1

PM-1 3 1/2" x 4" PHOTOGRAPHIC MICROCOPY TARGET
NBS 1010a ANSI/ISO #2 EQUIVALENT

PRECISIONSM RESOLUTION TARGETS

National Library
of Canada

Acquisitions and
Bibliographic Services Branch

395 Wellington Street
Ottawa, Ontario
K1A 0N4

Bibliothèque nationale
du Canada

Direction des acquisitions et
des services bibliographiques

395, rue Wellington
Ottawa (Ontario)
K1A 0N4

Source: Your reference

Our file: Notre référence

NOTICE

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

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

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

Reproduction in full or in part of this microform is governed by the Canadian Copyright Act, R.S.C. 1970, c. C-30, and subsequent amendments.

AVIS

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

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

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

La reproduction, même partielle, de cette microforme est soumise à la Loi canadienne sur le droit d'auteur, SRC 1970, c. C-30, et ses amendements subséquents.

Canada

**An ultrastructural, immunocytochemical and biochemical
study of the effects of the transcriptional inhibitor
DRB on nuclei and nuclear matrices of
concanavalin A-stimulated lymphocytes.**

by

Lynn Marie Davis, B.Sc.(Hons.)

**A thesis submitted to
the Faculty of Graduate Studies and Research
in partial fulfilment of
the degree of**

Master of Science

**Ottawa-Carleton Institute of Biology
Carleton University
Ottawa, Ontario.
March, 1993.
© copyright 1993
Lynn Marie Davis.**

National Library
of Canada

Acquisitions and
Bibliographic Services Branch

395 Wellington Street
Ottawa, Ontario
K1A 0N4

Bibliothèque nationale
du Canada

Direction des acquisitions et
des services bibliographiques

395, rue Wellington
Ottawa (Ontario)
K1A 0N4

Your file Votre référence

Our file Notre référence

The author has granted an irrevocable non-exclusive licence allowing the National Library of Canada to reproduce, loan, distribute or sell copies of his/her thesis by any means and in any form or format, making this thesis available to interested persons.

L'auteur a accordé une licence irrévocable et non exclusive permettant à la Bibliothèque nationale du Canada de reproduire, prêter, distribuer ou vendre des copies de sa thèse de quelque manière et sous quelque forme que ce soit pour mettre des exemplaires de cette thèse à la disposition des personnes intéressées.

The author retains ownership of the copyright in his/her thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without his/her permission.

L'auteur conserve la propriété du droit d'auteur qui protège sa thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

ISBN 0-315-84159-1

Canada

Thesis contains black & white
photographs &/or explanatory tables
which when microfilmed may lose their
significance. The hardcopy of the
thesis is available upon request from
Carleton University Library.

The undersigned recommend to the Faculty of Graduate Studies
and Research acceptance of the thesis

"An ultrastructural, immunocytochemical and biochemical
study of the effects of the transcriptional inhibitor DRB
on nuclei and nuclear matrices of concanavalin A-
stimulated lymphocytes"

submitted by Lynn Marie Davis, B.Sc. (Hons.)
in partial fulfilment of the requirements for
the degree of Master of Science

Thesis Supervisor

Chair, Department of Biology

Carleton University,

April, 1993.

ABSTRACT

The transcriptional inhibitor 5,6-dichloro-1- β -D-ribofuranosylbenzimidazole (DRB) inhibited ^3H -uridine incorporation in concanavalin A-stimulated murine lymphocytes to the level in unstimulated cells. Immunofluorescence labelling of nuclear matrix antigens and electron microscopic cytochemistry showed that DRB reversibly induced striking morphological changes. These changes included nucleolar disorganization, aggregation of condensed chromatin and interchromatin granules, and co-aggregation of the P11 and Sm antigens, and suggested that the in situ nuclear matrix was reorganized by DRB. Furthermore, although conventional electron microscopy showed no significant difference in the ultrastructure of isolated nuclear matrices, immunofluorescence labelling of isolated matrices indicated a change in the interaction of nuclear antigens with the matrix in DRB-treated cells. P11 antigen organization was altered, and the Sm antigen was no longer detectable in these samples. Within 8 hr recovery, P11 was distributed within isolated matrices as in controls, but the Sm antigen was still not detectable.

ACKNOWLEDGEMENTS

I would like to thank Dr. Nathalie Chaly for suggesting the project, and for her support, encouragement, advice and guidance throughout the course of my graduate studies. I also thank Drs. David L. Brown and Micheline Paulin-Levasseur for serving on my advisory committee, and for helpful discussions and troubleshooting.

Many thanks to Judy Little for technical assistance in teaching me the lymphocyte isolation, and to Bea Valentine for teaching me electron microscopy and for assistance with general technical problems. I cannot thank Joan Mallett enough for her invaluable help in the areas of light and electron microscopy, sectioning and staining, photography and printing.

I thank the donors of the antibodies, and Dr. Ken Storey, for the use of the Polaroid Cu-5 Land Camera.

I thank the staff, faculty and students at ELBA for making that building a wonderful place to work.

To my parents, Ralph and Marilyn, and my sister, Karen, thank you for your emotional, financial and moral support and encouragement, and for putting up with me while I complained about just about everything. I thank my father for his help in printing the final prints of the figures. Thanks also to Liz and Kathy, for getting me out of the house and the lab, and for taking my mind off my thesis every once in a while. Special thanks go to Denis for his

love, support and encouragement, and for his constant belief that I could do it, when I didn't think I could.

TABLE OF CONTENTS

TITLE	i
ABSTRACT	ii
ACKNOWLEDGEMENTS	iii
TABLE OF CONTENTS	v
LIST OF TABLES	viii
LIST OF FIGURES	ix
LIST OF ABBREVIATIONS	xi
CHAPTER 1 Literature Review	1
A. Structural Organization of the Nucleus	2
A.1 The Nuclear Envelope and Pore Complexes	3
A.2 The Lamina	10
A.3 The Nucleolus	14
A.4 Interchromatin Granules	17
A.5 Perichromatin Fibrils	24
A.6 Perichromatin Granules	25
A.7 Nuclear Bodies	26
B. The Nuclear Matrix	28
B.1 Structure and Composition	28
B.2 Domains and the Nuclear Matrix	33
B.2.1 Organization of DNA into Loop Domains	34
B.2.2 DNA Replication Domains and the Nuclear Matrix	38

B.2.3 Transcription, Processing, and Transport of RNA	40
C. The Relationship Between Transcriptional Activity and Nuclear Morphology	43
C.1 Naturally Occurring Changes in Transcriptional Activity and Nuclear Morphology	44
C.2 Effects of Transcriptional Inhibitors on Nuclear Morphology	48
D. Morphological and Biochemical Changes During Lymphocyte Activation	52
CHAPTER 2 Reversible Disassembly of Transcription Domains in Lymphocyte Nuclei During Inhibition of RNA Synthesis by DRB	60
SUMMARY	61
INTRODUCTION	64
MATERIALS AND METHODS	69
RESULTS	73
DISCUSSION	95
CHAPTER 3 Effects of Inhibition of Transcription by DRB on Nuclear Matrix Structure and Composition.	113
INTRODUCTION	114
MATERIALS AND METHODS	117
RESULTS	122
DISCUSSION	148
SUMMARY AND CONCLUSIONS	155

APPENDIX I: Protocol for isolation of lymphocytes from mouse spleen.	157
APPENDIX II: Comparison of stimulation indices between cultures supplemented with fetal bovine serum and CPSR-2.	159
APPENDIX III: Bismuth staining for electron microscopy.	162
APPENDIX IV: Recipes for solutions and running conditions for one-dimensional electrophoresis using the Bio-Rad Mini-Protean II system.	163
REFERENCES	167

LIST OF TABLES

Table 1:	Frequency of lobed nuclei in relation to DRB treatment	81
Table 2:	Comparison of stimulation indices between samples cultured with FBS and samples cultured with CPSR-2	160

LIST OF FIGURES

Figure 1: Histogram of ^3H -uridine incorporation in -Con A, +Con A, +DRB and samples recovering from DRB.	74
Figure 2: Immunofluorescence labelling with antibody PI1.	76
Figure 3: Immunofluorescence labelling of the Sm antigen with antibody Y12.	78
Figure 4: Lymphocytes labelled with anti-fibrillarin	80
Figure 5: Electron micrographs of morphotype I nuclei in -Con A lymphocytes	83
Figure 6: Electron micrographs of morphotype II and III nuclei in +Con A lymphocytes	85
Figure 7: Electron micrographs of nuclei in +DRB lymphocytes	88
Figure 8: Electron micrographs of nuclei in lymphocytes fixed after 2 hr of recovery from DRB	91
Figure 9: Electron micrographs of nuclei in lymphocytes fixed after 8 hr of recovery from DRB	93
Figure 10: Immunofluorescence labelling of PI1 in -Con A lymphocyte samples	125
Figure 11: Immunofluorescence labelling of PI1 in +Con A lymphocyte samples	126

Figure 12: Immunofluorescence labelling of PI1 in +DRB lymphocyte samples	127
Figure 13: Immunofluorescence labelling of PI1 in 8 hr recovery lymphocyte samples	128
Figure 14: Immunofluorescence labelling of the Sm antigen in -Con A lymphocyte samples	131
Figure 15: Immunofluorescence labelling of the Sm antigen in +Con A lymphocyte samples	132
Figure 16: Immunofluorescence labelling of the Sm antigen in +DRB lymphocyte samples	133
Figure 17: Immunofluorescence labelling of the Sm antigen in 8 hr recovery lymphocyte samples	134
Figure 18: Electron micrograph of a nuclear matrix isolated from -Con A samples	136
Figure 19: Electron micrographs of nuclear matrices isolated from +Con A samples	138
Figure 20: Electron micrographs of nuclear matrices isolated from +DRB samples	141
Figure 21: Electron micrograph of a nuclear matrix isolated from 8 hr recovered samples	143
Figure 22: One-dimensional SDS-PAGE gels of protein from isolated lymphocyte nuclei	145
Figure 23: One-dimensional SDS-PAGE gels of protein from nuclear matrices isolated from lymphocytes	145

LIST OF ABBREVIATIONS

ATP	adenosine triphosphate
Con A	concanavalin A
CPSR-2	controlled process serum replacement-2
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
DRB	5,6-dichloro-1- β -D-ribofuranosylbenzimidazole
EDTA	ethylenediaminetetraacetic acid
EM	electron microscope or electron microscopy
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
gp	glycoprotein
hnRNA	heterogenous nuclear RNA
ICG	interchromatin granule(s)
LMB	low magnesium buffer
mRNA	messenger RNA
PAGE	polyacrylamide gel electrophoresis
PMSF	phenylmethylsulfonylfluoride
p.s.	post-stimulation
PTA	phosphotungstic acid
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolutions per minute
rRNA	ribosomal RNA
RNP	ribonucleoprotein
SDS	sodium dodecyl sulfate

snRNP

small nuclear RNP

STM

sucrose-Tris-magnesium buffer

CHAPTER 1

Literature Review

I am studying the interrelationship between transcriptional activity, chromatin conformation and nuclear matrix organization. Using the lymphocyte nucleus as a model system, I have examined the structural and functional changes accompanying lymphocyte nuclear activation. In this literature review, I will discuss the features of the nucleus, its organization and functions, with emphasis on the relationship between transcriptional activity and nuclear organization.

A. Structural Organization of the Nucleus

Eukaryotic cells are distinguished from prokaryotic cells by the presence of a nucleus that houses the genetic material of the cell, as well as the enzymes and other factors necessary for the duplication and transcription of the genome. The nucleus is surrounded by a double phospholipid and protein membrane, the nuclear envelope, that separates the nuclear components from the cytoplasm. The nucleus and cytoplasm remain connected, however, by nuclear pore complexes through which transport between the nucleus and cytoplasm occurs.

This section of the literature review will describe the structure and function of the different components of the nucleus. The effects of transcriptional inhibitors on the various nuclear structures and the specific organization of lymphocyte nuclei are dealt with separately in sections C.2

and D respectively.

A.1 The nuclear envelope and pore complexes

The nuclear envelope is a double membrane, having two membrane bilayers. The outer membrane is continuous with the endoplasmic reticulum, and may have ribosomes bound to its surface (Gerace and Burke, 1988; Dingwall and Laskey, 1992). Between the inner and outer nuclear membranes is the perinuclear space, which is continuous with the lumen of the endoplasmic reticulum (Gerace and Burke, 1988). The inner and outer nuclear membranes are fused at intervals, forming pores into which are inserted the nuclear pore complexes (see below). The nucleoplasmic face of the nuclear envelope is lined with a network of fibres called the nuclear lamina (see below) (Gerace and Burke, 1988).

The nuclear pore complexes are supramolecular structures (Gerace and Burke, 1988) greater than 100 MD in size, that perforate both faces of the nuclear envelope, connecting the nucleus to the cytoplasm. Current models of the structure of the nuclear pore complex propose that it is composed of four components: the scaffold, the central transporter, short filaments bound to the cytoplasmic face of the pore, and the basket-like structure found on the nucleoplasmic face (reviewed in Forbes, 1992).

The structure of the pore complex scaffold was originally described by Unwin and Milligan (1982) as being

composed of three rings, one at the cytoplasmic face, one at the nucleoplasmic face, and a central ring made up of spokes. Each ring was described as having 8-fold symmetry, with the spokes of the central ring connected to each other and supporting the central transporter. Between the spokes are spaces of approximately 90 Å diameter. Recently, Hinshaw et al. (1992) have used 2- and 3-dimensional image analysis of electron micrographs to elucidate further the structure of the scaffold. Their model proposes that the spokes are composed of 4 different subunit types, with each spoke having 2 copies of each subunit. Within each spoke, there are several connections between the subunits, but there are few connections between individual spokes. Hinshaw et al. (1992) propose that the scaffold structure does not play a direct role in the active transport of macromolecules, but that it provides support for the transport machinery and maintains the connections between the inner and outer nuclear membranes. They also suggest that the spaces between the spokes provide channels for diffusion of small molecules.

The active transport of macromolecules between the nucleus and cytoplasm is thought to be carried out by the central transporter (Akey and Goldfarb, 1989; Akey, 1989; reviewed in Forbes, 1992). The structure of the transporter has been derived from image analysis of electron micrographs of cryosections of pores (Akey and Goldfarb, 1989; Akey,

1989). The central transporter has been visualized as a proteinaceous ring that can take on 4 different forms (Akey and Goldfarb, 1989). Each of these forms has 8 fold symmetry, and each is thought to represent a stage of nuclear import. Akey (1990) has proposed that the transporter is made up of 2 irises, each having 8 arms. These 2 irises are thought to be superimposed, and to function successively to transport macromolecules through the pore complex (Akey and Goldfarb, 1989).

Thick fibres have been observed on the cytoplasmic face of the pore complex (reviewed in Franke, 1974; Franke et al., 1981). These fibres are thought to serve as binding areas for nuclear proteins, prior to their translocation through the pores (Richardson et al., 1988). A basket-like structure on the nucleoplasmic face of the pore complex was recently observed using scanning electron microscopy (e. g. Jarnik and Aeby, 1991; Goldberg and Allen, 1992). The baskets are composed of 8 filaments that are connected to the nucleoplasmic ring of the pore, and are joined at a smaller ring (Jarnik and Aeby, 1991). The preservation of the baskets is dependent on the presence of divalent cations in the solutions used to prepare nuclear envelopes (Jarnik and Aeby, 1991). The function of the baskets is unclear (Jarnik and Aeby, 1991; Forbes, 1992).

The nuclear pore complex serves to facilitate transport of macromolecules between the nucleus and cytoplasm. Using

gold particles conjugated to nucleoplasmin, an abundant protein in Xenopus nuclei, Feldherr et al. (1984) demonstrated that import of protein into the nucleus takes place through the nuclear pore complexes. In these experiments, nucleoplasmin-gold microinjected into Xenopus oocytes accumulated quickly in the nucleus. The nucleoplasmin-gold particles were localized to the pore complexes, at both faces and within the pore channel (Feldherr et al., 1984). Two types of translocation can occur: passive diffusion of small molecules through the 90 Å channels in the pore complex, and active transport of proteins and RNA via the central transporter (reviewed in Dingwall and Laskey, 1992; Forbes, 1992).

For many proteins, transport into the nucleus requires that the protein to be transported contains a nuclear localization sequence (Dingwall et al., 1982). These sequences are usually positively charged, short, and contain proline or glycine residues, although no consensus sequence has as yet been identified (reviewed in Forbes, 1992). This localization sequence is recognized by the cytoplasmic face of the pore complex, and the protein is bound to the pore complex. Some nuclear proteins, however, do not contain nuclear localization sequences, and are imported into the nucleus with another protein that does (Dingwall et al., 1982; reviewed in Silver, 1991). Transport of proteins through the nuclear pores has been shown to be a two step

process (Newmeyer and Forbes, 1988; Richardson *et al.*, 1988). The first step is binding of the protein to be translocated at the cytoplasmic face of the pore complex. It is believed that nuclear localization sequences on nuclear proteins are recognized by receptors at the nuclear pore (reviewed in Silver, 1991). Translocation of the protein through the pore complex requires ATP and is inhibited by wheat germ agglutinin (Newmeyer and Forbes, 1988; Richardson *et al.*, 1988). Wheat germ agglutinin has also been shown to bind to a family of nuclear proteins that contain N-acetylglucosamine residues (see below), and so it has been suggested that these proteins may be involved in translocation of proteins through the nuclear pores (Richardson *et al.*, 1988). Finlay and Forbes (1990) have shown that nuclear pore complexes that do not contain these wheat germ agglutinin-binding proteins can neither transport nor bind proteins at the cytoplasmic face, suggesting that these proteins also mediate the initial binding step of transport (reviewed in Miller *et al.*, 1991).

The exact mechanism of translocation through the pore complexes is unclear. A model for translocation has been proposed based on the interpretation of the central transporter as two irises (Akey, 1990; reviewed in Dingwall and Laskey, 1992 and Forbes, 1992). In this model, the recognition of the nuclear localization sequence would cause the first iris to open, admitting the protein. The second

iris would then open, causing the protein to move further into the pore. The first iris would then close. After the protein had successfully passed through the pore, the second iris would then close.

Dworetzky and Feldherr (1988) have shown, using gold particles conjugated to tRNA or poly-adenylated RNA, that export of RNA from the nucleus to the cytoplasm also takes place through the nuclear pore complex. When RNA-gold particles were injected into Xenopus oocyte nuclei, they were rapidly exported from the nucleus, and when injected into the cytoplasm, remained there (Dworetzky and Feldherr, 1988). Dargemont and Kühn (1992) have shown that export of mRNA from the nucleus has similar properties to import of protein, in that both are temperature sensitive, require ATP and are inhibited by wheat germ agglutinin.

Although several proteins associated with the nuclear pore complex have been identified, it has been estimated that those proteins make up a small fraction of the composition of the pore complex (Gerace and Burke, 1988; Forbes, 1992). An integral membrane protein, gp210, has been identified in rat and *Drosophila* nuclear envelopes (Gerace et al., 1982; Filson et al., 1985). Each pore complex contains approximately 25 copies of gp210, and so it is assumed to be an essential protein. The protein has two transmembrane domains (Wozniak et al., 1989), and most of gp210 is localized to the lumen of the nuclear envelope

(Greber et al., 1990). gp210 is thought to function in securing the pore complex within the nuclear envelope (reviewed in Forbes, 1992), and may be part of one of the subunits of the nuclear pore scaffold described above (Hinshaw et al., 1992).

A number of other pore complex proteins have been identified using monoclonal antibodies raised against pore complex-lamina fractions from rat liver (see Snow et al., 1987). These proteins, ranging in molecular weight from 45 to 210 kD, contain O-linked N-acetylglucosamine residues, and have been termed nucleoporins (Davis and Blobel, 1986). Nucleoporins have been shown to bind the lectin wheat germ agglutinin at the N-acetylglucosamine residue, which results in inhibition of nucleocytoplasmic transport (Finlay et al., 1987; Dabauvalle et al., 1988). These results indicate that nucleoporins are involved in transport via the pores. One of the best characterized nucleoporins is p62, a protein localized to the cytoplasmic face of the pore complex. Antibodies against p62 have been shown to inhibit import into the nucleus (Featherstone et al., 1988; Dabauvalle et al., 1988), demonstrating that p62 is essential to protein import. The location of p62 on the cytoplasmic face of the pore complex indicates that it is involved in binding to the pore complex proteins to be imported. The peripheral component of the nuclear matrix antigen PI1 has been localized to the cytoplasmic face of the nuclear pores, and

has also been shown to inhibit transport of proteins into the nucleus (Dabauvalle *et al.*, 1988). The peripheral component of P11 is thought to be a homologue of p62 (Benavente *et al.*, 1989).

Nuclear pore complex proteins specific to yeast, that do not contain N-acetylglucosamine residues, have also been identified. NSP1, an 86 kD protein, was identified using polyclonal antibodies against yeast nuclear skeletons (Hurt, 1988). NSP1 is similar to p62 in composition and structure, suggesting that it is the yeast equivalent of p62 (reviewed in Forbes, 1992). Davis and Fink (1990) have identified another yeast nuclear pore protein termed NUP1, using a monoclonal antibody raised against rat nuclei. These two yeast pore proteins have been shown to be essential for growth in yeast (Hurt, 1988; Davis and Fink, 1990).

Recently, Sukegawa and Blobel (1993) identified a rat liver nuclear pore protein, nup153, that is localized to the nucleoplasmic face of the pore complex. This protein contains a zinc finger motif and has been shown to bind DNA.

A.2 The Lamina

Underlying the nuclear envelope is the nuclear lamina, a filamentous layer closely associated with the nuclear envelope. The lamina is composed mainly of a family of polypeptides, the lamins, that range in molecular weight

from 60-80 kD (Krohne and Benavente, 1986). Mammalian nuclei have been found to contain three major lamin proteins, A, B and C, that have been shown to be structurally, biochemically and immunologically very similar to the cytoplasmic intermediate filament proteins (Aebi *et al.*, 1986; McKeon *et al.*, 1986; Gerace and Burke, 1988). Association of the lamina with the inner nuclear membrane appears to be mediated in part by lamin B, with the participation of lamin A (Georgatos and Blobel, 1987). A receptor for lamin B has been identified in avian erythrocytes and localized to the inner nuclear membrane (Worman *et al.*, 1988, 1990). The lamin B receptor is thought to have several transmembrane domains, and a highly charged N-terminal domain that is thought to be located on the nucleoplasmic side of the nuclear envelope, and may act in binding lamin B (Worman *et al.*, 1990). During mitosis, the nuclear envelope breaks down, and the lamins are depolymerized. Lamins A and C are solubilized, while lamin B remains associated with membrane vesicles derived from the nuclear envelope (Gerace and Blobel, 1980).

Immunofluorescence labelling with anti-lamin antibodies shows prominent peripheral labelling in interphase nuclei. During mitosis, when the lamina is depolymerized, the staining pattern is diffusely cytoplasmic (Krohne and Benavente, 1986). Like other classes of intermediate filament proteins, lamins are highly insoluble. For

instance, during nuclear matrix preparation, in which the DNA is enzymatically cleaved in addition to high salt treatment of the samples (see below), anti-lamin labelling changes very little (Chaly et al., 1985). Although the exact function of the lamina is not clear, it appears to constitute an important structural component of the nucleus. For example, it has been shown in in vitro nuclear assembly systems that if the lamins are immunodepleted from the assembly system, the formation of the nuclear envelope is partially inhibited (Burke and Gerace, 1986). Injection of anti-lamin antibodies into live mitotic cells has shown that although envelope formation was not inhibited, growth of the newly formed envelope was inhibited (Benavente and Krohne, 1986). Newport et al. (1990) have also shown, using Xenopus oocyte extracts in nuclear assembly experiments, that while immunodepletion of lamins from the extract did not prevent formation of a nuclear envelope around the chromatin, growth of such nuclear envelopes was inhibited. These results indicated that the lamina was essential for growth of the nuclear envelope after its formation (Newport et al., 1990).

A number of studies have shown that lamina composition varies with the developmental or differentiation state of the cell. Schatten et al. (1985) found that lamins A/C were expressed only in early stages of embryogenesis in chick and sea urchin embryos, but that lamin B was expressed at all stages of embryogenesis. Lamins A/C were, however,

expressed in somatic cells of adults. Studies examining the behaviour of the lamins during myogenesis have shown that in both chicken (Lehner *et al.*, 1987; Lourim and Lin, 1989) and rat (Wedrychowski *et al.*, 1989) tissues, type A lamins show a quantitative increase during muscle differentiation.

Cross-linking studies using cultured rat myoblasts also demonstrated that the lamina composition was altered during myogenesis, and that the interaction between DNA and the lamins was also altered during differentiation, with lamin B showing a higher level of cross-linkage in differentiated myotubes compared to undifferentiated myoblasts

(Wedrychowski *et al.*, 1989). In mature lymphocytes, lamins A and C are only weakly expressed, if at all, while lamin B is strongly expressed. In lymphoblasts, all three lamins are expressed (Bladon *et al.*, 1988; Hornbeck *et al.*, 1988)

The lamins have been implicated in anchoring chromatin to the nuclear periphery (reviewed in Paddy *et al.*, 1990). For example, in studies using anti-lamin antibodies to impair the function of lamins, chromosome condensation is prevented (Burke and Gerace, 1986; Benavente and Krohne, 1986). Paddy *et al.* (1990) performed immunolabelling experiments with anti-lamin antibodies and a DNA-specific dye to relate the organization of the lamins to the peripheral chromatin. It was found that the lamins in Drosophila tissue and HeLa cells were organized into a fibrillar network that did not completely line the inner

nuclear envelope; there were gaps in various parts of the network. It was also found that although much of the chromatin at the nuclear periphery was located near part of the lamin network, the chromatin seemed to be too distant from the lamins to be in direct contact with any of the fibres (Paddy et al., 1990). The authors have suggested that there may be other proteins associated with the lamin network that act as intermediates between the lamins and the peripheral chromatin (Paddy et al., 1990).

In contrast to the above hypothesis, Ludérus et al. (1992), have recently shown, that rat liver lamin B₁ binds to DNA sequences termed matrix attachment regions (see section B.2.1) at the nuclear periphery.

A.3 The Nucleolus

The nucleolus, the most obvious feature of the interphase nucleus, is the site of transcription and processing of the ribosomal RNA genes. Electron microscopy has shown that the nucleolus is composed of three components: the fibrillar centre, the dense fibrillar component and the granular component. Although the exact relationship between the structural components of the nucleolus and the processes involved in ribosome biogenesis is unclear, most models suggest that in each of the three components of the nucleolus a specific event in the transcription and processing of ribosomal RNA takes place

(reviewed in Scheer and Benavente, 1990; Fischer et al., 1991; Hernandez-Verdun, 1991; Jordan, 1991).

Until recently, there was some confusion over the location of the rRNA genes within the nucleolus, although it was generally accepted that transcription occurred in one of the fibrillar components, evidence was contradictory as to which component precisely was involved. Thiry and Goessens (1991) recently performed experiments using autoradiography of cells labelled very briefly with ^3H -uridine, and an in situ-in vitro transcription system, in which cells embedded in Lowicryl K4M resin were incubated with biotinylated dUTP which was subsequently immunodetected with gold particles. The results from these experiments showed that rRNA genes are transcribed in the fibrillar centres of Ehrlich tumor cell nucleoli. They have also shown that the large numbers of silver grains over the dense fibrillar components in such autoradiograms represent accumulated transcripts, and not actively transcribing DNA. Furthermore, Thiry (1992) has recently confirmed these results, using terminal deoxynucleotidyl transferase and bromodeoxyuridine triphosphate to label DNA at the surface of thin sections. In the nucleoli of several species, the rRNA genes were located in the fibrillar centres. These data are further supported by in situ hybridization studies using probes for specific transcribed spacers in the rDNA transcript to monitor the progress of the nascent transcripts through the

nucleolus (Puvion-Dutilleul et al., 1991; reviewed in Fischer et al., 1991). These experiments indicate that the nascent transcripts move from the fibrillar centres to the dense fibrillar component, where much of the splicing occurs (Fischer et al., 1991). The ribosome precursors then move into the granular component where the maturing ribosomes are stored (reviewed in Hernandez-Verdun, 1991).

Several major nucleolus-specific proteins have been identified, and these proteins will be discussed briefly here. RNA polymerase I is responsible for rRNA transcription in the nucleolus (Hernandez-Verdun, 1991) and has been localized to the fibrillar centres (Scheer and Benavente, 1990).

Nucleolin, also called C23, is a phosphorylated protein that is abundant in nucleoli that actively synthesize ribosomes (Lapeyre et al., 1987; reviewed in Hernandez-Verdun, 1991). It has been localized to the dense fibrillar component (Biggiogera et al., 1989; Noaillac-Depeyre et al., 1989), and is thought to be involved in the early stages of ribosome formation (reviewed in Hernandez-Verdun, 1991), although its exact function in the nucleolus has not been determined.

Protein B23 is a phosphoprotein associated with RNA that is thought to be involved in the assembly of preribosomes (Schmidt-Zachmann et al., 1987). B23 has been localized mainly to the granular component of the nucleolus

(Schmidt-Zachmann et al., 1987), associated with maturing preribosomes (Dumbar et al., 1989).

