ASSOCIATION OF DNASE HYPERSENSITIVE CHROMATIN DOMAINS WITH THE NUCLEAR ENVELOPE AND WITH NUCLEAR PORE COMPLEXES IN 3T3 FIBROBLASTS

by

Jonathan Ka Lok Chan

A thesis submitted in conformity with the requirements for the degree of Master of Science
Graduate Department of Physiology
University of Toronto

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Preface

The experimental results described here are derived from two projects, involving the investigation of the spatial distribution of DNAse hypersensitive chromatin (DHC) domains and nuclear pore complexes (NPCs) in interphase nuclei of 3T3 fibroblasts. The author contributed 100% of the work on DNAse hypersensitive chromatin domains. The work on NPCs is divided into two parts. The first part, involving light microscopic assessment of the spatial distribution of NPCs, was conducted with fellow student Jon Tomas to which the author contributed 50% of the work each on tissue culture, immunocytochemistry, light microscopy and morphometric analysis. The second part, involving ultrastructural examination of the distribution of NPCs was performed in collaboration with colleagues Paul Park and Neesha Dhani. The author's contribution in this section includes 100% of tissue culture and 50% of lanthanum labeling of NPCs and embedding of cells.
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ABSTRACT

DNAse hypersensitive chromatin (DHC), putative transcriptionally competent sequences, exists either as pan-nuclear speckles, or as a shell apposed to the nuclear envelope, in cells with "flat" or spheroidal nuclei respectively. We postulated that DHC also associated with the nuclear envelope in "flat" nuclei, but that such an association may not be resolvable, due to the interdigitation at the nuclear midplane, of DHC associated with the upper and lower nuclear surfaces respectively.

3T3 fibroblasts, characterized by "flat" nuclei, were therefore subjected to hypotonic expansion, followed by in situ nick translation to visualize DHC. Confocal analysis of unexpanded cells showed that DHC is present as a shallow dome predominantly at the apical aspect of the nucleus, i.e. the side facing the culture medium. This topology persisted after hypotonic expansion and argues for a polarized association of DHC domains with the nuclear membrane. Preliminary data also indicate a similar, polarized distribution of nuclear pore complexes (NPCs). We conclude that the nuclear periphery may function as a compartment for the spatial coupling of transcription and nucleo-cytoplasmic transport.
ACKNOWLEDGEMENTS

First and foremost, I would like to express my gratitude to Dr. Umberto De Boni for giving me the opportunity to work at the frontiers of one of the most important fundamental issue in science. I have learnt from you not only to think like a scientist, but to live as one. Live and learn!

I would also like to thank members of my supervisory committee, Dr. L. Mills and Dr. M. Opas, for their constructive criticism, helpful suggestions and support throughout the duration of this project.

To the past graduate students, Dr. Paul C. Park. and Dr. Jon Janevski, I should like to say thank you for helpful discussion and the sharing of your interesting accounts during your years as graduate students in Dr. De Boni's laboratory. Special thanks to Dr. Paul C. Park, for I have learnt from you, perseverance, discipline and dedication.

I must thank the following colleagues: George Xeroulis, Glyka Martou, Nesime Askin, Paul Grossman, Tania Bruno and Eun Kim. Your helpful discussion and moral support are mostly appreciated. Special thanks to Jon Tomas, for I shall remember him for his steadfast companionship and stimulating conversations.

I owe a debt of gratitude to my family members, especially my mother and brother Joannes for their patience and encouragement. For my late Grandma, I cherish all the moments with you and I thank you for your tender care during my upbringing. Me and Alisha will always miss you.

