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Ronald L. Hall

CHANGES IN NUCLEI OF MITOGENICALLY
STIMULATED LYMPHOCYTES

© by
Ronald Hall, B.Sc. (Hons)

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

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ABSTRACT

Nuclei of murine splenic lymphocytes stimulated in vitro with concanavalin A (conA) increase several fold in volume and condensed chromatin disaggregates into small clumps. The nuclear morphological changes are accompanied by a large increase in interchromatinic material consisting of granules and fine fibrils.

Nuclei isolated from lymphocytes using TritonX-100 retained their in situ structure. Sequential extraction of these nuclei with deoxyribonuclease 1, 0.2mM magnesium chloride, 2M sodium chloride, TritonX-100, deoxyribonuclease 1 and ribonuclease A yielded residual nuclear matrices. These matrices contained approximately half of the original nuclear protein, 4-22% of the deoxyribonucleic acid and 10-16% of the nuclear ribonucleic acid, and were composed primarily of protein (81-96%). Morphologically, the matrices consisted of a residual nuclear envelope, residual nucleoli and an internal network composed of granules, fine fibrils and amorphous material. Matrices retained the general morphology of the nuclei from which they were derived. Matrices from stimulated cells were larger in volume and contained more extensive ramified internal network than matrices from unstimulated cells. Microspectrophotometry of naphthol yellow S stained samples showed large stimulated nuclei and matrices both

had increases in total protein of 2x and increases in non-histone protein of 2.8x and 3.6x respectively compared to unstimulated samples. The increase in matrix protein occurred between 10 and 18h post conA-stimulation (PS). Analysis of unstimulated and stimulated matrix proteins with 1- and 2-D polyacrylamide gel electrophoresis showed major components of 35, 43, 55, 65-70 and 90kd and numerous minor bands. Only minor changes in the electrophoretic pattern were found after stimulation. Substantial synthesis of matrix proteins occurred between 8 and 36h PS, much of this protein being stable during a 24h chase. The results substantiate previous cytological and cytochemical studies which indicated that interchromatinic matrix proteins are a major responding nuclear element during early stages of mitogenesis. These proteins may be responsible for the morphological changes of the nuclei and may also be involved in chromosome replication and/or gene transcription.

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LIST OF ABBREVIATIONS

Am	α -amanitin
AMD	actinomycin D
BSA	bovine serum albumin
CH	cycloheximide
DNA	deoxyribonucleic acid
DNase I	deoxyribonuclease I
EDTA	ethylenediaminetetraacetic acid
EM	electron microscope
FBS	fetal bovine serum
HB	homogenization buffer
HS	high salt buffer
LM	low magnesium buffer
conA	concanavalin A
LPS	lipopolysaccharide
ME	β -mercaptoethanol
NaTT	sodium tetrathionate
nm	nanometers
NYS	naphthol yellow S
PAGE	polyacrylamide gel electrophoresis
PHA	phytohemagglutinin
PMSF	phenylmethylsulfonyl fluoride
PS	post-stimulation
PWM	pokeweed mitogen

RNA	ribonucleic acid
RNaseA	ribonuclease A
RNP	ribonucleoprotein
SDS	sodium dodecylsulfate
STM	sucrose-tris-magnesium buffer
μm	micrometers

SOURCES OF SPECIAL CHEMICALS

conA	- Sigma Chemical Co.
BSA	"
DNA	"
DNaseI, RNaseA	- Worthington Biochemical Corp.
FBS	- Flow Labs. Inc.
³ H-leucine, ³ H-thymidine	- New England Nuclear
Mice, Balb/c, male	- Can. Breeding Farms Ltd.
PMSF	- Sigma Chemical Co.
NYS	- Polysciences Inc.
NATT	- Fluka Ab., Sweden
RNA	- Sigma Chemical Co.
RPMI 1640	- Flow Labs. Inc.
Soybean trypsin inhibitor	- Sigma Chemical Co.

INTRODUCTION

The eukaryotic nucleus plays a central role in control of cellular metabolism and differentiation. The nucleus is itself, however, an organelle which can exhibit substantial metabolic and morphological variation. Some nuclear changes, such as mitosis, are relatively transient while others are more permanent, eg. the differentiation of nuclei during maturation of avian erythrocytes (Dardick et al., 1981).

The factors which control volume and shape of nuclei and relative distribution of condensed chromatin and interchromatinic materials, ie. the gross organization of nuclei, are poorly understood. Virtually all nuclear components including DNA (Singer et al., 1978), RNA (Pogo, 1981), histones (Isenberg, 1979, Thoma et al., 1979, Corbett et al., 1980) and non-histone proteins (Paulson and Laemmli, 1977, Adolph, 1980) have been implicated in nuclear structure. Non-histone proteins have also been proposed as central agents in maintenance of metaphase chromosome structure (Adolph et al., 1977). However, no specific role in gross nuclear structure has as yet been assigned to any of these substances.

Recently, interest in gross nuclear structure has focused on non-chromatin components of nuclei which form a nuclear matrix (Berezney and Coffey, 1977) or cage

(McReady et al., 1980). These extra-chromosomal structures have been implicated in maintenance of gross nuclear structure (Berezney, 1979, Shaper et al., 1979, Brasch, 1982), control of nuclear volume (Wunderlich and Herlan, 1977), DNA synthesis (Comings and Wallack, 1978, Smith and Berezney, 1980, Berezney, and Buchholtz, 1981a, 1981b), RNA synthesis and processing (Miller et al., 1978, Long et al., 1979, Maundrell et al., 1981), function of hormone receptors (Agutter and Birchall, 1979, Barrack and Coffey, 1980) and organization of viral proteins (Chin and Maizel, 1977, Deppert, 1978, Buckler-White et al., 1980). Changes in the protein composition of nuclear matrices in relation to the cell cycle (Berezney et al., 1979a, 1979b), and with viral infection have been observed (Hodge et al., 1977), and it has been suggested that the matrix plays a central role in modulating interphase nuclear organization (Berezney, 1979, Shaper et al., 1979, Agutter et al., 1980).

Lymphocytes stimulated with mitogen undergo a large increase in nuclear volume and extensive morphological changes prior to DNA synthesis and mitosis (Tokuyasu et al., 1968, Milner, 1969a, 1969b, Setterfield et al., 1983). Morphometric analysis has shown that while the total volume of condensed chromatin remains essentially constant throughout G1, it is extensively

redistributed. On the other hand, the volume of interchromatinic space, containing fibrogranular ribonucleoprotein structures, increases markedly. Concomitant with these morphological changes, nuclear RNA and proteins are metabolized and relatively stable nuclear proteins appear (Setterfield et al., 1983). The lymphocyte thus provides a model system for study of processes underlying changes in nuclear structure during nuclear activation and preparation for cell division.

This thesis presents data which extends previous cytological and cytochemical work (Hall, 1980, Setterfield et al., 1983) in an attempt to describe more precisely macromolecular components involved in nuclear changes during lymphocyte stimulation. The main experimental approach has been to isolate and characterize nuclei and nuclear matrices from unstimulated and conA-stimulated lymphocytes. These preparations were characterized by light and electron microscopy, and were analyzed biochemically for gross chemical composition, by microspectrophotometry for protein content and by gel electrophoresis for protein composition.

LITERATURE REVIEW

A. CHANGES IN MITOGENICALLY STIMULATED LYMPHOCYTES

1. Biochemical Events

Mature lymphocytes in vivo are a population of small, non-dividing cells of great physiological importance because of their role in the immune system. There are two main subpopulations of lymphocytes based on embryonic origin. The thymus derived "T" lymphocytes are involved in cellular immunity and consist of several functionally distinct classes of cells including helper cells which stimulate B cell activation, suppressor cells, and cytotoxic or "killer" cells. The "B" cells, derived from bone marrow, are involved in humoral immunity and are the precursors of immunoglobulin secreting cells (Reinherz and Schlossman, 1980).

Lymphocytes isolated from peripheral blood, lymph nodes, or spleens can be cultured in a quiescent state for several days. Upon addition of either a foreign antigen or a non-antigenic mitogen, a population of cells enters the cell cycle with partial synchrony and divides 36 to 72 hours later. The mitogens most commonly used for in vitro lymphocyte stimulation are plant lectins such as concanavalin A (conA), poke weed mitogen (PWM), and phytohemagglutinin (PHA), although other substances such as bacterial lipopolysaccharide (LPS)

also have mitogenic action. These agents are preferred over simple antigens because they activate a large proportion of cells and are therefore called polyclonal mitogens.

The molecular basis of mitogenic stimulation has been studied extensively, although the complete story is still not known (for reviews see Ling and Kay, 1975, and Gottlieb, 1974). The mitogenic action of the lectins involves their binding to, and crosslinking of, cell surface glycoproteins. Since the T and B cell populations have different surface proteins their responses vary depending on the mitogen used. For example, conA activates only T cells, LPS only B cells, and PWM both T and B cells (Ling and Kay, 1975). It has been shown that removal of ConA by competition with α -methylmannoside up to 20 hours post conA-stimulation (PS) inhibited DNA synthesis at 48 hours (Rudd *et al.*, 1979).

Several events occur immediately after mitogen binding. Quastel and Kaplan (1970) have shown a ouabain-sensitive increase in K^+ uptake within minutes of activation which is not dependent on the synthesis of new K^+ -transport sites. This is balanced by an equal increase in K^+ efflux (Hamilton and Kaplan, 1976) so that the intracellular K^+ concentration remains constant. These conditions persist throughout activation and if

inhibited cell proliferation is prevented (Kaplan, 1978). Concomitant with the K^+ influx are an efflux of Na^+ (Averdunk and Lauf, 1975) and an influx of Ca^{++} (Freedman et al., 1975), resulting in a depolarization of the membrane (Kiefer et al., 1980).

There is also a transient rise in cAMP levels from 30-120 min after mitogen binding. Inhibition of this increase has been shown to prevent DNA synthesis (Foker et al., 1979). cGMP levels have also been shown to increase during this time (Hadden et al., 1972). Calcium ions and cyclic nucleotides are generally regarded as secondary messengers transmitting information from the cell membrane to the cytoplasm and nucleus (Cohen, 1978). Calcium ion concentration is controlled in many cell types by the ubiquitous Ca^{++} -binding protein calmodulin (Cheung, 1980, Means and Deadman, 1980, Pershadsingh et al., 1980) which has been shown to regulate the activity of protein kinases (Klee et al., 1980). cAMP-dependent protein kinases in CHO cells have also been shown to be translocated from the cytoplasm to the nucleus and bound to chromatin (Nesterova et al., 1981). This type of regulation has not yet been demonstrated in lymphocytes.

Increased protein synthesis, as indicated by incorporation of radioactive amino acids, has been observed within three to four hours PS and persists at

least until conclusion of the first mitosis (Kay, 1968). Inhibition of protein synthesis with cycloheximide during the first hour PS has been found to significantly reduce the proliferative response at later times (Varesio et al., 1980), suggesting that protein synthesis may begin very soon after activation. The activation of protein synthesis appears to involve translational controls since de novo RNA synthesis is not observed until approximately 6 hours PS (Mitchell et al., 1978). Concurrent with the increase in protein synthesis is an increase in processing and transport of presynthesized RNA transcripts to the cytoplasm (Mitchell et al., 1978).

The resting lymphocyte has a shortage of active initiation factors (Kay et al., 1978) and has other agents which inhibit protein synthesis in a cell-free system. Upon activation, the inhibitors lose activity while initiation factors become available (Kay et al., 1978). In addition, a ribosome-bound factor, elf3, necessary for the formation of the 80S initiation complex, becomes active a few hours after mitogen binding (Schreier et al., 1977, Thomas et al., 1979). Phosphorylation of two proteins (135kd and 150kd) was observed after 8 hours stimulation with ConA. This was inhibited by displacement of ConA from surface receptors with α -methylmannoside.

Pogo (1972) found that activity of RNA

polymerase-1 (nucleolar) and 2 (nucleoplasmic) increased after 15 min and 60 min exposure to PHA, respectively. Both activities increased linearly for six hours then remained constant for 48 hours. Inhibition of protein synthesis for the first six hours PS did not prevent these increases in activity suggesting that the enzymes were present but inactive in the resting cells.

Significant increases in total cellular RNA were observed beginning approximately 12 hours PS (Mitchell et al., 1978), and the levels increased significantly up to 48 hours. By 36 hours the first cells have entered S phase (Tokuyasu et al., 1968, Bard et al., 1978, Setterfield et al., 1983). The length of S phase appears to be variable from 5 to 12 hours (Ling and Kay, 1975). Slower cells continue to enter S phase until about 72 hours. Cell cycle dynamics become difficult to study beyond the first division because any partial synchrony is lost (Ling and Kay 1975).

2. Morphological Changes

The unstimulated lymphocyte is small (5-8 μ m diameter), and essentially spherical. In transmission electron micrographs it has a thin rim of cytoplasm with few organelles clustered adjacent to the centrioles at the so called "cell-center". The nucleus is filled with large clumps of heterochromatin, often has a deep cleft

and has very little interchromatinic space (Tokuyasu et al., 1968, Milner, 1969a, 1969b, Boutellier et al., 1978, Miragall and Renau-Piqueras, 1979, Vos et al., 1980, Dardick et al., 1981, Setterfield et al., 1983). A small nucleolus is seen either as ring-shaped with ribonucleoprotein (RNP) restricted to the periphery (Gani, 1976), or having concentric fibrillar and granular zones (Tokuyasu et al., 1968).

Immunofluorescent staining of unactivated lymphocytes with anti-tubulin antibodies revealed an organized network of microtubules radiating from a single microtubule organizing center (MTOC) at the cell center (Rogers et al., 1981). Within 10-20 min PS, immunoglobulin receptors migrated to one site on the cell surface forming a cap over the MTOC (Rudd et al., 1979, Oliver et al., 1980, Rogers et al., 1981). This process was inhibited by cytochalasin B (dePetris, 1975) but not by colchicine (Steen and Lindmo, 1978, Oliver et al., 1980, Pozzan et al., 1981, Rogers et al., 1981).

Between 16 and 24 hours PS with ConA substantial changes occur in cellular and nuclear morphology. The cell volume increases several fold, cytoplasmic organelles and villi on the cell membrane become more numerous, and a more extensive network of microtubules is observed (Tokuyasu et al., 1968, Milner, 1969a, Rudd

et al., 1979, Dardick et al., 1981, Rogers et al., 1981). Nuclear volume increases from 2 to 6 fold, and the distribution of condensed chromatin changes from a few large clumps to numerous smaller clumps. The total volume of condensed chromatin, however, as judged by morphometric analysis of EM micrographs does not change significantly during this time (Bladon, 1980, Setterfield et al., 1983). The increase in nuclear volume is mainly a result of increased volume of the interchromatinic space (Valkov and Moyne, 1974, Setterfield et al., 1983). This is in contrast to some meta-stable cells such as sea urchin eggs, which can be triggered to proliferate but whose nuclear volumes do not increase (Epel, 1980). The lymphocyte situation is the reverse of that observed in developing avian erythrocytes, where the interchromatinic volume decreases as nuclear volume decreases during cell differentiation (Dardick and Setterfield, 1976).

The regressive uranyl-EDTA stain of Bernhard (1969) preferentially stains ribonucleoprotein (RNP), free RNA and proteins but not chromatin. This stain revealed a small amount of interchromatinic material in nuclei of unstimulated lymphocytes and a substantial increase in such material in nuclei of stimulated cells (Valkov and Moyne, 1974, Setterfield et al., 1983). High magnification views of such preparations show that the

interchromatinic space in nuclei of both unstimulated and stimulated cells consists of 25nm diameter globular particles, fine fibrils 3-10nm in diameter and amorphous material.

Between 12 and 36 hours PS, the nucleoli increased up to several fold in volume, and fewer ring shaped nucleoli were seen. Most nucleoli in stimulated cells had a fibro-granular appearance with a few small lacunae. Nucleoli of cells in G2 (60 hours PS) were diffuse, with no distinct lacunae (Gani, 1976).

3. Relationship Between Nuclear Morphological Changes And Biochemical Events

Studies from our laboratory and others have shown that the nuclear morphological changes accompanying lymphocyte stimulation occurred in the presence of inhibitors of DNA synthesis such as hydroxyurea and cytosine arabinoside (Milner, 1969b, Conforti et al., 1978, Setterfield et al., 1983). However, both the nuclear morphological changes and DNA synthesis were prevented by inhibiting protein synthesis with cycloheximide (CH) or RNA synthesis with α -amanitin (Am) if the inhibitor was given prior to the morphological changes (ie. before 16hr PS). The nuclear morphological changes which precede DNA synthesis, therefore, can occur independently of but are probably a necessary

prerequisite for DNA synthesis.

Inhibition of protein synthesis with cycloheximide (CH) has been shown to inhibit lymphocyte proliferation (Kay and Korner, 1966). Addition of CH prior to 16 hours PS completely abolished nuclear morphological changes in cells examined at 28 hours PS. However, addition of CH at 24 hours PS did not prevent the nuclear changes (again assayed at 28 hours PS). Addition of CH between 16 and 24 hours gave a graded inhibition of the nuclear changes. The nuclear changes, therefore, have a transient dependence upon protein synthesis probably because proteins necessary for the nuclear morphological changes are made as the morphological changes occur. Similar results were obtained when RNA synthesis was inhibited with Am during the period of nuclear morphological changes. Electron-microscope autoradiography indicated that substantial amounts of nuclear proteins synthesized during the 16 to 24 hour period PS remain stable over 60 hours and most of these were located in the interchromatinic space (Hall, 1980). These results suggest that the nuclear morphological changes are dependent in part on the synthesis of stable proteins prior to and during these changes. Addition of either CH or Am for 8 hours to cells stimulated for 32 hours with conA caused no change in nuclear morphology.

(Hall, 1980). This indicates that the morphological changes in nuclei, once achieved are not dependent on further protein or RNA synthesis.

B. NON-CHROMATIN NUCLEAR STRUCTURES

1. The Isolated Nuclear Matrix

A powerful method for study of nuclear structure and composition is fractionation of isolated nuclei into sub-components followed by combined biochemical and cytological analysis. Early studies by Mayer and Gulick (1942) involved the extraction of isolated nuclei with 1.0M NaCl. They found that significant amounts of protein were not solubilized by the high salt treatment and they termed these "residual nuclear proteins". Electron microscopy of such salt extracted nuclei revealed fibrous structures similar in size and gross morphology to the intact nuclei (Mirsky and Ris, 1951). More detailed studies of these residual nuclear proteins were performed in the mid 1970's when several workers used procedures to extract DNA, RNA and chromatin-associated proteins from nuclei (for reviews see Bereznay, 1979, Shaper et al., 1979, and Agutter and Richardson, 1980).

Brasch, Setterfield, and Neelin (1972) treated isolated chicken liver and erythrocyte nuclei with 0.01M citric acid and various concentrations of HCl to

sequentially extract histones. They found that removal of all histones changed chromatin fibril diameter from 20nm to <10nm but did not disrupt the overall nuclear structure.

Berezney and Coffey (1974,1977) extracted isolated rat liver nuclei sequentially with DNase 1, a low magnesium buffer (0.2mM MgCl₂), a high salt buffer (2.0m NaCl), 1% TritonX-100, and DNase 1 and RNase A. The DNase 1, low magnesium and high salt buffers extracted approximately 80-90% of the total protein and most of the RNA and DNA. The final nuclease extraction left less than 1% of the total DNA and 2% of the total nuclear RNA in the residual nuclei. This final preparation was termed the proteinaceous nuclear matrix.