Fibrillarin has been localized to the dense fibrillar component (Ochs et al., 1985), and is associated with the U3 (Lapeyre et al., 1990), U8 and U13 snRNAs (Tyc and Steitz, 1989; reviewed in Hernandez-Verdun, 1991), that are involved in splicing of the rRNA transcripts (Kass et al., 1990). Fibrillarin has recently been shown to be part of the residual nucleolus in HeLa cell nuclear matrices (Ochs and Smetana, 1991).

A.4 Interchromatin Granules

Interchromatin granules (ICG) are small granules, approximately 200-250 Å in diameter, linked together by fibrils and organized as clusters distributed throughout the nucleus (Monneron and Bernhard, 1969). ICG were first described in early EM studies of nuclear organization (reviewed in Monneron and Bernhard, 1969). However, although their composition and function have been extensively investigated over the years, their role within the nucleus has remained elusive.

The regressive-EDTA technique (Bernhard, 1969) in which chromatin is unstained but RNP-containing structures are strongly stained, has demonstrated that ICG contain RNP (Monneron and Bernhard, 1969). Studies attempting to confirm this conclusion, using RNase digestion alone

(Monneron and Moulé, 1968; reviewed by Monneron and Bernhard, 1969) or in conjunction with proteases (Monneron and Bernhard, 1969) have shown that ICG are susceptible to RNase and protease digestion prior to, but not after, embedding in glycol methacrylate. These early EM studies concluded that ICG contain a class of RNP in which the RNA was protected from digestion (Monneron and Moulé, 1968; reviewed in Monneron and Bernhard, 1969). Bismuth staining of ICG (Locke and Huie, 1977) indicates that they contain phosphoproteins. In cytochemical studies using acetylation, methylation and oxidative deamination, Wassef (1979) also concluded that phosphoproteins were responsible for the staining properties of ICG. ICG have also been shown to be insoluble components of the nuclear matrix (Berezney, 1979).

Several studies using immunolabelling at the light and electron microscope levels have been performed in order to identify the protein components of ICG. Many studies have associated small nuclear RNP (snRNPs), part of the splicing machinery (reviewed in Lührmann et al., 1990), and other non-snRNP splicing components with ICG.

Spector et al. (1983) used light and electron microscope immunolabelling with antibodies against the Sm and RNP antigens. Both of these antigens were identified via autoimmune sera (e.g. Lerner et al., 1981). The Sm antigen has been detected in U1, U2 and U4-U6 snRNPs, but not the nucleolar U3 snRNP (Lerner et al., 1981). The RNP

antigen is specific to the U1 snRNP (Fakan et al., 1984). At the light microscope level, Sm labelling was distributed in a speckled pattern throughout the nucleus, with the exception of the nucleolus. The RNP antigen was localized in a similar pattern, although its label was not as extensive as that of Sm (Spector et al., 1983). By immunoperoxidase labelling at the electron microscope level, the Sm antigen was found to be distributed in a network within the nucleoplasm, extending from the nucleolus to the nuclear envelope (Spector et al., 1983; Spector, 1990). The RNP antigen was similarly distributed in a network that overlapped the Sm network. The patches of snRNP labelling within these networks, termed "speckles" by Spector (1990) were similar in size and location to ICG clusters, suggesting that snRNPs may be part of the nucleoplasmic RNP network detected by regressive staining (Spector et al., 1983). This study also used the transcriptional inhibitor DRB, which induces aggregation of ICG in treated cells (Puvion, 1982). In DRB-treated cells, the snRNP labelling was also aggregated in the interior of the nucleus. Spector (1984) has also demonstrated that U1 and U2 snRNPs are colocalized in clusters in the nucleus of kangaroo rat cells, and that these clusters also contain Sm.

Puvion et al. (1984b) used antibodies to Sm and RNP antigens of snRNPs to detect snRNPs by immunoperoxidase labelling and immunogold labelling, both at the EM level.

As had been reported by Spector et al. (1983), immunoperoxidase labelling revealed that the snRNPs were organized in a fibrous network throughout the nucleoplasm, excluding the nucleolus. Immunogold labelling localized the snRNP antigens mainly to ICG clusters, with some labelling over perichromatin fibrils and perichromatin granules. This study also used cells treated with DRB, to inhibit RNA polymerase II transcription, and CdCl_2 , which slows processing of nascent hnRNA transcripts and blocks their transport out of the nucleus (Puvion et al., 1984b). In DRB-treated cells, the large clusters of ICG induced by DRB were labelled by antibodies to both Sm and RNP. The label in CdCl_2 -treated cells was confined to the rim of condensed chromatin aggregates, that are the known sites of active transcription (see section A.5). These results indicate that RNP elements that contain hnRNA are associated with snRNPs (Puvion et al., 1984b), corroborating data showing that transcripts are spliced as they are being synthesized (Beyer et al., 1980).

Using immunogold labelling, Fakan et al. (1984) demonstrated that ICG labelled strongly with anti-Sm, but only occasionally with anti-hnRNP core protein, which they suggested indicated a storage role for ICG. Recent studies have also shown that non-snRNP spliceosome components co-localize with snRNP proteins. Fu and Maniatis (1990) have shown that the non-snRNP splicing factor SC-35 is localized

to the nuclear speckles, and recently Spector *et al.* (1991) have shown, using immunofluorescence and immunoelectron microscopy, that snRNPs and SC-35 co-localize to ICG and perichromatin fibrils. On the basis of these observations it was suggested that ICG may be sites of spliceosome assembly (Spector *et al.*, 1991).

Despite the cytochemical results suggesting that ICG contain RNA, it has proven difficult to demonstrate the presence of RNA unambiguously in these granules (reviewed in Fakan and Puvion, 1980). Fakan and Bernhard (1971) used a combination of ^3H -uridine EM autoradiography and regressive-EDTA staining of monkey kidney cells to visualize the RNP-containing sites that incorporate ^3H -uridine. Using labelling periods of 2 min to 1 hr, and post incubation with unlabelled uridine, or chase, of up to 3 hr, they found that ICG were unlabelled or only weakly labelled. ICG clusters that were labelled exhibited silver grains only at the periphery of the clusters (Fakan and Bernhard, 1971). In a subsequent study, Fakan and Bernhard (1973) used longer labelling and chase times: cells were labelled for 1 to 6 hr and chased for up to 96 hr. As in the previous study, ICG clusters were only weakly labelled by ^3H -uridine. Samples labelled for 6 hr and then chased for 24 hr showed slightly higher labelling of ICG clusters than other samples, but the label was still concentrated at the periphery of the ICG clusters (Fakan and Bernhard, 1973).

On the other hand, snRNA has been localized to speckles by Reuter et al. (1984) using immunofluorescence labelling of mammalian cells with antibodies against the 2,2,7-trimethylguanosine cap common to U1-U5 snRNAs. The labelling was distributed in a speckled pattern throughout the nucleus, excluding the nucleolus, similar to the labelling of snRNP antigens described above. Since the U3 snRNP contains a 2,2,7-trimethylguanosine cap, the lack of nucleolar fluorescence indicated that the cap on the U3 snRNP was not accessible to the antibody under the conditions used (Reuter et al., 1984). Double labelling experiments using anti-2,2,7-trimethylguanosine and either anti-Sm or anti-RNP showed that the 2,2,7-trimethylguanosine cap was colocalized with both Sm and RNP antigens (Reuter et al., 1984). Since speckles have been shown to be analogous to the ICG clusters seen at the EM level, these results imply that snRNA is present in the ICG clusters. The reason that the snRNA does not incorporate ³H-uridine even after prolonged labelling remains unresolved. The autoradiographic, cytochemical and immunocytochemical data have led to the hypothesis that ICG clusters are either sites of snRNP (spliceosome) storage, or of assembly (Spector et al., 1991).

One approach to investigating the function of ICG has been to examine their behaviour after treatment of the cells with transcriptional inhibitors. Simard and Duprat (1969)

showed that actinomycin D, at concentrations inhibiting essentially all RNA synthesis, induced nuclear inclusions that resembled ICG clusters, but that these inclusions did not label with ^3H -uridine administered either prior to or after actinomycin D treatment. Puvion *et al.* (1979) have shown that DRB, an inhibitor of RNA polymerase II, induces extensive clustering of ICG in treated cells. However, in contrast to the results with actinomycin D above, when rat liver cells pulsed for 30 min with ^3H -uridine were chased for 2 to 6 hr in the presence of DRB, the clusters of ICG were heavily labelled (Puvion, 1982). Cells treated with DRB prior to labelling did not incorporate label. These results suggested that the aggregates of ICG induced by DRB treatment contained RNA that had been synthesized in the nucleus before the drug was administered (Puvion, 1982). In subsequent experiments, Puvion *et al.* (1984a) treated rat liver cells with actinomycin D, at doses that inhibited primarily ribosomal RNA synthesis, prior to labelling with ^3H -uridine and chase in the presence of DRB. In these experiments, ICG were not labelled with ^3H -uridine. From these results, they concluded that the RNA accumulated in ICG in DRB-treated cells was ribosomal RNA (Puvion *et al.*, 1984a), and not hnRNA. Whether rRNA is a normal component of ICG is unknown.

The data of Carter *et al.* (1991) have further confused the issue of the function of ICG. They used *in situ*

hybridization to detect poly-adenylated RNA in the nucleus of mammalian cells, as a marker for pre-mRNA transcripts. Double labelling experiments, using in situ hybridization of poly-adenylated RNA and anti-Sm, showed that poly-adenylated RNA was concentrated in the speckles that also contained the Sm antigen (Carter et al., 1991). These results indicated that snRNP clusters within the nucleus were not solely sites of snRNP assembly or storage, but might also be sites of hnRNA processing (Carter et al., 1991). However, these observations appear to contradict early EM autoradiography studies showing no ^3H -uridine incorporation in the ICG clusters, whereas hnRNA, the precursor to poly-adenylated mRNA, would be rapidly and intensely labelled. The function of ICG in the nucleus therefore remains unclear.

A.5 Perichromatin Fibrils

Perichromatin fibrils were first visualized using the regressive-EDTA staining technique (Monneron and Bernhard, 1969), indicating that perichromatin fibrils contain RNP. They are of variable size, with their length ranging from 30 to up to 200 Å, and are found at the periphery of condensed chromatin aggregates (Monneron and Bernhard, 1969; Puvion and Moyne, 1981). Autoradiography of cells pulse-labelled with ^3H -uridine and cell fractionation studies have shown that perichromatin fibrils represent nascent hnRNA transcripts (reviewed in Puvion and Moyne, 1981).

Immunoelectron microscopy using antibodies to the Sm antigen of snRNPs and to the hnRNP core protein have shown that perichromatin fibrils contain both snRNP and hnRNP antigens, further supporting the notion that they represent nascent hnRNA transcripts (Fakan et al., 1984). The results of Spector et al. (1991) described above, showing that snRNPs and the splicing factor SC-35 are co-localized in ICG and perichromatin fibrils also lends support to the hypothesis that perichromatin fibrils represent nascent transcripts, as it has been proposed that these transcripts are spliced as they are being transcribed (Beyer et al., 1980).

A.6 Perichromatin Granules

Perichromatin granules are granules of approximately 400-500 Å in diameter, characteristically surrounded by a clear area of approximately 250 Å in radius (Monneron and Bernhard, 1969; Puvion and Moyne, 1981). They have been observed at the edges of condensed chromatin aggregates, where they are found as individual granules or in small clusters (Fakan et al., 1984). Like the perichromatin fibrils, perichromatin granules are stained by the regressive-EDTA staining method (Monneron and Bernhard, 1969), indicating the presence of RNP. They are also stained with bismuth (Locke and Huie, 1977), indicating that they contain phosphoproteins. Fakan et al. (1984) demonstrated that perichromatin granules are only rarely

labelled by anti-Sm, but are preferentially labelled with antibodies against hnRNP core protein.

The function of perichromatin granules within the nucleus is unclear. There is a relationship between the number of perichromatin granules within the nucleus and the metabolic state of the nucleus. For instance, it has been shown that when transcription is inhibited, the number of perichromatin granules increases (Puvion et al., 1979). On this basis, it has been proposed that they may play a role in transport of pre-mRNA within the nucleus or in storage of these molecules, but no direct evidence for these hypotheses has been obtained (Fakan et al., 1984; Puvion and Moyne, 1981).

A.7 Nuclear Bodies

Nuclear bodies are structures within the nucleus first described in pathological tissues (Bouteille et al., 1967). They have since been found in normal tissues as well. Bouteille et al. (1967) proposed a classification system for nuclear bodies, based on their structural characteristics. Five types were described, each having a characteristic size range and structure. Nuclear bodies characteristically exhibit an outer shell of fibrillar material, but the exact structure of the fibrillar components varies with nuclear body type. Nuclear bodies are differentiated primarily by the structure and amount of granular material within the

shell.

The function of nuclear bodies is unclear. It has been shown that the presence of large numbers of nuclear bodies is correlated with increased metabolic activity (reviewed in Bouteille et al., 1974; Chaly et al., 1983a; Brasch et al., 1989). Chaly et al. (1983a, b) have shown that the fibrillar material of nuclear bodies in mouse lymphocytes is non-chromatinic and is probably proteinaceous. The cores of nuclear bodies were shown to contain RNP and some chromatin (Chaly et al., 1983b).

Brasch et al. (1989) have studied nuclear bodies in estrogen-stimulated rooster liver. They found that the number and variety of nuclear bodies in liver nuclei increased with stimulation, to a peak at 48 hr after estrogen stimulation. After this time, as the response to the hormone subsided, the number of nuclear bodies per nucleus also decreased. These results further indicate that increased numbers of nuclear bodies correlate with the activity of the cell. This study also showed that nuclear bodies in estrogen-stimulated chicken livers were primarily proteinaceous, with traces of RNA and DNA. One-dimensional SDS-PAGE of isolated nuclear bodies from these cells (Brasch et al., 1989) showed that these nuclear bodies exhibit complex polypeptide patterns, with some unique proteins. Chaly et al. (1983b) demonstrated that nuclear bodies in lymphocytes were not nucleolus-derived, nor were they

associated with centromeres. These nuclear bodies were also not labelled in autoradiograms of cells labelled with ^3H -uridine, ^3H -thymidine, or ^3H -leucine, suggesting that nuclear bodies are not involved in macromolecular synthesis. Nuclear bodies have also been shown to isolate with the nuclear matrix (Chaly et al., 1983b).

B. The Nuclear Matrix

B.1 Structure and Composition

The nuclear matrix was first described in rat liver (Berezney and Coffey, 1974) as a proteinaceous structural framework that was revealed after extraction of isolated nuclei with low concentrations of Mg^{++} , high concentrations of NaCl, nonionic detergent, and digestion of nucleic acids with DNase and RNase. This treatment resulted in extraction of more than 90% of the total nuclear protein and nucleic acids, leaving a structure that was similar in morphology to the non-chromatin components of intact nuclei. The nuclear matrix has since been described in many other tissues (see Berezney, 1984, 1991; Brasch, 1990 for review).

The morphology of the isolated nuclear matrix varies, depending on the type of nuclei used and the isolation method. Most of the earlier studies on the nuclear matrix used extraction procedures based on that of Berezney and

Coffey (1974). Various other extraction methods have been used in recent years (reviewed in de Jong et al., 1990), and the different methods used resulted in residual nuclear structures with varied properties. Some workers have stabilized the structure of the matrix by using buffers containing sodium tetrathionate (Kaufmann and Shaper, 1984) or Cu^{++} ions (Kaufmann et al., 1981), or by exposing nuclei to heat shock at 37°C or 42°C prior to matrix isolation (Gasser and Laemmli, 1986).

There have also been variations in the methods used to extract chromatin. Protocols based on that of Berezney and Coffey (1974) use high concentrations of NaCl (e. g. Kaufmann and Shaper, 1984; Bladon et al., 1988; He et al., 1990; Stuurman et al., 1990). Laemmli and co-workers substituted high NaCl concentrations with the chaotropic agent lithium diiodosalicylate (Mirkovitch et al., 1984), producing structures they have termed "nuclear scaffolds". A milder extraction method uses cells embedded in agarose beads in a physiological buffer, and electrophoresis at near physiological conditions to extract chromatin (Jackson and Cook, 1985).

All of the methods described above result in a residual nuclear fraction containing a residual nuclear envelope and an internal matrix structure. (Stuurman et al., 1992). Comparison of their protein compositions has shown that all of these structures contain many common proteins (Belgrader

et al., 1991a, b), leading to the conclusion that these structures may be functionally similar to each other (Stuurman et al., 1992).

Different preparation techniques have been used to examine nuclear matrix structure. These techniques include whole mounts (Capco et al., 1982), embedded thin sections (e. g. Kaufmann and Shaper, 1984) and resinless thick sections (Fey et al., 1986; He et al., 1990). However, all of the results have indicated that a fibrogranular internal matrix structure does exist within extracted nuclei (de Jong et al., 1990).

Structurally, the isolated nuclear matrix consists of three components: the residual pore complex-lamina, the residual nucleolus, and an internal fibrogranular network (Brasch, 1990). The internal matrix is believed to correspond to residual components of the in situ interchromatinic regions of the intact nucleus (Brasch, 1990; Berezney, 1991). Regressive-EDTA staining for RNP has shown a similarity between the two regions (Berezney, 1984;

terfield et al., 1985). He et al. (1990), using resinless thick sections, have reported that the interior matrix is made up of thick core filaments of 8-10 nm in diameter. These core filaments formed a highly branched network that was disrupted with RNase treatment, suggesting that RNA may form an integral part of the nuclear matrix (He et al., 1990).

The polypeptide composition of the nuclear matrix has been shown to vary with the method of preparation and the tissue of origin (e. g. Stuurman et al., 1989; Getzenberg et al., 1991; Nickerson and Penman, 1992). One-dimensional SDS-PAGE of nuclear matrix samples has not proven useful due to the complexity of the matrix samples. Two-dimensional gels, in which proteins are separated first by isoelectric point, and then by molecular weight (O'Farrell, 1975) facilitate identification and classification of individual proteins. Two-dimensional gels of nuclear matrix preparations emphasize the complexity of these samples (Peters and Comings, 1980; Crowley and Brasch, 1987; Bladon et al., 1988; Fey and Penman, 1988; Stuurman et al., 1989, 1990). Recently, it has been proposed that nuclear matrices are composed of two sets of proteins: proteins common to all matrices, and proteins that are specific for cell type and/or differentiation state (Fey and Penman, 1988; Stuurman et al., 1989, 1990; Berezney, 1991).

Stuurman et al. (1990) have defined a set of "minimal matrix" proteins, defined by their molecular weight and isoelectric point, that are common to mouse cells of various states of differentiation. Several of these proteins were also found in human and rat cells. The lamins A, B and C were identified in nuclear matrix preparations by immunoblotting (Stuurman et al., 1990). Lamin B was included in the set of minimal matrix proteins, but lamins A

and C, although found in many of the matrix samples, were excluded from the minimal matrix because they are not expressed at all stages of development in all types of cells (Schatten *et al.*, 1985; Lebel *et al.*, 1987; Lehner *et al.*, 1987; Stuurman *et al.*, 1989).

Two-dimensional gels of nuclear matrix preparations from a variety of studies have shown that matrices contain snRNP proteins (Crowley and Brasch, 1987; reviewed in Verheijen *et al.*, 1988), as well as actin (Crowley and Brasch, 1987), vimentin, thought to be a cytoplasmic contaminant (Peters and Comings, 1980; Brasch, 1990), and a set of approximately 12 other proteins (reviewed in Brasch, 1990; Berezney, 1991). Immunofluorescence labelling has shown that several nuclear antigens are associated with the nuclear matrix, such as snRNP proteins (Spector *et al.*, 1983), lamins (Chaly *et al.*, 1985; Lehner *et al.*, 1986), and several antigens identified using monoclonal antibodies against nuclear matrices (Chaly *et al.*, 1985).

Berezney and co-workers (reviewed in Berezney, 1991) have been attempting to characterize a set of 12 major Coomassie-stained proteins observed in two-dimensional gels of rat liver nuclear matrices. They have suggested that these polypeptides represent a set of matrix proteins common to all mammalian cells (Berezney, 1991). The common polypeptides were identified by screening nuclear matrix proteins separated by two-dimensional gels for DNA binding

capability using Southwestern blots. Twelve major proteins were detected that bind DNA, including lamins A and C, but excluding lamin B, as well as several proteins of the internal nuclear matrix that they have termed "nuclear matrins" (Hakes and Berezney, 1991a). The finding that lamin B in these assays did not bind DNA is contradictory to the recent findings that lamin B₁ in rat liver binds to matrix attachment region sequences in DNA (Ludérus *et al.*, 1992). The DNA binding proteins include matrins D, E, F, G, 4, and an as yet unidentified protein of approximately 48 kD. Studies are still underway to characterize these nuclear matrins (see Belgrader *et al.*, 1991a; Hakes and Berezney, 1991a, b; Nakayasu and Berezney, 1991).

B.2 Domains and the Nuclear Matrix

It has been proposed that the nucleoplasm is organized into functional domains, in which a particular nuclear function takes place. Each domain would then contain all of the necessary enzymes, cofactors and DNA sequences for that function. I will discuss in detail functions that are relevant to lymphocyte activation, specifically, organization of chromatin (B.2.1), DNA replication (B.2.2), RNA transcription, processing and export (B.2.3).

B.2.1 Organization of DNA into Loop Domains

The first order of organization of the DNA is the nucleosome chain, with a diameter of 10 nm. The DNA molecule is complexed with histone proteins, to form nucleosomes. Histone proteins H2A, H2B, H3 and H4 form an octamer, around which two turns of DNA are coiled (Kornberg and Klug, 1981). A second order of folding arises by the coiling of the nucleosomal chains into fibres 30 nm in diameter that is thought to be mediated by histone H1 (Kornberg and Klug, 1981). It is now accepted that the 30 nm fibre is then folded into loops, with each loop consisting of 50-100 kb of DNA constrained by its attachment to a non-chromatin skeletal framework (Benyajati and Worcel, 1976; Cook and Brazell, 1976; Vogelstein et al., 1980; McCready et al., 1980; Lebkowski and Laemmli, 1982a, b; reviewed in Garrard, 1990).

Much of the evidence in support of a loop model of chromatin organization has been obtained by treating nucleoids with the intercalating dye ethidium bromide (Cook et al., 1976; Vogelstein et al., 1980; Risley et al., 1986). Nucleoids are residual nuclear structures that are depleted of histones and other proteins, but that retain a full genomic complement of unbroken DNA (Cook et al., 1976; Adolph, 1980). Sedimentation (Crawford and Waring, 1967) and fluorescence (Vogelstein et al., 1980) experiments have shown that the DNA of nucleoids exhibits a biphasic response

as the concentration of ethidium bromide is increased. In the absence of ethidium bromide, all the DNA is contained within the nucleoid. As the ethidium bromide concentration increases, a progressively larger halo of DNA is formed. Further increases in ethidium bromide concentration reduced halo size, until the DNA was again fully contained within the nucleoid (Vogelstein et al., 1980). To interpret these data, it was proposed that negatively supercoiled loops of DNA are anchored to a nuclear skeleton (Vogelstein et al., 1980). In nucleoids, ethidium bromide, on intercalating into the DNA, unwinds the loops by introducing positive supercoiling. When the loops have completely uncoiled, higher concentrations of ethidium bromide continue to induce positive supercoiling, rewinding the DNA in the opposite direction, which decreases halo size (Vogelstein et al., 1980).

Hancock and Hughes (1982) examined histone-depleted nuclei using electron microscopy in order to visualize the loops. They found that most of the DNA did show a looped conformation with both ends of each loop attached to a skeletal structure, and that individual loops could be followed from beginning to end. Using immunodiffusion, they also found that antiserum against the lamins reacted strongly with skeletal proteins (Hancock and Hughes, 1982). There was no reaction of the antiserum with other fractions obtained during preparation of nucleosome-free chromosomes.

Based on these results, Hancock and Hughes (1982) proposed a model for chromatin organization in the interphase nucleus, in which the chromatin is attached in the form of loops to the peripheral lamina of the nucleus. They suggested that the loops bind to the lamins, or other lamina proteins, that form a three-dimensional structure underlying the pore complex-lamina network and nuclear membrane, by protein-protein interactions, and are then compacted by supercoiling into higher-order conformations. Regions of chromatin undergoing transcription, i. e. active genes, would have a less compact conformation than those not being transcribed, and would be longer (Hancock and Hughes, 1982).

This early work has led to the current model of nuclear organization in which DNA is organized into loops anchored at the nuclear periphery and within the nucleoplasm by interaction with components of the nuclear matrix (reviewed in Garrard, 1990). Research has focused on identifying the DNA sequences by which the DNA loops are attached to the matrix, and the proteins that bind to these matrix or scaffold attachment regions (MARs/SARs) (e.g. Mirkovitch *et al.*, 1984; Cockerill and Garrard, 1986; Gasser and Laemmli, 1986; Dickinson *et al.*, 1992). MARs/SARs are usually defined as DNA segments that remain associated with the nuclear matrix, despite digestion of the DNA and protein extraction that occur in nuclear matrix preparation (Cockerill and Garrard, 1986; Cook and Brazell, 1976;

Mirkovitch et al., 1984; Garrard, 1990). Some such sequences have been identified reproducibly associated in nuclear matrix preparations, and it has been suggested that these so-called matrix attachment regions (MARs) serve to outline the boundaries of chromosome loop domains (reviewed in Kellum and Schedl, 1991).

MARs have been found near a variety of different genes, and they are generally thought to anchor the bases of chromatin loops to the nuclear matrix (reviewed in de Jong et al., 1990). MARs (reviewed in de Jong et al., 1990; Dickinson et al., 1992) occur in non-transcribed regions that are rich in A/T sequences, usually several kilobase pairs away from reading frames. They are evolutionarily conserved, and MARs from one organism have been shown to bind to matrices from evolutionarily distant eukaryotes. Although no consensus sequence has been found for MARs, arbitrary A/T-rich sequences do not always act as MARs (Gasser et al., 1989), and DNA sequences containing MARs often contain sequences similar to the consensus sequence for DNA topoisomerase II binding (Cockerill and Garrard, 1986; Gasser and Laemmli, 1986; Dickinson et al., 1992). MARs have also been shown to have a tendency to unwind if exposed to superhelical strain (Bode et al., 1992; reviewed in Dickinson et al., 1992)

There is less information available as to the nature of MAR-binding proteins, with only 3 such proteins identified

so far. Recently, von Kries et al. (1991) identified an attachment region binding protein (ARBP) that binds MARs cooperatively and is part of the internal nuclear matrix. DNA topoisomerase II, a component of the nuclear matrix (Berrios et al., 1985; Earnshaw and Heck, 1985; Kaufmann and Shaper, 1991) has also been shown to bind MARs, but it is unknown whether this binding occurs in vivo. Ludérus et al. (1992) showed in rat liver that lamin B₁ bound to MARs specifically, lending support to the concept that the nuclear lamina is involved in the binding of chromatin loops at the nuclear periphery. Dickinson et al. (1992) have identified a tissue-specific DNA binding protein they have called SATB1, that binds MARs from different species along the minor groove of double stranded DNA. The protein is thought to bind via the sugar-phosphate backbone of double stranded DNA (Dickinson et al., 1992). Whether these proteins regulate loop attachment, and the mechanisms by which they might do so have not yet been determined.

B.2.2 DNA replication domains and the nuclear matrix

It is generally accepted that the nuclear matrix is involved in DNA replication. Autoradiography experiments have been performed using cells labelled with ³H-thymidine, and then extracted with high salt concentrations to remove the histones and cause the DNA to form loops around the residual nucleoid. These experiments have shown that newly

replicated DNA is associated with the matrix part of the nucleoid, and not with the DNA halo (Dijkwel et al., 1979; Pardoll et al., 1980). If the label is chased with cold thymidine before extraction and autoradiography, most of the labelled DNA is found in the DNA halos. It was suggested, based on these results, that, rather than DNA polymerases moving along the DNA molecules, replicating DNA is pulled through fixed replication complexes that are associated with the nuclear matrix (reviewed in de Jong et al., 1990).

Fluorescence microscopy experiments in which replicating DNA was labelled with bromodeoxyuridine or biotinylated deoxynucleotides support this model, indicating that replication sites are organized into clusters (Nakamura et al., 1986; Nakayasu and Berezney, 1989; reviewed in Berezney, 1991). When the labelling of DNA occurred after extraction of the chromatin, the nuclear matrix still contained much of the newly replicated DNA, and the organization of the replication sites was similar to that in intact nuclei (Nakayasu and Berezney, 1989), providing further evidence that DNA replication is associated with the nuclear matrix (Jackson and Cook, 1986; Tubo and Berezney, 1987a).

Tubo and Berezney (1987b) have investigated the clusters of replication sites within the nucleus, and found that complexes of 100S and 150S, that contained DNA polymerase α and DNA primase, were released from nuclear

matrices by sonication. They have speculated that the replication machinery is organized into "clustersomes", which contain the enzymes and factors necessary for DNA replication (Berezney, 1991).