Last and definitely not least, I would like to dedicate this thesis to my dear Alisha. My M.Sc. project could not have been completed without her unconditional care and non-dwindling faith in me.
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<th>Description</th>
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<tbody>
<tr>
<td>μm</td>
<td>micrometer</td>
</tr>
<tr>
<td>°</td>
<td>degree</td>
</tr>
<tr>
<td>°C</td>
<td>degree Celsius</td>
</tr>
<tr>
<td>3-D</td>
<td>three-dimensional</td>
</tr>
<tr>
<td>A</td>
<td>adenine</td>
</tr>
<tr>
<td>ASF</td>
<td>alternative splicing factor</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>bio-16 dUTP</td>
<td>biotinylated 16 deoxyuridine triphosphate</td>
</tr>
<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>Br-UTP</td>
<td>bromo-uridine triphosphate</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>C</td>
<td>cytosine</td>
</tr>
<tr>
<td>dATP</td>
<td>deoxyadenine triphosphate</td>
</tr>
<tr>
<td>dCTP</td>
<td>deoxycytosine triphosphate</td>
</tr>
<tr>
<td>dGTP</td>
<td>deoxyguanine triphosphate</td>
</tr>
<tr>
<td>dUTP</td>
<td>deoxyuridine triphosphate</td>
</tr>
<tr>
<td>dTTP</td>
<td>deoxythymidine triphosphate</td>
</tr>
<tr>
<td>DHC</td>
<td>DNAse hypersensitive chromatin</td>
</tr>
<tr>
<td>DMSO</td>
<td>dimethylsulfoxide</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>EBV</td>
<td>Epstein Barr virus</td>
</tr>
<tr>
<td>FBS</td>
<td>fetal bovine serum</td>
</tr>
<tr>
<td>FC</td>
<td>fibrillar center</td>
</tr>
<tr>
<td>FISH</td>
<td>fluorescent in situ hybridization</td>
</tr>
<tr>
<td>FITC</td>
<td>fluorescein iso-thiocynate</td>
</tr>
<tr>
<td>g</td>
<td>gravity</td>
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<tr>
<td>G</td>
<td>guanine</td>
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ix
<table>
<thead>
<tr>
<th>Term</th>
<th>Definition</th>
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<tbody>
<tr>
<td>GABA</td>
<td>gamma-aminobutyric acid</td>
</tr>
<tr>
<td>GFP</td>
<td>green fluorescent protein</td>
</tr>
<tr>
<td>hsp</td>
<td>heat-shock protein</td>
</tr>
<tr>
<td>h</td>
<td>hour</td>
</tr>
<tr>
<td>HBSS</td>
<td>Hank's balanced salt solution</td>
</tr>
<tr>
<td>HSTF</td>
<td>heat-shock transcription factor</td>
</tr>
<tr>
<td>ICD</td>
<td>interchromosomal compartment domain</td>
</tr>
<tr>
<td>IGC</td>
<td>interchromatin granule cluster</td>
</tr>
<tr>
<td>kb</td>
<td>kilobase pair</td>
</tr>
<tr>
<td>kDa</td>
<td>kilodalton</td>
</tr>
<tr>
<td>LCR</td>
<td>locus control region</td>
</tr>
<tr>
<td>m</td>
<td>meter</td>
</tr>
<tr>
<td>MDa</td>
<td>megadalton</td>
</tr>
<tr>
<td>MEM</td>
<td>Minimum essential medium</td>
</tr>
<tr>
<td>min</td>
<td>minutes</td>
</tr>
<tr>
<td>mL</td>
<td>millimeter</td>
</tr>
<tr>
<td>MW</td>
<td>molecular weight</td>
</tr>
<tr>
<td>NGF</td>
<td>nerve growth factor</td>
</tr>
<tr>
<td>nm</td>
<td>nanometer</td>
</tr>
<tr>
<td>NPC</td>
<td>nuclear pore complex</td>
</tr>
<tr>
<td>NOR</td>
<td>nuclear organizing region</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>Pf</td>
<td>perichromatin fibril</td>
</tr>
<tr>
<td>rDNA</td>
<td>ribosomal DNA</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger RNA</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>s.e.m</td>
<td>standard error of the mean</td>
</tr>
<tr>
<td>SC35</td>
<td>splicing component 35</td>
</tr>
<tr>
<td>sDNA</td>
<td>satellite DNA</td>
</tr>
<tr>
<td>sRNP</td>
<td>small ribonucleoprotein</td>
</tr>
<tr>
<td>SR</td>
<td>serine/arginine</td>
</tr>
<tr>
<td>x</td>
<td></td>
</tr>
</tbody>
</table>
T  thymidine

tDNA  telomeric DNA

TRITC  tetramethyl rhodamine isothiocyanate

U  uracil

v/v  volume per volume

w/v  weight per volume
INTRODUCTION

The interphase nucleus is structurally and functionally compartmentalized (Borden & Manuelidis, 1988; Jackson, 1991; Cremer et al., 1993; Xing et al., 1993; De Boni, 1994; Marshall and Sedat, 1999). Specific chromatin domains, as well as non-chromatin components involved in pre-mRNA synthesis, processing and transport, display non-random, spatial distributions within the nucleus which are altered during changes in gene expression (Holowacz and De Boni, 1991; Park and De Boni, 1992; Billia et al., 1992; Sahlas et al., 1993; Janevski et al., 1995, Choh and De Boni, 1996). We have previously proposed that this intranuclear rearrangement of chromatin domains serves to transpose actively transcribed and/or transcriptionally competent genes to transcriptionally competent nuclear compartments (De Boni and Mintz, 1986; De Boni, 1994; Park and De Boni, 1998; 1999).