Electron microscopy (Berezney and Coffey,1977) showed that these matrices resembled intact nuclei, with three major components visible; a residual nuclear envelope at the periphery of the nuclear spheres, residual nucleoli and a fibro-granular internal network. In cross section the residual nuclear envelope was a continuous electron-dense layer 10-15nm in thickness which contained 90nm diameter annular structures resembling nuclear pores. In glancing sections the nuclear pores were well defined annuli with central openings of 30nm. The residual nucleoli were similar to in situ nucleoli but slightly more compact and

electron-dense. No nucleolar-associated chromatin was seen in the matrices; nucleoli were surrounded by spaces similar in size to the associated condensed chromatin of intact nuclei. The internal fibrillar network was composed of 15-25nm diameter globular particles and fibrils as small as 3-5nm diameter. The internal matrix appeared as a ramified network of fibro-granular material extending from the residual envelope to the residual nucleoli. Small regions of dense 15-25nm particles seen in nuclei in situ were also seen attached to the internal network. Also prominent in the matrices were numerous spaces adjacent to the nuclear envelope and in the internal network. These spaces were of similar sizes, positions and number as the regions of condensed chromatin seen in intact nuclei. Berezney and Coffey suggested that the spaces represented the locations of the extracted condensed chromatin while the internal network represented the euchromatic or interchromatinic regions. These studies suggested that chromatin is not essential for maintenance of gross nuclear structure.

Several other workers have isolated nuclear matrices from a wide range of tissues using procedures similar to Berezney and Coffey (1977). Matsuura et al. (1981) isolated rat-liver matrices consisting of a residual nuclear envelope, electron-dense residual

nucleoli and an internal network. Their internal network had fibrillar and granular components but was less extensive than that isolated by Berezney. Kuzmina and coworkers (1981) also isolated rat liver nuclear matrices similar to those of Berezney and Coffey (1977). Scanning EM of these matrices revealed the extent of the internal network; the overall appearance was that of a porous solid with numerous large internal spaces. Nuclear pores were clearly visible on the surface of the residual envelope which appeared as a continuous sheet covering the matrices. Fibrils as small as 5-10nm diameter were seen in the internal network but not in the large empty spaces (Kuzmina et al., 1981). Similar preparations have also been made by Cocca et al. (1980).

Comings and Okada (1976) isolated matrices from mouse liver using a procedure similar to Berezney and Coffey (1977). Their matrices consisted of a residual envelope, residual nucleoli and internal network, but they also observed disrupted matrices consisting of only residual nuclear envelopes. They suggested that the internal network is fragile and its loss may have been due to physical damage.

Bouvier and coworkers (1980) used stereo reconstruction from serial electron micrographs to examine the three dimensional organization of the

nuclear matrix from HeLa cells. They found that the residual nuclear envelope was a 13nm thick layer which contained nuclear pores and formed a continuous envelope surrounding each nuclear matrix. The internal fibrillar network consisted of 5nm thick fibrils and 8-10nm diameter granules, which formed a complex network of 'trabeculae' throughout the nuclear matrix. Some of the trabeculae were attached to the nuclear envelope and others to the residual nucleoli.

Nuclear matrices have also been isolated from chicken liver (Brasch, 1982), Drosophila melanogaster embryos (Fisher et al., 1982), Friend erythroleukemia cells (Grebanier and Pogo 1979), HeLa cells (Hodge et al., 1977, Herman et al., 1978), 3T6 cells (Buckler-White, 1980), Tetrahymena macronuclei (Wunderlich and Herlan, 1977, Herlan et al., 1979), Xenopus oocytes (Snead et al., 1979), Zajdela ascites hepatoma cells (Berezney et al., 1979a), and others (for review see Shaper et al., 1979).

To date, the only lymphocyte nuclear matrices reported have been prepared from bovine lymph nodes (Nakayasu and Ueda, 1981). Nuclei from unstimulated lymphocytes extracted with DNase, 0.4M NaCl, 2M NaCl and DNase and RNase gave matrices similar to those of Berezney and Coffey (1977). The matrices had residual nuclear envelopes, nucleoli and an internal network

which was electron-dense. Peripheral empty spaces corresponding to the large clumps of condensed chromatin of unstimulated lymphocytes were also seen.

Nuclear matrix proteins have been analyzed extensively by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Bovine lymphocyte matrices revealed three major proteins of 68kd, 53kd and 43kd and approximately 50 minor proteins of 15 to 150kd. Histone proteins were present in relatively small amounts indicating nearly complete extraction of chromatin from the nuclei (Nakayasu and Ueda, 1981). The 43kd protein accounted for 12% of the total matrix protein and was thought to be actin (Nakayasu et al., 1981).

Liver nuclear matrices contained three major proteins with molecular weights between 60kd and 70kd. These proteins accounted for up to one half of the total protein in the gels (Berezney and Coffey, 1974, 1977, Comings and Okada, 1976, Todorov and Hadjiolov, 1979, Kuzmina et al., 1981, Matsuura et al., 1981, Brasch, 1982). Numerous minor proteins were also observed from 15kd to 150kd. Tetrahymena nuclear matrices had major proteins of 67kd, 53kd, 43kd, 33kd and 26kd, and in addition to trace amounts of DNA and RNA contained 1% carbohydrate (Wunderlich and Herlan, 1977, Herlan et al., 1979). HeLa-cell matrices consisted of approximately 30

proteins with several major proteins from 45kd to 75kd (Hodge et al., 1977, Herman et al., 1979), while D. melanogaster matrices contained three major proteins of 174kd, 74kd and 42kd (Fisher et al., 1982).

Two dimensional electrophoresis using isoelectric focusing in the first dimension and SDS-PAGE in the second dimension (O'Farrell, 1975) has been used to analyze nuclear matrix proteins. Comings and Peters (1981) found that 2-D electrophoresis of nuclear matrix preparations from rat liver revealed approximately 50 proteins. The 60kd to 70kd proteins prominent in 1-D gels were resolved into approximately 25 proteins with slightly different pI's indicating the possibility of charge isomers due to postsynthetic modifications. Other major proteins were observed at approximately 50kd molecular weight. Most of the matrix proteins were in the acidic region of the gel with pI's from 5.5 to 7.0.

Two-D gels of HeLa nuclear matrices were also found to have numerous acidic proteins (Detke and Keller, 1982) but only minor proteins in the 60kd to 70kd range. The major polypeptides had molecular weights from 40 to 55kd, and pI's from 5.2 to 6.0. Histones were either absent or were minor components in most of these gels indicating that chromatin had been effectively extracted. Analysis of the matrix proteins showed that they had an acidic to basic amino acid ratio

of 1.46 (Berezney and Coffey, 1977).

Berezney (1980) further fractionated nuclear matrices from rat liver by sonicating samples and centrifuging at 5000xg to pellet a protein fraction termed "matricin" and centrifuging the resulting supernatant at 100,000xg to pellet a ribonucleoprotein fraction containing all of the RNA. SDS-PAGE of the matricin fraction showed only the three polypeptides of 62kd, 66kd, and 70kd which represented approximately 40% of the total matrix protein. Electron microscopy of these proteins showed only fine fibrils (3-5nm diameter) similar to those seen in the interior of the whole matrices. No RNA was found but 70% of the matrix-associated DNA was associated with the matricin fraction.

The RNP fraction also had substantial amounts of the 60kd to 70kd proteins but in addition contained numerous other proteins, especially between 25kd and 55kd. This fraction also contained 99% of the matrix-associated RNA and 30% of the matrix associated DNA. Berezney suggested that the fibrillar matricin proteins formed the fibrous nuclear matrix to which some DNA and the globular 25nm RNP particles were attached.

The overall morphological and biochemical similarities of nuclear matrices isolated from different cell types indicates that they are ubiquitous structures

which could be important for the overall organization of the eukaryotic nucleus. However, it should be kept in mind that the methods of matrix isolation are relatively crude and could result in aggregation or precipitation of nuclear components which are not direct parts of a single structural entity.

Clark and Rosenbaum (1979) have shown that gels of Xenopus oocyte nuclei with nuclear envelope removed contained a meshwork of randomly arranged microfilaments. They used myosin subfragment-1 from rabbit skeletal muscle to decorate the microfilaments indicating the presence of F actin. Bovine lymphocyte matrices have been found to contain significant amounts of protein with the same molecular weight as actin (Nakayasu et al., 1981). Nuclear actin bundles have been induced in Amoeba, Dictyostelium, and HeLa cells with dimethylsulfoxide (Katsumaru, 1979). However, electrophoretic analysis of nuclei from other sources revealed only small amounts of protein comigrating with actin (Peters and Comings, 1981, Berezney and Coffey, 1977).

2. The In Situ Nuclear Matrix

Treatment of nuclei with low and high ionic strength buffers, detergents and nucleases are relatively harsh treatments which may cause artifactual

aggregation or rearrangement of nuclear components. While nuclear matrices are morphologically similar to in situ nuclei, some nuclear structure appears to be lost during matrix isolation. In addition to structurally intact matrices a mixture of nuclear fragments, ghosts and structurally distorted matrices are also observed (Comings and Okada, 1976). Therefore, the precise architecture of the nuclear matrix in situ is not clear.

There is, however, indirect evidence for an in situ nuclear matrix. The regressive uranyl-EDTA EM staining procedure of Bernhard (1969) preferentially stains ribonucleoproteins (RNP), RNA and other proteins but not chromatin. The nuclei of guinea pig lymphocytes (Valkov and Moyne, 1974) and rat liver cells stained by this procedure had a staining pattern resembling the isolated nuclear matrix (Bernhard, 1969, Monneron and Bernhard, 1969, Derenzini et al., 1978). Between unstained chromatin masses a network of stained material, corresponding to the interchromatinic material seen in conventionally stained nuclei, contained globular particles of 25nm diameter, fibrils of 3-10nm diameter and amorphous material.

Treatment of rat liver cells with α -amanitin inhibited RNA synthesis and resulted in movement of chromatin from the interior of the nucleus to the periphery (Brasch and Sinclair, 1978). This resulted in a

large decrease in the number of fine fibrils observed in the interior of the nucleus. These preparations stained only weakly with conventional uranyl-lead but stained intensely with the phosphotungstic acid stain for proteins, showing an extensive network of fine fibrils as small as 10-15nm diameter throughout the entire nucleus (Brasch and Sinclair, 1978, Ghosh et al., 1978, Brasch, 1982). It was suggested that this fibrillar network represented the in situ nuclear matrix. No large spaces similar to those seen in isolated nuclear matrices, corresponding to regions of extracted condensed chromatin, were observed suggesting that the in situ nuclear matrix may be more extensive than was indicated by the isolated nuclear matrices. High voltage EM of unembedded thick sections of resting lymphocytes showed an extensive "trabecular" matrix within the nucleus (Guatelli et al., 1982). It was not clear, however, how much of this matrix was chromatin fibrils. While this evidence for an in situ nuclear matrix is circumstantial, it does indicate that a highly ramified fibrillar network exists in nuclei. The composition of this matrix, however, was not determined by these studies.

Other evidence that the isolated nuclear matrix is not grossly artifactual comes from cells which can be stimulated to undergo nuclear morphological

changes. Nuclei of Friend erythroleukemia cells stimulated to differentiate decrease in volume and large clumps of heterochromatin form at the nuclear periphery. Nuclear matrices from undifferentiated cells were large and contained large amounts of internal fibrillar network. Nuclear matrices from differentiated cells were smaller and contained less internal network and large empty spaces presumably representing the regions of extracted heterochromatin (Long et al., 1979).

Tetrahymena cells contain numerous small nucleoli while after treatment with actinomycin D (AMD) giant fusion nuclei form. Nuclear matrices from untreated cells had only small residual nucleoli while matrices from AMD treated cells had giant residual nucleoli (Herlan et al., 1978). Therefore, isolated nuclear matrices reflect in situ nuclear morphology.

3. Nuclear Lamina

The nuclear matrix as isolated by Berezney and Coffey (1977) has been criticized as being artifactual (Gerace et al., 1978, Krohne et al., 1978a, 1978b, Sticker and Hausen, 1980). This criticism is based on the fact that several nuclear fractionation procedures give only a residual nuclear envelope preparation, which has been termed the "nuclear lamina" or "nuclear pore-lamina complex" (for review see Franke et al., 1981a).

Aaronson and Blobel (1975) extracted rat liver nuclei with DNase, 2.5% Triton X-100, and a high salt buffer (0.3M $MgCl_2$). The procedure removed approximately 98% of total nuclear protein, >90% of DNA and >99% of nuclear RNA. The remaining material was composed of 98% protein. Electron microscopy of the preparation revealed little morphological similarity to intact nuclei. Three structures were identified; annuli 70-90nm in diameter resembling nuclear pores in cross-section, goblet-shaped structures 65nm long similar to nuclear pores in longitudinal section and an amorphous lamina 15nm thick and up to several micrometers in length. The lamina structures were interpreted as being residual nuclear envelope consisting of nuclear pores and integral membrane-associated proteins. SDS-PAGE of the lamina revealed three major proteins of 69kd, 68kd and 66kd termed lamins A, B, and C respectively. Several minor proteins were also detected as well as small amounts of core histones.

A modified procedure for isolation of the lamina was subsequently reported by Dwyer and Blobel, (1976). They treated rat liver nuclei sequentially with DNase 1 at pH8.5, DNase 1 at pH7.5, Triton X-100, and a high salt buffer (2M NaCl). In the EM they observed a triple layered lamina derived from the inner and outer nuclear

membranes, with residual nuclear pores. Negatively stained whole mounts showed sheets of fibrous proteins with randomly arranged nuclear pores. SDS-PAGE again revealed three major polypeptides of 60-70kd.

Giese and Wunderlich (1979) isolated lamina from Tetrahymena macronuclei by treatment with DNase and RNase in a low ionic-strength buffer, followed by high salt (1M NaCl). This procedure extracted 87% of the total nuclear protein and >99% of DNA and nuclear RNA. Electron microscopy of this preparation revealed circular lamina structures with residual nuclear pores. Krohne and coworkers (1978b) mechanically isolated nuclear envelopes from Xenopus oocytes. These were extracted with buffers containing 1% Triton X-100 and 1.5M KCl. They observed nuclear pores very similar to those in the unextracted nuclear envelopes, and amorphous proteins connecting the pore complexes. The pore density in the envelopes was lower after extraction suggesting that some rearrangements may have occurred. SDS-PAGE of the pore-lamina fractions revealed two major proteins of 150kd and 73kd, and several minor proteins. Rat liver pore-lamina preparations isolated with TritonX-100 and high salt buffers also contained these two proteins and two additional proteins of 78kd and 66kd. When rat liver laminae were extracted with TritonX-100 and high salt buffers the 66kd protein was

removed (Krohne et al., 1978a). It was suggested that this protein may be part of the fibrous lamina or part of the internal network. Richardson and Maddy (1980) iodinated proteins in isolated rat liver nuclei using conditions which only allowed labelling of the outer (cytoplasmic) surface of the nuclear envelope. They isolated the pore-lamina fraction and identified 12 proteins with molecular weights of 33kd to 200kd. Extraction of the isolated nuclei with a low magnesium buffer and DNase 1 (ie. isolation of pore-lamina) prior to iodination allowed labelling of nuclear proteins on both sides of the nuclear envelope. When this was done, a 58kd protein was detected in addition to the 12 proteins previously detected. This indicated that the 58kd protein was located on the inner (ie. nuclear) surface of the nuclear envelope.

The nuclear lamina preparations of Aaronson and Blobel (1975) have been extensively characterized by 2-D electrophoresis (Peters and Comings, 1980, Comings and Peters, 1981). The pore-lamina consisted of a prominent group of proteins at 68kd differing slightly in isoelectric points (approximately pI 6.1) which accounted for 68% of the total stained protein. Actin (43kd) comprised 13% of the total pore-lamina protein, and several other minor proteins of 45-55kd were present. Comparison with 2-D gels of nuclear matrices as per

Berezney and Coffey (1977) clearly showed that the pore-lamina proteins were present in the matrix preparations, albeit much less prominently.

Lam and Kasper (1979) electrophoresed nuclear envelopes in the presence of β -mercaptoethanol (ME) to analyze the positional relationships of major proteins. The 74kd protein was crosslinked by disulfide bridges to form two electrophoretically distinct polymeric forms. The 68kd protein remained exclusively in a monomeric state. The 49-60kd proteins were not crosslinked in the native membrane. They also found that the 78kd and 68kd proteins had close sequence homology while the 74kd protein was distinct. Shelton and coworkers (1980) heated (100°C) the pore-lamina proteins from chicken erythrocyte nuclear envelopes at pH 3.5 for up to 20 min and observed a decrease in the relative amount of 75kd protein (lamin A) and an increase in the 68kd protein (lamin C). It was suggested that lamin C might be a cleavage product of lamin A. Lamins A and C had similar tryptic peptide maps while that of lamin B was different.

The three major lamin proteins of the pore-lamina fraction have been localized cytochemically using polyclonal antibodies. Antibody preparations against the separate lamin proteins gave similar immunofluorescent staining patterns in whole cells; the nuclear periphery stained intensely while the interior

showed diffuse fluorescence (Ely et al., 1978, Gerace et al., 1978, Krohne et al., 1978a, Stick and Hausen, 1980). Purification of anti-70kd antibodies on an immunoaffinity column reduced staining of the nuclear interior without diminishing peripheral staining. Electron micrographs of immunoperoxidase staining with anti-70kd or anti-67kd antibodies showed exclusive perinuclear staining (Gerace et al., 1978).

Krohne and coworkers (1978a) also used indirect immunofluorescent staining and immunoperoxidase to localize the 67kd protein (lamin B). Staining in rat kangaroo PtK2 and murine 3T3 cells was confined to the nucleus and was very strong at the periphery. Some nuclei showed little staining of the interior while others showed considerable localized internal staining. Frozen rat liver sections stained only at the nuclear periphery with anti-70kd. In contrast, anti-histone H2b serum stained the nuclear interior intensely. Stick and Hausen (1980) purified the three major 60-70kd proteins from pore-lamina but polyclonal antibodies to each of these proteins crossreacted with several other nuclear envelope proteins. Although it was claimed that these antibodies stained the nuclear periphery exclusively the micrographs presented showed considerable staining of the nuclear interior. Their antibodies (which reacted with all three lamins) clearly stained an

intricate internal nuclear network in frozen Xenopus oocyte sections as well as the peripheral lamina.

The three major pore-lamina proteins have, therefore, been localized in the nuclear envelope. It is not yet clear, however, whether the same, or antigenically related, proteins are located in the internal network of the nucleus.

4. Differences Between Nuclear Matrix And Pore-Lamina

Using similar procedures several groups have isolated nuclear matrices (see Shaper et al., 1979 for review) while other groups have isolated the pore-lamina fractions (see Krohne et al., 1982 for review). From morphological (Berezney and Coffey, 1977, Aaronson and Blobel, 1975) and electrophoretic data (Peters and Comings, 1980) the pore-lamina appears to represent the residual nuclear envelope described by Berezney and Coffey (1977).

Is the internal component of the matrix an artifact, or has it been lost from the lamina preparations? Comings and Okada (1976) observed morphologically intact matrices and pore-laminae in the same preparations. This implies that the lamina is derived from part of the nuclear matrix and they

suggested that physical damage during isolation produced free laminae.