B.2.3 Transcription, processing, and transport of RNA

There is much evidence that transcription is associated with the nuclear matrix. Pulse labelling with ^3H -uridine has shown that newly transcribed RNA remains associated with the nuclear matrix (Jackson et al., 1981; Ciejek et al., 1982), and not with DNA loops (Jackson et al., 1981). Similarly, the residual DNA that remains associated with the nuclear matrix is rich in active sequences, and the same active sequences are extracted from matrices prepared from cells in which those genes are not expressed (Ciejek et al., 1983; de Jong et al., 1990; Getzenberg et al., 1991).

The association of active genes and their transcripts with the nuclear matrix is, however, to some extent dependent on the matrix isolation procedure used (reviewed in de Jong et al., 1990; Jackson et al., 1992). For example, lithium diiodosalicylate extraction removes actively transcribed sequences unless stabilized by incubation at 37°C (Mirkovitch et al., 1984), as do preliminary treatments with low ionic strength buffers (Razin et al., 1985).

Components of the transcriptional machinery have also

been identified in nuclear matrices. Proteins associated with snRNPs and some hnRNP proteins have been shown to be associated with the nuclear matrix (Lehner et al., 1986; Turner and Franchi, 1987; Bladon et al., 1988; Verheijen et al., 1988). Zeitlin et al. (1989) have demonstrated that when nuclear matrices were isolated using moderate salt concentrations, the matrices retained functional spliceosomes.

There is growing evidence that transcription/splicing, like DNA replication, is compartmentalized within nuclear domains. Electron microscope autoradiography and cytochemistry studies have shown that transcription is restricted to areas of the nucleoplasm that do not contain condensed chromatin, and to the borders of condensed chromatin aggregates (Fakan, 1986). Immunocytochemical studies have demonstrated that hnRNP and snRNP antigens are distributed discontinuously in the nucleoplasm (Fakan et al., 1984; Spector et al., 1983). As noted in section A.5, Spector and co-workers have shown that several splicing factors are concentrated into nucleoplasmic regions they have termed "nuclear speckles" (Spector, 1990; Huang and Spector, 1991; Spector et al., 1991, 1992). Using autoradiography, Spector (1990) found that transcribed DNA is located near the speckles, but not within them. Poly(A) RNA (Carter et al., 1991) and nascent pre-mRNA transcripts (Huang and Spector, 1991) have also been localized to the

nuclear speckles by in situ hybridization. It has also been demonstrated that the speckles are not associated with replicating DNA during S phase (Spector, 1990; Carter et al., 1991), and that they become dispersed in the cytoplasm during mitosis (Spector and Smith, 1986; Leser et al., 1989). These results suggest that transcript/processing domains are related to the level of transcriptional activity of the cell during the cell cycle.

There is also some evidence that RNA transport out of the nucleus may also be organized in domains associated with the nuclear matrix. In situ hybridization of transcripts from an Epstein-Barr virus genome integrated into a human fibroblast cell line (Lawrence et al., 1989) has provided results that support this concept. The viral transcripts accumulate in the nucleus and are transported to the cytoplasm after processing. The transcripts were localized within the nucleus in what appeared to be curvilinear tracks, stretching from the interior of the nucleus to the periphery (Lawrence et al., 1989). Lawrence and co-workers (Xing and Lawrence, 1989) further demonstrated that the transcripts were associated with the nuclear matrix, and that their distribution within the matrices was similar to that in intact cells. Meier and Blobel (1992) have also observed evidence of tracks of proteins within the nucleus, directed toward the nuclear pores. In their experiments, immunogold labelling at the electron microscope level showed

that a nucleolar protein appeared to shuttle between the nucleolus and the cytoplasm on curvilinear tracks (Meier and Blobel, 1992), although the nature of the tracks was unclear. They have suggested that transport of macromolecules into and out of the nucleus may occur on such tracks. They have further suggested that these tracks may serve to connect the nuclear pores to transcription or RNP assembly sites (Meier and Blobel, 1992).

C. The Relationship Between Transcriptional Activity and Nuclear Morphology

It has been widely demonstrated that nuclear morphology is affected by the metabolic state of the cell, and especially by transcriptional activity. Transcriptionally active nuclei exhibit morphologies distinct from those of transcriptionally inactive nuclei. The differences in nuclear morphology, and related differences in nuclear matrix structure and composition, are similar, whether as a result of natural changes in transcriptional activity, or due to the effect of transcriptional inhibitors.

C.1 Naturally Occurring Changes in Transcriptional Activity and Nuclear Morphology

It has been established in many systems that the organization of the nucleus in transcriptionally active cells differs significantly from that of inactive cells. It has also been shown that the structure of the nuclear matrix is dependent on transcriptional activity. The following are several examples of this phenomenon.

During avian erythrocyte maturation, the nuclei and nucleoli become progressively smaller in size and more regular in shape (Dardick and Setterfield, 1976). The condensed chromatin within the nucleus becomes increasingly aggregated, with an accompanying decrease in the amount of dispersed chromatin (Dardick and Setterfield, 1976). In fully mature avian erythrocytes, the condensed chromatin is aggregated at the nuclear periphery, with the aggregates extending into the nuclear interior. LaFond and Woodcock (1983) prepared nuclear matrices from mature adult chicken erythrocytes and from embryonic chicken erythroblasts. They observed that mature avian erythrocytes appeared to have no internal nuclear matrix, while embryonic erythroblasts had an internal matrix similar to that described in other tissues. They suggested that the presence of an internal nuclear matrix is directly related with nuclear activity (LaFond and Woodcock, 1983).

To test this hypothesis, Woodcock and associates (LaFond et al., 1983; Woodcock et al., 1984; Woodcock and Woodcock, 1986) performed studies on the reactivation of mature avian erythrocytes in enucleated mouse L cells, examining the status of the nuclear matrix in these reactivated nuclei. When mature avian erythrocyte nuclei were fused with the enucleated L cells, the chick nuclei enlarged, the condensed chromatin became disaggregated, the nuclei imported proteins from the L cell cytoplasm, and transcription was initiated (LaFond et al., 1983). During this reactivation, a nuclear matrix was developed in the erythrocyte nucleus. As they had proposed, the amount of internal matrix was found to vary with the state of reactivation of the erythrocyte nucleus (LaFond et al., 1983).

During spermatogenesis, the DNA in the sperm nucleus becomes highly compacted and transcriptionally inactive. In many mammalian species, the organization of the DNA is altered during spermatogenesis, as the histones are replaced by sperm-specific protamines, and organized into linear arrays (Balhorn, 1982). Despite this rearrangement of DNA in mammalian sperm, the DNA remains organized into loop domains attached to the nuclear matrix (Ward et al., 1989), as is the DNA in somatic cells. However, the loop domains of sperm DNA are smaller than those of somatic cells (Ward et al., 1989), and sperm DNA loops are not supercoiled

(Risley et al., 1986). Recently, Ward and Coffey (1990) have examined the attachment of sperm DNA loops to the sperm nuclear matrix. They used hamster sperm from which the protamines had been extracted and the DNA digested with restriction enzymes, to produce either soluble loop DNA or nuclear matrix structures containing matrix-associated DNA. These two DNA fractions were then hybridized to probes for 6 hamster genes in Southern blots, to determine which, if any, genes were associated with the nuclear matrix. It was found that 5 of the genes were not associated with the matrix at all, while the remaining gene was located within 2 kb of the nuclear matrix (Ward and Coffey, 1990). They suggested that the results fit current models of nuclear organization in which only active genes are thought to be associated with the nuclear matrix. They further suggested that sperm DNA is specifically organized with respect to the nuclear matrix (Ward and Coffey, 1990).

In plant cells as well, there appears to be a direct relationship between the organization of the nuclear matrix and transcriptional activity. Stoilov and co-workers have been studying maize to compare the transcriptionally active germinating embryo with the inactive dry embryo (Stoilov et al., 1989; Krachmarov et al., 1991). They have shown that the DNA in the dry embryo is reorganized during germination, as transcription is activated. Sedimentation studies (Stoilov et al., 1989) showed that dry embryo nucleoids did

not exhibit a biphasic response to ethidium bromide concentration, while nucleoids prepared from actively transcribing germinating embryos did. They interpreted these results to mean that dry embryo nuclear DNA is not organized in topologically constrained supercoiled loops, but that such loops do exist in germinating embryo nuclei. They have also prepared nuclear matrices from meristematic root cells and dry embryos of maize (Krachmarov *et al.*, 1991). It was found that meristematic root cells contained a residual nuclear lamina and an internal nuclear matrix similar to that described for animal cells. Dry embryos, on the other hand, contained only a residual lamina, with no internal matrix. The authors suggested that, based on their results, transcriptionally inactive nuclei contain DNA that is not topologically constrained, while DNA in active nuclei is supercoiled (Krachmarov *et al.*, 1991). They further suggested that the organization of DNA into constrained loop domains is dependent on the presence of an internal nuclear matrix structure.

A similar situation is seen during activation of mature lymphocytes. This phenomenon is outlined only briefly here, and will be discussed in more detail in section D, below. The unstimulated lymphocyte nucleus is small, and contains large amounts of condensed chromatin adjacent to the nuclear envelope (Tokuyasu *et al.*, 1968; Valkov and Moyne, 1974; Setterfield *et al.*, 1983). The interchromatinic regions in

unstimulated lymphocytes are sparse. When stimulated to proliferate, the lymphocyte nuclei enlarge, the condensed chromatin is dispersed throughout the nucleoplasm in small aggregates, and the interchromatinic regions increase in size dramatically. Nuclear matrix preparations from both populations of lymphocytes demonstrate that the amount of internal matrix present is correlated to the amount of interchromatinic regions in the nucleus in situ (Bladon et al., 1988). Unstimulated lymphocytes, then, do not have much internal matrix, whereas the nuclear matrix in stimulated cells contained an elaborate internal fibrogranular network.

C.2 Effects of Transcriptional Inhibitors on Nuclear Morphology

Transcriptional inhibitors have been used in a variety of studies as a means of investigating the functional organization of the nucleus (reviewed in Brasch, 1990). These inhibitors have been shown to induce dramatic structural changes in nuclei, revealing a structure within the nuclei that has been proposed to represent the nuclear matrix in situ (Brasch, 1982, 1990). The inhibitors most commonly used are α -amanitin, actinomycin D and 5,6-dichloro-1- β -D-ribofuranosyl-benzimidazole (DRB). These three inhibitors have been shown to induce similar

structural changes in affected nuclei (reviewed in Brasch, 1990), and so their effects will be discussed together.

Inhibition of transcription induces a reorganization of the condensed chromatin within the nucleus. In affected cells, the condensed chromatin becomes aggregated into large clumps located mainly at the nuclear periphery, with some smaller aggregates located in the nucleoplasm (Brasch and Sinclair, 1978; Sinclair and Brasch, 1978; Ghosh *et al.*, 1978; Nickerson *et al.*, 1989; Crowley and Brasch, 1987; Andersson *et al.*, 1984). In some studies, it has been suggested that the dispersed or active chromatin becomes condensed on inhibition of transcription, and is incorporated into the aggregated condensed chromatin at the nuclear periphery (Derenzini *et al.*, 1979; Andersson *et al.*, 1984). Derenzini *et al.* (1979) have further suggested, based on experiments using cortisol-stimulated hepatocytes in adrenalectomized rats that were then treated with α -amanitin, that changes in chromatin conformation are the result of, and not the cause of, transcriptional activity.

The nucleolus is fragmented during inhibition of transcription with DRB, and has been described as taking on a "nucleolar necklace" formation (Granick, 1975a, b; Scheer *et al.*, 1984; Noaillac-Depeyre *et al.*, 1989). Studies using DRB have also shown that the fibrillar and granular components of the fragmented nucleolus become segregated (Puvion *et al.*, 1979).

The fibrillar and granular components of the nucleoplasm are also affected by transcriptional inhibitors. There is generally a clearing of much of the fibrillar and granular material (Brasch and Sinclair, 1978; Sinclair and Brasch, 1978; Crowley and Brasch, 1987; reviewed in Brasch, 1990). When specific structures within the nucleoplasm were examined, it was found that perichromatin fibrils, thought to represent nascent transcripts, were generally absent from affected nuclei (Puvion et al., 1979, 1984a). The behaviour of ICG in response to transcriptional inhibitors has already been described (see section A.4).

Perichromatin granules were also largely absent from nuclei treated with α -amanitin (Brasch and Sinclair, 1978; Sinclair and Brasch, 1978); however, with DRB treatment, Puvion and co-workers (Puvion et al., 1979, 1981, 1984a, b) have noted the formation of granules similar to perichromatin granules, located near the fragmented nucleolus. Autoradiography using cells labelled with ^3H -uridine, and then chased with non-radioactive uridine in the presence of DRB showed accumulation of radioactivity in these newly formed granules. Treatment of the cells with actinomycin D prior to DRB treatment prevents the formation of these granules, and so it has been suggested that these granules contain RNA of nucleolar origin (Puvion et al., 1979). Preparations of nuclei containing nucleoli that have been "loosened", or partially decondensed, using a borate

buffer containing formaldehyde, Kodak Photo-flo and sucrose (Puvion et al., 1981) have shown that these DRB-induced granules form in the fibrillar regions of the nucleolus, at or near the sites of rRNA transcription. It has been further suggested that these granules represent accumulations of abnormally synthesized or processed ribosomal transcripts (Puvion et al., 1981).

Chaly et al. (1988) have studied the effects of DRB on RNA export in lymphocyte nuclei using autoradiography. They found that in partly stimulated lymphocytes (morphotype II, see section D), RNA was not transported normally to the cytoplasm, but was degraded while still in the nucleus. In fully stimulated nuclei (morphotype III, see section D), DRB had no effect on RNA export, but that RNA did not accumulate in the cytoplasm. These results suggested that RNA was degraded in the cytoplasm of morphotype III cells treated with DRB (Chaly et al., 1988).

The effect of transcription inhibitors on the organization of individual nuclear proteins has so far received little attention. Spector et al. (1983) have shown that the Sm antigen is reorganized from a speckled labelling pattern to large aggregates of label during DRB treatment. Using antibodies against nuclear proteins, Chaly et al. (1988) have shown that the nuclear matrix antigen P11 was also reorganized from a patchy nucleoplasmic distribution in untreated cells to large clumps of label in the nucleoplasm,

that co-localized with aggregates of Sm label.

The effects of DRB and, in live animals, α -amanitin have been shown to be reversible. When live roosters were treated with sub-lethal doses of α -amanitin, the animals showed essentially complete recovery 24-48 hr after administration of the drug (Brasch and Sinclair, 1978; Sinclair and Brasch, 1978). Similarly, once DRB has been removed from the culture medium of the treated cells, the effects are reversed within hours of its removal (Chaly et al., 1988).

D. Morphological and Biochemical Changes During Lymphocyte Activation

Lymphocytes are the cells of the immune system that are most intimately involved in its control and regulation. Two main subpopulations exist, divided on the basis of their developmental origins. T lymphocytes mature in the thymus, are involved in cell-mediated immunity and have been further subdivided into several different classes, including helper cells which secrete lymphokines that mediate stimulation of B cells and other cells involved in the immune response, suppressor cells and cytotoxic cells. B cells, derived from bone marrow in mammals, are involved in humoral immunity via secretion of antibody (MacDonald and Nabholz, 1986).

Unstimulated lymphocytes in vivo exist as small cells (5-8 μm in diameter) (Setterfield et al., 1983), and are somewhat irregular in shape. Much of the volume of unstimulated lymphocytes is taken up by the nucleus, which is surrounded by a thin layer of cytoplasm containing few organelles. The nucleus is also roughly spherical, but has a deep cleft in one side. There are few pore complexes in the nuclear envelope of unstimulated cells, and these are found in areas of the nuclear envelope that are not in contact with condensed chromatin (Maul et al. 1971; Setterfield et al., 1983). These nuclei contain large aggregates of condensed chromatin located at the nuclear periphery and extending into the nuclear interior. Interspersed between the condensed chromatin aggregates are small interchromatinic regions. In unstimulated lymphocytes, regressive-EDTA staining has shown that the interchromatinic regions are mainly granular, with some fibrillar material (Setterfield et al., 1983, 1985). Nucleoli in unstimulated lymphocytes are also small and lack granular regions. These cells are also essentially metabolically inactive, exhibiting very little RNA synthesis and no DNA synthesis (Setterfield et al., 1983).

Mature lymphocytes can be stimulated to proliferate with antigen or mitogens, such as phytohemagglutinin or concanavalin A (Con A). Mitogenic stimulation results in dramatic changes in lymphocyte nuclear morphology between 12

and 24 hr post stimulation (p.s.) (Setterfield et al., 1983). Nuclear volume increases up to 8 fold, and the volume of the cytoplasm increases as well. Stimulated nuclei are more regular in shape, with mainly spherical or elliptical outlines. The number of nuclear pores also increases during stimulation (Maul et al., 1971; Maul et al., 1972; Setterfield et al., 1983).

At the periphery of stimulated lymphocyte nuclei, there are few large aggregates of condensed chromatin; most of these have been broken up into smaller aggregates and dispersed throughout the nucleoplasm. Morphometric studies (Setterfield et al., 1983) have shown that the volume of condensed chromatin in lymphocyte nuclei does not change significantly during stimulation, but that this chromatin is indeed redistributed.

The volume and complexity of the interchromatinic regions increases dramatically during stimulation. Whereas these regions account for approximately 50% of nuclear volume in unstimulated lymphocytes, in fully stimulated cells, the interchromatinic regions make up more than 80% of the nuclear volume (Setterfield et al., 1983). These observations indicate that the increase in nuclear volume that occurs during stimulation is largely due to increases in the volume of the interchromatinic regions. When visualized by regressive-EDTA staining, the interchromatinic regions of stimulated lymphocyte nuclei were shown to be

composed mainly of fine fibrillar and granular material (Setterfield et al., 1983, 1985). Nucleoli in stimulated lymphocyte nuclei are large and more complex than in unstimulated nuclei, having well-developed fibrillar and granular regions (Valkov and Moyne, 1974; Setterfield et al., 1983).

Lymphocytes of mouse, human and bovine origin have been classified by morphological characteristics into three categories or morphotypes (Castellanos et al., 1982; Setterfield et al., 1983). This classification system was introduced because the response of lymphocytes to mitogen is asynchronous, and so a population of lymphocytes at a given time p.s. is likely to contain lymphocytes at various stages of activation. Morphotype I nuclei are unstimulated nuclei. Morphotype III nuclei are morphologically fully stimulated nuclei, and morphotype II nuclei are intermediate between types I and III (Setterfield et al., 1983). In freshly isolated, unstimulated samples of lymphocytes, the cells are almost exclusively of morphotype I. During stimulation with mitogens, morphotype I nuclei are progressively transformed into type II and then III.

Autoradiographic studies using cells labelled with ^3H -thymidine at 24, 36, 48 and 72 hr p.s. indicate that DNA synthesis in human peripheral blood lymphocytes does not begin until between 24 and 36 hr p.s., and that incorporation of ^3H -thymidine is limited to morphotype III

nuclei (Setterfield et al., 1983). Inhibitors of DNA synthesis administered at various times during stimulation showed that the morphological changes occurring during lymphocyte activation are independent of DNA synthesis (Setterfield et al., 1983).

Setterfield et al. (1983) also showed that if the protein synthesis inhibitor cycloheximide is administered with Con A, no stimulation occurs, and that even if the inhibitor was administered between 16 and 24 hr p.s., the number of morphotypes II and III that developed was significantly reduced. The effects of protein synthesis inhibition were lessened only if cycloheximide was given after 24 hr p.s. These experiments demonstrated that the nuclear structural changes that occur with lymphocyte stimulation are dependent on protein synthesis within the first 24 hours p.s. Autoradiography of cells labelled with ³H-leucine from 16 to 24 hr p.s., and then chased with cold leucine for various times indicated that there was a significant amount of protein synthesized during stimulation, and that this protein was relatively stable (Setterfield et al., 1983). Most of the labelled nuclear protein was in the interchromatinic regions, further emphasizing that much of the increase in volume of the stimulated lymphocyte nucleus occurs in the interchromatinic regions.

RNA synthesis was also shown to be necessary for the morphological changes accompanying lymphocyte activation to

occur. When RNA synthesis was inhibited by α -amanitin given concurrently with Con A, the numbers of morphotypes II and III did not increase (Setterfield et al., 1983). When α -amanitin was added between 8 and 16 hr p.s., the formation of morphotype II nuclei appeared unaffected, but the number of morphotype III nuclei was reduced. Lymphocyte activation was not affected by RNA synthesis inhibition after 24 hr p.s.. Autoradiography of cells pulsed with ^3H -uridine from 16 to 24 hr p.s., and then chased with cold uridine up to 72 hr p.s. indicated that nuclear RNA in stimulated lymphocytes has a high turnover rate. Setterfield et al. (1983) suggested, based on these results, that transcription is necessary for the morphological changes that occur during stimulation of lymphocytes, but that RNA may not be a significant structural component of the lymphocyte nuclear matrix. They further suggested that transcriptional activity may be required for the disaggregation of the condensed chromatin in activated lymphocytes, as inhibition of transcription induced re-aggregation of the condensed chromatin.

Nuclear matrices have been isolated from unstimulated and stimulated human (Setterfield et al., 1983), mouse (Hall, 1982) and bovine (Bladon, 1986; Bladon et al., 1988) lymphocytes, and examined by electron microscopy with similar results. It was observed that unstimulated lymphocytes contain very little internal nuclear matrix,

while stimulated lymphocytes contain extensive fibrogranular internal matrices. When protein profiles on two-dimensional gels of nuclear matrices from unstimulated and stimulated bovine lymphocytes were compared (Bladon *et al.*, 1988), several differences in the profiles were noted. The protein profiles of stimulated matrices were generally more complex than profiles of unstimulated matrices. Differences in the profiles included the appearance of lamins A and C in stimulated matrices but not in unstimulated matrices, decreases in high molecular weight basic proteins, and increases in RNP proteins in stimulated matrices. The increased complexity of protein profiles after stimulation reflects the extensive synthesis of new proteins during stimulation (Bladon *et al.*, 1988).

Bladon (1986) and Chaly *et al.* (1988) labelled unstimulated and stimulated lymphocytes with antibodies generated against mouse lymphocyte nuclear matrix to determine the distribution of these antigens in intact nuclei. Three antigens were localized. P1, a peripheral nuclear antigen described by Chaly *et al.* (1984), showed a uniform peripheral labelling in both unstimulated and stimulated lymphocytes. P11, which has both peripheral and internal components (Chaly *et al.*, 1984), showed very weak labelling in unstimulated cells, with faint peripheral labelling and little or no internal labelling. In stimulated cells, there was a punctate peripheral labelling,

with a bright internal label organized into patches of granules. PI2, which had been described with both peripheral and internal labelling (Chaly et al., 1984), showed only peripheral labelling in both unstimulated and stimulated cells.

Chaly et al. (1988) treated stimulated mouse lymphocytes with DRB to determine its effects on the localization of the above nuclear matrix antigens. P1 and PI2 were not affected by the drug. PI1 was redistributed into large aggregates of label within the nucleoplasm. DRB-treated cells were also labelled with anti-Sm, and this antigen was also redistributed by DRB. In stimulated cells, Sm was localized to the nucleoplasm, excluding the nucleolus, in a distribution that was co-localized with the patches of PI1 internal labelling. Double labelling experiments with PI1 and anti-Sm showed that these antigens were also co-localized during DRB treatment (Chaly et al., 1988). The effects of DRB were essentially reversed within 2 hr of its removal, and the PI1 and Sm antigen labelling patterns in recovered samples were similar to those in untreated, stimulated samples (Chaly et al., 1988).

CHAPTER 2

**Reversible disassembly of transcription domains in
lymphocyte nuclei during inhibition of RNA synthesis by DRB**

SUMMARY

We have been investigating the roles of RNA synthesis, chromatin structure and nuclear matrix organization in establishing and maintaining transcription domains, using mitogen-activated lymphocytes as a model system. Earlier studies showed transcription was required for chromatin disaggregation and nuclear matrix elaboration during activation. They also showed that inhibiting transcription in activated lymphocytes with the RNA polymerase II inhibitor DRB resulted in restructuring of the nuclear matrix. In this study, we have examined the effect of DRB treatment on lymphocyte nuclear ultrastructure by EM cytochemistry, using conventional uranyl/lead staining, regressive-EDTA staining for RNP, bismuth staining for phosphoproteins and phosphotungstic acid (PTA) staining for total protein. We have also investigated the reassembly of transcriptionally active nuclei during recovery from DRB treatment by immunofluorescence labelling of the nuclear matrix P11, Sm and nucleolar fibrillarin antigens, and by electron microscopy. After DRB treatment, chromatin was aggregated into large ramified masses that resembled chromatin in unstimulated lymphocytes by conventional staining but exhibited the PTA staining characteristics of chromatin in partially stimulated lymphocytes. In addition, interchromatin granules were gathered into a few large

clusters, proteinaceous tufts of material lay at the cytoplasmic face of nuclear pores, and nucleoli were disrupted. The nucleoplasmic fibrogranular RNP network was not significantly rarefied in DRB-treated samples when visualized by regressive-EDTA staining, but after PTA, interchromatinic regions were less textured and stained less intensely than in control stimulated lymphocytes. After removal of DRB, nuclear morphology and ^3H -uridine incorporation levels recovered to a large extent within 2 hr but required 8 hr for complete recovery. Immunofluorescence labelling showed that the DRB-induced large discrete patches of P11 and Sm were replaced by several smaller patches, as in controls, within 2 hr. As well, the many small foci of anti-fibrillarin labelling in DRB-treated samples were replaced by two to three large bodies. Electron microscopy confirmed these observations, showing that nucleoli had reformed, interchromatin granules had dispersed into numerous smaller clusters, pore-associated PTA-stained tufts had disappeared, and interchromatinic regions were once again textured after PTA staining within 2 hr DRB removal. However, chromatin was only partly disaggregated after 2 hr, and its recovery of control PTA staining characteristics required at least 8 hr. These results indicate that the chromatin, RNP and in situ matrix components of transcription domains are all affected to varying degrees when transcription is inhibited. The data further indicate,

however, that a nuclear matrix is retained in the absence of transcription, and that absence of transcription is not solely accountable for the highly aggregated state of the chromatin in resting lymphocytes.

INTRODUCTION

In current models of nuclear organization, functions such as transcription and replication are visualized as taking place in specialized nucleoplasmic compartments or domains, within which are concentrated the chromatin, enzymes and accessory proteins related to that function. It is generally believed that the chromatin in the domains consists of uncoiled loops that are extended from condensed chromatin in a regulated manner, and that the domains are assembled as part of a solid phase that is conceptually analogous to the nuclear fractions referred to as nuclear matrix, nuclear scaffold or nucleoskeleton (Berezney, 1984, 1991; Brasch, 1990; Carter and Lawrence, 1991; Dingwall, 1992; Garrard, 1990; Jackson, 1991; Jackson et al., 1992; Nigg, 1988).

With respect to transcription, early autoradiographic and cytochemical electron microscope studies established that RNA synthesis was compartmentalized, restricted to nucleoplasmic regions free of condensed chromatin and to the margins of condensed chromatin aggregates (Fakan, 1986; Puvion and Moyne, 1981). More recently immunocytochemical, in situ hybridization and autoradiographic studies have identified nucleoplasmic regions enriched in snRNPs, hnRNP, poly(A) RNA and nascent transcripts (Carter et al., 1991; Huang and Spector, 1991; Spector, 1990; Spector et al.,

1991, 1992). These regions are associated with the nuclear matrix and their organization is cell cycle-dependent (Carter et al., 1991; Xing and Lawrence, 1991). The data thus support the existence of specific transcription/processing domains whose formation is temporally related to the level of transcriptional activity in the cell. The mechanism by which these domains are assembled has not been characterized but there is considerable evidence that transcriptional activity and the nuclear matrix are involved in regulating the architecture of the chromatin (Brasch, 1990; Chaly et al., 1988; Nickerson et al., 1989).

We have been using the mitogen stimulated lymphocyte as a model system in which to examine the assembly of transcriptionally active nuclei. Nuclei in resting lymphocytes (morphotype I) display a morphology common to other types of inactive cells, such as nucleated erythrocytes (Brasch et al., 1971; Dardick and Setterfield, 1976). They are small and relatively inactive, and have small nucleoli and large aggregates of condensed chromatin. When stimulated by antigen or mitogen, they undergo major changes in structure and composition. In fully stimulated lymphocytes, morphotype III nuclei are up to 10-fold larger in volume than morphotype I nuclei, have well developed nucleoli, many small chromatin aggregates and a larger and more complex population of nuclear bodies (Brown et al., 1989; Chaly et al., 1983 a, b; Setterfield et al., 1983;

Tokuyasu et al., 1968; Valkov and Moyne, 1974). Morphotype II nuclei exhibit intermediate structure (Setterfield et al., 1983).