The nuclear periphery, by its proximity to the cytoplasm, has been implicated in playing a role in gene expression (Blobel, 1985; de Graaf et al., 1990; Sahlas et al., 1993; Park and De Boni, 1996; 1998) by facilitating nucleo-cytoplasmic transport of nascent mRNA. Controversy exists, however, whether gene expression is restricted to the nuclear periphery or whether it takes place in foci which are distributed throughout the nuclear space. One method to detect and localize transcriptionally competent sequences relies on in situ nick translation of DNAse hypersensitive chromatin (DHC) domains. Although DHC domains structurally represent all nucleosome-free DNA, regardless of transcriptional state, they do include transcriptionally active sequences (Weintraub, 1984; Eissenberg et al., 1985; Felsenfeld, 1992).
Use of this technique has shown that, in several cell types, DHC domains preferentially occur in a spatial domain juxtaposed to the nuclear envelope (de Graaf et al., 1990; Park and De Boni, 1998). From this peripheral domain, in some cell types, DHC domains may extend as fine channels into the nuclear interior (Hutchison and Weintraub, 1985). In other cell types, DHC domains can be dynamically redistributed, depending on the state of differentiation of the cell (Puck et al., 1991; Puck and Krystosek, 1992; Haag et al., 1994; Park and De Boni, 1996). In fact, the induction of differentiation of PC12 cells, by nerve growth factor, is associated with an apparent redistribution of DHC domains and of small nuclear ribonucleoprotein particles (snRNPs) from a co-localized, pan-nuclear, speckled pattern, to a 3-dimensional (3-D) shell at the periphery of the nucleus, with the repositioning of the DHC domains temporally preceding that of snRNPs (Park and De Boni, 1996).

In contrast, when assessed by an alternate technique which employs pulse-labeling of nascent RNA by incorporation of brominated or tritiated uridine triphosphate, transcription has consistently been shown to occur throughout the nucleus in multiple loci, both in mammalian (Fakan et al., 1976; Fakan and Puvion, 1980; Jackson et al., 1993, 1998; Wansink et al., 1993, 1996a; de Jong et al., 1996; Fay et al., 1997; Zeng et al., 1997; Cmarko et al., 1999) and in plant cells (Straatman et al., 1996). Similarly, an alternative method which involves incorporation of bromodeoxyuridine triphosphate to label early replicating, presumably active, sequences, showed that active sequences are distributed throughout the nuclear space (Ferreira et al., 1997). Thus, depending on the techniques employed or the cell type used, contradictory results are obtained.
The potential role of the geometry of the nucleus in the spatial organization of nuclear domains has been previously addressed (Ferreira et al., 1997; Park and De Boni, 1999). In fact, it has been stated that nuclei with a flat geometry would spatially constrain chromatin and that such constraints would be minimized in nuclei with a spherical geometry (Ferreira et al., 1997). In this context, it must be noted that the nerve growth factor-induced, apparent relocation of DHC domains from a pan-nuclear distribution to the nuclear periphery of PC12 cells is followed by a similar relocation of small ribonucleoprotein (snRNPs) splicing factors (Sahlas et al., 1993). The occurrence of these events is associated with a concomitant change in the geometry of the nucleus from a "flat" ellipsoid to a spheroidal ellipsoid (Park and De Boni, 1996). Moreover, in cell types in which nuclei are spheroidal, DHC domains are invariably associated with the nuclear periphery (Park and De Boni, 1998). Therefore, it may be speculated that DHC domains in flat nuclei are similarly associated with the upper and lower surfaces of the nucleus but are unresolvable as such by virtue of their interdigitation at the mid-nuclear plane (Fig. 1).

The work presented here was carried out to test the hypothesis that: DHC domains, detected by in situ nick translation, are spatially associated with the nuclear periphery in cell types with nuclei of a very flat geometry. NIH 3T3 fibroblasts, characterized by very flat nuclei in vitro, were chosen as a model system. Hypotonic solutions were employed to acutely alter the geometry of their nuclei from a very flat ellipsoid to a spheroid and thus to permit enhanced microscopic resolution of intranuclear detail along the optical axis (Fig. 1). In addition, to further test the hypothesis that the interphase nucleus is topologically organized for the spatial coupling of nuclear processes that are contingent in time, the spatial distribution of molecular factors involved in events downstream of transcription, such as
small ribonucleoprotein (snRNPs) splicing factors and nuclear pore complexes (NPCs), was also examined.
Hypotonic expansion

Hypothesis correct: DHC domains remain tethered to the nuclear envelope by attachment to nuclear lamina.

Hypothesis incorrect: DHC domains remain speckled throughout the nucleus.