There were differences in the isolation procedures for matrix and lamina. Berezney (1979) showed that without protease inhibitors (such as PMSF or NATT), proteolysis significantly decreased the yield of matrix proteins and changed protein profiles on SDS-PAGE gels. None of the lamina procedures (Aaronson and Blobel, 1975, Giese and Wunderlich, 1979, etc.) used these inhibitors and this may account for the absence of some proteins in lamina preparations. Kaufmann et al. (1981) have shown that inclusion of mercaptoethanol (ME) in matrix isolation buffers also leads to a large loss of matrix proteins. Matrices treated with ME lost all of their internal matrix and appeared similar to pore-lamina preparations. Both Aaronson and Blobel (1975) and Giese and Wunderlich (1979) used ME. Aaronson and Blobel (1975) isolated the lamina at room temperature while the groups isolating matrices performed their isolations at 0-4°C. Aaronson and Blobel also centrifuged the lamina preparations at high speed through buffers of low viscosity. We have observed that lymphocyte matrices are fragile and great care must be taken when resuspending the pellets, Aaronson and Blobel (1975) vortexed their preparations to suspend them. It appears, therefore, that there are sufficient technical

differences between the procedures used for isolating matrices and lamina to account for the different products. Interestingly, Blobel's group (Fisher et al., 1982) have recently isolated nuclear matrices from Drosophila embryos using a procedure similar to Berezney and Coffey (1977). These matrices had extensive internal fibrillar structure and residual nucleoli.

Additional evidence suggests that the matrix proteins are functionally distinct from the pore-lamina. Agutter and Richardson (1979) found that nuclear matrices isolated from rat endometrium contained high affinity estradiol binding sites whereas pore-laminas from the same cells did not. Also, rat liver nuclear matrices contained 85% of the rapidly labelled hnRNA, while pore-lamina preparations contained virtually none. Therefore, there is reasonable evidence to support the hypothesis that the pore-lamina is a fraction of the nuclear matrix representing the residual nuclear envelope.

5. Other Nuclear Structures

a) Nuclear Cage

Structures termed "nuclear cages" or "nucleoids" have been isolated by lysing HeLa nuclei in buffers containing 0.5% TritonX-100 and 1-2M NaCl (Cook

et al., 1976, McReady et al., 1980). These nuclear cages retained all of the DNA, most of the nuclear RNA and variable amounts of protein. The major effect of the extractions was removal of histones and chromosomal proteins as judged by SDS-PAGE (Cook et al., 1976). Four major proteins were detected with molecular weights of 45kd to 65kd. Whole mount electron microscopy of these nuclear cages revealed an electron-opaque core with radiating loops of DNA. The DNA appeared to be highly supercoiled and digestion with EcoRI released this supercoiling (McReady et al., 1980). The EcoRI digested cages contained 10% of the total DNA and when pulse labelled with ³H-thymidine, contained virtually all of the newly synthesized DNA.

Berezney and Buchholtz (1981a) have isolated a similar structure from rat liver cells by eliminating nuclease treatment from the matrix isolation procedure. These "DNA-rich" matrices contained between 60 and 90% of the total DNA. Most of the DNA, in contrast to the previously described nuclear cage, was in the interior of the structure in clumps and in groups of fine fibrils.

b) Nuclear Ghosts

A structure very similar to the nuclear cage and the nuclear matrix is the nuclear ghost isolated from

HeLa cells (Riley et al., 1975, Keller and Riley, 1976, Riley and Keller, 1978). The nuclear ghosts were isolated by extracting nuclei with 0.5M MgCl₂ and sedimenting them on continuous sucrose gradients containing 0.5M MgCl₂. This extraction removed 95% of the DNA, 71% of the RNA, and 87% of the nuclear protein. The composition of ghosts was 72% protein, 14% DNA, 10% phospholipid and 4% RNA (Riley et al., 1975). Whole mount electron microscopy of the ghosts showed a fibrous network composed predominantly of 50nm diameter rods up to 260nm in length. Treatment of ghosts with DNase 1 did not significantly alter their morphology. SDS-PAGE showed two major proteins of 20kd and 35kd. Nuclear ghosts from hen oviduct nuclei were more electron-opaque, with a dense core surrounded by loose fibres (protein or DNA). These appeared similar to the nuclear cages of McReady et al., (1980).

c) Nucleolar Matrices

Isolated nucleoli extracted with low and high salt buffers and digested with RNase and DNase revealed a fibrous matrix similar to the residual nucleoli seen within nuclear matrices (Todorov and Hadjiolov, 1979, Comings and Peters, 1981, Franke et al., 1981b). Electron microscopy of nucleolar matrices from Xenopus oocytes showed a fibrous internal structure with densely packed

peripheral aggregates. The smallest fibrils were 4nm in diameter although numerous larger fibrils were also seen. SDS-PAGE detected a very prominent 145kd protein, no RNA appeared in the electropherograms (Franke et al., 1981b). Rat liver nucleolar matrices contained several proteins of 82kd, 70kd, 56kd, 40kd and 30kd (Todorov and Hadjiolov, 1979). Comings and Peters (1981) also observed numerous proteins in the 40-95kd range from rat liver nucleolar matrices. 2-D electrophoresis revealed more than 20 proteins with a prominent basic group (pI 8.0) of 25-35kd.

d) Chromosome Scaffold

Adolph, Cheng, and Laemmli (1977) extracted isolated HeLa cell metaphase chromosomes with dextran sulfate and heparin. This removed approximately 90% of the total protein, >99% of the histones and 10-20% of the DNA. These residual chromosomes could be dissociated by further digestion with trypsin, chymotrypsin or by treatment with 4M urea, but not by 2M NaCl, indicating that residual non-histone proteins were structural components of metaphase chromosomes. SDS-PAGE of the residual chromosomes revealed three major proteins of 53kd, 51kd and 35kd, and approximately 20 minor proteins, predominantly >40kd.

Electron microscopy of these histone-depleted chromosomes revealed a scaffold or core of dense interconnected fibres from which extended numerous 10-30um loops of DNA (Paulson and Laemmli, 1977). Similar structures were observed when chromosomes were extracted with 2M NaCl instead of dextran sulfate. The scaffold had the basic shape of a metaphase chromosome and it was suggested that a small group of non-histone proteins were responsible for the structural organization of chromosomes. Similar structures were also reported by Wray et al., (1979).

Okada and Comings (1979) isolated histone depleted chinese hamster (CHO) chromosomes and compared the proteins with nuclear matrix proteins. The profiles were very similar, especially in the 40kd to 70kd range. Detke and Keller (1982) compared scaffold and nuclear matrix proteins using 2-D electrophoresis. Both preparations consisted of approximately 25 proteins with molecular weights from 40kd to 70kd and pI's from 5.5 to 6.5. The protein patterns were not identical but were very similar. Therefore, non-histone proteins similar to those of the nuclear matrix may be involved in the maintenance of condensed chromosome structure during mitosis.

C. POSSIBLE FUNCTIONS OF THE NUCLEAR MATRIX

1. Association With DNA

Although most of the chromatin is extracted during matrix isolation, a small amount of DNA (1-10%) remains tightly bound to the matrix. This matrix-bound DNA is only removed by extensive digestion with DNase I (Berezney and Coffey, 1977). Several studies have shown that the nuclear matrix may be involved in DNA replication. Berezney and Coffey (1975) injected ³H-thymidine into partially hepatectomized rats and isolated matrix-associated DNA and the extracted bulk-DNA after various periods of time. They found that for the first 10 to 20 min after injection, the specific activity of the matrix-bound DNA was several fold higher than that of the bulk DNA. The percentage of total label in the matrix fraction decreased linearly during the first 30 min after injection while the label in the bulk DNA fraction increased linearly during this time. These results suggested that the matrix might be the site of DNA replication.

In a repeat study, (Berezney and Buchholtz, 1981a) which included protease inhibitors during matrix isolation the matrix-bound DNA was found to contain approximately 50% of the total label after a one-minute pulse, and this quickly decreased to 5% after a 10 minute pulse. The bulk-DNA contained <5% of the total label

after one-minute and this increased linearly to 65% after 10-minute. This again suggested that newly-synthesized DNA was initially associated with the nuclear matrix and was subsequently transported to the bulk DNA fraction. The average size of DNA fragments attached to the nuclear matrix was estimated at 1.6kb by sedimentation. The labelled matrix-associated DNA was highly sensitive to exogenous DNase I digestion, as has been reported for replicating chromatin (Burgoyne et al., 1976, Hewish, 1977). Similar results have been reported for matrices from mouse 3T3 cells (Pardoll et al., 1980) and Physarum polycephalum (Hunt and Vogelstein, 1981). To investigate the possibility of artifactual DNA binding to the matrix, Berezney and Buchholtz (1981b) added labelled DNA obtained from matrices to unlabelled matrices, during and after matrix isolation, and found only trace amounts of bound label. This indicated that it was unlikely that DNA bound to the matrix fortuitously during matrix isolation.

By eliminating nuclease digestion during matrix isolation Berezney and Buchholtz (1981a) isolated "DNA-rich" matrices which contained 65% of the total nuclear DNA. The matrix-bound DNA had an average size of 500kb and contained >95% of label after a one min pulse with ³H-thymidine. Replication intermediates from 4-5S up to >50S were present at this time. After a one hour pulse, the amount of label sedimenting at <50S was reduced from 60% to 10% while there was a corresponding

increase in labelled DNA >50S.

Nuclear matrices isolated from mouse 3T6 cells lytically infected with polyoma virus contained approximately 10% of the total polyoma DNA (Buchler-White et al., 1980). The matrix bound polyoma DNA included both rapidly labelling and bulk viral DNA, therefore the matrix may be involved in replication of viral DNA. Robinson and coworkers (1982) gently digested chicken oviduct nuclei with DNase I, and isolated the matrix-bound DNA. This DNA was enriched in ovalbumin gene sequences as assayed by hybridization with a labelled probe containing the ovalbumin gene. Hybridization with a β -globin DNA probe showed no enrichment in matrix-bound DNA. Matrix-bound DNA from chicken liver showed no enrichment for ovalbumin sequences.

Pardoll and Vogelstein (1980) have shown that rat liver matrix-bound DNA is enriched 4 to 6 fold in ribosomal DNA sequences compared to total nuclear DNA. Matsumoto (1981) analyzed matrix-bound DNA from rat liver nuclei and found that it consisted of 60% satellite DNA, representing a 4 fold enrichment compared to total DNA. However, Cot analysis of rat liver matrix-bound DNA gave identical results to total DNA (Pardoll and Vogelstein, 1980).

Comings and Wallack (1978) studied binding of

mouse liver matrix proteins to DNA. They found that matrix proteins bound mouse DNA and E.coli DNA with similar affinities. However, AT-rich double stranded DNA bound to matrix proteins more effectively than GC-rich DNA. Kavenoff and Zimm (1981) reported that a 24kd protein from Physarum polycephalum matrix remained tightly bound to DNA after extensive pronase digestion. They suggested that this protein may be responsible for binding DNA to the matrix. Treatment of rat liver matrix with phospholipase C released most of the matrix-bound DNA while not altering the matrix morphology significantly, indicating that phospholipids may be involved in matrix protein-DNA interaction (Gilmour and Lang, 1980).

Berezney and Buchholtz (1981a) have estimated that there are 10^5 DNA-matrix attachment sites per nucleus and approximately 10^8 total matrix proteins per nucleus. Therefore, it is possible that only a very small percentage (<1%) of the matrix proteins are involved in DNA attachment. Small amounts of histones remain with matrices and these could be involved in the attachment. Grebanier and Pogo (1979) have shown by chemical crosslinking studies on isolated nuclei from Friend erythroleukemia cells that histone H3 is in close association with a few matrix proteins. Little work has been done on the association of histones with the

nuclear matrix.

The specific activity of DNA polymerase- α (α POL) in matrices from regenerating rat liver cells was more than two fold higher than in isolated nuclei from the same cells. In contrast, normal liver matrices and nuclei contained only trace amounts of α POL activity, and both normal and regenerating matrices and nuclei contained only trace amounts of DNA polymerase β activity (Smith and Berezney, 1980).

Taken overall, considerable evidence suggests that DNA replication occurs at the nuclear matrix. Various models for this have been proposed (Dijkwel et al., 1979, Pardoll et al., 1980, Berezney and Buchholtz, 1981a, 1981b).

2. ASSOCIATION WITH RNA

Nuclear matrices contain varying amounts of RNA (for review see Pogo, 1981). Several workers have isolated nuclear matrices which contain >30% of the total nuclear RNA prior to digestion with RNase (Berezney and Coffey, 1977, Herman et al., 1978, Miller et al., 1978, Herlan et al., 1979). Herman et al., (1978) labelled HeLaS3 cells with ^3H -uridine and using EM autoradiography found that the nuclear matrix contained substantial amounts of label. Extensive digestion of the matrices with RNase substantially

reduced the amount of label. Interestingly, the radioactivity was exclusively over the residual internal network and not over the residual nucleoli. A possible explanation for this is that hnRNA in the internal matrix may be protected from nuclease digestion by associated proteins (Pogo, 1981). hnRNA (labelled for 3 hours with ^3H -uridine) was associated with the matrix (unless digested with RNase). Also, >80% of newly synthesized RNA (labelled for 15 sec) remained with the matrix fraction (Herman *et al.*, 1978). These results suggest that RNA synthesis may involve the nuclear matrix and that hnRNP particles may also be associated with the matrix. This is consistent with the observation of 25nm RNP particles in isolated matrices (Berezney and Coffey, 1977).

After RNase digestion, approximately one half of the poly(A)-containing hnRNA remained attached to the nuclear matrix, especially to the residual envelope, in an EDTA-sensitive linkage (Herman *et al.*, 1978). After release from the matrix the poly(A)-containing hnRNA sedimented as a 12S particle. The particles were not eluted from the matrix with 0.4M NH_4Cl suggesting that their binding to the matrix was not fortuitous. Also 56% of the double stranded RNA regions were associated with the matrix after RNase digestion.

Herman and coworkers (1979) isolated nuclear

matrices from Tetrahymena macronuclei which contained approximately 60% of the total nuclear RNA. This RNA was fractionated by electrophoresis and contained ribosomal RNA precursors (Eckert et al., 1978). One of these RNAs (designated "A") was enriched in the matrix preparations compared to nuclei. Rat liver nuclear matrices contained all of the nuclear hnRNA if nucleases were omitted and proteinase inhibitors included during the isolation (Berezney and Buchholtz, 1981a). Rapidly labelled RNA was also associated with the nuclear matrix (Herlan et al., 1979). When labelled RNA was artificially added to unlabelled matrices from Tetrahymena macronuclei no binding was observed, suggesting that hnRNA binding to the matrix was not artifactual (Herlan et al., 1979). RNase treatment of the matrices removed most of the hnRNA but did not significantly alter the morphology of the matrices. VanEekelen and Venrooij (1981) prepared matrices from HeLa S3 cells which appeared very similar to those of Berezney and Coffey (1977) and contained high molecular weight RNA. They found that when commercial "RNase-free" DNase I was used during the isolation rapidly labelled RNA was quickly degraded. After purifying the DNase I they obtained rapidly labelled hnRNA from the nuclear matrices. Analysis of the RNA on glycerol density gradients revealed sedimentation profiles very similar

to those given by RNA from whole nuclei, suggesting that most of the hnRNA was isolated with the nuclear matrices in undegraded form. UV irradiation of nuclei was used to crosslink matrix proteins to hnRNA and two matrix proteins (41.5kd and 43kd) were found to be associated with the hnRNA.

Nuclear matrices isolated from undifferentiated and differentiated Friend erythroleukemia cells also retained most of the hnRNA. This RNA was removed only by chemical extractions, such as 8M urea + 10mM EDTA, which also disrupted the matrices (Long et al., 1979). HnRNA therefore appears to be attached to the nuclear matrix in a relatively stable association.

Small molecular weight RNAs (snRNAs) have also been detected in nuclear matrices. Miller et al. (1978) detected snRNAs designated N1-N8, 5S, 4.5S, and 4S in rat liver matrices. They estimated that approximately 80% of N3 and N4 and 70% of N1 and N2 were recovered in the matrix fractions. To test for fortuitous binding of the snRNAs to the matrix proteins, nuclei were disrupted in a nitrogen cavitation bomb and the snRNAs analyzed. This procedure did not release RNP complexes containing rapidly labelled RNA and no significant difference was observed between the snRNA profiles of the disrupted nuclei and the nuclear matrices.

Five snRNAs from Tetrahymena nuclear matrices were

detected by Herlan and coworkers (1979), a 5S, three snRNAs comparable to mammalian N1, N2, and N3, and another unknown higher molecular weight snRNA. The 5S snRNA incorporated a small amount of ^3H -uridine in 30 min but the other snRNAs did not.

The involvement of snRNAs in hnRNA processing is well established (Avvedimento et al., 1980, Gurney and Elicieri, 1980, Lerner et al., 1980) hence the association of snRNAs and hnRNAs with the nuclear matrix suggests that the matrix may be involved in transcription, processing and/or transport of RNA. In addition to these roles, RNAs may be necessary for the structural integrity of the matrix. Bouvier and coworkers (1982) digested HeLa nuclei with RNase A prior to extraction with 2M NaCl and observed no residual nucleoli or internal network (i.e. only lamina). Extraction of nuclei with 2M NaCl prior to RNase yielded normal matrices. There was, however, no noticeable difference in SDS-PAGE profiles of these preparations. Similar results were obtained by Kaufmann et al. (1981). They found that digestion of rat liver nuclei with RNase A prior to extraction with NaCl resulted in matrices with no residual nucleoli and reduced internal network while RNase digestion after NaCl caused no such effects. Although these results suggest a structural role for nuclear RNAs, it is not clear why the matrix structure

is only sensitive to RNase prior to NaCl extraction.

3. Association With Viral Proteins

Chin and Maizel (1977) fractionated HeLa cell nuclei infected with adenovirus type 2 (Ad2) to determine the location of the virally coded protein E3 (11kd). They found that E3 was enriched threefold in nuclear matrix as compared to isolated nuclei. Nuclear matrices from HeLa cells infected with Ad2+ND 1 (an Ad2-SV40 hybrid virus) contained 9 virus specific proteins (Deppert, 1978). The SV40 specific 28kd protein was especially prominent in the nuclear matrix protein profile. In Ad2+ND2 infected cells the 42kd and 56kd SV40 specific proteins were predominantly associated with the matrix, and these proteins were stable during a 3 hour chase indicating a specific association with the nuclear matrix. Some SV40 specific proteins (72, 74 and 95kd) were extracted by the high salt washes during matrix isolation suggesting that binding of the viral proteins to the matrix was not artifactual.

Immunofluorescent staining of HeLa cells with SV40 tumor serum (called U-antigen which reacts to most SV40 specific proteins) revealed only perinuclear staining. However, isolated matrices stained with U-antigen serum showed internal staining. The significance of this difference is unclear, and could be

due to relocation of proteins during matrix isolation, masking of antigenic sites on the viral proteins in the interior of the in situ matrix, or possibly a fixation artifact (Deppert, 1978).