We have shown that the isolated nuclear matrix fraction, and its in situ counterpart, the interchromatinic regions, closely parallel the overall changes in nuclear organization and function during mitogenic activation. Increased levels of RNA and protein synthesis are paralleled by a large increase in the volume of interchromatinic regions, by elaboration of a complex fibrogranular network visible by regressive-EDTA staining of intact cells and in isolated nuclear matrices, and by the appearance of numerous new polypeptides detected in nuclear matrices analyzed by 2D SDS-PAGE (Bladon et al., 1988; Setterfield et al., 1983, 1985). Immunofluorescence studies further showed that individual nuclear matrix components, such as P11 (Chaly et al., 1985) and the snRNP Sm antigen, increased in quantity and were re-distributed in response to mitogen (Chaly et al., 1988). The dependence of the mitogen-induced morphological transformation of lymphocyte nuclei on protein and RNA synthesis, but not on DNA replication (Setterfield et al., 1983), suggests that these changes are related to the assembly of transcription/processing domains.

As an approach to examining the role of nuclear matrix components in organizing nuclei for transcription, we

carried out a comparative study of the behaviour of nuclear matrix antigens during mitogen activation and during inhibition of transcription by the RNA polymerase II inhibitor DRB (Chaly et al., 1988). Staining of the P11 and Sm antigens increased substantially in response to mitogen, apparently in parallel with the previously described increase in transcriptional activity and nuclear matrix elaboration. DRB treatment of stimulated populations reduced RNA synthesis to the levels in resting lymphocytes and caused significant restructuring of the nuclear matrix, as evidenced by co-aggregation of P11 and Sm antigens. DRB treatment did not appear, however, to induce the loss of these antigens from the nuclei. These data support the hypothesis that a nuclear matrix is required for transcription to take place and that matrix organization is related to the level of ongoing transcription.

In the present study, we have applied EM cytochemistry to extend our understanding of nuclear reorganization during DRB treatment, using conventional staining to observe changes in overall nuclear morphology; regressive-EDTA staining for ribonucleoprotein (RNP) (Bernhard, 1969) to monitor the organization of RNP components in the nucleoplasm; bismuth staining to identify phosphoproteins, that are concentrated in the nucleolus and inter- and perichromatin granules (Locke and Huie, 1977); and PTA staining (Davies et al., 1974) to examine the organization

of total protein, with particular reference to interchromatinic regions and chromatin.

We have also investigated the recovery of morphology and function after removal of DRB. EM cytochemistry as above served to characterize the behaviour of various nuclear elements as transcription resumed, and individual nuclear matrix components were monitored by immunofluorescence labelling with antibodies to P11, Sm and the nucleolar protein, fibrillarin (Chaly *et al.*, 1984; Lerner *et al.*, 1981; Ochs *et al.*, 1985; Ochs and Smetana, 1991).

The data show that chromatin and other components of transcription domains are extensively disorganized by DRB treatment but return to a near control configuration as transcription resumes. The results further indicate that the fibrillar *in situ* nuclear matrix is retained in the absence of transcription, although granular components are extensively redistributed. Furthermore this study demonstrates that absence of transcription is not solely accountable for the highly aggregated state of the chromatin in resting lymphocytes.

This chapter has been submitted as a manuscript by L. Davis, M. Cadrin, D. L. Brown and N. Chaly for publication to *Biology of the Cell*. Modifications were made to the Materials and Methods section for inclusion in this thesis.

MATERIALS AND METHODS

Cell isolation and culture

Splenocytes from male Balb/c mice (Charles River) were isolated as previously described (Setterfield et al., 1983; Chaly et al., 1983a) (see Appendix I for isolation protocol) and resuspended at a density of 2.5×10^6 cells/ml in RPMI 1640 medium supplemented with 6% CPSR-2 (Sigma) (see Appendix II for preliminary experiments substituting CPSR-2 for fetal calf serum), 100 U/ml penicillin, 100 μ g/ml streptomycin, 0.25 μ g/ml fungizone and 2-mercaptoethanol (1 μ l/ml). The cells were cultured at 37°C with 5% CO₂ for 1 hr and then an aliquot was withdrawn for further culturing without mitogen. Samples cultured without mitogen will be referred to as -Con A samples. The remaining cells were stimulated with concanavalin A (Con A) (Sigma) (4 μ g/ml), and all samples were returned to the incubator for 24 hr. Lymphocytes at 24 hr post-stimulation (p.s.) will be referred to as +Con A samples.

DRB treatment

DRB (Sigma), at a final concentration of 50 μ g/ml (stock solution, 5 mg/ml in 95% ethanol), was added to aliquots of +Con A cultures at 24 hr p.s. DRB-treated and control lymphocytes were cultured for a further 6 hr. +Con A samples cultured in the presence of DRB for a further 6 hr will be referred to as +DRB samples. For recovery from

DRB, +DRB cultures were washed three times in DRB-free complete medium at 30 hr p.s., and then were cultured for up to 8 hr, i.e. up to 38 hr p.s., in the absence of DRB. These samples will be referred to as recovering samples. +DRB and recovering samples were compared to control samples cultured continuously with Con A.

RNA synthesis

To measure RNA synthesis, 5,6-³H-uridine (10 μ Ci/ml) (spec. act. 37.5 Ci/mmol) (NEN DuPont) was added to 2 ml aliquots of cultures and the cells returned to the incubator for a 2 hr labelling period. Incorporation was stopped by freezing the samples at -20°C overnight. Three 0.5 ml aliquots of each sample were collected on separate GF/C filters, washed with 0.2 M sodium chloride and 5% trichloroacetic acid, and dried. Radioactivity was counted in a LKB Rackbeta 1217 liquid scintillation counter. Counts were corrected for cell viability, as determined by Trypan Blue exclusion for each sample, and are presented as percent of controls. ³H-uridine was added to DRB-treated and control samples at 22, 24, 26 and 28 hr p.s. to measure the inhibitory effect of DRB on RNA synthesis. These times correspond to -2, 0, 2 and 4 hr of DRB treatment. Recovery of RNA synthesis from DRB treatment was measured by adding ³H-uridine to control samples and to samples from which DRB had been washed out at 30, 32, 34 and 36 hr p.s. These

times correspond to 6 hr of DRB treatment (0 hr of recovery), and 2, 4, and 6 hr of recovery. An aliquot of lymphocytes was also labelled for 2 hr after 22 hr of culture in the absence of Con A in order to determine the level of RNA synthesis in unstimulated cells.

Indirect immunofluorescence labelling

Cells settled on poly-L-lysine-coated coverslips were fixed, permeabilized and labelled as previously described (Chaly *et al.*, 1984; 1988).

Antibody PI1 (lyophilized tissue culture supernatant, 1:10) is a mouse monoclonal IgM antibody (Chaly *et al.*, 1984). Antibody Y12 (anti-Sm) (used as tissue culture supernatant, diluted 1:10; hybridoma gift of J. Steitz, Yale University, New Haven, Conn., USA) is a mouse monoclonal IgG (Lerner *et al.*, 1981). Anti-fibrillarin (undiluted tissue culture supernatant, gift of R. L. Ochs, The Scripps Research Institute, La Jolla, Calif., USA) is a mouse monoclonal IgG (Reimer *et al.*, 1987).

Secondary antibodies were one of the following, as appropriate: fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgM (μ -chain specific) (1:150, Cappel); FITC-conjugated goat anti-mouse IgG (heavy- and light-chain specific) (1:500, Cappel).

Samples were counterstained with 1.5 μ g/ml Hoechst 33258 and mounted as described in Chaly *et al.* (1984).

Preparations were examined with a Zeiss Photomicroscope III equipped for epifluorescence, using a x100/1.3 n.a. Neofluar phase objective and were photographed using Ilford XP1-400 film.

Electron Microscopy

For conventional electron microscopy, regressive-EDTA staining for RNP (Bernhard, 1969) and phosphotungstic acid (PTA) staining for proteins (Davies *et al.*, 1974), samples were fixed in 2% glutaraldehyde/2% formaldehyde in 0.05 M sodium phosphate buffer, for 4 hr at room temperature. For conventional uranyl-lead staining, samples were subsequently post-fixed in 1% OsO_4 for 1 hr at 4°C. For bismuth staining (see Appendix III for protocol), samples were fixed in 4% formaldehyde in 0.1 M sodium cacodylate buffer and stained according to the method of Locke and Huie (1977), and subsequently post-fixed in 1% OsO_4 at 4°C. All samples were dehydrated on ice through a graded ethanol series, and embedded in Epon-Araldite. Thin sections were cut with a diamond knife and mounted on copper (conventional, regressive and bismuth) or nickel (PTA) grids that had been etched by dipping in 70% nitric acid, rinsed twice in filtered water, washed in chloroform and rinsed twice again with filtered water. The sections were observed at 60 kV (regressive, bismuth) or at 80 kV (conventional, PTA), using a Philips 420 EM.

RESULTS

RNA SYNTHESIS

The effect of DRB and of its removal on RNA synthesis is shown in Figure 1. ^3H -uridine incorporation is expressed as percent of control (+Con A), with control values at 24 hr p.s. set at 100%. Also included is the incorporation by unstimulated cells cultured for 24 hr in the absence of mitogen. -Con A cells incorporated an average of 26.5% of the ^3H -uridine incorporated by +Con A cells stimulated for 24 hr. After 2 hr of DRB treatment, ^3H -uridine incorporation was reduced to an average of 27% of control, with slightly more reduction of incorporation after 6 hr, to an average of 18% of control. Incorporation in cells treated with DRB for 6 hr was comparable to that in -Con A cells, indicating that DRB inhibited most of the RNA synthesis that had occurred with stimulation. These results are similar to those previously reported (Chaly et al., 1988).

When DRB was removed, incorporation increased to nearly 75% of control within 2 hr (Fig. 1). Unexpectedly, there was a consistent drop in incorporation to approximately 45% of control at 4 hr after DRB removal. However, after 6 hr of recovery, incorporation had increased back to greater than 60% of control, and by 8 hr after DRB removal, incorporation was consistently at or above control levels.

Figure 1: Histogram of ^3H -uridine incorporation in -Con A, +Con A, +DRB and samples recovering from DRB. Cultures were labelled for two hours prior to sampling. Incorporation was measured by scintillation counting and is expressed as % of Con A samples. The results presented are average values from three separate experiments, with error bars representing the standard deviation.

NUCLEAR MATRIX ORGANIZATION: IMMUNOFLOUORESCENCE LABELLING

PI1 - As shown previously (Chaly *et al.*, 1988), PI1 staining was faint at the nuclear periphery with little or no internal labelling in nuclei of -Con A cells (Fig. 2A), and was much brighter in +Con A samples (Fig. 2B), with a punctate peripheral pattern and some nucleoplasmic granular patches. After 6 hr of DRB (Fig. 2C), PI1 labelling was as bright as in control (+Con A) samples, but was aggregated into one or two large clumps in the nucleoplasm, and a few clumps at the nuclear periphery. After removal of DRB, PI1 labelling returned to a control pattern. Within 2 hr (Fig. 2D), both peripheral and internal label had largely re-dispersed. Samples at 4 and 6 hr of recovery (not shown) showed progressively less clumping and by 8 hr (Fig. 2E), the treated samples were essentially indistinguishable from controls, whether labelled at 24 hr p.s. (Fig. 2B), when DRB treatment began, or at 38 hr p.s. (Fig. 2F), when recovery from DRB was terminated.

Sm - The Sm antigen was distributed in patches in the nucleoplasm of nuclei of -Con A cells (Fig. 3A). In +Con A samples (Fig. 3B), labelling with anti-Sm was brighter and consisted of a diffuse component and numerous brighter patches of diverse size. In +DRB samples (Fig. 3C), Sm was clumped in the nuclear interior and similar in appearance to

Figure 2: Immunofluorescence labelling with antibody P11.

(A) -Con A nuclei; (B) +Con A nucleus, 24 hr p.s.;
(C) +DRB nucleus, showing clustered peripheral
labelling (arrowheads); (D) nuclei after 2 hr of
recovery from DRB; (E) nucleus after 8 hr of
recovery from DRB; (F) +Con A nuclei (38 hr p.s.).

X 2800

the internal PI1 label described above (Fig. 2C). These observations are consistent with previous studies in which nucleoplasmic patches labelled by PI1 and by Sm were shown to be co-localized, both in control and DRB-treated samples (Chaly et al., 1988). As for the internal PI1 labelling, the clumps of Sm antigen were mostly re-dispersed within 2 hr of DRB removal (Fig. 3D), with samples labelled at 4 and 6 hr recovery showing progressively less clumping (not shown). By 8 hr after DRB removal (Fig. 3E), the treated samples were similar to controls labelled at 24 (Fig. 3B) and 38 (Fig. 3F) hr p.s.

Fibrillarin - In nuclei of unstimulated cells, anti-fibrillarin (Fig. 4A) labelled one to three small ring-like areas in each nucleus that corresponded to areas of less intense Hoechst 33258 staining (Fig. 4A'). In stimulated nuclei (Figs 4B-B'), the number of labelled rings appeared to be the same as in resting cells, but the rings were much enlarged. DRB treatment disrupted this labelling, with +DRB samples consistently exhibiting many very small rings in the nucleoplasm (Figs 4C-C'''). Within 2 hr of removal of DRB (Figs 4D-D'), however, the labelled areas resembled those in +Con A nuclei (Fig. 4B), although they were smaller. The labelled structures gradually enlarged over the next 4 hr of recovery, and 8 hr after DRB removal (Figs 4E-E'), labelling with anti-fibrillarin was like that in +Con A nuclei.

Figure 3: Immunofluorescence labelling of the Sm antigen with antibody Y12. (A) -Con A nuclei; (B) +Con A nuclei, 24 hr p.s.; (C) +DRB nucleus, showing large clump of label (arrowheads); (D) nuclei after 2 hr of recovery from DRB; (E) nuclei after 8 hr of recovery from DRB; (F) +Con A nuclei (38 hr p.s.). X 2800

2

PM-1 3 1/2" x 4" PHOTOGRAPHIC MICROCOPY TARGET
NBS 1010a ANSI/ISO #2 EQUIVALENT

PRECISIONSM RESOLUTION TARGETS

In examining these samples, it was noted that some of the nuclei in all samples had an irregular lobed appearance (e.g. Figs 4C'-C''), and that the proportion of lobed nuclei seemed to be higher after DRB treatment than in untreated samples. To test this observation, samples from two experiments were examined for nuclear shape, and nuclei were scored as either lobed or unlobed (Table 1). Although there was a difference in the absolute values obtained in the two experiments, the overall trend was similar. The proportion of lobed nuclei did not change in the first 24 hr after addition of mitogen, increased dramatically after 6 hr of DRB, and gradually dropped during recovery from DRB. At 8 hr after removal of DRB, the percent of lobed nuclei was still higher than in untreated samples, but was only about half of that in +DRB samples. In experiment 2, the percent lobed nuclei was greater in +Con A samples at 38 hr p.s. than at 24 hr p.s., whereas these percentages were very similar in experiment 1. The reason for this difference is not clear, but is not due to a difference in the degree of activation (data not shown). It may reflect the range of natural variability in the extent of nuclear lobing in normal lymphocytes populations.

Figure 4: Lymphocytes labelled with anti-fibrillarin (A-C, C', D-E) and counterstained with Hoechst 33258 (A', B', C'', C''', D'-E'). Hoechst-negative regions (arrowheads) correspond to nuclear regions labelled by anti-fibrillarin. (A, A') -Con A nuclei; (B, B') +Con A nuclei (24 hr p.s.); (C-C'') +DRB nuclei; (D, D') nuclei after 2 hr of recovery from DRB; (E, E') nuclei after 8 hr of recovery from DRB. Note lobing of nucleus in C', C'''. X 2800

Table 1: Frequency of lobed nuclei in relation to DRB treatment.

Sample	% Lobed Nuclei (experiment 1) ²	% Lobed Nuclei (experiment 2) ³
-Con A, 24 hr ps ¹	5.4	1.4
+Con A, 24 hr ps	6.5	2.6
+Con A, 38 hr ps	7.0	11.6
+DRB	27.5	41.7
2 hr recovery	19.5	30.0
4 hr recovery	14.2	32.6
6 hr recovery	10.5	24.6
8 hr recovery	13.5	18.2

¹ Samples of -Con A cells were taken after 24 hr of culture in the absence of mitogen. See Materials and Methods for descriptions of other samples.

² 200 nuclei counted per sample.

³ 500 nuclei counted per sample.

NUCLEAR ULTRASTRUCTURE AND CYTOCHEMISTRY

To investigate in more detail the changes in nuclear morphology during treatment with and recovery from DRB, samples were examined by electron microscopic cytochemistry. Samples stained conventionally with uranyl acetate and lead citrate provided information about the general appearance of the nuclei. The ribonucleoprotein (RNP) components, primarily nucleoli, ICG, perichromatin granules and perichromatin fibrils, were investigated in samples stained by the regressive-EDTA method (Bernhard, 1969). The organization of chromatin, unstained after this type of staining, was also highlighted. The behaviour of inter- and perichromatin granules, and of fibrillar regions of the nucleolus was also visualized in samples stained with bismuth, which binds to phosphoproteins in these structures (Locke and Huie, 1977). Phosphotungstic acid (PTA) staining (Davies *et al.*, 1974) was used to study the distribution of total nuclear protein.

-Con A - Nuclei in -Con A samples are almost exclusively of morphotype I (Setterfield *et al.*, 1983, 1985). Conventional (Fig. 5A) and regressive-EDTA (Fig. 5B) staining of these nuclei showed an organization similar to that previously described (Chaly *et al.*, 1983a, b; Setterfield *et al.*, 1983, 1985; Tokuyasu *et al.*, 1968; Valkov and Moyne, 1974); that is, large chromatin

The following is the list of symbols used on the electron micrographs: Nu, nucleolus; Ch, chromatin; NuR, nucleolar remnant; large arrowheads, nuclear pores; ICG, interchromatin granules; small arrowheads, perichromatin granules; NB, nuclear body; large arrow, nuclear lamina. In micrographs of PTA-stained nuclei, asterisks indicate regions of unstained chromatin, and open arrows point to densely stained chromatin.

Figure 5: Electron micrographs of morphotype I nuclei -Con A lymphocytes stained (A) conventionally; (B) by the regressive-EDTA method; (C) with bismuth tartrate; and (D) with PTA. (A) X 27,000; (B) X 28,000; (C), (D) X 30,000

aggregates, very small nucleoli and few nuclear pores. As well, the interchromatinic regions were small, with sparse fibrogranular material (Figs 5A-C). A few small clusters of ICG and some perichromatin granules were visible in bismuth-stained profiles (Fig. 5C), and perichromatin fibrils were occasionally observed near condensed chromatin aggregates (Fig. 5B).

In PTA-stained sections (Fig. 5D), the body of the condensed chromatin masses was not stained, but was edged with a fine rim of stained material facing the nucleoplasm. Although this result was surprising, as the histones should have bound PTA (Davies *et al.*, 1974), it was highly reproducible and characteristic of morphotype I nuclei in all samples (-Con A, +Con A, +DRB, and DRB recovery).

PTA did, however, stain intensely a thin layer lining the nuclear envelope, that is most likely the nuclear lamina. Both the shells and the granular contents of nuclear bodies were also stained by PTA (Fig. 5D), as were small tufts of material at the cytoplasmic face of nuclear pores. The interchromatinic regions were only lightly stained, exhibiting a fairly uniform fibrillar appearance.

+Con A - In +Con A samples stained conventionally (Fig. 6A) and by the regressive-EDTA method (Fig. 6B), nuclei of morphotypes II and III were larger, showed increased numbers of nuclear pores, large, well-developed

Figure 6: Electron micrographs of morphotype II (D, D') and III (A-C) nuclei in +Con A lymphocytes stained (A) conventionally; (B) by the regressive-EDTA method; (C) with bismuth tartrate; and (D), (D') with PTA. The nucleus in (D) is only slightly stimulated, whereas the nucleus in (D') is a fully developed morphotype II. (A), (B) X 29,000; (C) X 21,000; (D), (D') X 30,000

nucleoli and numerous small chromatin aggregates distributed throughout the nucleoplasm (Chaly *et al.*, 1983a, b; Setterfield *et al.*, 1983, 1985; Tokuyasu *et al.*, 1968; Valkov and Moyne, 1974). The interchromatinic regions exhibited a rich fibrogranular network (Figs 6A-B), and perichromatin fibrils were more abundant (Fig. 6B) than in morphotype I nuclei. The increase in granular material was particularly clear in bismuth stained samples (Fig. 6C), where perichromatin granules seemed more numerous than in morphotype I nuclei, and many small clusters of ICG were distributed throughout the nucleoplasm.

In PTA stained sections, chromatin exhibited a range of staining patterns, from unstained morphotype I nuclei (see Fig. 5D), through partly stained in slightly stimulated nuclei (Fig. 6D), to completely stained in fully developed morphotype II (Fig. 6D') and III (not shown) nuclei. In the slightly stimulated nuclei (Fig. 6D), the rim of stained chromatin was broader than in morphotype I nuclei (Fig. 5D), but extensive portions of the chromatin mass were still not stained. On the other hand, the appearance of chromatin in fully developed morphotype II (Fig. 6D') and in morphotype III nuclei after PTA staining was indistinguishable from that in conventionally stained preparations (cf. Fig. 6A).

The presumptive lamina, a thin layer of densely stained material adjacent to the nuclear envelope, was clearly visible in all nuclei in PTA-stained sections. However, the

nuclear pores in morphotype II and III nuclei (Figs 6D-D') appeared to lack the stained material seen at the cytoplasmic face of pores in morphotype I nuclei (Fig. 5D).

+DRB - After six hours of treatment with DRB, nuclear morphology in many lymphocytes was highly reorganized. Morphotype I nuclei seemed unaffected, and many normal morphotype II nuclei were also observed. However normal, fully stimulated morphotype III nuclei were rare. Conventional, regressive and PTA staining (Figs 7A-B, 7D) showed that the chromatin had become aggregated, mainly at the nuclear periphery. There were also some smaller profiles of chromatin aggregates in the nucleoplasm. In PTA-stained sections (Fig. 7D), the condensed chromatin was only partly stained, closely resembling chromatin in slightly stimulated nuclei (Fig. 6D).

Intact nucleoli were not evident in +DRB samples. However, what appeared to be small nucleolar remnants were identified in bismuth- (Fig. 7C) and conventionally stained (Fig. 7A) sections.

The intensely PTA-stained layer next to the nuclear envelope was also present in +DRB nuclei, and PTA-stained tufts of material were once again visible at the cytoplasmic face of nuclear pores. Conventionally stained samples also showed clusters of pore complexes in some profiles (Fig.

Figure 7: Electron micrographs of nuclei in +DRB

lymphocytes stained (A) conventionally; (B) by the regressive-EDTA method; (C) with bismuth tartrate; and (D) with PTA. Asterisks in (B) indicate highly bleached structures that may be part of nucleolar remnants. Barred arrows in (C) indicate small bismuth-stained structures of unknown origin. (A) X 27,000; (B) X 23,000; (C) X 28,000; (D) X 30,000

D

7A). This observation is consistent with the aggregation of peripheral PI1 labelling noted in +DRB samples (Fig. 2D), since the antigen detected by PI1 at the nuclear periphery is the p68 nuclear pore glycoprotein (Benavente *et al.*, 1989; Dabauvalle *et al.*, 1988).

Conventional (Fig. 7A) and regressive (Fig. 7B) staining of +DRB lymphocytes showed little change in the organization of the fibrillar and granular material in the nucleoplasm, although the +DRB samples exhibited fewer perichromatin fibrils than the +Con A controls. This observation is consistent with inhibition of hnRNA synthesis, as perichromatin fibrils are believed to represent nascent transcripts (Fakan, 1986). Bismuth staining, however, showed that ICG were gathered into a few very large clusters in the nucleoplasm (Fig. 7C), and that the number of perichromatin granules per profile had increased. Notably, in PTA-stained sections (Fig. 7D), the interchromatinic regions of nuclei affected by DRB exhibited less intense and more uniform staining than was seen in control samples.

In addition, nuclei affected by DRB contained some structures of uncertain origin. In regressive-EDTA stained samples, many nuclear profiles contained small areas that were very bleached (Fig. 7B). They were generally observed in clusters, embedded in condensed chromatin aggregates. Small, round structures were also observed in bismuth-

stained sections (Fig. 7C). Each structure consisted of a granular bismuth deposit in an amorphous background. These structures were smaller than the EDTA-bleached areas described above, and were also more uniform in size and shape. The bismuth-stained structures tended to occur singly, and were commonly associated with the surface of condensed chromatin. Although the bleached bodies in regressive-EDTA stained samples may be portions of the nucleolar remnants, the identity of the small bismuth-stained bodies is unknown.

Recovery from DRB, 2hr - Recovery from the effects of DRB occurred rapidly for all nuclear components in the first 2 hours following DRB removal (Fig. 8). The aggregates of condensed chromatin were considerably reduced in size (Figs 8A-B), with most nuclei having primarily a band of condensed chromatin at the nuclear periphery, as in +Con A samples (Fig. 6). In PTA-stained samples (Fig. 8D), a larger proportion of the condensed chromatin at the nuclear periphery was stained than in +DRB nuclei, and the smaller chromatin aggregates in the nuclear interior were fully stained. These observations are consistent with a partial recovery of chromatin organization within 2 hr.

Nucleoli had reformed (Fig. 8), and appeared similar in structure to those observed in +Con A samples, though somewhat smaller. The clusters of ICG had begun to disperse

Figure 8: Electron micrographs of nuclei in lymphocytes fixed after 2 hr of recovery from DRB, stained (A) conventionally; (B) by the regressive-EDTA method; (C) with bismuth tartrate; and (D) with PTA. (A) X 27,000; (B), (C) X 23,000; (D) X 30,000

(Fig. 8C), with most nuclei exhibiting primarily small clusters of ICG distributed throughout the nucleoplasm. As in +DPB samples, there seemed to be more perichromatin granules after 2 hr of recovery (Fig. 8C) than in untreated (+Con A) samples. In PTA-stained sections (Fig. 8D), the interchromatinic regions were more textured 2 hr after DRB removal than in +DRB samples (cf. Fig. 7D), though perhaps still less so than in control stimulated nuclei (cf. Fig. 6D'). However, perichromatin fibrils were more apparent than after DRB treatment (Fig. 8B), and the pore-associated PTA-stained tufts were no longer visible (Fig. 8D).

Neither of the structures of unknown origin that were seen in +DRB samples (Figs 7B-C) was observed in recovering samples.

Recovery from DRB, 4-8 hr - Complete recovery from DRB occurred more gradually over the next 4 to 6 hr, with samples at 8 hr recovery (Fig. 9A-D) largely indistinguishable from +Con A controls.

The size and disposition of condensed chromatin aggregates in conventionally (Fig. 9A) and regressive-EDTA (Fig. 9B) stained nuclei were very similar to that in +Con A controls (Figs 6A-B). PTA staining (Fig. 9D), however, showed that the chromatin had not fully recovered its original organization, as staining of chromatin masses near the nuclear envelope was still not quite uniform. However,

Figure 9: Electron micrographs of nuclei in lymphocytes fixed after 8 hr of recovery from DRB, stained (A) conventionally; (B) by the regressive-EDTA method; (C) with bismuth tartrate; and (D) with PTA. (A) X 27,000; (B) X 23,000; (C) X 21,000; (D) X 30,000

the interchromatinic regions appeared as textured (Fig. 9D) and rich in fibrogranular material (Figs 9A-C) as in controls (Fig. 6).

Perichromatin granules still appeared to be more prominent (Fig. 9C) than in +Con A samples, and nuclear bodies were more numerous than in any other samples (Fig. 9A).

DISCUSSION

Transcription-related alterations in the organization and composition of nuclei and nuclear matrices are not restricted to mitogen activated lymphocytes (Brasch, 1990). For instance, similar changes in nuclear morphology, function and composition are seen during reactivation of chick erythrocyte nuclei, including chromatin disaggregation, initiation of nucleic acid synthesis, nuclear import of proteins, and elaboration of a complex nuclear matrix (LaFond and Woodcock, 1983; LaFond et al., 1983; Woodcock and Woodcock, 1986). On the other hand, cessation of transcription, whether due to differentiation (Dingman and Sporn, 1964) or the use of transcriptional inhibitors (Brasch and Sinclair, 1978; Derenzini et al., 1981; Nickerson et al., 1989; Sinclair and Brasch, 1978) is invariably accompanied by extensive chromatin aggregation. In vivo α -amanitin treatment also clears granular material from the nucleoplasm of rooster livers, leaving behind a PTA-stainable fibrillar component interpreted as representing the nuclear matrix in situ (Brasch, 1982, 1990). Later studies showed that α -amanitin induced similar structural differences in isolated rooster liver nuclear matrices and also found that these matrices exhibited a less complex polypeptide profile by 2D SDS-PAGE (Crowley and Brasch, 1987).

A correlation between the level of transcription, the

extent of chromatin disaggregation, and the complexity of the nuclear matrix is therefore well established. The causal relationships among these three factors is less clear. The presence of an elaborated matrix in naturally active nuclei, and the elaboration of a matrix prior to, or in parallel with, nuclear activation would seem to indicate that a nuclear matrix is required for transcription to take place. A major role for the matrix might then be to anchor loops of active chromatin as they uncoil and extend from the disaggregating condensed chromatin masses.