Expanded nuclei with spheroidal geometry

- Elements which putatively tether DHC domains to the nuclear envelope.
- DHC domains which associate with the apical aspect of the nuclear envelope.
- DHC domains which associate with the basal aspect of the nuclear envelope.

Figure 1. Hypothesis: DNAse hypersensitive chromatin domains, i.e. putative transcriptionally active sequences, are associated with the nuclear envelope.
CHAPTER 1

STRUCTURE AND FUNCTION OF THE INTERPHASE NUCLEUS:
FROM DNA TO NUCLEOCYTOPLASMIC TRANSPORT OF mRNA

1.1) HIERARCHICAL FOLDING OF CHROMATIN

In eukaryotic cells, deoxyribonucleic acid (DNA), typically amounting to $3 \times 10^9$ base pairs (bp) and measuring over two meters in its linear form, is contained within a nucleus barely 10 μm wide. This scarcity of available space within the nucleus requires a highly co-ordinated scheme of chromatin folding not only to accommodate all the chromatin material and nuclear factors per se, but also to still allow essential nuclear processes such as transcription and DNA replication to occur. Six independent levels of chromatin organization have been proposed to facilitate this compaction, ranging from strands of naked duplex DNA molecules to the highly condensed chromatin evident in chromosomes at metaphase (Pienta and Coffey, 1984). In fact, it becomes increasingly clear that the organization of chromatin within the interphase nucleus plays a direct role in regulating differential expression of genes, that is cell-type, cell cycle- and developmental stage specific.

1.1.1) Conformation of DNA

The first level of chromatin organization is the formation of two anti-parallel polynucleotide strands, spaced 0.34 nm apart, which are stabilized by stacks of hydrogen bonding between purines and pyrimidines. This helix, coiled in the right-handed direction and with a pitch of 10.5 bp/turn (Horowitz and Wang, 1984), is known as B-DNA. Because
the glycosidic bonds of base pairs are not positioned diametrically opposite to each other, two helical grooves, one major and one minor, transverse the length of the helical axis. Such a configuration allows potential hydrogen donor and acceptor domains of base pairs to be faced outside the helix for sequence specific, trans-acting protein-DNA interactions.

B-DNA is the most stable conformation of DNA in the form of crystals (Prive et al., 1991; Yanagi et al., 1991), or in solutions of physiological ionic strength (Rhodes and Klug, 1980; Tullius and Dombroski, 1985). Other conformations of DNA, such as the A form, which has a higher pitch (11bp/turn) and the left-handed Z form (12 bp/turn), are DNA duplex structures formed in non-physiological conditions *in vitro*. Although the biological relevance of these alternative structures remains unclear, there is evidence that DNA rich in guanine (G) and cytosine (C) bases may adopt a Z-like conformation with cells. It is known that GC rich sequences or “islands” surrounds promoters of “housekeeping genes” *in vivo* (Zhang et al., 1992; Shirozu et al., 1995; Di Fruscio et al., 1998).

Studies of the conformation of synthetic AT-rich oligonucleotides (Berman, 1991; Travers and Klug, 1987; Travers, 1989) revealed that, depending on the local sequence content, the double helical DNA can assume structures that are intrinsically rigid, flexible, or curved. Although all these sequences can be assembled into nucleosomes (Hayes et al., 1991a), changes in the “bendability” of DNA produce kinks along its path around the core histones (Hogan et al., 1987). These structural discontinuities are preferred sites of integration of the human immunodeficiency virus (HIV) (Pruss et al., 1994). This explains why naturally occurring DNA sequences with inherent curvature, for example, the 5S ribosomal RNA (rRNA) genes (Hayes et al., 1990), are energetically favorable to be
incorporated into nucleosomes. The phenomena underlying such sequence preference for nucleosome formation is known as translational nucleosomal positioning (see section 1.2.1).

1.1.2) Structure of the nucleosome

The second level of chromatin folding involves the packaging of the DNA backbone by histones to form a linear array of nucleosomes (Kornberg, 1974; Simpson, 1978), which results in a reduction in the linear length of DNA by approximately a factor of 6 (Getzeberg et al., 1991). When observed by electron microscopy, upon induction of chromatin unraveling by low ionic strength from a more compact structure (Clark and Kimura, 1990), DNA at this level of organization gives a “beads-on-a-string” appearance (Woodcock, 1973; Olins and Olins, 1974). The fact that DNA wraps around the surface of core histones was revealed, directly and indirectly, by studies using different biophysical and biochemical techniques, including chemical and nuclease cleavage studies (Drew, 1984; Noll, 1974), X ray crystallography (Finch et al., 1977, Richmond et al., 1984; Arents and Moudrianakis, 1993), neutron scattering (Pardon et al., 1975) and chemical cross-linking studies (Pruss and Bavrykin, 1997).