Nuclear matrices from mouse 3T6 cells lytically infected with polyoma virus retained significant amounts of the 100kd T-antigen as detected by SDS-PAGE (Buckler-White et al., 1980). Immunofluorescent staining of 3T6 cells with anti-T antigen sera showed similar staining patterns in whole nuclei and in nuclear matrices. The staining was diffuse over the entire nucleus with only some nucleoli unstained. In addition to the T-antigen, viral capsid proteins VP1, VP2 and VP3 were also present on the nuclear matrix. By 30 hours post infection VP1 was the most abundant protein in the matrix preparations (Buckler-White et al., 1980). Therefore, the nuclear matrix appears to be a site of viral protein accumulation, and may be involved in viral encapsulation.

4. Association With Steroid Receptors

One hour after injecting radioactive estradiol into immature female rats labelled estrogen was recovered from isolated uterine nuclei (Barrack et al., 1977). Most of this estrogen was extractable with 0.6M KCl, but when nuclei extracted with 2M NaCl were

incubated with estradiol, additional steroid receptors were detected in amount equal to those removed by 0.6M KCl. Treatment of nuclei with 0.6M KCl and DNase 1 solubilized >90% of the DNA but 50% of the total estradiol binding sites remained insoluble. Uterine nuclear matrices (prepared as per Berezney and Coffey, 1977) also retained some high affinity estradiol binding sites although it is not known if these sites are occupied in vivo (Barrack et al., 1977).

Nuclear matrices from liver of immature chickens also retained approximately 60% of the total high-affinity estradiol binding sites (Barrack and Coffey, 1980). Liver matrices from mature roosters bound one eighth as much estradiol as those from mature females, indicating that the binding was not fortuitous. Rat ventral prostate nuclear matrix was shown to retain androgen specific receptors which were unaffected by estrogens (Barrack and Coffey, 1980). The physiological significance of these binding sites in matrices is still unknown although their relatively large number suggests that they may play a significant role in hormone function.

D. Cell-Cycle Changes In The Nuclear Matrix And Pore-Lamina

Changes in nuclear matrix proteins during the

cell cycle have been studied with SDS-PAGE. No major qualitative differences were observed in matrix proteins from HeLa S3 cells in early G1, mid-S and late S/G2 as compared to randomly dividing cells (Hodge et al., 1977). The early G1 and late S/G2 matrices had relatively more protein from 40kd to 65kd than the mid-S matrices and a 73kd protein was more prominent in mid-S matrices. Synchronized HeLa cells labelled with radioactive amino acids during G1 and allowed to proceed through mitosis retained a significant amount of label in the 50kd to 75kd range indicating that some matrix proteins survive mitosis (Hodge et al., 1977).

Nuclear matrices from non-dividing rat liver nuclei had a similar protein profile to regenerating liver matrices (Berezney et al., 1979b). However, several differences were detected between matrix from rat liver and from Zajdela ascites hepatoma cells. The hepatoma matrices had relatively less 68kd protein and more 58kd protein as well as minor differences (Berezney et al., 1979a, 1979b). It is not known whether these changes were due to cell cycle differences or were related to the malignant state of the hepatoma cells.

Morphological changes in the nuclear matrix during the cell cycle of Physarum polycephalum, which has intranuclear mitosis, have been studied (Bekers et al., 1981). Matrices from interphase cells consisted

of a nuclear envelope, internal network and residual nucleoli. In matrices from prophase cells the residual nucleoli were located at the nuclear periphery and were partially dispersed, the residual nuclear envelope was intact and the internal network was separated from the residual nucleoli and nuclear envelopes by empty space. Matrices from meta- and anaphase retained the nuclear envelope but the internal matrix was condensed and centrally located in the nucleus and contained DNase-sensitive fibrils. This suggested that the matrix may be organizing the chromatin in a manner analogous to the chromosome scaffold. It was not possible to isolate matrices from telophase cells.

Immunofluorescent staining of mitotic mouse 3T3 cells with anti-lamin serum showed that the perinuclear staining is dispersed throughout the cytoplasm in mid-prophase, and remains this way throughout mitosis (Gerace et al., 1978, Krohne et al., 1978a). Gerace and Blobel (1980) immunoprecipitated the three lamin proteins from synchronized CHO cells and found that mitotic cells contained approximately 60% of the total lamin protein present in interphase cells and that post-mitotic G1 cells contained the same amount of lamins as the mitotic cells. Therefore, the lamins appeared to be conserved through mitosis. The isoelectric points of lamins from mitotic cells were

more acidic than those of lamins from interphase cells, as judged by 2-D electrophoresis, suggesting postsynthetic modification of the lamins during mitosis (Gerace and Blobel, 1980). The lamins were slightly labelled with ^{32}P during interphase and significantly labelled just prior to or during mitosis. Lamin B was especially labelled in mitotic cells but not in interphase cells. Phosphorylation of the lamins, therefore, may be involved in the dispersion of the nuclear envelope during mitosis, and could account for the multiple charge isomers seen in 2-D gels from random cell samples.

Jost and Johnson (1981) studied the distribution and synthesis of the 67kd lamin B in synchronized HeLa cells. During S phase and to the end of G2, lamin B was located only in the nucleus, predominantly at the periphery. In late G2-prophase, lamin B was distributed diffusely throughout the cytoplasm indicating dispersion of the nuclear envelope. During telophase-G1 lamin B was reorganized in the nucleus, predominantly at the periphery. Nuclear fluorescence increased from G1 to S indicating synthesis of lamin B. This occurred in the presence of hydroxyurea and is therefore not directly coupled with DNA synthesis. It was also not inhibited by cycloheximide suggesting that lamin B may be in cytoplasmic pools and transported to the nucleus prior to S phase (Jost and Johnson, 1981).

MATERIALS AND METHODS

A. ISOLATION AND CULTURE OF MOUSE LYMPHOCYTES

For each experiment, five to ten 8-week old Balb/c mice were used. The mice were killed by cervical dislocation. Spleens were excised aseptically and placed on a fine wire screen in a 30mm petri dish containing 2ml of complete medium (RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 100U/ml of penicillin and 100µg/ml of streptomycin). Lymphocytes were released by crushing the spleens against the screen with a sterile rubber stopper. The stopper and screen were then rinsed with medium and the cells suspended by gentle pipetting, larger tissue fragments remained on the screen.

The cell suspension was transferred to a glass vial, underlain with 2ml FBS and cooled on ice for five min. The medium above the FBS was then transferred to a 15ml conical centrifuge tube and centrifuged at 600xg for 5 min. The supernatant was discarded and the pellet resuspended in 10-15ml of ice cold 0.83% (w/v) NH_4Cl and kept on ice for 12 to 15 min to lyse erythrocytes.

The cells were then centrifuged as before and washed twice with complete medium. Cell concentration was determined with a hemocytometer and cell viability by trypan-blue dye. 50µl samples of cells in medium were

added to 450 μ l of 0.05% (w/v) trypan blue in 0.15M NaCl.

The cells were suspended in complete medium to a concentration of 2×10^6 cells/ml and were cultured in plastic rectangular flasks of 25, 75 or 150 ml culture capacity at 39°C. Cultures were maintained for up to three days and were supplemented with complete medium (10% of the original culture volume) after each day in culture. Cells were mitogenically stimulated with concanavalin-A (conA) at a final concentration of 6 μ g/ml. Some cells were incubated with 3 H-Leucine (60-80Ci/mmol) or 3 H-thymidine (80Ci/mmol) as described in RESULTS.

B. ISOLATION OF LYMPHOCYTE NUCLEI AND NUCLEAR MATRICES

Various methods used for isolation of lymphocyte nuclei are described in the RESULTS section. The procedure presented here gave the best preparations and was used in all analytical experiments.

All steps in the nuclear isolation were performed at 0-4°C; all centrifugations were $600 \times g$ for 5-10 min. All solutions contained 1mM phenylmethylsulfonylfluoride (PMSF), 10 μ g/ml soybean trypsin inhibitor and 1mM sodium tetrathionate (NaTT) to inhibit endogenous proteinases.

Cells were centrifuged from the medium and washed once in STM buffer (20mM Tris-HCL, pH 7.4 containing 0.25M sucrose + 5mM $MgCl_2$). The cells were resuspended in 8 ml of ice-cold STM buffer containing 0.25% (v/v)

TritonX-100 and homogenized with 10 strokes of a tight fitting Dounce homogenizer. The homogenate was quickly diluted to 15ml with STM buffer and the nuclei pelleted. After two washes with STM buffer the nuclei appeared relatively clean as assessed by both light and electron microscopy (see RESULTS). The nuclear isolations were routinely monitored using Nomarski interference contrast microscopy. If more than 10% of the nuclei showed cytoplasmic contamination the homogenization was repeated, although this was rarely necessary.

Nuclear matrices were isolated from samples of $1-5 \times 10^8$ isolated nuclei by a procedure similar to that of Berezney and Coffey (1977). Nuclei were sequentially extracted as below, with centrifugation at 600xg for 10 min between extractions:

1. STM buffer + 50 μ g/ml DNase 1 for 15 min at 37°C (gives Dnase 1 spheres).
2. Low-magnesium (LM) buffer (10mM Tris-HCl, pH 7.4 + 0.2mM MgCl₂) for 10 min at 4°C (gives LM spheres).
3. High-salt (HS) buffer (LM buffer + 2M NaCl) for 10 min at 4°C (gives HS spheres).
4. LM buffer + 0.5% (w/v) TritonX-100 for 5 min at 4°C.
5. LM buffer + 250 μ g/ml DNase 1 and RNase A for 30 min at 37°C.
6. LM buffer, two washes at 4°C (gives nuclear matrices).

C. MICROSCOPY AND AUTORADIOGRAPHY

For light and electron microscopy samples of approximately 15×10^6 cells, nuclei and matrices were fixed for one hour at room temperature in 2% glutaraldehyde in 0.05M sodium cacodylate buffer, pH 7.4, containing 15mM NaCl and 5mM MgCl₂. Following three washes in buffer samples were post-fixed for one hour at 4°C in 1% osmium tetroxide in the same buffer. The samples were again washed three times in buffer, dehydrated and embedded in Epon-Araldite (Mollenhauer, 1964).

For light microscopy, 0.5-1.0 μ m thick sections were cut with glass knives on a Reichert OmU2 ultramicrotome, mounted on glass slides coated with 0.1% gelatin and stained for one to two minutes at 50°C with 0.05% (w/v) toluidine blue in 2.5% (w/v) sodium bicarbonate. Diameters of structures in sections were measured directly in the light microscope with a calibrated ocular micrometer and volumes calculated.

For electron microscopy, silver sections were cut with a diamond knife, mounted on formvar coated grids and stained for 30-60 min with 25% (w/v) uranyl acetate in methanol followed by 15 min with aqueous lead citrate (Reynolds, 1963). These were examined with a Siemens 101 electron microscope at 80kV.

Light microscope autoradiographs were prepared by dipping slides carrying 0.5 μ m thick sections in Ilford L4 emulsion diluted 1:1 with distilled water. After exposure (1-10 days), slides were developed in Dektol diluted 1:1 with water for 4 min at 20°C. Following fixing and washing sections were stained for 1 min at room temperature with toluidine blue as above.

D. CHEMICAL ANALYSIS

Samples of cells, nuclei and nuclear matrices were analysed for total protein, RNA and DNA content. Average values were based on triplicate measurements for each of three independent experiments. Typically, 1×10^7 structures were used for each determination. Protein

content was assayed by the method of Lowry et al. (1951) on samples suspended in STM buffer, and by the trichloroacetic acid turbidity assay of Comings and Tack (1972). Protein samples for the TCA assay were dissolved in 1% (w/v) SDS, 20 μ l of this solution was added to 1ml of water and 0.5ml of 50% (w/v) of TCA was then added. The solution was mixed and allowed to stand for 20 min. Turbidity was measured at 400nm against a blank containing no added protein. Both protein assays were calibrated with bovine serum albumin as a standard. The two methods gave similar results and the values from both were averaged.

RNA content was determined by the modified orcinol method of Almog and Shirley (1978) using yeast RNA as the standard. Samples were washed three times with 2-5 ml of 0.15M NaCl and a 0.25ml aliquot was incubated with 1ml of 85% (v/v) H_2SO_4 in a capped test tube for 24 hours at 40°C. To this, 25 μ l of 0.4% (w/v) orcinol in concentrated HCl was added and the mixture was heated at 100°C for 30 min. Absorbance was measured at 500nm. The initial washes with 0.15M NaCl were important because H_2SO_4 can react with sucrose in the STM buffer and give false readings.

DNA was determined by the diphenylamine reaction (Burton, 1956) using calf thymus DNA as the standard. 400 μ l samples were digested in 0.5N perchloric acid for

30 min at 80°C. Two volumes of diphenylamine reagent were then added and the mixture incubated at 30°C for 15 to 17 hours. Blanks were prepared identically but without added DNA. Absorbance was measured at 600nm.

All chemical analyses were normalized by counting the number of cells, nuclei or matrices in each sample using a hemocytometer and expressing the results as pg per structure.

E. ELECTROPHORESIS

Samples for one dimensional SDS-PAGE were washed twice in distilled water, (centrifugations were at 1000xg for 15min.), frozen at -80°C and lyophilized in a Virtis Uni-Trap freeze dryer. The proteins were solubilized in 50-100µl of 10mM Tris-HCl, pH7.4, containing 1%(w/v) SDS and 1%(v/v) β-mercaptoethanol (ME), in a 15ml plastic centrifuge tube with sonication in a Branson sonicator bath for 1-10 min at room temperature. Samples were placed in a boiling water bath for 1 min and 20-40µg of each sample was loaded onto 10% polyacrylamide slab gels (with 2.5% stacking gel) containing 1%(w/v) SDS, exactly as described by Comings and Harris (1975). The gels were 15x15cm, 0.75mm thick with slot widths of approximately 2mm. Samples were electrophoresed at 60V for one hour followed by 120V for three hours. The gels were stained overnight in 0.05% (w/v) coomassie blue R in

10% acetic acid and 25% isopropyl alcohol, destained one hour in 10% acetic acid and 25% isopropyl alcohol and then 24 hours in 10% acetic acid. Gels were photographed and the negatives scanned with a Joyce-Lobel densitometer. After staining and photographing, gels containing radioactivity were washed in dimethylsulfoxide (DMSO) twice for 30 min each, placed in 22.2% (w/v) 2,5-diphenyloxazole (PPO) in DMSO for three hours, rinsed in water and dried onto filter paper. The gels were placed in contact with Cronex-4 X-Ray film and exposed for 1-7 days at -80°C . The film was developed for five min at 20°C in Kodak GBX developer, rinsed and fixed for 5-10 min in Kodak RPX-OMAT fixer.

Samples for two dimensional equilibrium electrophoresis were solublized in Tris-SDS-ME as above, dialyzed overnight against two 2l changes of 5mM ME overnight at room temp., precipitated by dialysis against 95% ethanol at 4°C , and lyophilized. The electrophoresis was carried out as described in detail by Peters and Comings (1980). In the first dimension each sample was isofocused to equilibrium on a 3.2mmx19cm gel (8 hr at 200-1000V). Four of these gels were then electrophoresed simultaneously in the second dimension on a 20cmx86cm slab gel containing 1% (w/v) SDS for approximately 6 hours at 120V. After

electrophoresis, the slab gel was cut into four 20cmx20cm gels and stained and destained as per the one-D gels.

A set of protein standards was run on every 20x86cm gel to establish a reference grid of known pI's and molecular weights.

F. MICROSPECTROPHOTOMETRY

Microspectrophotometry was used to measure protein amount in samples stained with naphthol yellow S (Deitch, 1955). Samples of $10-20 \times 10^6$ cells, nuclei or matrices were fixed in methanol-40% formalin-acetic acid (85:15:5 by volume) for 30 min at room temperature, washed twice with 1% acetic acid and air dried on acid cleaned slides (Fredricks et al., 1980). The slides were stained by either of the following procedures.

1. NYS: Slides were stained for 20 min in 1% (w/v) naphthol yellow S in 1% acetic acid, rinsed for 2 hours in several changes of 1% acetic acid, rinsed once with tertiary butyl alcohol and air dried.
2. TCA-NYS: Slides were extracted with 5% trichloroacetic acid for 2 hours at 60°C to remove DNA (Fredricks et al., 1980) washed several times with water and stained as described in procedure 1.

Coverslips were mounted using oil of R.I.=1.556.

Measurements were made using a Zeiss microphotometer with rotating fixed diaphragms mounted on a Photomicroscope 2. A 100x plan achromatic objective (N.A. 1.3) with integral iris was used. Illumination was with a 60W DC lamp and a Zeiss variable interference-filter monochromator. Absorbance measurements were by the two-wavelength method (Mendelsohn, 1958a, 1958b) using light of 468nm and 480nm. Relative amount of protein per structure (cell, nucleus or matrix) was calculated using the tables of Mendelsohn (1968b). Diameters of individual structures were measured directly in the light microscope with a calibrated ocular micrometer and volumes calculated.

RESULTS

A. Terminology

Electron micrographs of interphase lymphocyte nuclei stained with conventional uranyl acetate and lead citrate show two distinct regions (excluding the nucleolus) corresponding to condensed (hetero-) chromatin and dispersed (eu-) chromatin. The condensed chromatin is electron-dense and consists of closely packed 10-30nm diameter chromatin fibrils. The region of dispersed chromatin contains relatively few widely spaced chromatin fibrils as well as a variety of non-chromatin fibrils and granules. This area has been called interchromatin (Bernhard and Monneron, 1969) or interchromatinic space (Dardick et al., 1981), terms which emphasize the relative lack of chromatin fibrils. In this thesis, the term condensed chromatin refers to regions containing tightly packed chromatin fibrils, and interchromatinic space refers to areas which are weakly stained by conventional uranyl-lead and contain few chromatin fibrils.

The terms condensed, aggregated, disaggregated, dispersed, and decondensed are often encountered in descriptions of chromatin distribution, and their use follows no set convention. In this thesis condensed chromatin is used as described above, dispersed

chromatin refers to widely spaced individual chromatin fibrils while aggregation and disaggregation relate to the relative size of condensed chromatin masses. Aggregated chromatin describes relatively large clumps of condensed chromatin while disaggregated chromatin refers to more widely spaced, smaller clumps (Setterfield et al., 1983).

B. Morphological Changes During In Vitro

Lymphocyte Stimulation

Figure 1 shows unstimulated murine splenic lymphocytes in the light microscope. Such cells were relatively uniform in structure having a prominent nucleus surrounded by a thin rim of cytoplasm. The nuclei contained darkly stained regions of condensed chromatin predominantly at the nuclear periphery, lightly stained regions of interchromatinic space and a nucleolus. Nucleoli were quite small and out of the plane of section in some cells. Figure 2 is an electron micrograph of an unstimulated lymphocyte. The regions described above are visible, as well as a cluster of cytoplasmic organelles at the cell center adjacent to a small nuclear cleft. Unstimulated nuclei often had one or more nuclear clefts, some of which intruded almost to the center of the nucleus. High magnifications of the interchromatinic regions of unstimulated nuclei revealed

Figure 1.

Light micrograph of unstimulated murine splenic lymphocytes. Note homogeneity of nuclear structure, clumps of condensed chromatin and nucleoli (arrows) are seen. $\times 1,600$ Bar = $10\text{ }\mu\text{m}$.

Figure 2.

Electron micrograph of unstimulated lymphocyte showing thin rim of cytoplasm, prominent clumps of condensed chromatin and small amount of interchromatinic material. $\times 21,000$ Bar = $1\text{ }\mu\text{m}$. (m) - mitochondria; (arrow) - nuclear cleft; (c) - condensed chromatin; (i) - interchromatinic material.