DRB as a Reversible Inhibitor of Transcription in Lymphocytes

^3H -uridine incorporation, immunofluorescence labelling, and EM cytochemistry all indicate that DRB is indeed a reversible inhibitor of transcription in lymphocytes. Transcription was reduced to resting cell levels after 6 hr of DRB treatment, as shown previously (Chaly et al., 1988). Within 2 hr of DRB removal, transcription was greatly increased but required 8 hr for full recovery. The increase in ^3H -uridine incorporation was steady, other than for a small but consistent decrease at 4 hr. The reason for the decrease is uncertain, but may be related to the effect of DRB and DRB removal on nucleolar components.

Both anti-fibrillarin labelling and EM cytochemistry showed that nucleoli were disrupted by DRB treatment.

Fibrillarin, a protein associated with the U3 snRNP particle, is localized to the dense fibrillar component of the nucleolus, and has also recently been detected in residual nucleoli of HeLa nuclear matrices prepared in situ (Jansen et al., 1991; Lapeyre et al., 1990; Ochs and Smetana, 1991; Ochs et al., 1985). Nucleolar disruption by DRB has been previously observed by light and electron microscopy in other cell types (Granick 1975a, b; Noaillac-Depeyre et al., 1989; Ochs et al., 1985; Scheer et al., 1984). Since DRB is known to be a specific inhibitor of RNA polymerase II (Tamm et al., 1976; Zandomeni et al., 1982, 1986), its effect on nucleolar organization is believed to arise from a secondary inhibition of rRNA synthesis (Tamm et al., 1976; Puvion et al., 1984b).

Nucleoli were the first nuclear components to recover when DRB was removed. Although perhaps still smaller than in controls, they were indistinguishable from controls in ultrastructure and anti-fibrillarin labelling within 2 hr of recovery. There is evidence that pre-ribosomal precursors are degraded during DRB treatment (Granick, 1975a), and RNA export appears unaffected by DRB treatment (Chaly et al., 1988). It is therefore probable that new rRNA synthesis is required for nucleolar reformation once DRB is removed. If there is a brief surge in rRNA synthesis in the first 2 hr of recovery, rRNA would be over-represented in the ³H-uridine incorporation values at that time point. Once

nucleoli had reformed, the rate of rRNA synthesis would be expected to drop somewhat, so that ^3H -uridine incorporation values 4 hr after DRB removal would represent more exactly the extent of recovery of hnRNA synthesis at that point.

DRB and Nuclear Organization

Results from this study are consistent with earlier reports on mitogen-induced changes in lymphocyte nuclei (Chaly *et al.*, 1988; Setterfield *et al.*, 1983, 1985; Tokuyasu *et al.*, 1968; Valkov and Moyne, 1974). Conventional and cytochemical stains showed chromatin disaggregation, elaboration of large interchromatinic regions rich in ICG, perichromatin granules and perichromatin fibrils, and reorganization of individual nuclear matrix components. Bismuth and PTA staining at the EM level has also allowed us to characterize stages in chromatin disaggregation and decondensation more fully, and to monitor the fibrillar and granular components of interchromatinic regions independently.

Similar techniques applied to lymphocytes after DRB treatment and during recovery from DRB tested the role of transcription and of the nuclear matrix in lymphocyte nuclear organization and function. The results show that DRB induced major structural changes in treated nuclei, including chromatin aggregation, redistribution of nuclear matrix components, nucleolar disruption and changes in

nuclear envelope organization. The changes were largely reversed within 8 hr after DRB removal, but nuclear compartments reassumed the control appearance at different rates. For instance, whereas nucleoli appeared normal within 2 hr of recovery (see above), nuclei with less intensely PTA-stained condensed chromatin aggregates were still visible at 8 hr of recovery.

Nuclear Envelope and Pores

Nuclei in resting lymphocytes are characteristically spherical, with a single pronounced invagination of the nuclear envelope forming a cleft that harbours the microtubule-organizing centre and other cytoplasmic organelles (Brown et al., 1992). As in other metabolically inactive cells (Miller et al., 1991), these nuclei exhibit only a few pores that are in areas of the nuclear envelope not in contact with aggregates of condensed chromatin (Maul et al., 1971, 1972). With stimulation, the cleft becomes less pronounced as the nucleus enlarges but nuclear shape remains roughly spherical. Pores increase in number and become distributed relatively evenly over the nuclear envelope, in parallel with the redistribution of disaggregating chromatin throughout the nucleoplasm (Maul et al., 1971, 1972).

The peripheral component of antibody PI1 labelling has been previously shown to be due to a conserved 68kD member

of the nucleoporin family of nuclear pore complex glycoproteins (Benavente *et al.*, 1989; Chaly *et al.*, 1989; Dabauvalle *et al.*, 1988). In this study, immunofluorescence P11 staining has verified the increase in lymphocyte nuclear pore number in response to mitogen, with the nuclear periphery barely detectable in resting cells and exhibiting a bright punctate fluorescence pattern in stimulated cells. Similarly electron microscopy showed a few pores located between condensed chromatin aggregates in resting lymphocytes, with many pores punctuating the envelope at frequent intervals in morphotype II and III nuclei. After DRB treatment, however, peripheral P11 staining was gathered into small clumps, and clusters of nuclear pores were frequently observed by EM. Pores became evenly distributed over the nuclear surface once again when DRB was removed.

Pore clustering may be an indirect result of DRB-induced chromatin aggregation. During DRB treatment, the nuclear envelope became extensively lined with chromatin, as in morphotype I nuclei. In +DRB samples, pores may perforce form clusters in the small portions of envelope that remain free of condensed chromatin. Clustering of pore complexes during treatment with DRB suggests that the nuclear envelope has a certain degree of plasticity that allows for reorganization of structures associated with it, although the mechanisms involved in this reorganization are unclear. These observations also imply that the pores assembled

during response to mitogen are largely retained during DRB treatment, even though transcription and, probably, nucleocytoplasmic exchange in these cells becomes severely reduced (see below).

DRB treatment also led to a greatly increased proportion of aberrantly shaped nuclei, with the proportion of lobed nuclei gradually declining towards control levels when DRB was removed. The exact cause and significance of the lobes are uncertain. Peripheral blood lymphocytes have been previously reported to lobulate transiently in the absence of mitogen under the effect of cyclosporin A (Simons et al., 1986), with the proportion of lobed mononuclear leukocytes rising from 2% in controls to a peak at 20% after a 60 min drug treatment, and falling back to control levels within four hours of continued incubation in the drug. Although a similar proportion of lymphocytes became lobed during DRB treatment, the lobing occurred over a much longer time period, was not returned to control levels even after 8 hr of incubation without DRB, and appeared to depend on mitogen stimulation, as lobed nuclei were not observed in resting cells. In our studies, lobing may be related to a reduction in nucleoplasmic volume during DRB treatment. We have previously reported that RNA, presumably as RNP, continues to be exported at control rates during DRB treatment (Chaly et al., 1988), and, in the absence of ongoing transcription, it is reasonable to suppose that

protein import is reduced. This implies a net loss of nuclear contents, of nucleoplasmic volume, and of nuclear volume. Nuclear volume could be reduced while maintaining the same nuclear envelope surface area by nuclear involution, *i.e.* lobing.

The structure of nuclear pores is reported to be essentially identical in all tissues and species, and no differences in pore complex organization attributable to differential nuclear activity have been previously reported (Miller *et al.*, 1991). In this study, however, PTA-stained tufts of material lay at the cytoplasmic face of nuclear pores in transcriptionally inactive cells, *i.e.* resting and DRB-affected lymphocytes, and were not observed in transcriptionally active cells, *i.e.* morphotype II and III lymphocytes, and lymphocytes recovering from DRB. The stained material may represent components that have been transferred or that are waiting for transfer through the complex. Alternatively, the tufts may be integral parts of the pore complex, perhaps representing the arrays of fibrils visualized at the cytoplasmic face of nuclear pores (Franke, 1974; Richardson *et al.*, 1988). Nucleus-targeted proteins have been shown to bind to these fibrils, and it has been proposed that they participate in accumulating such proteins around the nuclear periphery prior to translocation through the pore (Richardson *et al.*, 1988). Under conditions of reduced nucleocytoplasmic exchange such as are found in

resting and DRB-treated lymphocytes, the fibrils may collapse and become visible as PTA-stained tufts.

Chromatin

Perhaps the most obvious change in nuclear ultrastructure during these experiments was the cycle of disaggregation, reaggregation, and, once again, disaggregation exhibited by the chromatin as lymphocytes were in turn mitogen-activated, DRB-treated, and allowed to recover from DRB. Conventional and regressive-EDTA staining did not distinguish between the chromatin in these samples, other than to demonstrate the differences in size of individual aggregates. PTA staining, however, suggested that the condensed chromatin aggregates in resting lymphocytes are fundamentally different from the aggregates in other lymphocyte samples, and that they undergo disaggregation, decondensation and decompaction as the cell is activated.

Whereas chromatin was essentially unstained by PTA in morphotype I nuclei, it was intensely stained in mid-morphotype II, morphotype III and recovered nuclei. A loss of PTA staining with a decrease in transcriptional activity has been observed in other systems. Chromatin aggregates in α -amanitin-treated chicken liver nuclei were only partially stained by PTA (Brasch, 1982), and a progressive lessening of chromatin staining by PTA has been described during avian

erythropoiesis (Davies et al., 1974) and during spermatogenesis (Courstens and Loir, 1981a, b). Davies et al. (1974) proposed that closer packing of chromatin fibrils in mature transcriptionally-inactive erythrocytes would account for the differential staining of chromatin in these samples by PTA and uranyl-lead. Binding sites accessible to the uranyl ion might not be accessible to the 10-fold larger PTA molecule. On the other hand, Courstens and Loir (1981a) demonstrated that PTA bound to amino groups in lysine residues under their staining conditions, and suggested that loss of staining during spermatogenesis was due to replacement of somatic nucleoproteins with sperm-specific proteins lacking lysine (Courstens and Loir, 1981b). It appears more likely that the absence of PTA staining in avian erythrocytes and lymphocytes is a result of poor accessibility due to chromatin compaction, since both cell types contain lysine-rich histones (van Holde, 1989; Lennox and Cohen, 1983).

Although the regulated unfolding of chromatin loops for transcription is well-documented in numerous systems (Alberts et al., 1989), whether this unfolding is sufficient in itself to transform large, compact, transcriptionally inactive chromatin aggregates into the numerous smaller, more loosely woven aggregates in active cells has been little investigated. Observations from this and earlier reports (Setterfield et al., 1983), however, indicate that

this process is complex. Morphometric studies on lymphocytes have shown that the total volume of condensed chromatin does not decrease during stimulation, although individual chromatin masses become smaller (Setterfield et al., 1983). The authors concluded that chromatin in morphotype I nuclei undergoes both disaggregation, i.e. breaking up of a large chromatin mass into several smaller ones, and decondensation, i.e. reduction of chromatin supercoiling, in its conversion to the chromatin morphology characteristic of morphotype III nuclei. As well as corroborating these conclusions, our study indicates that the chromatin in addition goes through decompaction, i. e. loosening of the weave of chromatin fibrils in the aggregates, making them more accessible to PTA.

Acquisition of PTA staining seemed to occur in two stages. The first stage consisted of: limited disaggregation of the chromatin masses in morphotype I nuclei; decondensation at the margins of the aggregates, making them fully accessible to PTA; some decompaction, so that aggregates were no longer totally stain negative. These nuclei would be classified as early morphotype II by conventional staining (see Fig. 6D), and would likely still exhibit relatively low levels of transcription (Setterfield et al., 1983). In mid-morphotype II nuclei, chromatin had passed through a second stage in which aggregates became even smaller and fully accessible to PTA. Such nuclei have

been shown to be active in transcription (Setterfield et al., 1983). We interpret this second transition as involving extensive decompaction in addition to disaggregation and further decondensation.

A multi-step model of chromatin folding during formation of condensed chromatin, with chromatin compaction as one distinct step, is consistent with data from in vitro studies. Observing nuclear assembly in interphase extracts, Newport (1987) reported that naked DNA went through an initial condensation as it became histone-associated, but then continued to condense, finally forming tightly packed spheres free of nuclear membranes and lamins. Only after compaction did the DNA begin to decondense and acquire a lamin-containing membranous nuclear envelope. Further, Wood and Earnshaw (1990) showed that chromatin condensation in mitotic extracts occurs in at least two stages: a DNA topoisomerase II-independent compaction of chromatin, and the resolution of condensed chromatin into individual chromosomes that is apparently topoisomerase II-dependent (Wood and Earnshaw, 1990).

However, the degree of PTA staining of lymphocyte nuclei was not related solely to transcriptional activity. For instance, chromatin was unstained in -Con A cells but remained partially stained in DRB-affected nuclei, even though the ^3H -uridine incorporation levels were similar in these samples. The highly compacted state of chromatin in

-Con A lymphocytes may be due to an intrinsic biochemical feature, a difference in histone H1 subtype (Lennox and Cohen, 1983) or post-translational histone modification (Boulikas *et al.*, 1990), for instance. Alternatively it may be related to the composition and organization of the nuclear matrix.

A major role proposed for the interphase nuclear matrix is anchoring chromatin loops within nuclear domains. As the matrix is assembled during activation, it may function not only to fasten extended chromatin loops, according to current models, but may also play a role in anchoring and decompacting chromatin aggregates. The fate of the uncoiled chromatin loops during DRB treatment is unknown, but by analogy with transcription-dependent puffing of loci on polytene chromosomes (Alberts *et al.*, 1989), the loops would be expected to retract towards the aggregates. The release of loops from the matrix in +DRB lymphocytes, and reorganization of the matrix itself during transcription arrest may then result in the observed partial reaggregation and recompaction of chromatin. However, the persistence of a matrix may not allow recompaction to the PTA-inaccessible state of resting lymphocyte chromatin.

Nuclear Matrix

Isolated nuclear matrices consist of residual nucleoli suspended in a fibrogranular network that includes ICG

clusters and is bounded by a residual nuclear envelope. Nuclear matrix organization in this study was assessed by immunofluorescence labelling and by EM cytochemistry.

As shown previously, internal PI1 labelling and the Sm antigen increased in intensity and quantity as the nuclear matrix was assembled in response to mitogen, as did the nucleolar matrix antigen fibrillarin. These components were then reversibly disorganized by DRB. PI1 and Sm formed co-localized nucleoplasmic aggregates, and fibrillarin dispersed into multiple small foci, resembling previously described DRB-induced "nucleolar necklaces" (Granick *et al.*, 1975a, 1975b; Scheer *et al.*, 1984).

The behaviour of the PI1 and Sm antigens mirrored the organization of ICG clusters visualized by bismuth staining. During lymphocyte stimulation, both the size and the number of ICG clusters increased, and DRB treatment reversibly induced the formation of a few very large ICG clusters. Unexpectedly, although ICG were clearly visible by bismuth staining, they were not contrasted within the nucleoplasm by regressive staining. This phenomenon has been noted with lymphocytes elsewhere (Krzyzowska-Gruca *et al.*, 1988). The changes in ICG organization are consistent with the EM localization of PI1 and Sm. Sm and other snRNP antigens have been localized primarily in association with ICG and perichromatin fibrils (Fakan *et al.*, 1984; Leser *et al.*, 1989; Spector *et al.*, 1991). Although the nature of the

antigen detected by the P11 antibody in the nucleoplasm is unknown, it is widely conserved (Chaly et al., 1986; Schatten et al., 1985), and its behaviour during lymphocyte activation (Chaly et al., 1988; Setterfield et al., 1985), early embryogenesis (Prather and Schatten, 1992) and mitosis (Chaly et al., 1984) indicate that it carries out a transcription-related function. EM immunoperoxidase and immunogold labelling studies of 3T3 (Chaly et al., 1989) and HeLa (Chen and Chaly, in preparation) cells have shown that, like snRNP antigens, the internal P11 component is associated with ICG clusters.

Other studies using transcriptional inhibitors (Brasch and Sinclair, 1978; Crowley and Brasch, 1987; Puvion et al., 1984a) have also reported that inhibition of transcription caused the aggregation of ICG in the nucleoplasm. Puvion et al. (1984b) reported finding RNA of nucleolar origin in ICG clusters in DRB-treated cells, and proposed that ICG in these nuclei may be associated with accumulation or transport of nucleolar RNA.

An additional effect reported for the transcriptional inhibitors α -amanitin and actinomycin D is a dramatic clearing of fibrogranular material from the nucleoplasm (Brasch and Sinclair, 1978; Ghosh et al., 1978; Herlan et al., 1978; Brasch, 1982; Crowley and Brasch, 1987; Nickerson et al., 1989; Brasch, 1990). Again unexpectedly, the nucleoplasm in +DRB lymphocyte samples showed only slight

clearing. This was particularly surprising in view of the continuing export of RNA during DRB treatment that we have previously demonstrated. The reason for this difference in transcription-arrested morphology is uncertain. The modes of action of these three drugs differ. α -Amanitin binds to RNA polymerase II and inhibits synthesis of new hnRNA, while pre-existing hnRNA continues to be processed and exported from the nucleus (Sinclair and Brasch, 1978), whereas actinomycin D intercalates into DNA and so inhibits elongation of transcripts. Although the mode of action of DRB is not entirely clear, it appears that it inhibits initiation of transcription (Zandomeni *et al.* 1983; 1986) or induces premature termination of transcripts initiated by RNA polymerase II (Chodosh *et al.*, 1989) by a mechanism involving casein kinase II (Chodosh *et al.*, 1989; Zandomeni *et al.*, 1984, 1986).

EM of PTA stained samples revealed the organization of the proteinaceous component of interchromatinic regions. These regions became very extensive during mitogen stimulation and, though fibrillar, acquired a textured appearance, perhaps from the accumulation of fibrogranular RNP. DRB treatment did not reduce the extent of these regions, but did result in a loss of texture that was reversed when DRB was removed. If, as suggested by Brasch (1982), the PTA-stained component of interchromatinic regions represents the subtending *in situ* nuclear matrix,

these data demonstrate that, once assembled in response to mitogen, a fibrillar nuclear matrix component is maintained despite transcription arrest and loss and/or redistribution of granular components.

Conclusions

This study provides evidence: (1) that assembly of transcription domains is dependent on the presence of a matrix, but that the matrix can persist in the absence of transcription; (2) that granular components of the matrix can reorganize independently of the fibrillar components; (3) that chromatin disaggregation is maintained by transcription but is not a requirement for it; (4) that chromatin decondensation may proceed independently of chromatin disaggregation; and (5) that condensed chromatin aggregates in resting lymphocytes and the aggregates induced by DRB are not strictly analogous. (6) The data also indicate that in lymphocyte nuclei with a low rate of transcription the nuclear pores have a characteristic morphology.

CHAPTER 3

Effects of inhibition of transcription by DRB on nuclear matrix structure and composition.

INTRODUCTION

The mitogen-stimulated lymphocyte is a good example of a system in which nuclear morphology varies with the metabolic activity of the cell. Mature, unstimulated lymphocytes contain sparse interchromatinic regions, but when stimulated to proliferate by antigen or mitogen, the interchromatinic regions enlarge up to 10 fold, and become more complex (Setterfield et al., 1983; 1985; Tokuyasu et al., 1968; Valkov and Moyne, 1974). This has been reviewed in detail in Chapter 1, and only key points relevant to this chapter will be summarized here.

The nuclear matrix is a residual nuclear structure that is resistant to extraction with detergents, low and high ionic strength buffers and nucleases (reviewed in Berezney, 1984; Brasch, 1990), and has been implicated in several functions of the nucleus, such as in organizing replication and transcription domains (reviewed in Chapter 1, section B). The morphology of the interchromatinic regions appears to be reflected in nuclear matrix morphology in murine, human and bovine lymphocytes (Setterfield et al., 1983; 1985; Bladon et al., 1988), estrogen-stimulated chicken livers (Brasch and Peters, 1985) and reactivated chick erythrocyte nuclei (LaFond et al., 1983). The morphology of the nuclear matrix has also been shown to mirror the morphology of the interchromatinic regions in situ in cells

in which transcription was inhibited by α -amanitin (Crowley and Brasch, 1987). Nuclear matrix composition has also been shown to vary with cellular function (see examples above). This phenomenon is presumed to be a reflection of the role of the nuclear matrix in DNA replication (reviewed in Berezney, 1984) and RNA synthesis (Jackson and Cook, 1985).

In the previous chapter, the effects of the transcriptional inhibitor DRB on the structure, organization and function of the lymphocyte nucleus were discussed. It was shown that although chromatin and RNP components of transcription domains were affected to varying degrees by the inhibition of transcription, the in situ nuclear matrix did not appear to be significantly reorganized. In the studies described in this chapter, the effect of DRB on isolated nuclear matrix organization has been investigated. Nuclear matrices were isolated from -Con A, +Con A, +DRB and 8 hr recovery samples and examined by immunofluorescence labelling, electron microscopy and one-dimensional SDS-PAGE in order to study more directly the effects of inhibition of transcription on nuclear matrix structure and composition.

Electron microscopy of isolated nuclear matrices from the above samples suggests that the structure of the isolated nuclear matrix in both transcriptionally active and transcription-arrested samples is indeed similar to the morphologies of the in situ interchromatin regions in corresponding intact cells. These results also seem to

indicate that the structure of the internal matrix is not severely altered by inhibition of transcription by DRB. Preliminary one-dimensional SDS-PAGE of proteins from isolated nuclei and nuclear matrices showed few qualitative or quantitative differences between +Con A, +DRB and 8 hr recovery samples.

Immunofluorescence labelling with antibodies to the nuclear matrix antigens PI1 and Sm revealed, on the other hand, that while the PI1 antigen remained associated with the nuclear matrix in all samples, its organization was somewhat disrupted in +DRB nuclear matrices and that the Sm antigen was extracted from +DRB and 8 hr recovered samples. Taken together, these results suggest that while inhibition of transcription may not significantly alter the structure of the nuclear matrix, it alters the organization of the components of transcription domains associated with the nuclear matrix.

MATERIALS AND METHODS

Cell isolation, culture and DRB treatment

Splenocytes from male Balb/c mice (Charles River) were isolated, cultured, mitogen-stimulated and treated with DRB as previously described (see Chapter 2 and Appendix I).

Isolation of Nuclei and Nuclear Matrices

Isolation protocols were based on methods developed by R. Hall (Hall, 1982; Setterfield et al., 1983) and T. Bladon (Bladon, 1986; Bladon et al., 1988) for lymphocytes, and on the method originally described for rat liver by Berezney and Coffey (1974). All steps in the isolation of nuclei and nuclear matrices were performed at 4°C. Centrifugations were performed using a Sorval RC5-B refrigerated centrifuge with a HB-4 swinging bucket rotor. All solutions contained 1 mM phenylmethylsulfonylfluoride (PMSF), 0.05 mM sodium tetrathionate dihydrate and 0.01 mM soybean trypsin inhibitor.

To isolate nuclei, cells were pelleted at 700-800 g for 5 minutes. The cells were washed once in sucrose-Tris-magnesium (STM) buffer (0.25 M sucrose, 10 mM TRIZMA base, 2 mM MgCl₂·6H₂O, 25 mM KCl, pH 6.2), and pelleted at 700-800 g for 5 min. The cells were then resuspended in STM buffer, pH 7.4, to a density of 1-2 x 10⁷ cells/ml. A 7 ml aliquot of the cell suspension was homogenized with 350 µl of 10%

Triton X-100, using a 15 ml Dounce homogenizer with a tight-fitting pestle. Eight to ten strokes were used to break cells of -Con A samples, while 12-15 strokes were used to break cells of +Con A, +DRB and 8 hr recovery samples. The degree of breakage and extent of cytoplasmic tagging was monitored by phase-contrast microscopy. If extensive cytoplasmic tags were observed, the homogenization was repeated. The homogenates were then transferred to 15 ml round-bottomed centrifuge tubes and nuclei were pelleted at 150-180 g (1000 rpm) for 4 min. The nuclei were washed again with STM, pH 7.4, and then were either used to prepare isolated nuclear matrices (see below), were processed for immunofluorescence labelling, or were stored at -70°C for electrophoresis.

Isolated nuclei were resuspended in 2 ml STM, pH 7.4, with DNase I added to a final concentration of 10 µg/ml. After 20 min on ice, the nuclei were pelleted at 200-400 g (1500 rpm) for 5 min, and then were resuspended, very slowly, in 2 ml of low magnesium buffer (LMB; 0.2 mM MgCl₂·6H₂O, 10 mM TRIZMA base), pH 7.4. After 20 min incubation on ice, 2 ml of 2 M high salt buffer (2 M NaCl in LMB) were added slowly, and the suspension was incubated on ice for 15 min. 2 ml of 4 M high salt buffer (4 M NaCl in LMB) were then added, and the suspension was kept on ice for 15 min. The salt treated nuclei were then pelleted at 400-500 g (1750 rpm) for 5 min. The pellet was resuspended in 2

ml of LMB, with RNase A and DNase I added to concentrations of 50 and 100 μ g/ml, respectively. This suspension was incubated on ice for 25 min. The nuclear matrices were then pelleted at 400-500 g (1750 rpm) for 5 min, and processed for immunofluorescence labelling, electron microscopy, or were frozen for use in gels.

Indirect immunofluorescence labelling

Aliquots of intact cells, isolated nuclei and isolated nuclear matrices were settled on poly-L-lysine-coated coverslips and fixed, permeabilized and labelled as previously described (Chaly *et al.*, 1984; 1988). For samples of isolated nuclei and isolated nuclear matrices, the 20 min permeabilization in Triton X-100 was omitted.

The primary antibodies used, P11 and anti-Sm, and their appropriate FITC-conjugated secondary antibodies, as well as the staining procedure, are described in Chapter 2.

Electron Microscopy

Nuclear matrices isolated simultaneously from 4 aliquots of cells were pooled and fixed in 2% glutaraldehyde in 0.05 M sodium cacodylate buffer (pH 7.4) for 1-2 hr on ice. The matrices were then washed once in 0.05 M sodium cacodylate buffer, and formed into pellets in 50 μ l microcapillary tubes and centrifuged in a hematocrit rotor for 20 min at 3000 rpm in an IEC Centra-4 centrifuge. The

pellets were then washed in 0.05 M sodium cacodylate buffer for a further 30 min. The pellets were post-fixed, dehydrated and embedded in Epon-Araldite as described in Chapter 2. Sections mounted on nitric acid-etched copper grids were stained with uranyl acetate and lead citrate, and examined at 80 kV using a Philips 420 EM.

Electrophoresis

Samples of isolated nuclei or nuclear matrices were suspended in distilled water, pooled and dried using a Savant Speed-Vac SC 100, and stored at -70°C . Dried samples were resuspended in 1-2 ml of buffer (SDS-reducing buffer without β -mercaptoethanol, see Appendix IV) and sonicated for 1-2 min. using a Sonics Materials Vibra-Cell sonicator with a probe. The amount of protein in each sample was determined using the bicinchoninic acid assay (Sigma) (Smith *et al.*, 1985). The protein in the samples was concentrated prior to electrophoresis by acetone precipitation as follows: acetone at room-temperature was added to the protein samples to a concentration of 80%. After 10 min incubation at room temperature, the protein was pelleted at 1500 rpm for 5 min at room temperature (Buecker, 1992). The supernatant was removed and the samples were resuspended in SDS-reducing buffer to a concentration of 2 μg protein per ml solution.

One dimensional SDS-PAGE was performed using the Bio-

Rad Mini Protean II electrophoresis cell (see Appendix IV for details on recipes and running conditions). Samples of isolated nuclei and nuclear matrices prepared from -Con A, +Con A, +DRB and 8 hr recovery cells were separated through 5% stacking and 12% separating gels, with 20 μ g of protein loaded per sample. The gels were run at 150 V constant voltage, and were stained with 0.1% Coomassie Brilliant Blue R-250, or with silver using the Bio-Rad silver staining kit (Bio-Rad). Gels were photographed using a Polaroid Cu-5 Land camera with type 665 film.

RESULTS

Immunofluorescence Labelling of Nuclear Matrices

Hoechst 33258 Staining and Phase Contrast Images

Hoechst 33258 binds specifically to DNA. In intact cells, the Hoechst staining reflected the organization of the nuclei in each sample. In -Con A samples (Figs. 10A, 14A), the nuclei were small, with intense Hoechst staining at the nuclear periphery, corresponding to the aggregates of condensed chromatin at the nuclear periphery in these nuclei. In +Con A samples (Figs. 11A, 15A), the nuclei were larger than nuclei in -Con A samples, with a more homogeneous staining of the nucleus with Hoechst. In +DRB samples (Figs. 12A, 16A), the Hoechst staining was slightly more intense at the nuclear periphery than in +Con A nuclei, due to the aggregation of condensed chromatin at the nuclear periphery in +DRB nuclei. In 8 hr recovered samples (Figs. 13A, 17A), the Hoechst staining resembled that in +Con A nuclei.

The pattern of Hoechst staining in isolated nuclei (Figs. 10B, 11B, 12B, 13B, 14B, 15B, 16B, 17B) was similar to that described in intact cells for all samples, but the intensity of the staining was generally lower in isolated nuclei than in intact cells. Hoechst staining was not detectable in nuclear matrices in any of the samples (see

Figs. 10C, 11C, 12C, 13C, 14C, 15C, 16C, 17C), indicating that DNA had been largely removed from these structures.