Nucleosomes consist of the histone core particles, the DNA that wraps around each nucleosome and spans between particles (linker DNA), and the linker histones. The nucleosomal core particle is a complex of histone proteins in precise stoichiometry (Kornberg and Thomas, 1974), and is composed of a central tetramer of histones (H3/H4)2, bordered on both sides by a histone dimer of H2A/H2B (Arents et al., 1991; Moudrianakis
and Arents, 1993). The amino acid sequence of all core histones and the general quaternary structure thus formed by their interactions are evolutionarily conserved across species (De Lange et al., 1969a,b; Arents and Moudrianakis, 1995). Core histones are small proteins (11-16kDa), consisting of a carboxyl terminal histone-fold domain that is involved in histone-histone and histone-DNA interactions, and a highly basic amino acid terminal tail (Arents et al., 1991). The arginine and lysine residues residing on the latter terminal tail are sites where extensive post-translational modifications occur, including acetylation, phosphorylation, methylation and ubiquitination (see Section 1.1.3). Approximately 146 bps of DNA are wound around the core histones 1.75 times in a left-handed direction, resulting in a superhelical coil which measures approximately 11.0 nm in diameter and 5.6 nm in thickness (Finch et al., 1977, Richmond et al., 1984).

Kinetic studies reveal that the mechanism responsible for organizing DNA in the nucleosome primarily involves electrostatic interaction between the phosphodiester backbone of DNA and arginine residues present on the histone fold domains of the octamer (Ichimura et al., 1982, Arents and Moudrianakis, 1993). Specifically, high resolution x-ray crystallography of the nucleosomal core (Arents and Moudrianakis, 1993) reveals that on the centrally located histone (H3/H4)$_2$ tetramer, 14 arginine residues are strategically positioned such that their interaction with the DNA backbone results in a reduction of the average helical periodicity of DNA from 10.5 bp/turn in solution (Hayes et al, 1990, 1991b), to 10.2bp/turn (Klug and Lutter, 1981). This reduction, which contributes to the overwinding of DNA around core histones (Finch et al., 1977), determines which face of the DNA is available for the association of sequence-specific trans-acting factors, an aspect of nucleosome formation known as rotational positioning (see section 1.2.1).
1.1.3) Post-translational modification of histones

Nuclear magnetic resonance data reveals that core histone tails interact with DNA in the core particle at physiological strength (<600 mOsm NaCl) (Cary et al., 1982). However, this association is not essential in facilitating the primary folding of DNA around the nucleosome (Ausio et al., 1989, Hayes et al., 1991b). Instead, core histone tails are extensively modified post-translationally leading to subtle changes in nucleosome conformation (Oliva et al., 1990; Bode et al., 1983). These changes may then have much larger effects on transcription. Specifically, modifications such as acetylation and phosphorylation are correlated with enhanced transcriptional activity of genes associated within the modified nucleosomal array (see below).

Acetylation at lysine residues occurs at all four core histone tails in all animal and plant species examined (Csordas, 1990). Histone acetylation improves chromatin solubility (Perry and Chalkley, 1981), and is correlated with reduced amount of histone H1 in nucleosomal arrays, (Reeves et al, 1985; Ridsdale et al., 1990) and fewer superhelical turns around nucleosomes (Norton et al., 1989, 1990). The resulting instability of histone-DNA contacts may allow trans-acting factor access to DNA in the nucleosomes (Bauer et al., 1994). For example, in vitro experiments have shown upon acetylation of core histones that are positioned at the intrinsically curved promoter region of Xenopus 5S rRNA gene (see section 1.1.1), the binding of transcription factor TFIIIA to the underlying sequence is enhanced (Simpson and Stafford, 1983; FitzGerald and Simpson, 1985; Rhodes, 1985; Lee et al, 1993). In fact, actively transcribed and potentially active chromatin domains are selectively enriched in hyperacetylated histones (Sterner et al., 1987; Hebbes et al., 1988;
Csordas, 1990, Hebbes et al., 1992), whereas transcriptionally inactive chromatin contains hypoacetylated histones (Braunstein et al., 1993, Jeppesen and Turner, 1993). It has been suggested that acetylation is not a consequence of transcription but a prerequisite and that it may be responsible for either generating or maintaining the open structure of potentially active and of active genes (Crane-Robinson et al. 1997).

Another core histone modification which also represents a focus of research is phosphorylation. For example, phosphorylation of histone H3 at serine residues at the basic amino terminal domain was observed to be concomitant with phorbol ester-induced proliferation of ras-transformed NIH 3T3 cells (Chen and Allfrey, 1987; Chen et al., 1990, Laitinen, J. et al, 1990). It was suggested that phosphorylation of H3 serves to disrupt nucleosome integrity, leading to decondensation of chromatin and permitting transcriptional activation of proto-oncogenes such as c-jun and c-myc through the ras-raf pathway (Mahadevan et al., 1991).