2

—20-30nm diameter granules and amorphous material (Figure 3).

Lymphocytes stimulated with conA for 36h are shown in Figures 4 and 5. These cells had greatly increased cytoplasmic volume with a larger number of cytoplasmic organelles, including polysomes. The nuclei were substantially larger than nuclei in unstimulated cells and were generally smooth in outline although shallow clefts were often seen. The distribution of condensed chromatin changed from large clumps in the unstimulated cells to more numerous, smaller clumps dispersed throughout the nucleus in stimulated cells. Some condensed chromatin remained associated with the nuclear envelope. The interchromatinic space was much larger in the stimulated cells and with conventional staining had approximately the same electron density as in the unstimulated cells. Higher magnifications of this interchromatinic space revealed small fibrils, and granular particles (Figure 6).

Nucleoli in stimulated cells were very prominent and contained numerous lacunae. Fibrillar and granular regions in the nucleoli of both unstimulated and stimulated cells were intermingled and not distinctly defined (Figure 3).

Figures 4 and 5 also demonstrate the variability of the response of lymphocytes to mitogen. In any sample

Figure 3.

High magnification view of nucleus of unstimulated cell. Interchromatinic material contains 20-30 nm diameter granules and a few fine fibrils. $\times 100,000$ Bar = 0.1 μm . (c) - condensed chromatin; (i) - interchromatinic material; (n) - nucleolus.

Figure 4.

Light micrograph of autoradiograph of 0.5 μm section of cells stimulated with con A for 36h. Cells supplied 10 $\mu\text{Ci/ml}$ ^3H -thymidine (80 Ci/mM) for 1h prior to fixation. Exposure 5 days. Note asynchrony of mitogenic response; morphotypes 1, 2 and 3 are shown. Only type 3 cells are labelled (large arrows) and unlabelled type 3s are also seen (small arrows). $\times 1600$ Bar=10 μm .

Figure 5.

Electron micrograph of cells stimulated with con A for 24h. Morphotypes 1, 2 and 3 are shown. Note increase in nuclear volume and decrease in size of clumps of condensed chromatin (c) in type 3 cell. x 10,600 Bar = 1 μ m.

Figure 6.

High magnification of interchromatinic region of type 3 nucleus, granules and fine fibrils are seen between clumps of condensed chromatin x 80,000 Bar = 0.1 μ m, (c) - condensed chromatin; (i) interchromatinic material.

stimulated for longer than 16h, cells at various stages of activation were observed and average measurements of parameters on the total population are therefore misleading. To avoid this problem of asynchronous response, lymphocytes have been classified into three morphotypes based on cell size and nuclear morphology (Dardick et al., 1981, Castellanos et al., 1982, Setterfield et al., 1983) and measured parameters are related to morphotype rather than just time of stimulation. Type 1 cells are the small lymphocytes present in unstimulated samples, with small nuclei (3-4 μ m diameter) containing highly aggregated condensed chromatin. Type 2 cells are larger in size, have a larger nucleus (4-5 μ m), prominent nucleolus and show partially disaggregated clumps of condensed chromatin. Many type 2 cells still have substantial aggregates of condensed chromatin at the nuclear periphery. Type 3 cells are large lymphoblasts with large nuclei (>5 μ m) containing prominent nucleoli and numerous small disaggregated clumps of condensed chromatin. Chromatin clumps in type 3 nuclei are not well resolved in the light microscope making such nuclei appear devoid of condensed chromatin (Figure 4).

Samples stained with the regressive uranyl-EDTA stain of Bernhard (1969) (which preferentially stains ribonucleoproteins and not chromatin) are shown in

Figures 7 and 9. In nuclei of type 1 cells the interchromatinic space and nucleoli were intensely stained while the condensed chromatin was seen as large unstained areas primarily at the nuclear periphery. In nuclei of type 2 and 3 cells, the interchromatinic material was greatly increased and the unstained clumps of condensed chromatin were much less prominent. Higher magnifications of the interchromatinic material stained by uranyl-EDTA revealed granules of various sizes (20-50nm) and fine fibrils of >5nm diameter (Figures 8 and 10).

C. Time Course of Nuclear Morphological Changes and DNA Synthesis

The time course of nuclear morphological changes after conA stimulation was assessed by fixing cells at various times post stimulation with conA (PS) and scoring the frequency of each morphotype in light microscope sections. These results are shown in Table 1. Unstimulated cells cultured for 48h showed no significant nuclear activation, ie. all type 1 nuclei. At 12h PS with conA, approximately 20% type 2 nuclei were observed while at 24h PS there were 39% type 3 and 29% type 2. At 48 hours PS up to 52% of the cells were type 3 and 20% type 2, although substantially fewer type 3 cells were sometimes observed. Type 2 and 3 cells

Figures 7 - 10. Electron micrographs of cells stained with regressive Uranyl - EDTA to reveal interchromatinic material. Condensed chromatin is unstained.

Figure 7.

Morphotype 1 (unstimulated) cell. x 19,000.

Figure 8.

High magnification view of interchromatinic region of a type 1 nucleus showing numerous granules and some fibrils. x 160,000.

Figure 9.

Morphotype 3 cell showing much greater amount of interchromatinic material than type 1. x 9,000.

Figure 10.

High magnification of interchromatinic region of type 3 nucleus also showing granules and fibrils. x 160,000.

Bars in Figs. 7 and 9 = 1 μ m; in Figs. 8 and 10 = 0.1 μ m. (c) - condensed chromatin; (i) - interchromatinic material; (n) - nucleolus.

7

8

9

Table 1
The Time Course of Nuclear Morphological Changes
and DNA Synthesis During Lymphocyte Activation

Sample	Time in Culture (h)	Morphotype (%) ¹			% Labelled ²
		1	2	3	
-ConA	0	98	2	0	2
	12	97	2	0	0
	24	98	2	0	0
	36	97	2	1	2
	48	98	2	0	2
+ConA	0	98	2	0	2
	12	77	23	0	1
	24	32	29	39	9
	36	32	20	48	25
	48	28	20	52	29
+LPS	0	97	3	0	2
	12	94	4	2	3
	24	93	5	2	3
	36	79	13	8	14
	48	81	10	10	15

1. Based on counts of 300 cells per sample from each of three experiments.
2. Cells cultured with ³H-thymidine for one hour prior to fixation and labelled cells scored in autoradiographs.

were also seen in cultures stimulated with LPS for 36h (10% each).

DNA synthesis was detected by incubating cells with ^3H -thymidine for one hour prior to fixation and scoring the frequency of labelled cells in autoradiographs. These results are also shown in Table 1. Unstimulated cells showed only a constant low frequency of labelled nuclei, probably due to a small number of macrophages. Cells stimulated with conA showed a slight increase in labelled nuclei by 24h PS and considerable increase by 36h and 48h PS. Approximately 30% of the cells showed label at these later times. 14% of cells stimulated with LPS (36h) showed label.

Figure 4, an autoradiograph of conA stimulated cells (36h PS) labelled with ^3H -thymidine for one hour prior to fixation illustrates two important observations on the relation between nuclear morphology and DNA replication. Only type 3 cells are labelled, but unlabelled type 3 cells are also present. This illustrates that the nuclear change from morphotype 1 to 3 is a mitogenic response which precedes DNA synthesis.

Cell death during culture was assessed by trypan blue dye exclusion. Table 2 shows that for both stimulated and unstimulated cultures, approximately 20-25% of the original cells died each day of culture.

Table 2
Lymphocyte Death During Culture as
Determined by Trypan Blue Dye Exclusion

Sample	Time in Culture (h)	% Dead Cells ^{1.}
-ConA	0	4+2 ^{2.}
	24	19+5
	48	47+4
	120	67+5
+ConA	0	4+2
	24	24+8
	48	50+10
	120	74+10

1. Based on counts of 300 cells per sample from
each of three experiments.

2. +SD

D. Nuclear Isolation

Several experimental protocols for the isolation of lymphocyte nuclei were tested; the results are summarized in Table 3. The quality of the resulting nuclei was judged in electron microscope sections of pellets. The main criteria considered important for a good preparation were minimum cytoplasmic contamination and nuclear morphology comparable to that of in situ nuclei. Light microscopy of sections or whole mounts was inadequate for assay of nuclear quality. Several preparations which appeared excellent by phase and interference contrast showed structurally damaged nuclei or cytoplasmic contaminants when examined by EM (Figures 11 and 12).

The isolation procedures can be divided into two groups based on whether or not homogenization was used. Procedures 1-5 (Table 3, based on Mitchell et al., 1978). involved incubating cells in a homogenization buffer containing a detergent (TritonX-100 or Tween-80) for a specified time without homogenization and then washing the nuclei in buffer without detergent. Procedures 1, 2 and 3 used buffers with TritonX-100 concentrations of 0.005-0.2% (w/v). These procedures yielded nuclei with extensive cytoplasmic contamination (Figure 13).

Procedures 4 and 5 used buffers with

Table 3
Summary of Nuclear Isolation Procedures and Results

Homogenization Buffer (HB)*	Procedure	Observations
1. 10mM HEPES, pH 7.8, 50mM KCl, 5mM Mg(CH ₃ -COOH), 0.005% Triton X-100, 50µg/ml polyvinylsulfate, 100µg/ml spermine.	Cells washed in saline (0.15M NaCl, resuspended in HB for 13 min, 0°C, washed in saline.	Extensive cytoplasmic contamination, stimulated cells very poor.
2. As per 1.	Procedure 1 repeated twice.	As above.
3. As per 1., but with 0.01, 0.05, 0.1, or 0.2% Triton X-100.	As per procedure 1, 10-30min in HB.	Unstimulated nuclei appeared good at 0.1 and 0.2% Triton X-100, but stimulated nuclei had cytoplasmic contamination. Nuclei extracted after 30min in HB.
4. As per 1. but with 0.25M sucrose, and 0.01, 0.1, and 0.2% Tween-80 instead of Triton X-100.	Cells spun out of medium, resuspended in HB for 15-30 min at room temp., nuclei spun at 15K rpm for 60 min at 4°C, and washed in saline. As above, but nuclei spun on 10-30% Ficoll density gradient at 15K for 60min at 4°C after suspension in HB.	Nuclei free from cytoplasmic contamination, but appeared extracted especially at 0.2% Tween.
5. As per 4.	Cells washed in saline+0.25M sucrose, resuspended in 8ml HB and homogenized with 5-10 strokes in a Dounce at 4°C. Nuclei washed in saline.	Nuclei free of cytoplasm but appeared extracted.
6. As per 4. but without detergent.		Nuclei had cytoplasmic tags but were morphologically good.

*All HBs contained 1mM Phenylmethylsulfonylfluoride (PMSF).

2

Table 3 (Continued)

Homogenization Buffer (HB)*	Procedure	Observations
7. 0.15M NaCl, 0.25M sucrose, no detergent.	As per 6.	Extensive cytoplasmic contamination.
8. As per 7., + 0.05% TritonX-100.	As per 6.	Nuclei clean and morphologically good.
9. 20mM Tris-HCl, pH 7.4, 0.25M sucrose, 5mM MgCl ₂ (STM), +0.1, 0.25, or 0.5% Triton X-100.	Cells washed in STM (no Triton), resuspended in 8ml HB, homogenized 5-10 strokes in Dounce at 4°C. Nuclei mixed 1:2 with STM+2.2M sucrose, spun at 20K, 60min 4°C, washed in STM.	Nuclei free of cytoplasm and morphologically excellent, especially at 0.25% Triton X-100.
10. As per 9 + 149g/ml soybean trypsin inhibitor, 1mM sodium tetrathionate.	Cells washed in STM, resuspended in 8ml STM+0.25% Triton X-100, homogenized 5-10 strokes with Dounce at 4°C. Nuclei diluted to 15ml with STM and washed x2 with STM.	Best nuclear preparation little cytoplasmic contamination, nuclear morphology excellent.

*All HBs contained 1mM PMSP.

Figure 11.

Light micrograph of isolated nuclei from con A stimulated (24h) cells. Nuclei were isolated by procedure 5 (Table 3) and appear similar morphologically to intact nuclei. x 1,800 Bar. = 10 μ m.

Figure 12.

Electron micrograph of isolated nuclei shown in Fig. 11. Nuclei appeared extracted and some nuclei were distorted. x 6,600. Bar = 5 μ m. (c) - condensed chromatin; (n) - nucleolus; (s) - empty spaces; (large arrow) - distorted nucleus; (small arrow) - cytoplasmic contamination.

Figures 13 - 15. Electron micrographs of isolated nuclei from unstimulated cells.

Figure 13.

Nuclei isolated by procedure 1, note extensive cytoplasmic contamination. x 8,000.

Figure 14.

Nuclei isolated by procedure 5, nuclei were free of cytoplasm but appeared extracted and had large empty spaces. x 8,500.

Figure 15.

Nuclei isolated by procedure 6, note extensive cytoplasmic contamination and extracted appearance. x10,000. All bars = 1 μ m. (c) - condensed chromatin; (s) - empty spaces; (arrows) - cytoplasmic contamination.

0.01-0.2% (w/v) Tween-80. The resulting nuclei were free of cytoplasm but appeared extracted and morphologically distorted (Figure 14).

Procedures 6 to 10 (based on Berezney and Coffey, 1977) involved suspending cells in buffer without or with detergent and gently homogenizing with either a Dounce or Potter-Elvehjem homogenizer. Procedures 6 and 7 used no detergents and the nuclei had substantial cytoplasmic contamination (Figure 15). Procedures 8 to 10 used a homogenization buffer containing 0.05-0.5% (w/v) TritonX-100. At 0.05% triton the cytoplasm was not fully removed, at 0.5% the nuclei were damaged. The procedure which consistently gave the best appearing nuclei was procedure 10 (Table 3) which was used in subsequent studies. This procedure involved homogenizing the cells in STM buffer containing 0.25% (w/v) TritonX-100. The Dounce homogenizer gave more consistent results than the Potter-Elvehjem. The number of strokes with the Dounce was not critical but it was important not to use excessive force during homogenization. Examples of nuclei obtained using this procedure are shown in Figures 16-18. Figure 18 shows a type 1 nucleus; highly aggregated condensed chromatin, interchromatinic space, and nucleolus are visible. Figure 19 shows a 3 nucleus; disaggregated clumps of condensed chromatin, large nucleolus

Figure 16.

Low magnification electron micrograph of type 1 nuclei isolated by procedure 10. Nuclei are free of cytoplasm and have well preserved morphology. x 9,000. Bar = 2 μ m.

Figure 17.

Low magnification electron micrograph of nuclei isolated (procedure 10) from con A stimulated cells (24h) showing good morphological preservation and removal of cytoplasm. x 5,800. Bar = 2 μ m. (c) - condensed chromatin; (i) - interchromatinic material; (n) - nucleolus; (arrows) - nuclear cleft.

Figure 18.

Electron micrograph of type 1 nucleus isolated by procedure 10 showing good morphological preservation. x21,800. Bar = 1 μ m. (c) - condensed chromatin; (n) - nucleolus; (arrow) - nuclear cleft.

Figure 19.

Electron micrograph of type 3 nucleus isolated by procedure 10. Note the large nucleolus and extensive interchromatinic material similar to in situ type 3 nuclei. x 15,000 Bar = 1 μ m. (c) - condensed chromatin; (i) - interchromatinic material; (n) - nucleolus.

and extensive interchromatinic space are seen.

The frequency of nuclear morphotypes in EM sections of unstimulated and stimulated cells and nuclei isolated from such cells is compared in Table 4. Unstimulated cells and nuclei isolated from them were almost all type 1. ConA stimulated cells (24 PS) and nuclei isolated from such cells had similar morphotype distributions although the isolated nuclei showed somewhat higher type 2 and lower type 1 morphotypes. Type 3 morphotypes were near 50% in both cells and nuclei from the stimulated cultures.

Cytoplasmic contamination of isolated nuclei was assessed by electron microscopy. Frequency of nuclei classified as having no, some or extensive cytoplasmic contamination was scored. Examples of these nuclei are shown in Figures 20, and the quantitative results in Table 5. Samples from several different nuclear isolations (Procedure 10) from both stimulated and unstimulated cells had approximately 85-90% nuclei with no visible cytoplasmic contamination. Most of the remainder were only partially contaminated. The cytoplasmic contamination appeared extracted and often consisted of only a fibrous network with no organelles visible. Attempts to further remove residual cytoplasm led to greater nuclear damage and it was decided that this degree of contamination was acceptable.

Table 4
Comparison of Morphotype Frequencies Between
in situ and Isolated Lymphocyte Nuclei

Sample	ConA ^{2.}	Frequency of Nuclear Morphotypes (%) ^{1.}		
		1	2	3
Cells	-	97	34	0
	+	32	20	47
Nuclei ^{3.}	-	98	2	0
	+	21	30	49

1. Based on scoring 300 cells or nuclei per sample in EM sections.
2. ConA stimulation for 24h.
3. Nuclear isolation procedure 10 was used.

Figure 20.

Electron micrograph of type 1 and 2 nuclei isolated by procedure 10 showing various degrees of cytoplasmic contamination. x 11,000. Bar = 1.0 μ m. (arrows) - cytoplasmic contamination; (A) - no cytoplasm; (B) - some cytoplasm; (C) - extensive cytoplasm.

Table 5
Evaluation of the Degree of Cytoplasmic
Contamination of Isolated Lymphocyte Nuclei

Sample	N ² .	% of total ¹ .		
		No Cytoplasm	Some Cytoplasm	Extensive Cytoplasm
-ConA Nuclei	200	87	11	2
	200	85	12	3
	200	93	6	1
	$\bar{x}$	89 $\pm$ 4 ³	9.3 $\pm$ 3	2 $\pm$ 1
+ConA Nuclei	100	86	11	3
	200	87	9	4
	200	91	8	1
	100	93	6	1
	100	83	12	5
	$\bar{x}$	88 $\pm$ 4	9.1 $\pm$ 3	3 $\pm$ 2

1. Each line represents a different experiment.

Nuclei were isolated by procedure 10.

2. Number of nuclei scored.

3. $\pm$ SD

Nuclear diameters from embedded samples are compared in Table 6. Both unstimulated and stimulated isolated nuclei were slightly smaller than the respective in situ nuclei, representing a maximum volume decrease of 10% during nuclear isolation. This difference was not significant at $\alpha=0.01$.

E. Isolation of Nuclear Matrix

The procedure for isolation of nuclear matrices was that of Berezney and Coffey (1977) with some modifications. The original procedure involved incubating the nuclei in STM buffer overnight at 0°C to allow endogenous digestion of the DNA. This was followed by three extractions for 10 min each in; LM buffer (0.2mM MgCl₂) and HS buffer (2M NaCl), one extraction for 10 min in LM buffer + 0.5% TritonX-100, and 60 min in LM buffer + 200µg/ml DNase I and RNase A. Unstimulated lymphocyte nuclei extracted by this procedure are shown in Figure 21. Only residual nuclear envelopes were observed. These were roughly circular, consisting of a 15nm thick lamina containing residual nuclear pores. Very little internal network was observed.