Phase contrast images of intact cells reflected the general nuclear morphology observed by conventional electron microscopy as described in Chapter 2. In -Con A samples (Figs. 10A'', 14A''), the cells were small, with their nuclei occupying most of the cell volume. There were no obvious nucleoli in these cells, and aggregates of condensed chromatin were visible at the nuclear periphery. In +Con A samples (Figs. 11A'', 15A''), the cells were much larger than -Con A cells. The nuclei were also larger than -Con A nuclei, and were surrounded by larger amounts of cytoplasm. Large nucleoli were visible, and there were no large aggregates of condensed chromatin observed at the nuclear periphery. In +DRB samples (Figs. 12A'', 16A''), condensed chromatin aggregates were visible at the nuclear periphery, but no organized nucleoli were observed. Cells in the 8 hr recovery samples (Figs. 13A'', 17A'') were similar in appearance to cells in the +Con A sample.

Phase contrast images of isolated nuclei (Figs. 10B'', 11B'', 12B'', 13B'', 14B'', 15B'', 16B'', 17B'') were similar to the images of nuclei in intact cells, but there was no visible cytoplasm around most isolated nuclei. Nuclear matrices (Figs. 10C'', 11C'', 12C'', 13C'', 14C'', 15C'', 16C'', 17C'') had little recognizable internal structure by phase contrast. Some matrices had visible

residual nucleoli, but the internal matrix did not have high contrast. In matrices isolated from +DRB cells, residual nucleoli were not observed.

Immunofluorescence Labelling

Cells and Nuclei

The labelling of PI1 and Sm antigens in intact cells of all samples (Figs. 10A', 11A', 12A', 13A', 14A', 15A', 16A', 17A') was as described in Chapter 2 (see Figs. 2 and 3). The labelling patterns for these antigens were not affected by nuclear isolation, and were indistinguishable from those observed in intact cells. However, the intensity of the fluorescence in isolated nuclei (Figs. 10B', 11B', 12B', 13B', 14B', 15B', 16B', 17B') was usually greater than the intensity seen in intact cells.

Matrices

In all of the nuclear matrix samples, the matrices tended to clump together and individual matrices were difficult to find. Many of the figures showing these samples, therefore, show clusters of several matrices.

PI1 - The PI1 labelling pattern of nuclear matrices prepared from -Con A cells (Fig. 10C') was similar to that in nuclei of -Con A cells (Fig. 10B'). The labelling pattern of PI1 in nuclear matrices isolated from mouse lymphocytes was also

Figure 10: Immunofluorescence labelling of PI1 in -Con A lymphocyte samples (A'-C'), counterstained with Hoechst 33258 (A-C), and phase contrast images of the samples (A''-C''). A-A'', samples of intact cells; B-B'', samples of isolated nuclei; C-C'', samples of isolated nuclear matrices. X 3500.

Figure 11: Immunofluorescence labelling of P11 in +Con A lymphocyte samples (A'-C'), counterstained with Hoechst 33258 (A-C), and phase contrast images of the samples (A''-C''). A-A'', samples of intact cells; B-B'', samples of isolated nuclei; C-C'', samples of isolated nuclear matrices. In the nuclear matrix sample, the structure on the right in C'', that is unstained in C', is a dead cell. X 3500.

C.

Figure 12: Immunofluorescence labelling of PI1 in +DRB

lymphocyte samples (A'-C'), counterstained with Hoechst 33258 (A-C), and phase contrast images of the samples (A''-C''). A-A'', samples of intact cells; B-B'', samples of isolated nuclei; C-C'', samples of isolated nuclear matrices. Arrowheads indicate nuclear matrices containing internal label. X 3500.

Figure 13: Immunofluorescence labelling of P11 in 8 hr recovery lymphocyte samples (A'-C'), counterstained with Hoechst 33258 (A-C), and phase contrast images of the samples (A''-C''). A-A'', samples of intact cells; B-B'', samples of isolated nuclei; C-C'', samples of isolated nuclear matrices. X 3500.

C

similar to that observed in matrices isolated from bovine lymphocytes (Bladon, 1986). There was little internal label, and a sparse peripheral label. The intensity of the labelling was as bright as or brighter than in intact cells or isolated nuclei.

Figure 11C' shows a nuclear matrix fraction prepared from +Con A cells, with several isolated nuclear matrices next to a matrix within an unbroken residual cell, emphasizing the shrinkage that took place during matrix preparation. The peripheral portion of the PI1 label was still intact, indicating that the pore complexes were not extracted during the matrix isolation. The internal label seemed to be as bright as or brighter than in nuclei. The organization of the label into patches in the interior was not as obvious in matrices as it was in nuclei, perhaps because of the small size of the matrices. These results are similar to those obtained for PI1 labelling of matrices isolated from bovine lymphocytes (Bladon, 1986).

In nuclear matrices prepared from +DRB cells, the peripheral component of the PI1 labelling (Fig. 12C') was slightly less bright than in +Con A samples. The peripheral label appeared to be clumped, as in the samples of intact cells and isolated nuclei. The internal component of PI1 was less brightly labelled than in +Con A matrices, and in some matrices, the internal label was quite faint. The internal label did not appear to be aggregated as in nuclei

in +DRB cells.

PI1 labelling in nuclear matrices prepared from 8 hr recovered cells (Fig. 13C') was similar in appearance to PI1 in matrices prepared from +Con A samples (Fig. 11C'), with the PI1 labelling pattern resembling a condensed version of the labelling in nuclei.

Sm - The anti-Sm labelling pattern in nuclear matrices prepared from -Con A cells (Fig. 14C') seemed slightly less bright than in nuclei. The matrices were small and compact in structure, as in the PI1-labelled samples. The general distribution of Sm, however, resembled that in nuclei of intact cells and isolated nuclei, that is, small patches of label throughout the nucleoplasm.

The anti-Sm labelling in nuclear matrices prepared from +Con A cells (Fig. 15C') was still very bright, although not quite as bright as in nuclei in intact cells. The label still seemed to be organized into patches in the interior, and the residual nucleolus was still devoid of label. In general, the Sm distribution in nuclear matrices appeared to be a compacted version of the distribution in intact nuclei.

In nuclear matrices prepared from +DRB cells (Fig. 15C') and from 8 hr recovered samples (Fig. 17C'), labelling of the Sm antigen was not observed. The only fluorescent signal was very faint fluorescence of the internal matrix, that was indistinguishable from control samples incubated

Figure 14: Immunofluorescence labelling of the Sm antigen in -Con A lymphocyte samples with antibody Y12 (A'-C'), counterstained with Hoechst 33258 (A-C), and phase contrast images of the samples (A''-C''). A-A'', samples of intact cells; B-B'', samples of isolated nuclei; C-C'', samples of isolated nuclear matrices. X 3500.

Figure 15: Immunofluorescence labelling of the Sm antigen in +Con A lymphocyte samples with antibody Y12 (A'-C'), counterstained with Hoechst 33258 (A-C), and phase contrast images of the samples (A''-C''). A-A'', samples of intact cells; B-B'', samples of isolated nuclei; C-C'', samples of isolated nuclear matrices. X 3500.

Figure 16: Immunofluorescence labelling of the Sm antigen in +DRB lymphocyte samples with antibody Y12 (A'-C'), counterstained with Hoechst 33258 (A-C), and phase contrast images of the samples (A''-C''). A-A'', samples of intact cells; B-B'', samples of isolated nuclei; C-C'', samples of isolated nuclear matrices. X 3500.

Figure 17: Immunofluorescence labelling of the Sm antigen in 8 hr recovery lymphocyte samples with antibody Y12 (A'-C'), or phosphate buffered saline in place of Y12 (D'), counterstained with Hoechst 33258 (A-D), and phase contrast images of the samples (A''-D''). A-A'', samples of intact cells; B-B'', samples of isolated nuclei; C-C'', D-D'', samples of isolated nuclear matrices. X 3500.

with PBS in the place of primary antibody (Fig. 17D').

Electron Microscopy

-Con A: No structures resembling isolated nuclear matrices were found in the blocks made from -Con A samples. The only examples of nuclear matrices obtained from unstimulated cells were matrix structures surrounded by large amounts of residual cytoplasm (see Fig. 18). Portions of a residual nuclear envelope were visible in some areas. These matrices resembled -Con A matrices previously isolated from mouse, human and bovine lymphocytes (Setterfield *et al.*, 1983; Bladon *et al.*, 1988). There were large, empty spaces resembling the size and distribution of condensed chromatin in intact cells (cf. Fig. 5, Chapter 2). The internal matrix regions contained small clumps of fibrogranular material, and some small patches of fibrillar material that did not contain granules. Measurements of the granular material (not shown) indicated that the granules were the same size as ICG in intact cells, i. e. 200-250 Å. The distribution of the granular material was also similar to that of ICG. These matrices occasionally contained a small residual nucleolus, which appeared very electron dense and showed little contrast (Fig. 18). The residual nucleoli

Figure 18: Electron micrograph of a nuclear matrix isolated from -Con A samples. This nuclear matrix is embedded within an extensive residual cytoplasm. RNu, residual nucleolus; arrowheads, residual nuclear envelope.
X 31 000.

seemed to be surrounded by a thin rim of fibrillar material.

+Con A: The isolated matrices were much smaller than the nuclei in situ (cf. Figs. 6A and 19A), indicating that the extraction process leads to shrinking and/or collapse of the residual nuclear structure. This phenomenon has been described previously for bovine lymphocyte nuclear matrices (Bladon et al., 1988). No clear residual nuclear envelope was observed in these samples. The presence of a residual nuclear envelope was expected, based on the immunofluorescence results with P11, the peripheral component of which recognizes the pore complexes (Benavente et al., 1989; Chaly et al., 1989; Dabauvalle et al., 1988). It may be that the residual nuclear envelope in these matrices was obscured by the peripheral fibrillar portions of the internal matrix. This phenomenon may be the result of compression of the matrices during centrifugation.

Figure 19A shows a typical example of the nuclear matrices obtained from the +Con A samples. There was a large, very electron dense residual nucleolus, with a homogeneous appearance to its structure. As in -Con A matrices, the residual nucleoli in these samples also appeared to be surrounded with a rim of fibrillar material, and the spaces within the nucleolus were lined with similar fibrillar material. The fibrils surrounding the nucleoli

Figure 19: Electron micrographs of nuclear matrices

isolated from +Con A samples. A, a typical +Con A nuclear matrix. B, a compacted nuclear matrix from the same sample. RNu, residual nucleolus; ICG, interchromatin granule clusters; arrows, cytoplasmic filaments; NB, nuclear body. A, X 46 000; B, X 50 000.

19A

19B

seemed to connect to the fibrillar material in the internal matrix. None of the fibrillar material in the internal matrices appeared to be very electron dense.

There were many empty spaces within the internal matrix that were approximately the same size as the small aggregates of condensed chromatin in intact nuclei (cf. Fig. 6, Chapter 2). The spaces around the nucleolus may be locations of extracted perinucleolar chromatin. The internal matrix in these samples was also fibrogranular, as in the unstimulated lymphocyte nuclear matrix (Fig. 18). The granules seemed relatively large, but most were the same size as ICG. The small clusters of granules appeared to be connected to one another in a network. When +Con A nuclear matrices were compared to nuclei in bismuth-stained +Con A samples (cf. Fig. 19A and Fig. 6C), there seemed to be more granular material in the internal nuclear matrix than can be accounted for solely by ICG. This result suggests that not all of the granular material in the matrices of +Con A samples is ICG.

Figure 19B shows a more compacted nuclear matrix, that may have collapsed during the extraction or during formation of the pellets for electron microscopy. This nuclear matrix contains nuclear bodies, which have previously been shown to be associated with the nuclear matrix (Chaly et al., 1983b). Nuclear bodies were found in some nuclear matrix profiles in all of the samples. In Figure 19B there is one large type

IVa nuclear body and two type II nuclear bodies (see Chaly et al., 1983a for nuclear body nomenclature and descriptions). The nuclear bodies were connected to the fibrogranular internal matrix by fibrillar extensions from the surfaces of the nuclear bodies.

+DRB: Figure 20A shows a typical nuclear matrix isolated from a +DRB cell. In contrast to +Con A matrices, no organized residual nucleolus was detected in +DRB nuclear matrices. Instead, there were several electron dense masses located in clusters in the interior of the matrices, resembling in morphology and distribution the structures interpreted as nucleolar remnants in intact nuclei (see Fig. 7A-D). The nucleolar remnants were also each surrounded by a thin layer of fibrillar material similar to the fibrillar material around the residual nucleolus in the +Con A samples.

Although there were large quantities of granular material in the +DRB matrices, this granular material was not organized in the same way as the ICG in intact cells. The granular material in +DRB matrices appeared to be connected in a network, as in +Con A matrices (Fig. 19A), and seemed to be organized into many small clusters of granules. The large clusters of ICG anticipated on the basis of bismuth staining of intact cells (see Fig. 7C) were not obvious.

Figure 20: Electron micrographs of nuclear matrices

isolated from +DRB samples. A, a nuclear matrix showing few cytoplasmic contaminants. B, a nuclear matrix prepared from an unbroken cell. RNu, residual nucleolus; ICG, interchromatin granule clusters; arrowheads, residual nuclear envelope; arrows, filamentous cytoplasmic contaminants. A, X 50 000; B, X 29 500.

Figure 20B shows a nuclear matrix prepared from a +DRB cell that was still surrounded by cytoplasm after homogenization. In these matrices, the shape and size of the nuclei of origin are maintained, and a residual nuclear envelope is clearly visible, surrounded by extensive cytoplasmic remnants. The internal matrix shown in Figure 20B was less densely aggregated than the internal matrix of cytoplasm-free isolated matrices. Other than this difference, the internal matrices of the two samples were similar. Judging from the size of these two matrices (cf. magnification of Fig. 20A and Fig. 20B), Figure 20B seems to have been less compacted during the extraction procedure than matrices isolated from nuclei that were not surrounded by large amounts of cytoplasm. This suggests that the presence of large amounts of cytoplasm around the nucleus protects the nucleus from the shrinking and compaction that seemed to occur with the extraction of soluble material. This phenomenon has been previously reported for nuclear matrices prepared from bovine lymphocytes (Bladon et al., 1988).

8 hr recovery: The isolated nuclear matrices prepared from these samples (see Fig. 21) were, in general, very similar in morphology to the matrices isolated from +Con A samples (see Fig. 19). The residual nucleolus was perhaps the only

Figure 21: Electron micrograph of a nuclear matrix isolated from 8 hr recovered samples. RNu, residual nucleolus; ICG, interchromatin granule clusters; arrows, filamentous cytoplasmic contaminants. X 38 500

structure that differed from that of the +Con A matrix. The overall morphology of the nucleoli from the two types of matrices was similar but the residual nucleoli in recovered matrices seemed to have more or larger spaces within them than the residual nucleoli of the +Con A samples. The internal matrix was also similar in appearance to that observed in the +Con A matrix, with the granular material appearing as a network of ICG and other granules in the internal matrix.

One-Dimensional SDS-PAGE

One-dimensional gels were run for samples of isolated nuclei and for isolated nuclear matrices. One gel of each group of samples was stained with Coomassie Brilliant Blue R-250 and one with silver.

In general, the protein separation patterns of +Con A, +DRB, and 8 hr recovery nuclei (Fig. 22) and nuclear matrices (Fig. 23) showed few differences in one dimensional gels. Differences between one-dimensional SDS-PAGE separations of -Con A and +Con A nuclei and matrices have been previously described for mouse lymphocytes (Hall, 1982) and the results presented here are consistent with those.

The lane containing the protein from +DRB samples of both nuclei and matrices seems to have been overloaded, as the intensity of the staining in most or all of the bands seemed higher than that in the lanes containing the other

3 of/de 3

PM-1 3½"x4" PHOTOGRAPHIC MICROCOPY TARGET
NBS 1010a ANSI/ISO #2 EQUIVALENT

PRECISIONSM RESOLUTION TARGETS

Figure 22: One-dimensional SDS-PAGE gels of protein from isolated lymphocyte nuclei stained with Coomassie Brilliant Blue R-250 (A) or Bio-Rad silver stain (B). Lane 1, -Con A nuclei; lane 2, +Con A nuclei; lane 3, +DRB nuclei; lane 4, 8 hr recovered nuclei. Molecular weight standards used are marked on the left side of A.

Figure 23: One-dimensional SDS-PAGE gels of protein from nuclear matrices isolated from lymphocytes stained with Coomassie Brilliant Blue R-250 (A) or Bio-Rad silver stain (B). Lane 1, +Con A nuclear matrices; lane 2, +DRB nuclear matrices; lane 3, 8 hr recovered nuclear matrices. Molecular weight standards used are marked on the left side of A.

samples. Apart from this apparent discrepancy in loading, there were few differences among the banding patterns of the +Con A, +DRB and 8 hr recovery samples. From these gels, it appears that inhibition of transcription by DRB caused no major changes in the composition of the nuclear matrix, and the composition of the matrix appeared to be maintained during recovery from DRB.

There were, however, a few differences in the protein separations between the +Con A, +DRB and 8 hr recovery samples, but the proteins cannot be identified based on information from one-dimensional gels alone. Differences between protein profiles of samples of nuclei (Fig. 22) were difficult to detect by either Coomassie staining or silver staining. There were some bands that were less intensely stained in some lanes than in others, but the loading problem mentioned above may account for these differences. In the gels of nuclear matrices stained with silver (Fig. 23B), the main difference between +Con A polypeptides and +DRB polypeptides occurred in the lower molecular weight region. Two intensely stained bands, one at 13.7 kD and one at 16.3 kD, were present in the +DRB matrices, but were not observed in the +Con A or 8 hr recovery matrices. There were also generally fewer distinct bands in the 8 hr recovery samples than in the +Con A samples. Due to the limitations of this technique, the results of the one-dimensional gels will not be discussed further. Two-

dimensional electrophoresis, in which the proteins are separated by isoelectric point, and then by molecular weight (O'Farrell, 1975), would facilitate identification of individual proteins. Data from two-dimensional gels were not obtained, as preliminary experiments showed that there was insufficient protein in the matrix samples available, and insufficient time to accumulate enough protein.

DISCUSSION

In this chapter, the effects of inhibition of transcription by DRB on the structure and composition of the nuclear matrix were examined. Electron microscopy of isolated nuclear matrices prepared from aliquots of -Con A, +Con A, +DRB and 8 hr recovery cells indicated that the structural integrity of the nuclear matrix was little affected by DRB, and that the structure of the isolated nuclear matrix does indeed mirror the structure of the interchromatinic regions in situ. However, immunofluorescence labelling with antibodies to the nuclear matrix antigens PI1 and Sm indicated that DRB released the association of the Sm antigen with the nuclear matrix, and perhaps also altered the association of PI1 with the matrix to a lesser extent.

The nuclear matrices obtained from samples of -Con A and +Con A nuclei were similar in morphology to those previously described for nuclear matrices prepared from murine and bovine lymphocytes (Setterfield et al., 1983, 1985; Bladon et al., 1988). Their structures reflected the organization of the in situ interchromatin regions of the respective nuclei of origin.

In nuclear matrices prepared from +DRB nuclei, the major structural features of the interchromatinic regions of the nuclei were also maintained. The residual nucleoli of

the matrices resembled the disorganized nucleoli in the intact nuclei, indicating that DRB had no effect on the association of the nucleolar remnants with the nuclear matrix. This was true of both the isolated nuclear matrices and the matrices prepared from unbroken cells. The large aggregates of granular material expected to be found in the internal matrix based on the bismuth staining results on intact cells were not observed, however, although +DRB matrices did contain substantial amounts of granular material. It may be that DRB had an effect on the association of the ICG with the nuclear matrix, so that they no longer remained aggregated during matrix preparation, but remained associated with the matrix.

The matrix of +DRB also seemed to be quite rich in fibrogranular material. This result was consistent with the EM cytochemistry data described in Chapter 2, in which the nucleoplasm was not cleared of most of its fibrogranular material during inhibition of transcription, as had been the case in other transcription-arrested systems (Brasch and Sinclair, 1978; Crowley and Brasch, 1987; Nickerson *et al.*, 1989; Sinclair and Brasch, 1978). The structure of the fibrogranular matrix in +DRB samples was comparable to that of the matrix from +Con A samples, aside from the residual nucleolus and the aggregates of granular material. This result suggested that the structure of the lymphocyte nuclear matrix itself was not drastically altered when

transcription was inhibited by DRB. In contrast, Crowley and Brasch (1987) found that nuclear matrices isolated from α -amanitin-treated chicken liver cells appeared to be more skeleton-like than matrices isolated from untreated cells.

The general trend of the results is, however, consistent with previous work relating isolated nuclear matrix structure to the morphology of the interchromatin regions in situ. It has been shown in several systems, including erythrocyte activation (LaFond and Woodcock, 1983; LaFond et al., 1983; Woodcock and Woodcock, 1986), α -amanitin treatment in liver cells (Crowley and Brasch, 1987), plant cell nuclear matrices (Stoilov et al., 1989; Krachmarov, 1991), and lymphocyte activation (Setterfield et al., 1983, 1985; Bladon et al., 1988), that the structure of the isolated nuclear matrix reflects the structure of the interchromatinic regions in situ, and the results presented here are consistent with this conclusion.

The results of the immunofluorescence labelling indicate, however, that the association of individual nuclear matrix antigens, specifically PI1 and Sm, with the nuclear matrix was affected by DRB.

In the case of PI1, both the peripheral and internal components of the label were retained during isolation of nuclear matrices from DRB-treated samples, although the internal label no longer appeared to be aggregated and the intensity of the label was diminished from that in +Con A

matrices. The peripheral component of PI1 labelling is due to a conserved 68 kd nucleoporin glycoprotein (Benavente et al., 1989; Chaly et al., 1989; Dabauvalle et al., 1988). The results in this study confirm that the pore complexes, and this nucleoporin specifically, remain part of the isolated nuclear matrix. Preparation of nuclear matrices in situ from fibroblasts (Chaly et al., 1985) has similarly shown that the distribution of PI1 is not affected by the extraction procedure.

Chaly et al. (1985) suggested that the organization of some nuclear matrix components in nuclear matrix preparations can be predicted from their organization in situ. The results presented here suggest that the above concept can be extended, in part, for the PI1 antigen, to include transcriptionally active cells, but may not always be true for transcriptionally arrested cells. Although the identity and function of the internal component of PI1 labelling are still not clear, it has been shown to co-localize with Sm in -Con A, +Con A, +DRB and recovery samples of intact cells (Chaly et al., 1988), and it was proposed that the internal component of PI1 may be involved in some step of RNA metabolism.

The distribution of the Sm antigen in -Con A and +Con A samples was not affected by the matrix isolation and extraction. However, in +DRB and 8 hr recovery samples, the Sm antigen was not detected. It was either extracted during

nuclear matrix preparation, or the antigen was made inaccessible to the antibody during matrix isolation.

I consider it more likely that the antigen was extracted, as the antigen has been detected in a wide range of cell types and nuclear fractions prepared for immunolabelling by various procedures. If indeed it was extracted, the explanation for these results remains unclear. Demonstration of an association of the snRNPs with the nuclear matrix has been variable. Some workers have reported that snRNPs remain associated with the matrix (e.g. Spector *et al.*, 1983; Reuter *et al.*, 1984), while others report that such an association is tenuous, and easily disrupted (Nakayasu *et al.*, 1982). It is possible that the association of snRNPs with the nuclear matrix is indeed tenuous, taking the form, perhaps, of an indirect association through other proteins that are more strongly matrix-associated. Sm has been shown to co-localize with other nuclear proteins and snRNA, and so its association with the nuclear matrix may be mediated by one or more of these other components. It is possible that the other component(s) has/have a stronger association to the matrix than does Sm, and so during isolation of nuclear matrices, Sm is disconnected from the matrix, but maintains its connection to the other component and is not extracted. During inhibition of transcription, the bond between Sm and the other component may be disrupted, and Sm is extracted

during nuclear matrix isolation.

In the 8 hr recovered samples, the association of the snRNPs factors with the nuclear matrix remained weakened or absent. This result is more difficult to explain, since the snRNP distribution in nuclei of intact cells after 8 hr of recovery was similar to that in +Con A samples, and since the structure of the internal nuclear matrix was also similar in +Con A and 8 hr recovery samples. Apparently, 8 hr of recovery was not long enough for the association of snRNPs with the nuclear matrix to return to its pre-DRB state.

No metabolic activity has yet been shown to occur within the ICG, although labelling with ^3H -uridine in cells that have been pulsed with label and chased with cold uridine in the presence of DRB has shown that they are sites of accumulation of rRNA in such cells (Puvion, 1982; Puvion *et al.*, 1984a). They have also been shown normally to contain the Sm antigen (Fakan *et al.*, 1984), the non-snRNP splicing factor SC-35 (Fu and Maniatis, 1990; Spector *et al.*, 1991), snRNA (Reuter *et al.*, 1984) and phosphoproteins (Locke and Huie, 1977; Chapter 2). These observations have led to the hypothesis that the ICG serve as storage sites for snRNPs (Fakan *et al.*, 1984), and more recently, that ICG may be sites of spliceosome assembly (Spector *et al.*, 1991). The mechanism of action of DRB is currently under dispute. Some workers report that it acts to inhibit initiation of

transcription (Egyházi, 1975; Egyházi et al., 1982; Zandomeni et al., 1982, 1983), allowing transcripts that have already been initiated to be completely transcribed. Others indicate that it causes premature termination of transcripts, with no effects on initiation (Tamm and Kikuchi, 1979; Laub et al., 1980; Chodosh et al., 1989). It has not been determined whether DRB affects splicing of pre-mRNA transcripts, and so the effect of DRB on the splicing components is unknown. It may be that DRB treatment affects the storage of snRNPs within ICG so that they are released during the nuclear matrix preparation, and it may take long periods of time to re-accumulate snRNPs within the ICG during recovery.

The results presented here suggest that the function of a nuclear domain is essential to its structural organization, and, perhaps, to its composition. When transcription was inhibited with DRB, the function of the transcription domains was inhibited. Cessation of the function of the domain resulted in a morphological restructuring of the components of that domain in intact cells, and also led to changes in the association of polypeptide components of the domain with the nuclear matrix fraction.

SUMMARY AND CONCLUSIONS

The EM ultrastructural data presented in Chapter 2 for intact nuclei indicated that lymphocyte nuclei were not fully recovered from the effects of DRB in 8 hr. The immunofluorescence experiments using anti-Sm and nuclear matrices also indicated that recovery of nuclear matrix function was not complete within 8 hr. Recovery of nuclear function from the effects of DRB has not been extensively studied; previous studies on recovery focused mainly on morphological recovery. It is possible that while morphology returns to normal after removal of the drug, DRB may have a less reversible effect on nuclear function. The absence of Sm from 8 hr recovered nuclear matrices may indicate an impairment in nuclear function caused by DRB. Recovery time could be extended further, and DNA replication be measured by ^3H -thymidine incorporation, to determine whether cells recovering from DRB can enter S phase. Recovery could be followed using light microscopy to determine if recovering cells can enter mitosis.

The immunofluorescence data, and the preliminary one-dimensional gel work indicated that some components of the nuclear matrix were affected by DRB. To identify other components of the nuclear matrix that may be affected by DRB treatment, two-dimensional gels should be run for +Con A, +DRB and 8 hr recovered samples. These gels would also

indicate whether the composition of the matrix truly recovers from the effects of DR3.

APPENDIX I: Protocol for isolation of lymphocytes from mouse spleen.

This protocol, adapted from Judy Little (University of Ottawa), was used to isolate splenocytes from Balb/c mice at least 8 weeks old.

1. Kill the mouse by cervical dislocation and lay it on its right side, on paper towel. Swab the mouse with 70% ethanol and place it in the tissue culture hood.
2. Using sterile scissors and forceps, cut a hole in the skin on the left side of the mouse, just below the ribs. Rip the skin of the mouse away from its body.
3. With a second set of sterile scissors and forceps, cut open the peritoneum to expose the spleen. Separate the spleen from blood vessels and connective tissue.
4. Place the spleen on a sterile metal screen in a sterile 60 mm glass petri dish. Cover the spleen with 4 ml of complete RPMI 1640 medium with 6% (CPSR-2 and antibiotics).
5. Push the spleen through the screen using the flat end of a sterile plastic 5 ml syringe plunger. Do not grind the tissue.
6. Transfer the cell suspension to a 15 ml conical centrifuge tube, using a Pasteur pipette. Rinse the screen with a further 4 ml of complete medium, and

transfer this medium to the centrifuge tube.

7. Underlay the cell suspension with 2-3 ml cold CPSR-2 and let sit on ice for 5 minutes. This step allows fat and large pieces of tissue to settle out of the cell suspension.
8. Carefully remove the cell suspension with a Pasteur pipette, and transfer it to another 15 ml centrifuge tube. Try to remove as little of the CPSR-2 underlay as possible. Centrifuge the suspension for 5 minutes at 1500 rpm in a benchtop clinical centrifuge.
9. Discard the supernatant. Resuspend the pellet in 2-3 ml cold 0.83% NH_4Cl . Let sit on ice 8-10 minutes, to lyse the erythrocytes.
10. Underlay the suspension with 2-3 ml cold CPSR-2, using a Pasteur pipette, taking care not to mix the two layers. Centrifuge at 1500 rpm for 5 minutes, as above.
11. Discard the supernatant and resuspend the cells in complete RPMI 1640 medium to a density of 2.5×10^6 cells/ml, after determining cell viability by Trypan Blue exclusion. If the cultures are to be stimulated, add 1 $\mu\text{l/ml}$ 5×10^{-2} M β -mercaptoethanol. Incubate at 37°C with 5% CO_2 for at least 1 hr before adding $4\mu\text{g/ml}$ concanavalin A.