1.1.4) Compaction of nucleosomal arrays into chromatin fibers

The number of base pairs of DNA that wrap around the core particles is remarkably constant across species. However, the overall length of the nucleosomal repeats, and thus, the linker DNA varies (Lohr and van Holde, 1979; Yaniv and Cereghini, 1987), and displays gene-, cell type, tissue- and species-specificity (Wu et al., 1979; Wu and Gilbert, 1981; Wu, 1984). This variability in repeat length, together with the negative charges on the phosphodiester backbone, might impose steric constraint on the further folding of the nucleosomal arrays into the 30-nm fibers; although the latter structures are routinely
observed in eukaryotic interphase nuclei (De Boni, 1988; Gross and De Boni, 1990). This compaction, which depends on the cation concentration in solution (Clark and Kimura, 1990; Hansen and Wolffe, 1992; Schwarz et al., 1996), is mediated by the binding of histone H1 onto linker DNA (Allan et al., 1980).

Although nucleosomal arrays can fold in the absence of histone H1 (Hansen et al., 1989; Garcia-Ramirez et al., 1992), histone H1 greatly facilitates this process by shielding the negative charges on the DNA backbone, thus conferring flexibility to the linker DNA (Lowary and Widom, 1989). In addition, linker histone induces allosteric change in the conformation of the histone octamer, resulting in the stabilization of the DNA winding the core particle (Simpson, 1978; Carruthers et al., 1998). The fact that histone H1 is deficient in transcriptionally active chromatin (Rose and Garrard, 1984; Rocha et al., 1984; Xu et al., 1986) implies that competition between histone H1 binding and post-translational modifications of core histones (see section 1.1.3) may represent a fundamental control mechanism in regulating gene expression.

Different biophysical models were proposed to describe the architecture of the 30 nm fibers as seen in vivo (Felsenfeld and McGhee, 1986; Woodcock et al., 1984; 1993; Bednar et al., 1995; Leuba et al., 1994; Horowitz et al., 1994). Of these, the solenoid model is the most widely accepted (Thoma et al., 1979; McGhee et al., 1980, 1983). According to this model, linker histones interact along a central axis (Losa et al., 1984; Dimitrov et al., 1987) such that six nucleosomes, with their long axes parallel to the fiber, are radially positioned with respect to the central axis in a helical manner (Suau et al., 1979; Widom and Klug, 1985). However, whether this symmetrical organization of nucleosomal array, deduced from studies of chromatin folding in vitro, is prevalent over the entire genome in vivo, or whether it is a local
phenomenon, is a subject of debate. In contrast, the twisted ribbon model, based on studies of chromatin conformation in fixed intact cells in situ, has drawn much attention in recent years (Horowitz et al., 1994). In this alternative model, linear arrays of dinucleosomes, interspersed by straight linker DNA, zig-zag along a highly convoluted and twisted path as 30-nm fibers. It remains unclear which model is correct. However, it is most likely that heterogeneity and flexibility of the 30-nm fiber should exist in vivo, with frequent transitions between each observed conformation.

1.1.5) The helical folding and radial loop models of mitotic chromosomes

Given the lack of an unifying structural model of the 30-nm chromatin fiber (see section 1.1.4), it is not surprising that different models exists to describe the folding of this fiber into the most condensed form of chromosomal organization: the mitotic chromosome (Pienta and Coffey, 1984; Saitoh and Laemmli, 1993; Rattner and Lin, 1985; Sedat and Manuelidis, 1978; Manuelidis and Chen, 1990). The radial loop model proposed by Pienta and Coffey (1984), as well as the similar loop/scaffold model advocated by Saitoh and Laemmli (1993), predict that the 30-nm fiber is organized into loops of varying length (60 to 200 kbps), such that when these loops are radially arranged along a central scaffolding axis and stacked together, such supramolecular configuration then gives rise to a metaphase chromosome.

A critical assumption in this model is that compaction occurs by association of chromatin fibers with nuclear scaffolding proteins, a process purported to be facilitated by topoisomerase II (Earnshaw et al., 1985; Gasser et al., 1986). However, whether
topoisomerase II actually forms the anchorage sites along the chromosomal axis remains controversial (Hirano and Mitchison, 1993). In an alternative model, Sedat and Manuelidis (1978) proposed that metaphase chromosomes are formed first by helical folding of 30-nm fiber, followed by a further helical folding of the resultant 250-nm fiber, thus alleviating a strict requirement for a central, solid scaffolding axis.