The original procedure was modified by replacing the first step, i.e. overnight incubation at 0°C, with a 10 min digestion in STM buffer + 50µg/ml DNase I at

Table 6
Comparison of Average Diameters and Volumes of
Unstimulated and Stimulated Isolated Lymphocyte
Nuclei and Nuclear Matrices

Sample 1.	Mean Diameter (μm)		Mean Volume (μm^3)	
	-ConA	+ConA	-ConA	+ConA
Nuclei in cells	4.1 $\pm$.2 ^{2.}	5.4 $\pm$.4	36 $\pm$ 2	82 $\pm$ 4
Isolated Nuclei	3.9 $\pm$.2	5.2 $\pm$.4	31 $\pm$ 2	76 $\pm$ 4
Nuclear Matrices	3.1 $\pm$.2	4.4 $\pm$.4	16 $\pm$ 2	45 $\pm$ 4

1. Based on 300 measurements per sample, samples stimulated for 24h with conA.

2. $\pm$ SD

Figure 21.

Electron micrograph of nuclear matrix isolated from type 1 nuclei by the method of Berézney and Coffey (1977). Only the residual nuclear envelope is seen. x 38,000. Bar = 1 μ m. (arrow) - residual nuclear envelope.

Figure 22.

Electron micrograph of nuclear matrix isolated from type 1 nuclei by a variation of the procedure used in Fig. 21. A small amount of internal network is seen in addition to the nuclear envelope. x 16,500. Bar = 1 μ m. (arrows) - residual nuclear envelope; (IN) - internal network.

25° C. PMSF was added throughout to reduce proteolysis. Nuclear matrices isolated by this procedure (Figure 22) contained the residual nuclear envelope, and some internal network consisting of 20-30nm granules and amorphous material. Up to 50% of the matrices, however, contained no internal network.

The extraction procedure was further modified by reducing the number of LM and HS extractions to one each and decreasing the final nuclease digestion to 30 min. Soybean trypsin inhibitor and NaTT were also added to further reduce proteolysis. The resulting matrices contained residual nuclear envelope, residual nucleoli and internal network similar to that of Berezney and Coffey (1977). It was felt that this procedure yielded relatively intact matrices and was used in subsequent analytical experiments. Hemocytometer counts indicated that approximately 30% of the starting nuclei were isolated as nuclear matrices.

The matrices were easily disrupted by physical forces. Excessive force by pipetting, centrifugation, etc., resulted in loss of the internal network and more empty residual nuclear envelopes resulted.

F. Morphology of Nuclear Matrices

Nuclei from unstimulated cells treated with DNase 1 and low magnesium buffer (designated LM spheres)

are shown in Figure 23. The condensed chromatin appeared opaque with little visible fine structure. Regions of interchromatinic space were visible but nucleoli were indistinguishable from the condensed chromatin.

After extraction of the LM spheres with 2M NaCl (HS spheres) much of the nuclear material was removed (Figure 24). Four main regions were visible: a 15nm thick residual nuclear envelope containing periodically spaced structures similar to nuclear pores; a residual nucleolus similar to the in situ nucleolus; an internal network connected to the nuclear envelope and to the residual nucleolus; large areas containing no visible structures. Higher magnifications of the internal network showed mainly amorphous material with some interspersed regions showing globular particles (20-40nm) and fine fibrils (3-10nm) (Figure 25). This network appeared similar to the interchromatinic material observed in unstimulated lymphocytes stained with uranyl-EDTA. The large empty spaces were similar in size and location to the large clumps of condensed chromatin of in situ and isolated nuclei of unstimulated cells.

Following digestion with DNase I and RNase A the resting nuclear matrices remained morphologically identical to the HS spheres. While a number of matrices

Figure 23.

Electron micrograph of unstimulated nuclei treated with LM buffer showing electron - opaque clumps of condensed chromatin. x 12,000. Bar = 1 μ m. (c) - condensed chromatin.

Figure 24.

Electron micrograph of type 1 LM spheres treated with HS buffer (2M NaCl). Condensed chromatin has been extracted leaving large empty spaces and a small amount of internal network. x 23,700. Bar = 1 μ m. (IN) - internal network; (arrow) - nuclear envelope.

Figure 25.

High magnification view of internal network of Fig. 24 showing numerous 20-50 nm granules and fine fibrils. x100,000. Bar = 0.1 μ m. (arrow) - fine fibrils.

had intact envelopes and were roughly spherical in shape, a large proportion (up to 50%) were distorted to some degree (Figures 26 and 27).

Extraction of nuclei from conA-stimulated cells gave morphologically variable residual nuclear structures resembling morphotypes 1, 2 and 3. Morphotype 1 derived structures behaved in the extractions like those from unstimulated cells. The following descriptions apply to residual structures judged to be derived from morphotype 2 and 3 nuclei.

LM spheres from stimulated cells are shown in Figure 28. The condensed chromatin appeared opaque as in the unstimulated LM spheres but the nucleoli were distinguishable from the condensed chromatin. The interchromatinic material was more extensive than in the unstimulated LM spheres and contained clusters of 20-30nm globular particles (Figure 28). Extraction of the LM spheres with 2M NaCl removed some nuclear material. A residual nuclear envelope, large residual nucleoli, and an extensive internal network were seen. In contrast to unstimulated HS spheres no large empty spaces were seen (Figure 29). Numerous smaller areas of relatively low electron density were observed, high magnifications of these regions often showed numerous 3-10nm fibrils (Figure 30).

Treatment of the HS spheres with DNase 1

Figure 26.

Electron micrograph of type 1 nuclear matrix showing nuclear envelope, internal network, residual nucleolus and large empty "chromatin" spaces. x 20,000. Bar = 1 μ m. (IN) - internal network; (n) - nucleolus; (s) - empty spaces.

Figure 27.

High magnification view of internal network of Fig. 26 showing 20-50nm granules and fine fibrils. x 100,000. Bar = 0.1 μ m. (arrows) - fine fibrils.

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Figure 28.

Electron micrograph of type 3 nucleus treated with LM buffer showing electron-opaque chromatin. x 14,000. Bar = 1 μ m. (c) - condensed chromatin.

Figure 29.

Electron micrograph of ConA stimulated LM spheres treated with HS buffer. Note; very small empty "chromatin" spaces; extensive internal network; large residual nucleoli. x 6,500. Bar = 2 μ m. (IN) - internal network; (n) - residual nucleolus.

Figure 30.

High magnification view of internal network of Fig. 29. Note many fine fibrils and a few granules. x 35,000. Bar = 0.5 μ m.

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and RNAase A removed a small amount of nuclear material. The resulting nuclear matrices (Figures 31-34) contained the four major regions described in unstimulated matrices. The nuclear envelope was 10-20nm thick and contained structures resembling nuclear pores (Figure 35). The residual nucleoli were large and contained lacunae. The internal network extended from the residual nuclear envelope to the residual nucleoli and occupied a proportionately greater amount of the total matrix volume than did the internal network in type 1 matrices (Figures 31-34). Higher magnifications of the internal network showed predominantly amorphous material similar to that seen in the type 1 matrices. Small fibrils (5-10nm, Figure 36) and globular particles of various sizes (10-50nm, Figure 37) were visible in some regions. The overall internal network appeared similar morphologically to the interchromatinic space in type 2 and 3 nuclei stained with uranyl-EDTA. Matrices stained with uranyl-EDTA appeared identical to matrices stained with conventional uranyl-lead (Figures 38 and 39).

Numerous small regions containing no visible structures were observed throughout the type 2 and 3 matrices. These were of similar size and distribution to the regions of condensed chromatin seen in type 2 and 3 nuclei. The empty regions also appeared to correspond to the regions of low electron density in

Figure 31. Electron micrograph of type 3 nuclear matrices showing residual nuclear envelopes; extensive internal network; residual nucleoli; small empty "chromatin" spaces. $\times 19,300$, Bar=1 μ m. (IN)- internal network; (n)- residual nucleolus; (s)- empty "chromatin" spaces; (arrows)- nuclear envelope.

Figures 32-33. Electron micrographs of type 3 nuclear matrices showing extensive internal network, small empty "chromatin" spaces and a nuclear body. (IN)- internal network; (n)- residual nucleolus; (s)- empty spaces; (NB)- nuclear body. Bars=1 μ m.

Figure 32. x19,000

Figure 33. x11,200

Figure 34.

Electron micrograph of type 3 nuclear matrix. x16,000.

Bar=1 μ m.

Figures 35 - 37. High magnification views of Figures 31

- 34.

Figure 35.

Showing internal network and nuclear envelope with nuclear pores. x 47,000. Bar = 0.5 μ m.

Figure 36.

Showing internal network and residual nucleolus. Note fine fibrillar network in space adjacent to nucleolus. x40,000. Bar = 0.5 μ m.

Figure 37.

Note clusters of interchromatinic granules within internal network adjacent to nucleolus. x 63,000. Bar = .25 μ m. (arrows) - nuclear pores; (f) - fine fibrils; (IG) - interchromatinic granules; (n) - residual nucleolus.

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Figure 38.

Electron micrograph of a type 3 nuclear matrix stained with regressive uranyl - EDTA to reveal RNP. Note intensely stained internal network and nucleolus. x16,000. Bar = 1 μ m. (IN) - internal network; (n) - residual nucleolus.

Figure 39.

High magnification view of internal network of Fig. 38 showing fine fibrils and granules. x 52,000. Bar = .25 μ m.

type 2 and 3 HS spheres. Absence of the fine fibrils seen in these low density regions in HS spheres suggests that such fibrils may have been chromatin or RNA.

The average diameters of the matrices were substantially smaller than either in situ or isolated nuclei (Table 6) indicating that although gross nuclear morphology was being retained in the matrices some artifactual collapse of the matrix occurred during isolation (Figure 40).

G. Chemical Analysis Of The Nuclear Matrix

To compensate for the loss of nuclei throughout the extraction procedure, the results of biochemical analyses were normalized by determining the number of structures in each sample with a hemocytometer and expressing the results as pg/ structure.

The mean protein, DNA and RNA contents of nuclei and nuclear matrices are shown in Table 7. Matrices from unstimulated cells contained approximately 48% of total nuclear protein, 22% of nuclear DNA and 16% of nuclear RNA. Matrices from stimulated cells (24h ~~PM~~) contained 59% of nuclear protein, 4% of the DNA and 10% of the RNA. The matrix isolation procedure, therefore, removed approximately 40-60% of nuclear protein, 80-96% of the DNA and 84-90% of nuclear RNA.

Figure 40.

Electron micrograph of nuclear matrices from stimulated cells (24h) showing distorted morphology. x 11,000. Bar = 1 μ m. (small arrow) - normal matrix; (large arrow) - distorted

Table 7
Chemical Composition of Nuclei and Nuclear Matrices

Sample ^{2.}	ConA ^{3.}	Protein		DNA		RNA	
		pg	% ^{1.}	pg	%	pg	%
Nuclei	-	14+1	64	6.0+0.5	27	2.2+0.2	9
	+	22+1.0	71	6.0+1.0	19	3.0+0.2	10
Matrix	-	7+1.0	81	1.3+0.2	15	0.4+0.1	4
	+	13+1	96	0.3+0.1	2	0.3+0.1	2
Matrix as % of Nucleus	-	48+10		22+8		16+9	
	+	59+6		4+2		10+9	

1. % values are % of total composition.
2. Based on three separate determinations.
3. Samples cultured for 24h with conA.

The composition of unstimulated matrices was 81% protein, 15% DNA and 4% RNA. Stimulated matrices were predominantly protein (96%).

H. Quantitative Changes in Proteins During Lymphocyte Stimulation

In order to assess variation in protein content between individual structures, particularly during the asynchronous response to conA, total cellular, nuclear, and nuclear matrix proteins were quantitatively assayed by microspectrophotometry of NYS stained samples. The relative values obtained by microphotometry were normalized with the biochemical values by arbitrarily setting the average microphotometric value for unstimulated cells (+TCA) equal to the equivalent biochemical value for total protein per cell, i.e., 27pg/cell (Table 8).

The biochemical and average microspectrophotometric results for total proteins (+TCA) were in reasonable agreement (Table 8). Total cellular protein doubled after stimulation (27 to 53pg/cell, 24h PS), nuclear protein increased from 14 to 22pg/nucleus (1.6x), and nuclear matrix proteins increased from 7 to 13pg/matrix (1.7x). Similarly, microspectrophotometric analysis of non-histone proteins (-TCA) showed that cellular proteins increased approximately 2.4x, nuclear

Table 8
Mean Protein Content of Cells, Nuclei and Nuclear
Matrices before and after 24h ConA Stimulation

Sample	ConA	Biochem. Analysis	Mean Vol. (μm^3)	Microspectrophotometry ^{2.}					
				All Structures			40% Largest Structures		
				+TCA _{3.}	-TCA	Hist. _{4.}	+TCA	-TCA	Hist.
Cells	-	27 $\pm$ 1	45	27	16	11	-	-	-
Nuclei	-	14 $\pm$ 1	22	16	9	7	-	-	-
Matrix	-	7 $\pm$ 1	15	8	5	3	-	-	-
Cells	+	53 $\pm$ 3	80	48	37	11	68	58	10
Nuclei	+	22 $\pm$ 1	45	25	16	9	32	25	7
Matrix	+	13 $\pm$ 1	40	12	13	0	16	19	0

1.Values in pg/individual structure, based on three determinations.

2.Analysis of Naphthol Yellow S stained samples.

3.+TCA=trichloroacetic acid treated samples.

4.+TCA values minus -TCA values gives a rough estimate of histone content.

proteins 1.9x, and matrix proteins 2.6x.

To further examine the changes in protein content during stimulation individual NYS protein values for cells, nuclei and matrices were plotted against the volume of the individual structure measured (Figures 41-46). The TCA-treated samples indicate total protein while the untreated samples (-TCA) are supposed to indicate non-histone protein (Fredricks et al., 1980).

As expected, unstimulated samples were relatively homogeneous in protein content (+ and -TCA) and volume. The TCA treated cells and isolated nuclei had substantially higher protein values than the -TCA samples giving values for "histone" proteins of 11 and 7pg/structure, respectively. The difference in values between + and -TCA unstimulated matrices was smaller than for cells and nuclei indicating reduced chromatin content.

Figures 41-46 also show the results for conA stimulated (24h PS) cells, nuclei and matrices respectively. These samples showed substantially greater heterogeneity of protein content and volume than the unstimulated samples. The protein values for all structures increased approximately linearly with volume. While the mean protein values (Table 8) for the complete samples indicated that cellular, nuclear and matrix protein increased about two

Figures 41 - 50. Graphs of protein content of individual cells, nuclei and matrices plotted against the volumes of individual structures. Protein content was measured by microspectrophotometry of naphthol yellow S stained samples and expressed as pg per structure.

Figure 41.

Graph of log non-histone protein content vs log volume for unstimulated and stimulated cells (24h). Note the heterogeneity of protein content and volumes of con A stimulated cells compared to unstimulated cells. (o) - unstimulated cells; (●) - stimulated cells.

Figure 42.

Graph of log total protein content vs log volume for unstimulated and stimulated cells (24h). Note the relatively higher protein values compared to Fig. 41. (o) - unstimulated cells; (●) - stimulated cells.

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Figure 43.

Graph of log non-histone protein content vs log volume of nuclei from unstimulated and con A stimulated cells. (○)- unstimulated; (●) - stimulated (24h).

Figure 44.

Graph of log total protein content vs log volume of nuclei from unstimulated and stimulated cells. (○)- unstimulated; (●)- stimulated (24h).

Figure 45.

Graph of log non-histone protein content vs log volume of matrices from unstimulated and stimulated cells. Note heterogeneity of protein content of stimulated matrices. (○) - unstimulated; (●) - stimulated (24h).

Figure 46.

Graph of log total protein content vs log volume of matrices from unstimulated and stimulated cells. Note similarity of proteip values to Fig. 45 indicating extraction of histones. (○)- unstimulated; (●)- stimulated (24h)

fold during stimulation the individual values show that at 24h PS some structures contained amounts of protein equivalent to the unstimulated structures while others had up to 6 fold higher amounts.

Nuclear morphotypes could not be recognized morphologically in these samples but cell and nuclear volume are well correlated with morphotype and are a reasonable indicator of stimulation (Bladon, 1980, Setterfield et al., 1983). The smallest structures therefore represented unstimulated type 1 morphotypes and the largest, type 3. Type 3 structures accounted for approximately 40% of the total structures in many preparations (Table 1). Average +TCA protein values for the 40% of the structures with the largest volumes were 68, 32 and 16pg per cell, nucleus and matrix respectively (Table 8). These represent increases of 2.5x, 2x and 2x for type 3 cells, nuclei and matrices, respectively, over unstimulated samples. Similarly, the average non-histone values for the largest 40% of the total structures were 58pg, 25pg and 19pg per cell, nucleus and matrix, respectively. These results indicate increases in amount of non-histone protein for stimulated morphotype 3 cells, nuclei and matrices of approximately 3.6x, 2.8x and 3.6x respectively. Such increases are substantially higher than the average values for the whole populations and are indicative of

the real increases in protein during mitogenic stimulation.

The +TCA protein values for stimulated cells and nuclei were relatively higher than the corresponding -TCA values giving values for "histone" proteins of 11 and 9 pg/structure respectively for the total population and 10 and 7pg/structure for the selected type 3s, respectively. The average protein values for +TCA matrices were lower than values for -TCA matrices indicating absence of chromatin and possible loss of acid-soluble proteins.

To examine the time course of increase in non-histone matrix proteins during stimulation, matrices were isolated at various times after stimulation and the NYS protein values measured. Figure 47-50 shows that matrices from cells stimulated for 0 and 10h had protein amounts and volumes similar to matrices from unstimulated cells. Matrices stimulated for 18h had protein amounts and volumes similar to those of matrices stimulated for 24h. This indicates, therefore, that the main increase in matrix proteins occurs between 10 and 18h PS.

Figure 47.

Graph of log non-histone protein content vs log volume
for matrices stimulated for 0h.

Figure 48.

Graph of log non-histone protein content vs log volume
for matrices from cells stimulated for 10h.

Figure 49.

Graph of log non-histone protein content vs log volume
for matrices from cells stimulated for 18h.

Figure 50.

Graph of log non-histone protein content vs log volume
for matrices from cells stimulated for 24h.

I. Proteins of the Nuclear Matrix

The results of one-dimensional SDS-PAGE of total nuclear and nuclear matrix proteins are shown in Figures 51 and 59. Histone proteins were very prominent in total nuclear proteins from both stimulated and unstimulated nuclei. A 43kd protein was also prominent and approximately 20 minor proteins between 20 and 150kd were visible. No major differences were observed between total nuclear proteins from unstimulated and conA stimulated (8-36h PS) cells, based on three separate preparations.

When loaded at the same total protein concentration, samples of nuclear matrix proteins from unstimulated and stimulated cells contained little or no histone H1 and substantially less core histone proteins compared to total nuclear proteins (Figure 52). The amount of core histones varied noticeably in different matrix preparations from unstimulated cells, but was always very low in matrices from stimulated cells. The 43kd protein and the minor proteins observed in the samples of total nuclear proteins were relatively more intense in the matrix preparations. In addition to the minor proteins, several strongly staining proteins not detected in the nuclear protein samples became evident in the matrix samples.

Figure 51.

1-D PAGE of total nuclear and nuclear matrix proteins. Lanes A1-6 were loaded with 20 μ g of protein and lanes B1-6 with 40 μ g of protein. Note the relative decrease in histone protein in the matrix samples compared to nuclear proteins.

Lanes A1-2 and B1-2 nuclear proteins from unstimulated bovine lymphocytes.

Lanes A3 and B3 - nuclear proteins from unstimulated murine lymphocytes.

