependent control of microtubule assembly-disassembly in a number of different biological systems (Marcum et al., 1978; Welsh et al., 1978; Schliwa et al., 1981; Job et al., 1981). In addition, it has been demonstrated in crude brain extracts that calcium dependent proteases act on MAP 2 and not on tubulin (Sandoval and Weber, 1978). Whether this is mediated by calmodulin has not yet been demonstrated.

Preliminary data from our laboratory indicates that formation of the CSHMWP and marginal band disassembly may involve calmodulin since it can be inhibited by trifluoperazine. At the calcium levels tested, no equivalent inhibition of microtubule depolymerization was observed. We are curious whether the production of this polypeptide is required for the demonstrated calcium induced change in cell morphology. Both

dogfish (Cohen et al.,1982) and chicken (Barrett and Dawson, 1974) erythrocytes retain their normal morphology in the absence of marginal bands.

Centrioles have been observed in blood cells from certain invertebrates but in contrast with those noted in the Xenopus erythrocte cytoskeleton, the former are associated with the marginal band (Nemhauser and Cohen, 1980). Nemhauser and Cohen (1981), have presented evidence which indicates that these structures may act as part of a microtubule organizing center during the in vivo reassembly of Noetia marginal bands. Dogfish erythrocyte marginal bands are also amenable to disassembly and reassembly in vivo (Cohen et al. 1981). It would be of value to know whether centrioles are present in the cytoskeleton of this organism and if so, the nature of their location.

CONCLUDING REMARKS

A major focus in cell biology in recent years has been the biochemical and ultrastructural characterization of the structural components of the nucleus and cytoplasm. Constituent proteins of several cytoskeletal components (microtubules, microfilaments, intermediate filaments) have been purified and the components reassembled *in vitro*. In some instances, accessory proteins which associate with these structures and possibly regulate them *in vivo*, have also been identified. This approach has considerably expanded our knowledge of the molecular organization of eukaryotic cells.

In this dissertation, nuclear and cytoskeletal structures were examined in a variety of *Xenopus* cell types. Spermatogenic cells were used to study nuclear matrix changes during meiotic prophase. From this study, it was demonstrated that in contrast to previous reports, nuclear matrices with NPLCs and SCs could be isolated from spermatocytes provided gentle isolation procedures were utilized. It was also shown that the NPLC becomes fragmented during chromosome synapsis.

Erythrocyte nuclear matrices were demonstrated to lack intranuclear matrix elements and consist solely of the nuclear lamina. The lamina was isolated in association with cytoskeletal elements (cyto-nuclear skeletons) such as centrioles and intermediate filaments. The later extends to the outer subsurface cytoskeletal shell. In erythrocyte cyto-nuclear skeletons, the nuclear lamina was often observed to have the overall shape and position of intact nuclei. Electron microscopic and electrophoretic characterization of these high salt treated structures suggests that although microtubules were no longer ultrastructurally observable, tubulin remained associated with the cyto-nuclear skeletons. This association may reflect unique interactions

between this protein and other cytoskeletal components as opposed to simply non-specific binding.

Xenopus erythrocyte marginal band microtubules were not depolymerized in intact cells or cytoskeletons by agents (cold, microtubule poisons, the direct action of calcium) which solubilize cytoplasmic microtubules. They were disassembled by calcium lysis. Calcium lysis appears to influence specifically tubulin and another high molecular weight cytoskeletal polypeptide since electrophoretic profiles of calcium lysed erythrocytes do not differ in other ways from those of cells lysed in the presence of EGTA. Moreover, the calcium released tubulin was not degraded. Therefore, it appears that calcium activates cytoplasmic factors, possibly mediated by calmodulin, which result in a sudden change in marginal band stability properties.

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