1.1.6) Chromosome territories

In view of the hierarchical folding of chromatin into metaphase chromosomes, an immediate question arises regarding the degree of compaction of chromatin during interphase. This question was initially pondered by cytologists over a century ago (Rabl, 1885; Boveri, 1909), who proposed that interphase chromosomes, though then not detectable by light microscopy, would still remain spatially confined within sub-nuclear regions at interphase. However, due to a lack of direct experimental evidence, their model was not given serious consideration. Instead, the view of a highly decondensed distribution of chromatin, with each chromosome expanding widely across the interphase nucleus, was prevalent for decades (Comings, 1968).

The resurgence of the idea that individual interphase chromosomes exists as discrete entities, now called chromosome territories, was derived from two types of experiment. First, following subchromosomal damage of metaphase chromosomes by UV microirradiation, it was observed that the repaired DNA was maintained as distinct patches during subsequent interphases (Cremer et al., 1984). Conversely, if the damage was directed
at a region of chromatin during interphase, the repaired DNA, as shown by autoradiography or immunolabeling of incorporated marker nucleotides, was shown to be confined to a few metaphase chromosomes only (Cremer et al., 1982a,b). These results point to the notion that individual interphase chromosomes retain to some degree their integrity as seen at metaphase. By using chromosome painting, a fluorescent in situ hybridization (FISH) technique that essentially labels all the unique sequences of a particular chromosome, it became apparent that a territorial organization of interphase chromosomes exists in nuclei of higher eukaryotes. This phenomenon is observed not only in cell hybrids (Manuelidis, 1985; Schardin et al., 1985), but also in non-hybrid mammalian cell lines (Cremer et al., 1988; Lichter et al., 1988; Bischoff et al., 1993; Park and De Boni, 1998) and in plant cells (Leitch et al., 1990).

Based on the above evidence, Cremer et al. (1993) proposed that the chromatin of individual territories during interphase is organized into chromosomal loops that are similar in size, but less compacted, or "fluffier" than the metaphase chromosome model put forth by Saitoh and Laemmli (1993, section 1.1.5). Chromosome territories, during interphase are discrete, non-intermingling entities, postulated to be separated from each another by repulsive electrostatic force between opposite territorial surfaces at physiological pH (Cremer et al., 1993; 1996). Spanning between territories are putative channels, a continuous reticulum of DNA-free space, wherein protein complexes involved in nuclear processes such as transcription, splicing, mRNA export and DNA replication (Spector, 1990; Cremer et al., 1993; Bridger et al., 1998) accumulate. This channel network is proposed to communicate
with nuclear pores and together with the surface area of the chromosome territories, is termed the interchromosomal domain (ICD) compartment (Zirbel et al., 1993).

It is unlikely that the supramolecular organization of chromosome territories and their structural integrity, can be assembled and maintained by histone proteins \textit{per se}. In fact, it has been postulated that a proteinaceous scaffold network, termed the nuclear matrix, exists within the nucleus (Berezney and Coffey 1974). This scaffold is postulated to not only provide the structural framework for chromosome territories, but also to act as functional foci for other nuclear processes (Hozak, 1996). The molecular basis of such a nuclear skeletal framework remains enigmatic (see Section 1.3.1), since the isolation of its constituent proteins usually requires non-physiological high-salt extraction. However, specific DNA sequences, known as matrix attachment regions (MARs) have been found to preferentially associate with the nuclear matrix (Gasser and Laemmli, 1986). Together with the fact that DNA loops of 5 to 200 kb can be isolated in relatively “physiological” conditions (Jackson et al., 1990), it has been proposed that a pair of MARs may serve as the "book-end" to anchor a chromosomal loop to the nuclear matrix (Mirkovitch et al., 1984; Cockerill and Garrard, 1986). Such an organization might allow each chromosome unit to be autonomously compacted into a higher-order chromatin structure, and might allow partitioning of chromatin into loops for differential gene regulation (Cremer et al, 1993; 1995).
1.2) THE ROLE OF CHROMATIN STRUCTURE IN THE CONTROL OF TRANSCRIPTION

In the past, studies of eukaryotic gene expression have put much focus on identifying the molecular identity of trans-acting factors and their role in the transcriptional activation or repression of gene(s). However, it has become increasingly apparent that any observed effect has to be viewed in the context of the hierarchical folding of chromatin (Felsenfeld, 1992). Specifically, chromatin structures at different levels of organization, whether it is the individual nucleosome, the 30-nm fiber, or the chromosomal loop domain, all have their unique roles in regulating transcription. This reflects the fact that chromatin is differentially assembled in order to fulfil the contrasting needs of DNA compaction and accessibility for transcription during interphase.