Lanes A4 and B4 - nuclear proteins from conA stimulated (24h) murine lymphocytes.

Lanes A5 and B5 - matrix proteins from unstimulated (24h) murine lymphocytes.

Lanes A6 and B6 - matrix proteins from conA stimulated murine lymphocytes.

Figure 52.

2-D PAGE of standard proteins used to establish reference grid for comparison of 2-D gels. Proteins used were; carbonic anhydrase (29kd), actin (43kd), 3-phospho- glycerate kinase (47kd), bovine serum albumin (68kd) and phosphorylase B (94kd).

Nuclear matrix proteins from unstimulated and conA stimulated cells were very similar in terms of total number of proteins resolved, their molecular weights and the relative staining intensity of individual protein bands. This situation held in three separate preparations. Major proteins of 23, 35, 43, 55, 65-70 and 90kd and approximately 30 minor proteins from 15-150kd were observed in matrices prepared from unstimulated and stimulated cells. No major qualitative differences were observed between matrix proteins from unstimulated and stimulated cells but several minor quantitative changes in individual protein bands were observed. Figures 51 and 59 show that proteins of 17, 22, 40, 49kd and three proteins between 60 and 70kd were relatively more prominent in matrices from stimulated cells while one protein of 28kd was less prominent in these matrices.

Equilibrium two-dimensional electrophoresis was also used to analyze nuclear matrix proteins. Two separate matrix preparations were analyzed and gave essentially the same results. A set of standard proteins with known molecular weights and pI's was run concurrently with the matrix samples and these were used to establish a reference grid (Peters and Comings, 1980) to aid in comparing gels (Figure 52).

Approximately 30 acidic proteins were resolved in

the matrix preparations from cells stimulated for 0h (unstimulated), 18h and 36h as shown in Figures 53-58. Consistent with the 1-D SDS-PAGE, the protein patterns from the three 2-D preparations were qualitatively very similar; no new major proteins were detected accompanying stimulation. Major proteins of 38kd (pI 5.8), 43kd (pI 6.1), 45kd (pI 6.1), 50kd (pI 5.9), 55kd (pI 5.8), 58kd (pI 6.0) and 69kd (pI 6.5) were detected in matrix preparations from unstimulated cells. Three minor proteins migrated close to the 43kd protein possibly indicating charge isomers. The 69 and 50kd proteins also had one or two minor charge isomers.

Several changes in the relative amounts of individual major proteins were observed between matrices from unstimulated and stimulated cells. In two experiments proteins designated 1(69kd), and 5(35kd) appeared to increase in amount, relative to the other proteins, during stimulation. In one experiment proteins 3 and 4 also showed increases on stimulation but this was not consistent in a second experiment. In contrast, protein 2(68kd) was less abundant in stimulated matrices than in unstimulated matrices. Some minor proteins may have changed qualitatively during stimulation but this was difficult to determine with certainty.

Figures 53 - 54.

2-D equilibrium PAGE of nuclear matrix proteins from unstimulated cells. Figures 53 and 54 show results of two independent experiments. Figures 53, 55 and 57 show samples from one experiment and Figures 54, 56 and 58 show samples from an independent experiment. Sample loading was 600 μ g per gel.

pH

7.4

6.7 6.1

53

-94

-68

-43

-29

MW x 10³

54

-94

-68

-43

-29

Figures 55 - 56.

2-D equilibrium PAGE of nuclear matrix proteins from conA stimulated cells (18h). Figures 55 and 56 show results of two independent experiments. Note overall similarity to Figures 53 and 54, and the relative changes in proteins 1 - 5. 600 μ g of protein per gel.

pH

7.4

6.7 6.1

55

-94

-68

-43

-29³
MW x 10³

56

-94

-68

-43

-29

Figures 57 - 58.

2 - D equilibrium PAGE of nuclear matrix proteins from cells stimulated with conA for 36h. Note; similarity to Figs. 53-56; the relative changes in amount of proteins 1-5. 600 μ g of protein per gel.

J. Synthesis and Stability of Matrix Proteins

The synthesis of total nuclear and nuclear matrix proteins was studied by culturing cells for various times with ^3H -leucine, isolating nuclei and matrices and analyzing the constituent proteins with 1-D SDS-PAGE and fluorography. Protein turnover was examined by washing labelled cells with culture medium containing 1mM unlabelled L-leucine and culturing without label for a further 24h. The proteins were then analyzed as above (Figures 59 and 60).

Unstimulated cells supplied label 0 to 16h PS showed little incorporation into nuclear proteins (lane A1, Figure 60). The 43kd protein and a few minor proteins were labelled. ConA-stimulated cells labelled for 8 to 16h (lane A2) and 16 to 24h (lane A3) PS also showed relatively little incorporation of radioactivity. The 43kd protein and some minor proteins were labelled, with the former showing slightly more intense radioactivity after the 24h chase. Substantial label was incorporated into nuclear proteins of cells labelled 28 to 36h PS (lane A4). Core histones were heavily labelled and histone H1, the 43kd protein and minor proteins of 15 and 38kd also showed substantial labelling. The histones and the 43kd protein retained their label throughout the chase but the minor proteins lost considerable activity.

Figures 59 - 60.

1 - D PAGE of total nuclear and nuclear matrix proteins from cells labelled for various periods with ^3H -leucine (20 $\mu\text{Ci/ml}$, 80Ci/mM). All lanes had 20 μg of protein.

Figure 59.

Gel stained with coomassie blue.

Figure 60.

Fluorogram of gel in Figure 59, exposed 7 days.

Lanes A1 - nuclear proteins from unstimulated cells labelled 0-16h PS.

Lanes A2-4 - nuclear proteins from conA stimulated cells labelled 8-16h (lanes A2), 16-24h (lanes A3), 28-36h (lanes A4).

Lanes A5-8 - as per lanes A1-4 respectively but the cells were cultured without radioactive label for 24h after labelling period (ie a 24h chase).

Lanes B1 - nuclear matrix proteins from unstimulated cells labelled 0-16h PS.

Lanes B2-4 - nuclear matrix proteins from conA stimulated cells labelled 8-16h (lanes B3), 16-24h (lanes B3), 28-36h (lanes B4).

Lanes B5-8 - as per lanes B1-4 but the cells were cultured without radioactive label for 24h after the labelling period.

Nuclear matrix proteins from unstimulated cells labelled 0 to 16h PS (lane B1, Figure 60) showed little incorporation of label. Matrix proteins from conA-stimulated cells labelled 8-16h PS (lane B2); however, had substantial incorporation of label into most matrix proteins seen in the stained gel. The patterns of labelling of matrix proteins from stimulated cells labelled 16-24 (lane B3) and 28-36h (lane B4) PS were similar to the proteins labelled 8-16h PS, but with less activity than in the 8-16h proteins.

After a 24h chase the matrix proteins of stimulated cells labelled 8-16h PS lost considerable activity but some of the label was stable during the chase. Proteins labelled from 16 to 24h and 28 to 36h PS retained most of their label during the chase and appeared very similar to the labelling pattern of the 8-16h proteins after the chase.

DISCUSSION

A. Cytological Changes in Nuclei During Stimulation

The cytological results presented show that a major response of mouse splenic lymphocytes to the mitogen conA is a 2 to 3 fold increase in cell volume involving enlargement of both cytoplasm and nucleus. Concurrent with the increase in nuclear volume nucleoli enlarge and condensed chromatin disaggregates from large continuous clumps located at the nuclear periphery to numerous smaller clumps dispersed throughout the nucleus. On the basis of size and nuclear morphology cells were arbitrarily classified into three morphotypes, type 1 being unstimulated, type 3 showing full morphological response and type 2 intermediate (Setterfield et al., 1983).

The nuclear responses were markedly asynchronous. Morphotype 2 and 3 nuclei appeared in cultures 12h PS, with the maximum number (20% type 2 and 50% type 3) being observed between 24h and 36h PS. At any time between 12 and 36h PS, however, substantial numbers of all three morphotypes were present. These data indicate that average measurements of parameters in mitogenically stimulated populations can severely underestimate changes occurring in cells actually responding to

mitogen. This is an unavoidable problem in biochemical analyses of the type here presented. Attempts to sub-fractionate stimulated cell samples into populations resembling types 1-3 prior to analyses were unsuccessful. As an alternative, where feasible, biochemical findings were related to cytological studies of samples. The changes in gross nuclear structure here described have been reported previously for human lymphocytes stimulated with PHA (Milner and Hahoe, 1968, Tokuyasu et al., 1968, Milner 1969a, 1969b) and conA (Dardick et al., 1981, Setterfield et al., 1982) and for guinea pig lymphocytes stimulated with PHA (Valkov et al., 1974). Tokuyasu et al., (1968) and Milner (1969a, 1969b) suggested that much of the condensed chromatin was dispersing to euchromatin during stimulation. However, morphometric study of stimulated human peripheral lymphocytes has shown that while nuclear and nucleolar volumes increased up to six fold the volume of condensed chromatin actually increased by 15 to 45% between morphotypes 1 and 3. Most of the increase in nuclear volume was due to a 10 fold increase in the volume of the interchromatinic region (Bladon, 1980, Setterfield et al., 1983).

A morphometric study of mouse lymphocytes stimulated for 4 days in vivo with dinitrochlorobenzene gave similar results. Nuclear volume increased

approximately 4 fold, the volume of condensed chromatin doubled and the volume of the "euchromatin compartment" (equivalent to interchromatinic space) increased 7 fold. The authors concluded that 70% of the increase in nuclear volume was due to the increase in euchromatin (Al-hamdani et al., 1979).

The increase in interchromatinic material during stimulation was visualized by the regressive uranyl-EDTA stain of Bernhard (1969). The stain revealed a small amount of interchromatinic material in type 1 nuclei compared to a substantially larger network of material in type 3. The interchromatinic material in both unstimulated and stimulated nuclei was composed of 25-50nm diameter granules, 3-10nm diameter fibrils and amorphous material. Type 3 nuclei also contained distinct clusters of 20-30nm interchromatinic granules (Puvion et al., 1979, Pogo, 1981).

Valkov and Moyne (1974) and Valkov et al., (1974) stained guinea pig lymphocytes with the uranyl-EDTA stain. They also observed a small network of interchromatinic material in unstimulated cells and a substantial increase in amount of interchromatinic material after 24h to 48h of culture with PHA. The interchromatinic material was composed of granules and fine fibrils similar to those presented here.

Increases in uranyl-EDTA positive

interchromatinic material and correlated disaggregation of condensed chromatin were also observed in nuclei of stimulated rat liver cells (Derenzini et al., 1981). In addition, however, use of specific EM stains for DNA indicated some increase in truly decondensed chromatin in the interchromatinic region of these nuclei (Derenzini et al., 1981). It is quite possible that a similar increase in decondensed chromatin occurs in stimulated lymphocytes, but it would be undetected by the analyses used to date. Application of specific stains for chromatin to lymphocytes would be of interest. Such decondensation of chromatin could be associated with onset of transcription which occurs about 12h PS in conA stimulated lymphocytes (Mitchell et al., 1978).

Changes in the volume of interchromatinic space correlated with directed variations in chromatin aggregation have been shown in several other nuclei (Dardick and Setterfield, 1976 etc) and it seems that this may be a general factor in modulating nuclear structure. As observed here for mouse, and in human lymphocytes (Setterfield et al., 1983) the increase in interchromatinic material precedes, and is probably prerequisite for, DNA synthesis. This is supported by the observation that morphotype 3 nuclei appear in normal frequencies in the presence of inhibitors of DNA

synthesis (Milner and Hahoe, 1968, Setterfield et al., 1983). It is also possible that changes in interchromatinic materials are important in disaggregating chromatin during replication in regularly cycling cells (Setterfield et al., 1983).

B. Nuclear Isolation

Some authors have stated that degradation of nuclear morphology during isolation results in morphologically distorted nuclear matrices (Comings and Okada, 1976, Berezney and Coffey, 1977, Berezney, 1979). As a prelude to matrix isolation, therefore, it was decided to examine isolated nuclei with the electron microscope to assess effectiveness of removal of cytoplasm and maintenance of nuclear morphology. Light microscopy was inadequate for such evaluation.

Two types of nuclear isolation procedures were evaluated. Procedures 1 to 5 (Table 3) involved incubating cells in a buffer containing a non-ionic detergent (TritonX-100 or Tween-80) without homogenization. These procedures worked reasonably well for unstimulated cells, removing most of the cytoplasm while retaining nuclear morphology. Stimulated cells, however, showed extensive cytoplasmic contamination unless they were incubated in the buffer for a relatively long time (30min, Procedure 5) resulting in

extraction of nuclear material. Tween-80 disrupted nuclear morphology more than TritonX-100, even at low concentrations (0.05%) and is not recommended. These procedures were judged unsuitable for isolation of lymphocyte nuclei.

The second group of procedures (6-10) involved gentle homogenization of the cells in a buffer without or with detergent. The cytoplasm was not effectively removed when the buffer contained no detergent (Procedures 6 and 7). A TritonX-100 concentration of 0.1-0.5% in the homogenization buffer gave nuclei with little cytoplasmic contamination and 0.25% (i.e. Procedure 10) gave optimal removal of cytoplasm and preservation of nuclear morphology. Inclusion of proteinase inhibitors in all buffers was necessary for preservation of nuclear morphology.

Various nuclear isolation procedures have been reported and vary widely in detail. Mitchell et al., (1978) isolated nuclei from unstimulated and conA stimulated human peripheral lymphocytes by incubating cells in a hypotonic buffer (10mM HEPES, 50mM KCl) containing 0.005% TritonX-100 for 13 min followed by washing in isotonic saline (ie. Procedure 1, Table 3). They reported on the basis of light microscopy that the nuclei were free of cytoplasm. Nakayasu and Ueda (1981) isolated nuclei from unstimulated bovine lymphocytes by

suspending the cells in STM buffer (see MATERIALS and METHODS) containing 1% TritonX-100 and passing the suspension through a syringe needle several times. Their nuclei were free of cytoplasmic contamination but in the EM appeared to be somewhat extracted. Berezney and Coffey (1977) isolated nuclei from rat liver by suspending cells in STM buffer without detergent and homogenizing in a Potter-Elvehjem apparatus. With lymphocytes the Dounce homogenizer gave better preservation of structure. In the EM the rat liver nuclei appeared free of cytoplasm and were morphologically well preserved. Although these nuclear isolation procedures were all reported to be reasonably effective the results presented here indicate that it may be necessary to try several different isolation procedures for each cell type in order to obtain optimally preserved nuclei. Even then some cytoplasmic contamination was unavoidable.

C. Nuclear Matrix Isolation

The procedure for isolation of nuclear matrix reported by Berezney and Coffey (1977), and two variations of this procedure, were used to isolate nuclear matrices from unstimulated and stimulated lymphocytes. Berezney and Coffey's procedure involved incubating nuclei overnight to allow endogenous

nucleases to partially digest the DNA. This was followed by successive incubations in buffers containing; 0.2mM $MgCl_2$ which partially dispersed the chromatin; 2M NaCl to remove chromatin; TritonX-100 to extract membrane lipids and DNase and RNase to further remove nucleic acids. Extraction of lymphocyte nuclei by this procedure resulted in isolation of only residual nuclear envelope lacking residual nucleoli or internal network. When the first step was replaced by a short digestion with DNase 1 many of the final matrices contained residual envelope, nucleoli and a small internal network. Decreasing the number of extractions in each buffer and shortening the time of the final nuclease treatment further improved retention of the internal network of the matrices. These matrices more closely resembled isolated nuclei than did matrices from the first two procedures and were, therefore, used for the analytical studies.

A possible explanation for the isolation of only the nuclear envelope with Berezney and Coffey's (1977) original procedure is that endogenous proteases may have degraded the internal network during the long isolation procedure. The nucleases used were not electrophoretically pure so that the shorter nuclease digestion times in the final procedure, along with inclusion of three protease inhibitors, may have reduced

proteolysis and preserved internal networks. Berezney (1979) and Kaufmann et al., (1981) have stressed the necessity for protease inhibitors during matrix isolation.

The matrices contained 40-60% of the original nuclear protein and 4-20% of the nuclear DNA and RNA. The extractions, therefore, removed approximately half of the nuclear protein and 80-90% of the nuclear DNA and RNA from unstimulated and stimulated nuclei. Slightly less DNA was extracted from unstimulated than stimulated nuclei possibly due to the highly aggregated state of chromatin in the type 1 nuclei. The composition of the nuclear matrices was predominantly protein (81-96%), with small amounts of DNA and RNA.

Approximately 30% of the starting nuclei were recovered as matrices. Wunderlich and Herlan (1977) reported recovering 30% of starting Tetrahymena cells as matrices while Berezney and Buchholtz (1981a) reported recovery of one third of rat liver nuclei as matrices. Some nuclei were presumably lost during the centrifugations but longer centrifugations would increase the time for isolation, favouring more proteolysis.

The amount of protein, DNA and RNA in individual nuclei and matrices was estimated by dividing total determinations by the number of structures in each

sample determined in hemocytometer counts. This estimation does not allow for small fragments of structures which would contribute to biochemical results but not to visual counts. Also, since matrices tend to form small clumps they may be underestimated in counting. It was felt, however, that this method was better than not correcting for loss of nuclei which would seriously overestimate the amount of material extracted from the nuclei.

Other workers have normalized their analyses in this way. Wunderlich and Herlan (1977) (and Herlan et al., 1979) isolated nuclear matrices from Tetrahymena macronuclei. They extracted 75-85% of the total nuclear protein, >95% of the DNA, and 40-90% of the nuclear RNA. The Tetrahymena matrices were composed of >95% protein (Wunderlich and Herlan, 1977). Berezney and Buchholtz (1981a) isolated rat liver matrices and reported extraction of 36% of nuclear proteins.

Most workers have not normalized their biochemical analyses in terms of individual nuclei and may therefore have overestimated the amount of material extracted. Typically, these workers have reported extraction of 80-90% of total nuclear protein, >95% of DNA, and 75-99% of the nuclear RNA. Compositions of such matrices are, however, similar to those found here eg., 70-95% protein, 0-20% DNA, and 0-20% RNA (Comings and

Okada, 1976, Berezney and Coffey, 1977, Berezney et al., 1979a, Berezney, 1980, and Fisher et al., 1982).

Nakayasu and Ueda (1981) isolated matrices from unstimulated bovine lymphocytes and without correcting for loss of nuclei reported extraction of 91% of total nuclear protein, >99% of DNA, and 83% of nuclear RNA. The composition of their final matrices was 85% protein, 1% DNA, and 14% RNA. Allowing for differences in isolation procedures and analysis of biochemical results, the reported results for the composition of matrices are in reasonable agreement with those here reported.

There was approximately a 50% decrease in nuclear volume during matrix isolation. Other workers have reported similar volume decreases, Nakayasu and Ueda (1981) reported an 80% decrease for bovine lymphocyte matrices, Hodge et al. (1977) a 70% decrease for HeLa S3 matrices, and Berezney and Coffey (1977) a 70% decrease for rat liver matrices.

Wunderlich and Herlan (1977) found that matrices from Tetrahymena macronuclei reversibly contracted when the total concentration of calcium and magnesium ions were lowered from 125mM to 5mM. The contraction resulted in a 50% decrease in matrix volume and did not depend on ATP, indicating that nuclear matrices may be sensitive to ionic conditions. Most matrix isolation procedures,

including the one used here, end with a wash in a low ionic strength buffer, which may account for some of the observed volume decrease.