APPENDIX II: Comparison of stimulation indices between cultures supplemented with fetal bovine serum and CPSR-2.

Previously, we had used RPMI 1640 culture medium supplemented with fetal bovine serum (FBS) to culture lymphocytes. Sigma makes a serum replacement, Controlled Process Serum Replacement type-2 (CPSR-2), that is specifically designed for lymphocyte proliferation assays. This serum replacement is made from bovine plasma, but is lower in mitogenic activity than serum (Sigma). CPSR-2 was tested as a replacement for FBS in the following experiments.

After lysis of the erythrocytes with NH_4Cl (see Appendix I), the lymphocyte pellet was resuspended in 10 ml of RPMI 1640, supplemented with 6% FBS and 1% antibiotics. The cell suspension was then divided into two 5 ml aliquots. One aliquot was centrifuged to pellet the cells, and the medium was removed. This pellet was then resuspended in RPMI 1640 supplemented with 6% CPSR-2 and 1% antibiotics. Both cell suspensions were then adjusted to densities of 2.5×10^6 cells/ml with the appropriate medium. After 1 hour in culture, the cells were stimulated with 4 $\mu\text{g/ml}$ Con A, as described in Chapter 2, Materials and Methods, and Appendix I.

The ^3H -thymidine assay for lymphocyte proliferation was performed as follows: during each experiment, aliquots of 2

ml each of -Con A and +Con A cells were reserved. At 46 hr p.s., cell viability of the aliquots was determined by Trypan Blue exclusion, and then the aliquots were fed 4 $\mu\text{Ci/ml}$ ^3H -thymidine. After 2 hr incorporation, the aliquots were harvested and counted in a liquid scintillation counter, as described in Chapter 1 for ^3H -uridine incorporation. The scintillation counts were corrected for cell viability, and the stimulation index for the experiment was determined by dividing the counts for the +Con A sample by the counts for the -Con A sample. Table 2 shows a comparison of stimulation indices between cells cultured with FBS and cells cultured with CPSR-2, for 2 separate experiments.

Table 2. Comparison of stimulation indices between samples cultured with FBS and samples cultured with CPSR-2.

	-Con A cpm/ 10^6 cells	+Con A cpm/ 10^6 cells	Stimulation index
FBS	1. 5889.3	76 605.3	13.0
	2. 6982.4	49 462.6	7.1
CPSR-2	1. 2211	53 976.4	24.4
	2. 3942.6	37 841.3	9.6

In each experiment, the stimulation index was higher in the cells cultured with CPSR-2. When the immunofluorescence labelling patterns of the cultures were examined (data not

shown), there was no observable difference due to CPSR-2. Because the stimulation index is higher with CPSR-2 than with FBS, and also because CPSR-2 is considerably less expensive than FBS, the experiments in this thesis were performed using lymphocytes that had been cultured with CPSR-2.

APPENDIX III: Bismuth staining for electron microscopy.

This protocol was adapted from those described by Locke and Huie (1976), and by Hayat (1986).

1. Fix in freshly prepared 4% paraformaldehyde, in 0.1 M sodium cacodylate buffer, pH 7.4, for 4 hr at room temperature.
2. Wash in 0.05 M sodium cacodylate buffer, with 10% sucrose, pH 7.4, 30 minutes to overnight, at room temperature.
3. Stain in bismuth staining solution (see below) for 3 hr at room temperature, on a rotary shaker.
4. Wash in several changes of 0.1 M triethanolamine-HCl, pH 7.0, for 30 minutes at room temperature.
5. Post-fix in 1% OsO₄ in 0.5 M sodium cacodylate buffer, pH 7.4, for 1 hr on ice.
6. Dehydrate in ethanol and embed in resin.

To prepare the bismuth staining solution:

Solution A: Dissolve 400 mg sodium tartrate in 10 ml of 1 N NaOH. Add this solution dropwise to 200 mg bismuth subnitrate (BiONO₃·H₂O).

Solution B: 0.2 M triethanolamine-HCl, pH 7.0.

Working solution: Add 1 part solution A to 2 parts solution B, i. e. 10 ml solution A are added to 20 ml solution B. Adjust the pH to 7.0. The staining solution is stable for only a few hours.

**APPENDIX IV: Recipes for solutions and running conditions
for one-dimensional electrophoresis using the Bio-Rad Mini-
Protean II system.**

Preparation of Solutions

1. Acrylamide/bis (30% T, 2.67% C)

29.2 g acrylamide

0.8 g N'N'-bis'methylene-acrylamide

Make to 100 ml with distilled water. Filter through
Whatman #1 paper and store at 4°C in the dark, 30 days
maximum.

2. 1.5 M Tris-HCl, pH 8.8

18.15 g Tris base

Add about 60 ml distilled water, and adjust the pH with
1M HCl. Make to 100 ml with distilled water and store
at 4°C.

3. 0.5 M Tris-HCl, pH 6.8

6 g Tris base

Add about 60 ml distilled water, and adjust the pH with
1M HCl. Make to 100 ml with distilled water and store
at 4°C.

4. 10% sodium dodecyl sulphate (SDS) (w/v)

Dissolve 10 g SDS in water with stirring, and adjust volume to 100 ml with distilled water.

5. SDS Reducing Buffer (8 ml total volume)

distilled water	4.0 ml
0.5 M Tris-HCl, pH 6.8	1.0 ml
glycerol	0.8 ml (1.01 g)
10% SDS	1.6 ml
β -mercaptoethanol	0.4 ml
0.05% bromophenol blue	0.2 ml

Store at room temperature. Dilute the sample at least 1:4 with SDS reducing buffer, and heat for 4 minutes at 95°C.

6. 5X Running Buffer, pH 8.3

Tris base	9 g
glycine	43.2 g
SDS	3 g

Make to 600 ml with distilled water. Store at 4°C.

Dilute 60 ml of 5X stock with 240 ml distilled water for one electrophoretic run.

7. Separating Gel Preparation (12% gel)

Distilled water	3.35 ml
1.5 M Tris-HCl, pH 8.8	2.5 ml
10% SDS stock	100 μ l
acrylamide/bis (30% stock)	4.0 ml
10% ammonium persulfate	50 μ l
TEMED*	5 μ l

This makes 10 ml of monomer solution. Any volume of monomer solution can be prepared by using multiples of the 10 ml recipe.

* TEMED is N,N,N',N'-tetramethylethylenediamine

8. Stacking Gel Preparation (5% gel)

Distilled water	5.7 ml
0.5 M Tris-HCl, pH6.8	2.5 ml
10% SDS	100 μ l
acrylamide/bis (30% stock)	1.7 ml
10% ammonium persulfate	50 μ l
TEMED	5 μ l

This makes 10 ml of monomer solution, enough for 2 minigels of all thicknesses.

Sample Loading

The Bio-Rad manual states that an ideal protein load for a complex sample is 5-10 μ g, but it was found that this amount of protein was not enough to give good staining

intensity of the bands. Both isolated nuclei and nuclear matrices were run on gels using 20 μ g of protein per sample, as this amount of protein gave good staining intensity of the major bands. Preliminary experiments (not shown) suggested that up to 300 μ g of nuclear protein are necessary to obtain good staining intensity with two-dimensional gels, in contrast to the 5-10 μ g of protein recommended by the Bio-Rad manual.

Running Conditions

The conditions recommended in the Bio-Rad manual are 200 V, constant voltage. Use of this voltage gave a "smile" pattern to the gels, with the bands in the outermost lanes curving upwards at the outer edges. Reduction of the voltage to 150 V, constant voltage, eliminated the "smile" effect.

REFERENCES

- Adolph, K. W. (1980). Organization of chromosomes in HeLa cells: Isolation of histone-depleted nuclei and nuclear scaffolds. *J. Cell Sci.* **42**, 291-304.
- Aebi, U., Cohn, J. B., Buhle, L. and Gerace, L. (1986). The nuclear lamina is a meshwork of intermediate type filaments. *Nature* **323**, 560-564.
- Akey, C. W. (1990). Visualization of transport-related configurations of the nuclear pore transporter. *Biophys. J.* **58**, 341-355.
- Akey, C. W. (1989). Interactions and structure of the nuclear pore complex revealed by cryo-electron microscopy. *J. Cell Biol.* **109**, 955-970.
- Akey, C. W. and Goldfarb, D. S. (1989). Protein import through the nuclear pore complex is a multistep process. *J. Cell Biol.* **109**, 971-982.
- Alberts, B., Dennis, B., Lewis, J., Raff, M., Roberts, K. and Watson, J. (1989). Molecular Biology of the Cell, 2nd edition. New York: Garland Publishing, Inc.

- Andersson, K., Bjorkroth, B. and Daneholt, B. (1984).
Packing of a specific gene into higher order structures
following repression of RNA synthesis. *J. Cell Biol.*
98, 1296-1303.
- Balhorn, R. (1982). A model for the structure of chromatin
in mammalian sperm. *J. Cell Biol.* **93**, 298-305.
- Belgrader, P., Dey, R. and Berezney, R. (1991a). Molecular
cloning of matrin 3. *J. Biol. Chem.* **266**, 9893-9899.
- Belgrader, P., Siegel, A. J. and Berezney, R. (1991b). A
comprehensive study on the isolation and
characterization of the HeLa S3 nuclear matrix. *J.*
Cell Sci. **98**, 281-291.
- Benavente, R., Dabauvalle, M.-C., Scheer, U., and Chaly, N.
(1989). Functional role of newly formed pore complexes
in postmitotic nuclear reorganization. *Chromosoma* **98**,
233-241.
- Benavente, R. and Krohne, G. (1986). Involvement of nuclear
lamins in postmitotic reorganization of chromatin as
demonstrated by microinjection of lamin antibodies. *J.*
Cell Biol. **103**, 1847-1854.

- Benyajati, C. and Worcel, A. (1976). Isolation, characterization and structure of the folded chromosome of Drosophila melanogaster. *Cell* 9, 393-407.
- Berezney, R. (1991). The nuclear matrix: a heuristic model for investigating genomic organization and function in the cell nucleus. *J. Cell. Biochem.* 47, 109-123.
- Berezney, R. (1984). Organization and functions of the nuclear matrix. In Chromosomal nonhistone proteins, vol. IV: Structural associations. (ed. L.S. Hnilica), pp 119-180, Boca Raton, Fla.: CRC Press, Inc.
- Berezney, R. (1979). Dynamic properties of the nuclear matrix. In The Cell Nucleus. Vol 7. (ed. H. Busch), pp413-456, New York: Academic Press.
- Berezney, R. and Coffey, D. S. (1974). Identification of a nuclear protein matrix. *Biochem. Biophys. Res. Comm.* 60, 1410-1417.
- Bernhard, W. (1969). A new staining procedure for electron microscopical cytology. *J. Ultrastruct. Res.* 27, 250-265.

- Berrios, M., Osheroff, N. and Fisher, P. A. (1985). In situ localization of DNA topoisomerase II, a major polypeptide component of the Drosophila nuclear matrix fraction. Proc. Natl. Acad. Sci. USA. **82**, 4142-4146.
- Beyer, A. L., Miller, O. L., Jr., and McKnight, S. L. (1980). Ribonucleoprotein structure in nascent hnRNA is nonrandom and sequence-dependent. Cell **20**, 75-84.
- Biggioera, M. Fakan, S., Kaufmann, S. H., Black, A., Shaper, J. H. and Busch, H. (1989). Simultaneous immunoelectron microscopic visualization of protein B23 and C23 distribution in the HeLa cell nucleolus. J. Histochem. Cytochem. **37**, 1371-1374.
- Bladon, T. S. (1986). A biochemical, ultrastructural and immunocytochemical analysis of nuclear matrix in concanavalin A-stimulated lymphocytes. PhD. Thesis, Carleton University, Ottawa.
- Bladon, T., Brasch, K., Brown, D. L. and Setterfield, G. (1988). Changes in structure and protein composition of bovine lymphocyte nuclear matrix during concanavalin A-induced mitogenesis. Biochem. Cell Biol. **66**, 40-53.

- Bode, J., Kohwi, Y, Dickinson, L., Joh, T., Klehr, D.,
Mielke, C. and Kohwi-Shigematsu, T. (1992). Biological
significance of unwinding capability of nuclear matrix-
associating DNAs. *Science* **255**, 195-197.
- Boulikas, T., Bastin, B., Boulikas, P. and Dupuis, G.
(1990). Increase in histone poly(ADP-ribosylation) in
mitogen-activated lymphoid cells. *Exp. Cell Res.* **187**,
77-84.
- Bouteille, M., Laval, M. and Dupuy-Coin, A. M. (1974).
Localization of nuclear functions as revealed by
ultrastructural autoradiography and cytochemistry. In
The Cell Nucleus. Vol 1., pp 5-64. (ed. H. Busch).
New York: Academic Press, Inc.
- Bouteille, M., Kalifat, S. R. and Delarue, J. (1967).
Ultrastructural variations of nuclear bodies in human
diseases. *J. Ultrastruc. Res.* **19**, 474-486.
- Brasch, K. (1990). Drug and metabolite-induced
perturbations in nuclear structure and function: a
review. *Biochem. Cell Biol.* **68**, 408-426.
- Brasch, K. (1982). Fine structure and localization of the
nuclear matrix in situ. *Exp. Cell Res.* **140**, 161-171.

- Brasch, K., Harrington, S. and Blake, H. (1989). Isolation and analysis of nuclear bodies from estrogen-stimulated chick liver. *Exp. Cell Res.* **182**, 425-435.
- Brasch, K. and Peters, K. E. (1985). Nuclear protein matrix and structural changes in rooster liver after estrogenic induction and vitellogenesis. *Biol. Cell* **54**, 109-122.
- Brasch, K. and Sinclair, G. D. (1978). The organization, composition and matrix of hepatocyte nuclei exposed to amanitin. *Virchows Arch. B. Cell Pathol.* **17**, 193-204.
- Brasch, K., Seligy, V. L. and Setterfield, G. (1971). Effects of low salt concentration on structural organization and template activity of chromatin in chicken erythrocyte nuclei. *Exp. Cell Res.* **65**, 61-72.
- Brown, D. L., Knox, J. D. and Paulin-Levasseur, M. (1992). The lymphocyte centrosome. In The Centrosome. (ed. V.I. Kalnins), pp. 261-268, San Diego, Ca.: Academic Press.
- Brown, D. L., Chaly, N. and Kaplan, J. G. (1989). Assembly of the lymphocyte nucleus for transcription and replication. In Cellular Basis of Immune Modulation.

pp. 67-77. New York: Alan R. Liss, Inc.

Buecker, J. (1992). Preparation of mammalian cell lysates for 2-D analysis. Millipore Bioforum.

Burke, B. and Gerace, L. (1986). A cell free system to study reassembly of the nuclear envelope at the end of mitosis. *Cell* 44, 639-652.

Capco, D. G., Wan, K. M. and Penman, S. (1982). The nuclear matrix: three dimensional architecture and protein composition. *Cell* 29, 847-858.

Carter, K. C. and Lawrence, J. B. (1991). DNA and RNA within the nucleus: How much sequence-specific spatial organization? *J. Cell. Biochem.* 47, 124-129.

Carter, K. C., Taneja, K. L. and Lawrence, J. B. (1991). Discrete nuclear domains of poly(A)RNA and their relationship to the functional organization of the nucleus. *J. Cell Biol.* 115, 1191-1202.

Castellanos, G., Owens, T., Rudd, C., Bladon, T., Setterfield, G. and Kaplan, J. G. (1982). The effect of ultraviolet radiation on early stages of activation of human lymphocytes: inhibition is independent of

effects on DNA. Can. J. Biochem. 60, 854-860.

Chaly, N., St. Aubin, G. and Brown, D. L. (1989).

Ultrastructural localization of nuclear antigens during interphase in mouse 3T3 fibroblasts. Biochem. Cell Biol. 67, 563-574.

Chaly, N., Cadrin, M., Kaplan, J. G. and Brown, D. L.

(1988). Modulation of lymphocyte nuclear matrix organization in vivo by 5,6-dichloro-1- β -D-ribofuranosyl benzimidazole: an autoradiographic and immunofluorescence study. Biol. Cell 63, 9-17.

Chaly, N., Sabour, M. P., Silver, J. C., Aitchison, W. A.,

Little, J. E. and Brown, D. L. (1986). Monoclonal antibodies against nuclear matrix detect nuclear antigens in mammalian, insect and plant cells: an immunofluorescence study. Cell Biol. Int. Rep. 10, 421-428.

Chaly, N., Little, J. E. and Brown, D. L. (1985).

Localization of nuclear antigens during preparation of nuclear matrices in situ. Can. J. Biochem. Cell Biol. 63, 644-653.

- Chaly, N., Bladon, T., Setterfield, G., Little, J. E., Kaplan, J. G. and Brown, D. L. (1984). Changes in the distribution of nuclear matrix antigens during the mitotic cell cycle. *J. Cell Biol.* **99**, 661-671.
- Chaly, N., Setterfield, G., Kaplan, J. G. and Brown, D. L. (1983a). Nuclear bodies in mouse splenic lymphocytes: I -Ultrastructural changes during stimulation by concanavalin A. *Biol. Cell* **47**, 275-284.
- Chaly, N., Setterfield, G., Kaplan, J. G. and Brown, D. L. (1983b). Nuclear bodies in mouse splenic lymphocytes: II - Cytochemistry and autoradiography during stimulation by concanavalin A. *Biol. Cell* **49**, 35-44.
- Chodosh, L., Fire, A., Samuels, M. and Sharp, P. A. (1989). 5,6-Dichloro-1- β -D-ribofuranosylbenzimidazole inhibits transcription elongation by RNA polymerase II in vitro. *J. Biol. Chem.* **264**, 2250-2257.
- Ciejek, E. M., Tsai, M.-J. and O'Malley, B. W. (1983). Actively transcribed genes are associated with the nuclear matrix. *Nature* **306**, 607-609.
- Ciejek, E. M., Nordstrom, J. L., Tsai, M.-J. and O'Malley, B. W. (1982). Ribonucleic acid precursors are

associated with the chick oviduct nuclear matrix.

Biochemistry 21, 4945-4953.

Cockerill, P. N. and Garrard, W. T. (1986). Chromosomal loop anchorage of the kappa immunoglobulin gene occurs next to the enhancer in a region containing topoisomerase II sites. Cell 44, 273-282.

Cook, P. R. and Brazell, I. A. (1976). Conformational constraints in nuclear DNA. J. Cell Sci. 22, 297-302.

Cook, P. R., Brazell, I. A. and Jost, E. (1976). Characterization of nuclear structures containing superhelical DNA. J. Cell Sci. 22, 303-324.

Courtens, J.-L. and Loir, M. (1981a). Ultrastructural detection of basic nucleoproteins: alcoholic phosphotungstic acid does not bind to arginine residues. J. Ultrastruct. Res. 74, 322-326.

Courtens, J.-L. and Loir, M. (1981b). A cytochemical study of nuclear changes in boar, bull, goat, mouse, rat, and stallion spermatids. J. Ultrastruct. Res. 74, 327-340.

Crawford, L. V. and Waring, M. J. (1967). Supercoiling of polyoma virus DNA measured by its intercalation with

ethidium bromide. J. Mol. Biol. 25, 23-30.

Crowley, K. S. and Brasch, K. (1987). Does the interchromatin compartment contain actin? Cell Biol. Int. Rep. 11, 537-546.

Dabauvalle, M.-C., Benavente, R. and Chaly, N. (1988). Monoclonal antibodies to a M_r 68 000 pore complex glycoprotein interfere with nuclear protein uptake in Xenopus oocytes. Chromosoma 97, 193-197.

Dardick, I. and Setterfield, G. (1976). Volume of condensed chromatin in developing primitive-line erythrocytes in chick. Exp. Cell Res. 100, 159-171.

Dargemont, C. and Kühn, L. C. (1992). Export of mRNA from microinjected nuclei of Xenopus laevis oocytes. J. Cell Biol. 118, 1-9.

Davies, H. G., Murray, A. B. and Walmsley, M. E. (1974). Electron-microscope observations on the organization of the nucleus in chicken erythrocytes and a superunit thread hypothesis for chromosome structure. J. Cell Sci. 16, 261-299.

Davis, L. I. and Fink, G. R. (1990). The NUP1 gene encodes an essential component of the yeast nuclear pore complex. *Cell* 61, 965-978.

Davis, L. I. and Blobel, G. (1986). Identification and characterization of a nuclear pore complex glycoprotein. *Cell* 45, 699-709.

de Jong, I., van Driel, R., Stuurman, N., Meijne, A. M. L. and van Renswoude, J. (1990). Principles of nuclear organization. *Cell Biol. Int. Rep.* 14, 1051-1074.

Derenzini, M., Thiry, M. and Goessens, G. (1990). Ultrastructural cytochemistry of the mammalian cell nucleolus. *J. Histochem. Cytochem.* 38, 1237-1256.

Derenzini, M., Pession-Brizzi, A., Betts-Eusebi, C. and Novello, F. (1981). Relationship between the fine structural organization of chromatin and nucleic acid synthesis in regenerating rat hepatocytes. *J. Ultrastruct. Res.* 75, 229-242.

Derenzini, M., Pession-Brizzi, S., Bonetti, E. and Novello, F. (1979). Relationship between ultrastructure and function of hepatocyte chromatin: a study with adrenalectomized rats after cortisol administration.

J. Ultrastruct. Res. 67, 161-179.

Dickinson, L. A., Joh, T., Kohwi, Y. and Kohwi-Shigematsu, T. (1992). A tissue-specific MAR/SAR DNA-binding protein with unusual binding site recognition. Cell 70, 631-645.

Dijkwel, P. A., Mullenders, L. M. F. and Wanka, F. (1979). Analysis of the attachment of replicating DNA to a nuclear matrix in mammalian interphase nuclei. Nucleic Acids Res. 14, 3241-3249.

Dingman, C. W. and Sporn, M. B. (1964). Studies on chromatin. I. Isolation and characterization of nuclear complexes of deoxyribonucleic acid, ribonucleic acid, and protein from embryonic and adult tissues of the chicken. J. Biol. Chem. 239, 3483-3492.

Dingwall, C. (1992). Soluble factors and solid phases. Current Biol. 2, 503-505.

Dingwall, C. and Laskey, R. (1992). The nuclear membrane. Science 258, 942-947.

Dingwall, C., Sharnick, S. V. and Laskey, R. A. (1982). A polypeptide domain that specifies migration of

nucleoplasmin into the nucleus. *Cell* 30, 449-458.

Dumbar, T. S., Gentry, G. A. and Olson, M. O. J. (1989).

Interaction of nucleolar phosphoprotein B23 with
nucleic acids. *Biochemistry* 28, 9495-9501.

Dworetzky, S. I. and Feldherr, C. M. (1988). Translocation
of RNA-coated gold particles through the nuclear pores
of oocytes. *J. Cell Biol.* 106, 575-584.

Earnshaw, W. C. and Heck, M. M. S. (1985). Localization of
topoisomerase II in mitotic chromosomes. *J. Cell Biol.*
100, 1716-1725.

Egyházi, E. (1975). Inhibition of Balbiani ring RNA
synthesis at the initiation level. *Proc. Natl. Acad.
Sci. USA.* 72, 947-950.

Egyházi, E., Pigon, A. and Rydlander, L. (1982). 5,6-
Dichloro-1- β -ribofuranosylbenzimidazole inhibits the
rate of transcription initiation in intact Chironomus
cells. *Eur. J. Biochem.* 122, 445-451.

Fakan, S. (1986). Structural support for RNA synthesis in
the cell nucleus. *Meth. Achiev. Exp. Pathol.* 12, 105-
140.

Fakan, S., Leser, G. and Martin, T. E. (1984).

Ultrastructural distribution of nuclear ribonucleoproteins as visualized by immunocytochemistry on thin sections. J. Cell Biol. **98**, 358-363.

Fakan, S. and Puvion, E. (1980). The ultrastructural visualization of nucleolar and extranucleolar RNA synthesis and distribution. Int. Rev. Cytol. **65**, 255-299.

Fakan, S. and Bernhard, W. (1973). Nuclear labelling after prolonged ^3H -uridine incorporation as visualized by high resolution autoradiography. Exp. Cell Res. **79**, 431-444.

Fakan, S. and Bernhard, W. (1971). Localisation of rapidly and slowly labelled nuclear RNA as visualized by high resolution autoradiography. Exp. Cell Res. **67**, 129-141.

Featherstone, C., Darby, M. K. and Gerace, L. (1988). A monoclonal antibody against the nuclear pore complex inhibits nucleocytoplasmic transport of protein and RNA in vivo. J. Cell Biol. **107**, 1289-1297.

Feldherr, C. M., Kallenback, E. and Schultz, N. (1984).

Movement of a karyophilic protein through the nuclear pores of oocytes. *J. Cell Biol.* **99**, 2216-2222.

Fey, E. G. and Penman, S. (1988). Nuclear matrix proteins reflect cell type of origin in cultured human cells.

Proc. Natl. Acad. Sci. USA. **85**, 121-125.

Fey, E. G., Krochmalnic, G. and Penman, S. (1986). The non-chromatin structure of the nucleus: The ribonucleoprotein (RNP)-containing and RNP-depleted matrix analyzed by sequential fractionation and resinless section microscopy. *J. Cell Biol.* **102**, 1654-1665.

Filson, A. J., Lewis, A., Blobel, G. and Fisher, P. A.

(1985). Monoclonal antibodies prepared against the major Drosophila nuclear matrix-pore complex-lamina glycoprotein bind specifically to the nuclear envelope in situ. *J. Biol. Chem.* **260**, 3164-3172.

Finlay, D. R. and Forbes, D. J. (1990). Reconstitution of biochemically altered nuclear pores: transport can be eliminated and restored. *Cell* **60**, 17-29.

- Finlay, D. R., Newmeyer, D. D., Price, T. M. and Forbes, D. J. (1987). Inhibition of in vitro nuclear transport by a lectin that binds to nuclear pores. J. Cell Biol. 104, 189-200.
- Fischer, D., Weisenberger, D. and Scheer, U. (1991). Assigning functions to nucleolar structures. Chromosoma 101, 133-140.
- Forbes, D. J. (1992). The nuclear pore complex. Ann. Rev. Immunol. 8, 495-527.
- Franke, W. W. (1974). Structure, biochemistry, and functions of the nuclear envelope. Int. Rev. Cytol. Suppl. 4, 71-236.
- Franke, W. W., Scheer, U., Krohne, G. and Jarasch, E.-D. (1981). The nuclear envelope and the architecture of the nuclear periphery. J. Cell Biol. 91, 39s-50s.
- Fu, X.-D. and Maniatis, T. (1990). Factor required for mammalian spliceosome assembly is localized to discrete regions in the nucleus. Nature 343, 437-441.
- Garrard, W. T. (1990). Chromosomal loop organization in eukaryotic genomes. Nucleic Acids Mol. Biol. 4, 163-

175.

Gasser, S. M., Amati, B. B., Cardenas, M. E. and Hofman, J. F. X. (1989). Studies on scaffold attachment sites and their relation to genome function. *Int. Rev. Cytol.* **119**, 57-96.

Gasser, S. M. and Laemmli, U. K. (1986). Cohabitation of scaffold binding regions with upstream/enhancer elements of three developmentally regulated genes of D. melanogaster. *Cell* **46**, 521-530.

Georgatos, S. D. and Blobel, G. (1987). Lamin B constitutes an intermediate filament attachment site at the nuclear envelope. *J. Cell Biol.* **105**, 117-125.

Gerace, L. and Burke, B. (1988). Functional organization of the nuclear envelope. *Ann. Rev. Cell Biol.* **4**, 335-374.

Gerace, L., Ottaviano, Y. and Kondor-Koch, C. (1982). Identification of a major polypeptide of the nuclear pore complex. *J. Cell Biol.* **95**, 826-837.

Gerace, L. and Blobel, G. (1980). The nuclear envelope lamina is reversibly depolymerized during mitosis. *Cell* **19**, 277-287.

- Getzenberg, R. H., Pienta, K. J., Ward, W. S. and Coffey, D. S. (1991). Nuclear structure and the three-dimensional organization of DNA. *J. Cell. Biochem.* **47**, 289-299.
- Ghosh, S., Paweletz, N. and Ghosh, T. (1978). Cytological identification and characterization of the nuclear matrix. *Exp. Cell Res.* **111**, 363-371.
- Goldberg, M. W. and Allen, T. D. (1992). High resolution scanning electron microscopy of the nuclear envelope: Demonstration of a new, regular, fibrous lattice attached to the baskets of the nucleoplasmic face of the nuclear pores. *J. Cell Biol.* **119**, 1429-1440.
- Granick, D. (1975a). Nucleolar necklaces in chick embryo fibroblast cells. I. Formation of necklaces by dichlororibobenzimidazole and other adenosine analogues that decrease RNA synthesis and degrade preribosomes. *J. Cell Biol.* **65**, 389-417.
- Granick, D. (1975b). Nucleolar necklaces in chick embryo fibroblast cells. II. Microscope observations of the effect of adenosine analogues on nucleolar necklace formation. *J. Cell Biol.* **65**, 418-427.

Greber, U. F., Senior, A. and Gerace, L. (1990). A major glycoprotein of the nuclear pore complex is a membrane-spanning polypeptide with a large lumenal domain and a small cytoplasmic tail. *EMBO J.* **9**, 1495-1502.

Hakes, D. J and Berezney, R. (1991a). DNA binding properties of the nuclear matrix and individual nuclear matrix proteins. *J. Biol. Chem.* **266**, 11131-11140.

Hakes, D. J. and Berezney, R. (1991b). Molecular cloning of matrin F/G: A DNA binding protein of the nuclear matrix that contains putative zinc finger motifs. *Proc. Natl. Acad. Sci. USA* **88**, 6186-6190.

Hall, R. (1982). Changes in nuclei of mitogenically stimulated lymphocytes. M.Sc. Thesis, Carleton University, Ottawa.

Hancock, R. and Hughes, M. E. (1982). Organization of DNA in the interphase nucleus. *Biol. Cell* **44**, 201-212.

Hayat, M. A. (1986). Basic Techniques for Transmission Electron Microscopy. p. 184. Academic Press Inc., Toronto.

- He, D., Nickerson, J. A. and Penman, S. (1990). Core filaments of the nuclear matrix. *J. Cell Biol.* **110**, 569-580.
- Herlan, G., Quevedo, R. and Wunderlich, F. (1978). Structural transformation of the nuclear matrix in situ. *Exp. Cell Res.* **115**, 103-110.
- Hernandez-Verdun, D. (1991). The nucleolus today. *J. Cell Sci.* **99**, 465-471.
- Hinshaw, J. E., Carragher, B. O. and Milligan, R. A. (1992). Architecture and design of the nuclear pore complex. *Cell* **69**, 1133-1141.
- Hornbeck, P., Huang, K.-P. and Paul, W. E. (1988). Lamin B is rapidly phosphorylated in lymphocytes after activation of protein kinase C. *Proc. Natl. Acad. Sci. USA.* **85**, 2279-2283.
- Huang, S. and Spector, D.L. (1991). Nascent pre-mRNA transcripts are associated with nuclear regions enriched in splicing factors. *Genes Develop.* **5**, 2288-2303.

Hurt, E. C. (1988). A novel nucleoskeletal-like protein located at the nuclear periphery is required for the life cycle of Saccharomyces cerevisiae. EMBO J. 7, 4323-4334.

Jackson, D. A. (1991). Structure-function relationships in eukaryotic nuclei. BioEssays 13, 1-10.

Jackson, D. A., Dolle, A., Robertson, G. and Cook, P. R. (1992). The attachments of chromatin loops to the nucleoskeleton. Cell Biol. Int. Rep. 16, 683-696.

Jackson, D. A. and Cook, P. R. (1986). Replication occurs at a nucleoskeleton. EMBO J. 5, 1403-1410.

Jackson, D. A. and Cook, P. R. (1985). Transcription occurs at a nucleoskeleton. EMBO J. 4, 919-925.

Jackson, D. A., McCready, S. J. and Cook, P. R. (1981). RNA is synthesized at the nuclear cage. Nature 292, 552-555.

Jansen, R. P., Hurt, E. C., Kern, H., Lehtonen, H., Carmo-Fonseca, M., Lapeyre, B. and Tollervey, D. (1991). Evolutionary conservation of the human nucleolar protein fibrillarin and its functional expression in

yeast. *J. Cell Biol.* **113**, 715-729.

Jarnik, M. and Aebi, U. (1991). Toward a more complete 3-D structure of the nuclear pore complex. *J. Struct. Biol.* **107**, 291-308.

Jordan, E. G. (1991). Interpreting nucleolar structure: where are the transcribing genes? *J. Cell Sci.* **98**, 437-442.

Kass, S., Tyc, K., Steitz, J. A. and Sollner-Webb, B. (1990). The U3 small nuclear ribonucleoprotein functions in the first step of preribosomal RNA processing. *Cell* **60**, 897-908.

Kaufmann, S. H. and Shaper, J. H. (1991). Association of topoisomerase II with the hepatoma cell nuclear matrix: The role of intermolecular disulfide bond formation. *Exp. Cell Res.* **192**, 511-523.

Kaufmann, S. H. and Shaper, J. H. (1984). A subset of non-histone nuclear proteins reversibly stabilized by the sulfhydryl cross-linking reagent tetrathionate. *Exp. Cell Res.* **155**, 477-495.

Kaufmann, S. H., Coffey, D. S. and Shaper, J. H. (1981).

Considerations in the isolation of rat liver nuclear matrix, nuclear envelope, and pore complex lamina.

Exp. Cell Res. **132**, 105-123.

Kellum, R. and Schedl, P. (1991). A position-effect assay for boundaries of higher order chromosomal domains.

Cell **64**, 941-950.

Kornberg, R. D. and Klug, A. (1981). The nucleosome.

Scientific American **244** (2), 52-64.

Krachmarov, C., Stoilov, L. and Zlatanova, J. (1991).

Nuclear matrices from transcriptionally active and inactive plant cells. Plant Sci. **76**, 35-41.

Krohne, G. and Benavente, R. (1986). The nuclear lamins.

Exp. Cell Res. **162**, 1-10.

Krzychowska-Gruca, S., Gruca, S., Zbored, A. and Filipowska, M. (1988). Interchromatin granules during

phytohemagglutinin stimulation of human lymphocytes. A cytochemical study. Histochem. **88**, 563-569.

LaFond, R. E. and Woodcock, C. L. F. (1983). Status of the nuclear matrix in mature and embryonic chick

erythrocyte nuclei. *Exp. Cell Res.* **147**, 31-39.

LaFond, R. E., Woodcock, H., Woodcock, C. L. F., Kundahl, E. R. and Lucas, J. J. (1983). Generation of an internal matrix in avian erythrocyte nuclei during reactivation in cytoplasts. *J. Cell Biol.* **96**, 1815-1819.

Lapeyre, B., Mariottini, P., Mathieu, C., Ferrer, P., Amaldi, F., Amalric, F. and Caizergues-Ferrer, M. (1990). Molecular cloning of Xenopus fibrillarin, a conserved U3 small nuclear ribonucleoprotein recognized by antisera from humans with autoimmune disease. *Mol. Cell. Biol.* **10**, 430-434.

Lapeyre, B., Bourbon, H. and Amalric, F. (1987). Nucleolin, the major nucleolar protein of growing eukaryotic cells: an unusual protein structure revealed by the nucleotide sequence. *Proc. Natl. Acad. Sci. USA* **84**, 1472-1476.

Laub, O., Jakobovits, E. B. and Aloni, Y. (1980). 5,6-Dichloro-1- β -ribofuranosylbenzimidazole enhances premature termination of late transcription of simian virus 40 DNA. *Proc. Natl. Acad. Sci. USA* **77**, 3297-3301.

- Lawrence, J. B., Singer, R. H. and Marselle, L. M. (1989).
Highly localized tracks of specific transcripts within
interphase nuclei visualized by in situ hybridization.
Cell 57, 493-502.
- Lebel, S., Lampron, C., Royal, A. and Raymond, Y. (1987).
Lamins A and C appear during retinoic acid-induced
differentiation of mouse embryonal carcinoma cells. J.
Cell Biol. 105, 1099-1104.
- Lebkowski, J. S. and Laemmli, U. K. (1982a). Evidence for
two levels of DNA folding in histone-depleted HeLa
interphase nuclei. J. Mol. Biol. 156, 309-324.
- Lebkowski, J. S. and Laemmli, U. K. (1982b). Non-histone
proteins and long-range organization of HeLa interphase
DNA. J. Mol. Biol. 156, 325-344.
- Lehner, C. F., Stick, R. Eppenberger, H. M. and Nigg, E. A.
(1987). Differential expression of nuclear lamin
proteins during chicken development. J. Cell Biol.
105, 577-587.
- Lehner, C. F., Eppenberger, H. M., Fakan, S. and Nigg, E. A.
(1986). Nuclear substructure antigens. Exp. Cell Res.
162, 205-219.

- Lennox, R.W. and Cohen, L.H. (1983). The histone H1 complements of dividing and nondividing cells of the mouse. *J. Biol. Chem.* **258**, 262-268.
- Lerner, E. A., Lerner, M. R., Janeway, C. A. and Steitz, J. A. (1981). Monoclonal antibodies to nucleic acid-containing cellular constituents: probes for molecular biology and autoimmune disease. *Proc. Natl. Acad. Sci. USA* **78**, 2737-2741.
- Leser, G. P., Fakan, S. and Martin, T. E. (1989). Ultrastructural distribution of ribonucleoprotein complexes during mitosis. snRNP antigens are contained in mitotic granule clusters. *Eur. J. Cell Biol.* **50**, 376-389.
- Locke, M. and Huie, P. (1977). Bismuth staining for light and electron microscopy. *Tissue & Cell* **9**, 347-371.
- Lourim, D. and Lin, J. J.-C. (1989). Expression of nuclear lamin A and muscle specific proteins in differentiating muscle cells in ovo and in vitro. *J. Cell Biol.* **109**, 495-504.
- Ludérus, M. E. E., de Graaf, A., Mattia, E., den Blaauwen, J. L., Grande, M. A., de Jong, L. and van Driel, R.

(1992). Binding of matrix attachment regions to lamin B₁. *Cell* **70**, 949-959.

Lührmann, R., Kastner, B. and Bach, M. (1990). Structure of spliceosomal snRNPs and their role in pre-mRNA splicing. *Biochim. Biophys. Acta.* **1087**, 265-292.

MacDonald, H. R. and Nabholz, M. (1986). T-cell activation. *Ann. Rev. Cell Biol.* **2**, 231-253.

Maul, G. G., Maul, H. M., Scogna, J. E., Lieberman, M. W., Stein, G. S., Hsu, B. Y.-L. and Borun, T. W. (1972). Time sequence of nuclear pore formation in phytohemagglutinin-stimulated lymphocytes and in HeLa cells during the cell cycle. *J. Cell Biol.* **55**, 433-447.

Maul, G. G., Price, J. W. and Lieberman, M. W. (1971). Formation and distribution of nuclear pore complexes in interphase. *J. Cell Biol.* **51**, 405-418.

McCready, S. J., Godwin, J., Maon, D. W., Brazell, I. A. and Cook, P. R. (1980). DNA is replicated at the nuclear cage. *J. Cell Sci.* **46**, 365-386.

McKeon, F. D., Kirschner, M. W. and Caput, D. (1986).

Homologies in both primary and secondary structure between nuclear envelope and intermediate filament proteins. *Nature* 319, 463-468.

Meier, U. T. and Blobel, G. (1992). Nopp140 shuttles on tracks between nucleolus and cytoplasm. *Cell* 70, 127-138.

Miller, M., Park, M. K. and Hanover, J. A. (1991). Nuclear pore complex: structure, function, and regulation. *Physiol. Rev.* 71, 909-949.

Mirkovitch, J., Mirault, M.-E. and Laemmli, U. K. (1984). Organization of the higher order chromatin loop: Specific DNA attachment sites on nuclear scaffold. *Cell* 39, 223-232.

Monneron, A. and Bernhard, W. (1969). Fine structural organization of the interphase nucleus in some mammalian cells. *J. Ultrastruct. Res.* 27, 266-288.

Monneron, A. et Moulé, Y. (1968). Etude ultrastructurale de particules ribonucléoprotéiques nucléaires isolées à partir du foie de rat. *Exp. Cell Res.* 51, 531-554.

- Nakamura, H., Morita, T. and Sato, C. (1986). Structural organization of replicon domains during DNA synthetic phase in the mammalian nucleus. *Exp. Cell Res.* **165**, 291-297.
- Nakayasu, H. and Berezney, R. (1991). Nuclear matrins: Identification of the major nuclear matrix proteins. *Proc. Natl. Acad. Sci. USA* **88**, 10312-10316.
- Nakayasu, H. and Berezney, R. (1989). Mapping replicational sites in the eucaryotic cell nucleus. *J. Cell Biol.* **108**, 1-11.
- Nakayasu, H., Mori, H. and Ueda, K. (1982). Association of small nuclear RNA-protein complex with the nuclear matrix from bovine lymphocytes. *Cell Struct. Funct.* **7**, 253-262.
- Newmeyer, D. D. and Forbes, D. J. (1988). Nuclear import can be separated into distinct steps in vitro: Nuclear pore binding and translocation. *Cell* **52**, 641-653.
- Newport, J. (1987). Nuclear reconstitution in vitro: stages of assembly around protein-free DNA. *Cell* **48**, 205-217.

- Newport, J., Wilson, K. L. and Dunphy, W. G. (1990). A lamin-independent pathway for nuclear envelope assembly. *J. Cell Biol.* **111**, 2247-2259.
- Nickerson, J. A. and Penman, S. (1992). The nuclear matrix: Structure and involvement in gene expression. In Molecular Approaches to the Control of Proliferation and Differentiation. pp 343-380. New York: Academic Press, Inc.
- Nickerson, J. A., Krochmalnic, G. and Penman, S. (1989). Chromatin architecture and nuclear RNA. *Proc. Natl. Acad. Sci. USA.* **86**, 177-181.
- Nigg, E. A. (1988). Nuclear function and organization: the potential of immunochemical approaches. *Int. Rev. Cytol.* **110**, 27-92.
- Noaillac-Depeyre, J., Dupont, M.-A., Tichadou, J.-L. and Gas, N. (1989). The effect of adenosine analogue (DRB) on a major nucleolar phosphoprotein nucleolin. *Biol. Cell* **67**, 27-35.
- Ochs, R. L. and Smetana, K. (1991). Detection of fibrillarin in nucleolar remnants and the nucleolar matrix. *Exp Cell Res.* **197**, 183-190.

- Ochs, R. L., Lischwe, M. A., Spohn, W. H. and Busch, H. (1985). Fibrillarin: a new protein of the nucleolus identified by autoimmune sera. *Biol. Cell* **54**, 123-134.
- O'Farrell, P. H. (1975). High resolution two-dimensional electrophoresis of proteins. *J. Biol. Chem.* **250**, 4007-4021.
- Paddy, M. R., Belmont, A. S., Saumweber, H., Agard, D. A. and Sedat, J. W. (1990). Interphase nuclear envelope lamins form a discontinuous network that interacts with only a fraction of the chromatin in the nuclear periphery. *Cell* **62**, 89-106.
- Pardoll, D. M., Vogelstein, B. and Coffey, D. S. (1980). A fixed site of DNA replication in eucaryotic cells. *Cell* **19**, 527-536.
- Peters, K. E. and Comings, D. E. (1980). Two-dimensional gel electrophoresis of rat liver nuclear washes, nuclear matrix, and hnRNA proteins. *J. Cell Biol.* **86**, 135-155.
- Prather, R. S. and Schatten, G. (1992). Construction of the nuclear matrix at the transition from maternal to zygotic control of development in the mouse: an

immunocytochemical study. Mol. Reprod. Develop. **32**, 203-208.

Puvion, E. (1982). Presence of RNA in the interchromatin granules of cells treated with dichlorobenzimidazole riboside (DRB). Biol. Cell **43**, 147-150.

Puvion, E., Viron, A., Assens, C., Leduc, E. H. and Jeanteur, Ph. (1984a). Immunocytochemical identification of nuclear structures containing snRNPs in isolated rat liver cells. J. Ultrastruct. Res. **87**, 180-189.

Puvion, E., Viron, A. and Xu, F. X. (1984b). High resolution autoradiographical detection of RNA in the interchromatin granules of DRB-treated cells. Exp. Cell Res. **152**, 37-367.

Puvion, E. and Moyne, G. (1981). In situ localization of RNA structures. In The Cell Nucleus. vol. VIII (ed. H. Busch), pp 59-115, New York: Academic Press.

Puvion, E., Puvion-Dutilleul, F. and Leduc, E. H. (1981). The formation of nucleolar perichromatin granules. J. Ultrastruct. Res. **76**, 181-190.

- Puvion, E., Bachellerie, J.-P. and Burglen, M.-J. (1979).
Nucleolar perichromatin granules induced by
dichlorobenzimidazole riboside. *J. Ultrastruct. Res.*
69, 1-12.
- Puvion-Dutilleul, F., Bachellerie, J. P. and Puvion, E.
(1991). Nucleolar organization of HeLa cells as
studied by in situ hybridization. *Chromosoma* **100**, 395-
409.
- Razin, S. V., Yarovaya, O. V. and Georgiev, G. P. (1985).
Low ionic strength extraction of nuclease-treated
nuclei destroys the attachment of transcriptionally
active DNA to the nuclear skeleton. *Nucleic Acids Res.*
13, 7427-7444.
- Reimer, G., Pollard, K. M., Penning, C. A., Ochs, R. L.,
Lischwe, M. A., Busch, H. and Tan, E. M. (1987).
Monoclonal autoantibody from a (New Zealand Black X New
Zealand White) F_1 mouse and some human scleroderma sera
target an M_r 34,000 nucleolar protein of the U3 RNP
particle. *Arthritis Rheum.* **30**, 793-800.
- Reuter, R., Appel, B., Bringmann, P., Rinke, J. and Lührmann,
R. (1984). 5'-Terminal caps of snRNAs are reactive
with antibodies specific for 2,2,7-trimethylguanosine

in whole cells and nuclear matrices. Exp. Cell Res. 154, 548-560.

Richardson, W. D., Mills, A. D., Dilworth, S. M., Laskey, R. A., and Dingwall, C. (1988). Nuclear protein migration involves two steps: Rapid binding at the nuclear envelope followed by slower translocation through nuclear pores. Cell 52, 655-664.

Risley, M. S., Einheber, S. and Bumcrot, D. A. (1986). Changes in DNA topology during spermatogenesis. Chromosoma 94, 217-227.

Schatten, G., Maul, G. C., Schatten, H., Chaly, N., Simerly, C., Balczon, R. and Brown, D. L. (1985). Nuclear lamins and peripheral nuclear antigens during fertilization and embryogenesis in mice and sea urchins. Proc. Natl Acad. Sci. USA 82, 4727-4731.

Scheer, U. and Benavente, R. (1990). Functional and dynamic aspects of the mammalian nucleolus. BioEssays 12, 14-21.

Scheer, U., Hügle, B., Hazan, R. and Rose, K. M. (1984). Drug-induced dispersal of transcribed rRNA genes and transcriptional products: immunolocalization and silver

staining of different nucleolar components in rat cells treated with 5,6-dichloro- β -D-ribofuranosyl-benzimidazole. J. Cell Biol. **99**, 672-679.

Schmidt-Zachmann, M. S., Hügler-Dörr, B. and Franke, W. W. (1987). A constitutive nucleolar protein identified as a member of the nucleoplasmin family. EMBO J. **6**, 1881-1890.

Setterfield, G., Bladon, T., Hall, R., Chaly, N., Brasch, K., ElAnsary, H. and Brown, D. L. (1985). Extrachromatin nuclear components and structural changes in nuclei. In Nuclear Envelope Structure and RNA Maturation. UCLA Symp. Mol. Cell Biol., New Series, vol. 26 (ed. E. A. Smuckler and G. A. Clawson), pp 63-86, New York: Alan R. Liss Inc.

Setterfield, G., Hall, R., Bladon, T., Little, J. and Kaplan, J. G. (1983). Changes in structure and composition of lymphocyte nuclei during mitogenic stimulation. J. Ultrastruct. Res. **82**, 264-282.

Silver, P. A. (1991). How proteins enter the nucleus. Cell **64**, 489-497.

- Simard, R. et Duprat, A. M. (1969). Action de l'actinomycine D sur les ribonucléoprotéines nucléaires de cellules d'amphibiens en différenciation. J. Ultrastruct. Res. **29**, 60-75.
- Simons, J. W., Noga, S. J., Colombani, P. M., Beschorner, W. E., Coffey, D. S. and Hess, A. D. (1986). Cyclosporine A, an in vitro calmodulin antagonist, induces nuclear lobulations in human T cell lymphocytes and monocytes. J. Cell Biol. **102**, 145-150.
- Sinclair, G. D. and Brasch, K. (1978). The reversible action of α -amanitin on nuclear structure and molecular composition. Exp. Cell Res. **111**, 1-14.
- Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J. and Klenk, D. C. (1985). Measurement of protein using bicinchoninic acid. Anal. Biochem. **150**, 76-85.
- Snow, C. M., Senior, A. and Gerace, L. (1987). Monoclonal antibodies identify a group of nuclear pore complex glycoproteins. J. Cell Biol. **104**, 1143-1156.

- Spector, D. L. (1990). Higher order nuclear organization: Three-dimensional distribution of small nuclear ribonucleoprotein particles. *Proc. Natl. Acad. Sci. USA* **87**, 147-151.
- Spector, D. L. (1984). Colocalization of U1 and U2 small nuclear RNPs by immunocytochemistry. *Biol. Cell* **51**, 109-112.
- Spector, D. L., Lark, G. and Huang, S. (1992). Differences in snRNP localization between transformed and nontransformed cells. *Mol. Biol. Cell* **3**, 555-569.
- Spector, D. L., Fu, X.-D., and Maniatis, T. (1991). Associations between distinct pre-mRNA splicing components and the cell nucleus. *EMBO J.* **10**, 3467-3481.
- Spector, D. L. and Smith, H. C. (1986). Redistribution of U-snRNPs during mitosis. *Exp. Cell Res.* **163**, 87-94.
- Spector, D. L., Schrier, W. H. and Busch, H. (1983). Immunoelectron microscopic localization of snRNPs. *Biol. Cell* **49**, 1-10.

Stoilov, L., Mirkova, V. and Zlatanova, J. (1989).

Transcriptional activity and DNA supercoiling during early germination in maize. *Plant Sci.* **63**, 59-66.

Stuurman, N., de Jong, L. and van Driel, R. (1992). Nuclear frameworks: Concepts and operational definitions.

Cell Biol. Int. Rep. **16**, 837-852.

Stuurman, N., Meijne, A. M. L., van der Pol, A. J., de Jong, L., van Driel, R. and van Renswoude, J. (1990). The nuclear matrix from cells of different origin. *J. Biol. Chem.* **265**, 5460-5465.

Stuurman, N., van Driel, R., de Jong, L., Meijne, A. M. L. and van Renswoude, J. (1989). The protein composition of the nuclear matrix of murine P19 embryonal carcinoma cells is differentiation-stage dependent. *Exp. Cell Res.* **180**, 460-466.

Sukegawa, J. and Blobel, G. (1993). A nuclear pore complex protein that contains zinc finger motifs, binds DNA, and faces the nucleoplasm. *Cell* **72**, 29-38.

Tamm, I., and Kikuchi, T. (1979). Early termination of heterogenous nuclear RNA transcripts in mammalian cells: accentuation by 5,6-dichloro-1- β -D-

ribofuranosylbenzimidazole. Proc. Natl. Acad. Sci. USA. 76, 5750-5754.

Tamm, I., Hand, R. and Caliguiri, L. A. (1976). Action of dichlorobenzimidazole riboside on RNA synthesis in L-929 and HeLa cells. J. Cell Biol. 69, 229-240.

Thiry, M. (1992). Ultrastructural detection of DNA within the nucleolus by sensitive molecular immunocytochemistry. Exp. Cell Res. 200, 135-144.

Thiry, M. and Goessens, G. (1991). Distinguishing the sites of pre-rRNA synthesis and accumulation in Ehrlich tumor cell nucleoli. J. Cell Sci. 99, 759-767.

Tokuyasu, K., Madden, S. C. and Zeldis, L. J. (1968). Fine structural alterations of interphase nuclei of lymphocytes stimulated to growth activity in vitro. J. Cell Biol. 39, 630-660.

Tubo, R. A. and Berezney, R. (1987a). Pre-replicative association of multiple replicative enzyme activities with the nuclear matrix during rat liver regeneration. J. Biol. Chem. 262, 1148-1154.

- Tubo, R. A. and Berezney, R. (1987b). Identification of 100 and 150 S polymerase alpha-primase megacomplexes solubilized from the nuclear matrix of regenerating rat liver. *J. Biol. Chem.* **262**, 5857-5865.
- Turner, B. M. and Franchi, L. (1987). Identification of protein antigens associated with the nuclear matrix and with clusters of interchromatin granules in both interphase and mitotic cells. *J. Cell Sci.* **87**, 269-282.
- Tyc, K. and Steitz, J. A. (1989). U3, U8 and U13 comprise a new class of mammalian snRNPs localized in the cell nucleolus. *EMBO J.* **8**, 3113-3119.
- Unwin, P. N. T. and Milligan, R. A. (1982). A large particle associated with the perimeter of the nuclear pore complex. *J. Cell. Biol.* **93**, 63-75.
- Valkov, I. et Moyne, G. (1974). *Cytochimie ultrastructurale des modifications du noyau de lymphocytes cultivés in vitro en présence du phytohémagglutinine.* *J. Microscopie* **20**, 133-144.
- van Holde, K.E. (1989). *Chromatin.* New York: Springer-Verlag.

- Verheijen, R., Van Venrooij, W. and Ramaekers. (1988). The nuclear matrix: Structure and composition. *J. Cell Sci.* **90**, 11-36.
- Vogelstein, B., Pardoll, D. M. and Coffey, D. S. (1980). Supercoiled loops and eukaryotic DNA replication. *Cell* **22**, 79-85.
- von Kries, J. P., Buhrmester, H. and Strätling, W. H. (1991). A matrix/scaffold attachment region binding protein: Identification, purification, and mode of binding. *Cell* **64**, 123-135.
- Ward, W. S. and Coffey, D. S. (1990). Specific organization of genes in relation to the sperm nuclear matrix. *Biochem. Biophys. Res. Comm.* **173**, 20-25.
- Ward, W. S., Partin, A. W. and Coffey, D. S. (1989). DNA loop domains in mammalian spermatozoa. *Chromosoma* **98**, 153-159.
- Wassef, M. (1979). A cytochemical study of interchromatin granules. *J. Ultrastruct. Res.* **69**, 121-133.
- Wedrychowski, A., Bhorjee, J. S. and Briggs, R. C. (1989). In vivo crosslinking of nuclear proteins to DNA by cis-

diamminedichloroplatinum (II) in differentiating rat myoblasts. *Exp. Cell Res.* **183**, 376-387.

Wood, E.R. and Earnshaw, W.C. (1990). Mitotic chromatin condensation in vitro using somatic cell extracts and nuclei with variable levels of endogenous topoisomerase II. *J. Cell Biol.* **111**, 2839-2850.

Woodcock, C. L. F. and Woodcock, H. (1986). Nuclear matrix generation during reactivation of avian erythrocyte nuclei: an analysis of the protein traffic in cybrids. *J. Cell Sci.* **84**, 105-127.

Woodcock, C. L. F., LaFond, R. E., Woodcock, H., Baldwin, L. A. and Bhorjee, J. S. (1984). Reactivation of avian erythrocyte nuclei in mammalian cytoplasts. *Exp. Cell Res.* **154**, 155-170.

Worman, H. J., Evans, C. D. and Blobel, G. (1990). The lamin B receptor of the nuclear envelope inner membrane: A polytopic protein with eight potential transmembrane domains. *J. Cell Biol.* **111**, 1535-1542.

Worman, H. J., Yuan, J., Blobel, G. and Georgatos, S. D. (1988). A lamin B receptor in the nuclear envelope. *Proc. Natl. Acad. Sci. USA.* **85**, 8531-8534.

- Wozniak, R. W., Bartnik, E. and Blobel, G. (1989). Primary structure analysis of an integral membrane glycoprotein of the nuclear pore. *J. Cell Biol.* **108**, 2083-2092.
- Xing, Y. and Lawrence, J.B. (1991). Preservation of specific RNA distribution within the chromatin-depleted nuclear substructure demonstrated by in situ hybridization coupled with biochemical fractionation. *J. Cell Biol.* **112**, 1055-1063.
- Zandomeni, R., Zandomeni, M. C., Shugar, D. and Weinmann, R. (1986). Casein kinase type II is involved in the inhibition by 5,6-dichloro-1- β -D-ribofuranosylbenzimidazole of specific RNA polymerase II transcription. *J. Biol. Chem.* **261**, 3414-3419.
- Zandomeni, R. and Weinmann, R. (1984). Inhibitory effect of 5,6-dichloro-1- β -D-ribofuranosylbenzimidazole on a protein kinase. *J. Biol. Chem.* **259**, 14804-14811.
- Zandomeni, R., Bunick, D., Ackerman, S., Mittleman, B. and Weinmann, R. (1983). Mechanism of action of DRB. III. Effect on specific in vitro initiation of transcription. *J. Mol. Biol.* **167**, 561-574.

Zandomeni, R., Mittleman, B., Bunick, D., Ackerman, S. and Weinmann, R. (1982). Mechanism of action of dichloro- β -D-ribofuranosylbenzimidazole: Effect on in vitro transcription. Proc. Natl. Acad. Sci. USA **79**, 3167-3170.

Zeitlin, S., Wilson, R. C. and Efstratiadis, A. (1989). Autonomous splicing and complementation of in vivo-assembled spliceosomes. J. Cell Biol. **108**, 765-777.

END

16-12-93

FIN