D. Structure of Nuclear Matrices

Electron microscopy showed that the final matrices derived from unstimulated and stimulated cell samples reflected the structure of intact nuclei in such cells. Matrices from unstimulated cells were reasonably consistent in size and morphology, showing three components; a residual envelope with pores, a small residual nucleolus and a tenuous fibro-granular internal network. Most of the interior of such matrices was electron transparent, representing large areas from which highly aggregated chromatin had been extracted.

Matrix preparations from stimulated cells showed some matrices similar to those of unstimulated preparations but in addition, contained matrices consistent with nuclear morphotypes 2 and 3. These matrices were larger (up to 3x larger volume) and the residual nucleoli were also larger and contained many spaces similar to the lacunae found in nucleoli of type 2 and 3 nuclei in situ. Importantly, the internal network of these matrices was highly ramified between dispersed small chromatin spaces, as in the interchromatinic material of intact type 2 and 3 nuclei.

At the fine structure level the internal network

of the matrices showed similarities to the interchromatinic material of intact nuclei. Granules 20-50nm in diameter, fibrils 3-5nm thick and amorphous material were present. Even nuclear bodies found in the interchromatinic region (Valkov and Moyne, 1974, Chaly et al., 1983) were observed in matrices. The amount of amorphous material was considerably greater in matrices than nuclei, however, indicating that some collapse of fine structure occurred during matrix isolation. Overall, however, the cytological analysis indicates that matrices which retain the general form of the non-chromatin proteins of the nucleus can be prepared.

Correlations between morphology of nuclear matrices and in situ nuclei have been previously reported. Long et al. (1979) isolated nuclear matrices from Friend erythroleukemia cells stimulated to differentiate. The undifferentiated cells had large nuclei and during differentiation nuclear volume decreased and condensed chromatin became more aggregated. Nuclear matrices isolated from undifferentiated cells were large and had an extensive internal network, while matrices isolated from differentiated cells were small and had reduced internal network. Herfan et al. (1978) treated Tetrahymena cells with actinomycin D (AMD) which induced large fusion nuclei. Nuclear matrices from untreated cells had numerous, small residual nucleoli while matrices from

AMD treated cells showed fewer large residual nucleoli.

The granules and fibrils found in isolated matrices are commonly seen in the interchromatinic region of nuclei in situ, particularly with regressive uranyl staining. The granules were classified cytologically as interchromatinic and perichromatinic (Monneron and Bernhard, 1969) and are presumed to be involved in RNA metabolism and/or transport (Pogo, 1982).

Fibrils are also commonly seen in the interchromatinic region of nuclei by electron microscopy using either sections (Derenzini et al., 1981) or freeze fracture (Capco et al., 1982). Many of the fibrils may represent extended transcripts in RNP associations (Derenzini et al., 1981) but Brasch (1982) has shown that when RNP fibrils disappear from liver nuclei treated with α -amanitin, a residual fibrillar matrix which stains for protein is visible. Such nuclei continue to show disaggregated chromatin although some local condensation may occur. Morphotype 3 lymphocyte nuclei treated with α -amanitin do not collapse to type 1 but retain their morphology (Setterfield et al., 1983). The presumption, therefore, is that at least part of the interchromatinic material is a fibrillar proteinaceous matrix which is involved in maintaining nuclear structure. This fibrillar material is presumed to form a major portion of isolated matrices (Berezney and Coffey,

1974, 1977, Comings and Okada, 1976, Kaufmann et al., 1981). Sonication of nuclear matrices removed granular components leaving isolated fibrils which were composed of proteins in the 65-70kd molecular weight range, termed matricin (Berezney, 1980).

It appears, therefore, that both the gross morphology and fine structure of isolated matrices parallel structures present in intact nuclei. This suggests that the matrix is not entirely artifactual but represents organized proteinaceous elements of the nuclear envelope, nucleolus and interchromatinic materials which are important in nuclear organization. It must be remembered, however, that the extraction procedure is harsh and some rearrangements of nuclear components may have occurred during matrix isolation. The 2M NaCl extraction in particular could have artifactually precipitated nuclear proteins onto the matrix. The occurrence in matrices of fibrils and granules of irregular sizes may support this. The extent of this artifact may be small, based on the similarity of the matrices to intact nuclei, but it should not be ignored. Several authors have expressed concern about the possibility of this artifact (Berezney, 1979, Shaper et al., 1979,

E. Quantitative Changes in Nuclear and Matrix Proteins

Protein content of cells, nuclei and nuclear matrices, before and after stimulation, was measured biochemically and by microspectrophotometry using the acid dye naphthol yellow S (NYS). The latter method used without DNA extraction (-TCA) is presumed to measure total non-histone proteins while following removal of DNA with TCA histones are also measured. The difference between + and - TCA should measure histone proteins (Tas et al., 1974, 1978; Fredericks et al., 1980).

Unstimulated lymphocytes, and nuclei and matrices derived from them, showed reasonably consistent protein contents, with or without TCA. When the mean microphotometric value for unstimulated cells (+TCA) was normalized to the equivalent biochemical value of 27 pg per cell, the other mean +TCA NYS values showed good agreement with equivalent biochemical measurements. These values showed increases in mean total protein, at 24h PS, of approximately 2x, 1.6x, and 1.7x for cells, nuclei and matrices, respectively. Equivalent increases for non-histone proteins (-TCA NYS values) were 2.3x, 1.8x and 2.6x. Since DNA replication had not commenced at 24h PS any protein increases should be non-histone. Unstimulated bovine lymphocytes, which are larger than mouse lymphocytes, stimulated with PHA showed a 6x increase in cell and a 4x increase in nuclear dry mass

at 48h PS (Steffan and Soren, 1968, Soren, 1969). NYS measurements for nuclei and matrices from stimulated cells also showed a wide range of values (15-60pg/nucleus and 5-30pg/matrix for +TCA).

In general, protein content increased in proportion to volume of structure indicating more or less constant protein concentration. Since about 40% of cells are morphotype 3 at 24h PS, protein values for the largest 40% of structures were averaged separately, to give mean values for cells actually responding to con A. Using these values the increases in non-histone protein (-TCA) for stimulated cells, nuclei and matrices were 3.6x, 2.8x and 3.6x respectively. the increase in these proteins for the 40% large structures was 14 pg/nucleus and 11 pg/matrix. This indicates that a large proportion (80%) of the increase in nuclear protein on stimulation was in proteins which purify as matrix.

Microspectrophotometric analysis proved to be very useful for analyzing the protein content of lymphocytes and isolated structures. There were, however, two anomalous aspects to this data. First, the "histone" values were estimated at between 7pg and 11pg/cell. The 2C DNA content of mouse cells is 6pg (Laird, 1971) and the histone proteins should have approximately the same value. This indicates that the NYS histone values are too high. One possible

explanation for this is that histone proteins have a high content of basic amino acids (Isenberg, 1979) and would, therefore, bind relatively more dye than most other proteins. Naphthol yellow S would thus overestimate relative content of histones. Second, TCA-treated matrices from stimulated cells showed a relatively small range of protein contents compared to the range of volumes, indicating that some matrices increased in volume without increasing their protein content. This was not observed in the -TCA stimulated matrices. One explanation for this is that the TCA treatment may have extracted some proteins preferentially from the matrices. Alternatively, TCA may have caused loosening of the matrices, although this was not observed in unstimulated samples. Therefore, although the NYS method is useful it should be used with care and in conjunction with other quantitative methods.

The time course of increases in non-histone matrix proteins was examined by NYS staining of -TCA matrices isolated at various times after stimulation. At 0h and 10h PS the matrices had protein and volume values equivalent to unstimulated matrices. Matrices stimulated for 18h, however, had protein and volume values identical to those at 24h PS. This indicates that a major increase in matrix protein occurred between 10 and 18h PS. Importantly, this time period corresponds to

the time when the nuclear morphological changes occur and to the time period when some proteins, essential for the nuclear changes, are synthesized (ie. 12-18h PS, Setterfield et al., 1983). This correspondence gives further evidence that the increases in size and amount of matrix represents a major component of the observed nuclear morphological changes.

F. Qualitative Changes in Nuclear and Matrix Proteins

Proteins from isolated nuclei and nuclear matrices were analyzed by one- and two-dimensional PAGE to reveal possible qualitative changes during mitogenic stimulation. The major proteins in 1-D gels of total nuclear protein samples were histones (core and H1) and a 43kd protein. Approximately 20 minor proteins of 20 to 150kd were also resolved but no substantial differences between proteins from unstimulated and stimulated nuclei were detected. When nuclear matrix proteins were run on 1-D gels at the same total concentration as nuclear proteins they had substantially less H1 and core histone proteins indicating extraction of chromatin. It should be noted, however, that the relatively 'gentle' procedure adopted to give matrices with good internal structure always left some residual chromatin elements, although these may have been structurally disorganized.

Major non-histone proteins of 23, 35, 43, 55 and 68-70kd, and approximately 30 minor proteins of 15-150kd were present.

The overall 1-D protein patterns of matrices from unstimulated and stimulated cells were very similar, only a few small quantitative differences were observed in individual protein bands.

Two dimensional equilibrium gels give high resolution of acidic ($pI < 7$) but not basic proteins, so that not all matrix proteins are resolved. Notably many nucleolar, ribonucleoproteins and contaminating histones are basic and would not run in the body of these gels (Suria and Liew, 1978, Peters and Comings, 1980). It is difficult, therefore, to directly compare the 1-D and 2-D results as basic proteins would appear on the 1-D gels but not in the body of 2-D gels.

Approximately 30 acidic matrix proteins were resolved by 2-D PAGE with major proteins of 35, 43, 50, 52, 55 and 68kd. The overall patterns of matrix proteins from unstimulated and stimulated (18 and 36h PS) cells were quite similar. No new major proteins were detected in the stimulated matrix samples but three proteins changed in amount during stimulation. Staining intensity of proteins at 69kd (protein 1) and 35kd (protein 5) appeared to increase during stimulation while a protein at 68kd (protein 2) appeared to

decrease. Other changes were observed but were inconsistent in replicate experiments. These changes may also be real but require further investigation. Due to the substantial fraction of type 1 matrices present in stimulated samples, the observed changes represent minimum estimates of protein changes in responding type 3 matrices.

Proteins; 1, 2, 4 and the 43kd protein had at least one or two minor charge isomers possibly indicating post-synthetic modifications. Some minor proteins were observed only in stimulated matrices but it was not certain if these proteins were appearing de novo or were merely below the level of detection of 2-D PAGE in unstimulated matrices.

Several authors have reported analyses of matrix proteins on one- and two-D PAGE. Nakayasu and Ueda (1981) analyzed matrix proteins from unstimulated bovine lymphocytes on 1-D gels. They observed major proteins of 43, 53 and 68kd and approximately 50 minor proteins of 30 to 100kd. The 43kd protein accounted for 12% of the total matrix protein and it was suggested that it was actin (Nakayasu et al., 1981). Fisher et al., (1982) found that rat liver matrices had major proteins of 38, 43 and 70kd and approximately 30 minor proteins from 20 to 200kd. Matrices from avian erythroblast nuclei contained four major proteins of 43, 52, 66 and 68kd and

approximately 30 minor proteins, mostly acidic (Maundrell et al., 1981). These results are reasonably consistent with the lymphocyte results presented here in terms of total number of proteins in the matrix (>30) and the individual major proteins (ie. 43, 50-55 and 65-70kd).

In contrast to these results, Berezney and Coffey (1974, 1977) found that matrices from rat liver contained three major proteins of 62, 66 and 69kd, which accounted for half of the total matrix protein, and a few minor proteins of 50kd and >100kd. Comings and Okada (1976) found that matrices from mouse liver contained major proteins of 65, 67 and 68kd and a few minor proteins. These results suggest that the nuclear matrix contains relatively few distinct proteins (i.e.<10), with the major ones being three 65-70kd proteins. These latter proteins are similar to those of the pore-lamina preparations (Krohne et al., 1982). Considerable emphasis has been placed on the 65-70kd proteins by most authors but it is clear from the first studies referred to and the results presented here that the nuclear matrix contains numerous proteins in addition to the 65-70kd proteins.

Peters and Comings (1980) analyzed rat liver matrices by 2-D equilibrium PAGE. They observed in excess of 50 proteins with major components of 43-55,

65, 68 and 70kd. Most of the proteins had several charge isomers. Detke and Keller (1982) similarly analyzed matrices from HeLa cells and observed more than 30 proteins with major proteins of 40-55kd but only minor proteins of 60-70kd. Brasch (personal communication) analyzed chicken liver matrix proteins on 2-D gels and observed protein patterns similar to those of Comings and Peters (1980) but with fewer charge isomers.

Proteins 1 and 2 correspond to the 65-70kd group from rat liver and there is some similarity between proteins in the 43-55kd range (i.e. proteins 3-5) of lymphocyte matrices and the 43-55kd group from rat liver. No proteins from lymphocyte matrices corresponded to the 65 and 70kd (pI 7-7.4) proteins of rat liver matrices.

The 2-D results presented here, therefore, show some similarity with matrix proteins from rat and chicken liver and HeLa cells in terms of number of proteins and individual proteins but there are substantial differences between the protein patterns. As relatively little work has been done on 2-D analysis of matrix proteins it is not possible at this time to identify the majority of the proteins resolved in these samples. Importantly, proteins 1 and 2 (69 and 68kd) correspond to at least one of the 60-70kd lamin proteins, which have been localized primarily at the

nuclear periphery (Krohne et al., 1978). Also, the 43kd protein comigrated with actin on 2-D gels. Nakayasu et al., (1981) have reported that the 43kd protein from bovine lymphocyte matrices comigrated with actin.

There appears to be some changes in lymphocyte matrices during stimulation but no drastic qualitative alteration occurs. This result suggests that some components of the nuclear matrix could be relatively ubiquitous, possibly conserved, structural elements which vary only quantitatively in relation to nuclear volume and activity. Other matrix components appear to be variable and could be metabolic components which change in relation to specialized nuclear function.

G. Synthesis of Nuclear and Matrix Proteins

The fluorographic analysis of total nuclear proteins labelled with ^3H -leucine showed that little labelling occurred until 28-36h PS. At this time synthesis of core histones began, corresponding to the onset of DNA synthesis (Tokuyasu et al., 1968, Milner, 1969, Setterfield et al., 1983). The 43kd protein and numerous minor proteins, which would include matrix proteins, from 15 to 150kd were also synthesized at this time, most of which were stable during the 24h chase.

While matrix proteins from unstimulated cells had very little label, matrix proteins from stimulated cells

labelled 8-16h, 16-24h and 28-36h PS all showed substantial label. Most stained proteins were labelled (especially the 43kd and 65-70kd proteins), indicating synthesis of most if not all matrix proteins. These labelled matrix proteins were not detected in the total nuclear protein samples because they would have been diluted by other nuclear proteins (i.e. histones) which did not incorporate label. The labelled minor proteins seen in nuclear samples labelled 28-36h PS probably represent matrix proteins, which at this time constitute a larger proportion of total nuclear proteins than in unstimulated cells. Therefore, substantial amounts of matrix proteins were synthesized between 8 and 36h PS, much of this protein being stable over the 24h chase.

The synthesis of matrix proteins between 8 and 16h PS corresponds well to the time of increase in total matrix protein detected by NYS-microspectrophotometry (ie 10-18h PS). This also corresponds to the time period of nuclear morphological changes and to the time of synthesis of some proteins essential for the nuclear changes (Setterfield et al., 1983). These results are also consistent with the results of an autoradiographic study of conA stimulated lymphocytes labelled with ³H-leucine for 8-16h PS. It was shown that some nuclear proteins synthesized during this time were stable for up to 48h and some of these proteins appeared to survive

mitosis (Setterfield et al., 1983). Hodge et al. (1977) have shown that a substantial amount of 50kd to 75kd matrix proteins synthesized during G1 in synchronized HeLa cells were stable throughout mitosis.

H. Possible Functions of the Nuclear Matrix

The nuclear matrix has been implicated in, virtually all functions associated with the nucleus including; control and maintenance of nuclear volume, shape and organization, DNA synthesis, RNA synthesis and processing, steroid hormone receptor function, viral protein function etc (Berezney, 1979, Shaper et al., 1979, Agutter et al., 1980). However, it is difficult to assess the validity of these proposed functions. Almost all studies involving nuclear matrices use harsh fractionation procedures which could lead to artifactual association of nuclear components.

The results presented here indicate that it is possible to extract most of the chromatin and RNA from lymphocyte nuclei without destroying their gross morphology. The resulting residual nuclei or matrices contained the nuclear envelope, nucleoli and an internal network ramified throughout the matrix. Matrices from unstimulated cells contained a tenuous internal network and large empty "chromatin" spaces. Type 3 matrices, however, were larger in size and had a more extensive

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internal network and smaller "chromatin" spaces. These results suggest two things. First, these residual components are sufficient to maintain the gross structure of lymphocyte nuclei. Second, the observed increase in nuclear volume is accompanied by an increase in the amount of material in the nuclear matrix roughly proportional to the increase in volume of the nucleus. This suggests that the increase in nuclear volume may be due to the increase in amount of matrix material. This is supported by the increase in amount of matrix material occurring at the time of increase in nuclear volume, the synthesis of relatively stable matrix proteins during this time and the similarity of protein composition between unstimulated and stimulated matrices. Indeed, it is tempting to suggest that nuclear volume is regulated by the amount of material in the nuclear matrix, especially in the internal network. This is consistent with the decrease in amount of internal network observed accompanying differentiation (i.e. decrease in nuclear volume) of Friend erythroleukemia cells (Long et al., 1979). This evidence is, however, circumstantial and further work will be necessary to substantiate this hypothesis.

The observed increase in size of the nuclear matrix during stimulation may be involved in

disaggregation of condensed chromatin in the intact nucleus. Considerable evidence suggest that some DNA is bound to the matrix (Berezney and Buchholtz, 1981a,1981b etc). If the chromatin in type 1 cells is attached to the matrix, then as the matrix increases in size during stimulation the clumps of condensed chromatin may be pulled further from each other. It is also possible that new chromatin matrix-binding sites are added to the matrix during stimulation and these may be involved in disaggregation. Further analysis of matrix components, especially the isolation of chromatin binding proteins would be necessary to establish these mechanisms for disaggregation.

It appears, therefore, that the nuclear matrix is a major component of the lymphocyte nucleus and may be involved in the observed gross morphological changes.

The lymphocyte matrices presented here contained DNA (5-20% of total DNA) which was resistant to extraction with 2M NaCl and DNase I. Other workers have shown that 1-10% of DNA is tightly bound to the nuclear matrix and newly synthesized DNA is transiently associated with the matrix (Pardoll et al.,1980, Vogelstein et al.,1980, Berezney and Buchholtz,1981a,1981b). If the nuclear matrix provides attachment sites for chromatin, then the increase in matrix material during conA stimulation may provide more

attachment sites. The evidence for this is, however, circumstantial and further analysis of matrix proteins to identify DNA-binding proteins is necessary.

The lymphocyte matrices contained 25nm globular particles, often in small clusters. It has been suggested that these particles are hnRNP particles containing newly synthesized hnRNA (Miller et al., 1978, Pogo, 1981). These particles have also been observed in matrices from rat liver (Berezney and Coffey, 1977). However, it will be necessary to isolate and characterize hnRNP-binding proteins in the nuclear matrix in order to establish this function more conclusively.

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