1.2.1) Nucleosome positioning

Numerous studies have shown that the organization of nucleosomes around regulatory regions of eukaryotic genes is specific (Li et al., 1997). This phenomenon, known as nucleosome positioning, affects which segment (translational positioning), and which rotational orientation (rotational positioning), brings a particular DNA sequence in contact with the nucleosomes (see sections 1.1.1 and 1.1.2). Nucleosomal positioning can be directed by DNA sequence preference, statistical (see below), or by active interaction between trans-acting factors and nucleosomes (Wolffe, 1995).

As mentioned earlier, sequence-directed nucleosome positioning is a consequence of the inherent curvature or rigidity possessed by different DNA sequences (see section 1.1.1).
For example, the matrix associated regions (MARs) of the chicken α-globin gene are cis-acting elements which exhibit, among other functions (see below), transcriptional enhancer activity via their interaction with transcription factors. It has been suggested that the competition between transcription factors and histones for the MARs of the chicken α-globin gene (the latter bind favorably due to the intrinsic curvature of these MARs), may serve to regulate the expression of chicken α-globin gene (Boulikas, 1994). Statistical nucleosome positioning refers to the generation of boundaries by sequence specific, non-histone proteins (Fedor et al., 1988), followed by phasing in of histones into the sequence of interest based on these borders (Kornberg, 1988). Likewise, sequence specific trans-acting factors can directly influence nucleosome positioning through protein-histone interaction. For example, in *Saccharomyces cerevisiae*, the α2-MCM1 protein complex is involved in specifically positioning a nucleosome over the TATA box of a-cell specific genes (Shimizu et al., 1991), resulting in transcriptional repression (Simpson, 1993). Mutations in histone H4 disrupt this positioning, suggesting that a direct interaction between the repressor complex and histones is required.

The classical view that nucleosomes function as repressors of transcription (Morse, 1989), is exemplified in the genes of mammalian X chromosomes. Specifically, promoters in the inactive X chromosome appear to be incorporated into positioned nucleosomes; whereas promoters in the active X chromosome are free of such structures and bind transcription factors (Riggs and Pfeifer, 1992). However, it is increasingly evident that nucleosome formation can also play an active role in gene activation (Li et al., 1997). Examples of nucleosome-facilitated transcriptional activation in eukaryotes are found in systems such as the *Drosophila* heat-shock protein (*hsp*) 26 gene (Thomas & Elgin, 1988), the rat prolactin
gene (Cullen, 1993) and the Xenopus vitellogenin B1 gene (Schild et al., 1993). In keeping with the loop-out hypothesis (Ptashne, 1986), it has been proposed that these positioned nucleosomes, observed at intervening sequences between promoter and enhancer sequences, serve to juxtapose trans-acting factors that bind to these sequences, thereby facilitating transcriptional activation and initiation of complex formation (Thomas and Elgin, 1988).

1.2.2) DNAse hypersensitive chromatin (DHC) domains

In eukaryotes and viruses, certain subsets of chromatin are a hundred times more susceptible to DNAse I digestion or to chemical modification than the remainder of bulk chromatin (Wu et al., 1979; Wu and Gilbert, 1981; McGhee et al., 1981; Burch and Weintraub, 1983). This type of chromatin, operationally defined as DNAse hypersensitive chromatin (DHC) domains, represents nucleosome-free DNA segments that encompass the length of single or multiple units of nucleosomal repeats. These sites are commonly mapped to cis-acting elements, sequences that exert regulatory functions in nuclear processes. They include promoters, enhancers, steroid response elements, locus control regions, silencers, replication origins and telomeres (Gross and Gerrard, 1988). In addition, a subset of these sites is associated with non-histone proteins, such as topoisomerase I and II, RNA polymerase II, and transcription factors.

Likewise, and frequently flanking the DHC domains are sequences known as regions of general DNAse sensitivity (Eissenberg, 1985). While not as sensitive as hypersensitive DNA, these regions exhibit an increased susceptibility to DNAse I digestion by an order of magnitude, compared to the remainder of chromatin. Structurally, these regions extend over
many kilobases, and are associated with the coding segments of transcriptionally active or potentially active genes (Weintraub and Groudine, 1976; Wood and Felsenfeld, 1982). Within the nucleus of an intact cell, DHC domains, labeled by a technique known as *in situ* nick translation, are generally interpreted to represent sites or compartments at which active or potentially active genes reside (Hutchison and Weintraub, 1985, see introduction). Although it is unclear what fraction of DHC domains are involved in transcriptional activation of genes within a cell, DHC domains are preferably found within the chromatin of active genes (Bonifer et al., 1991). In fact, Levitt et al. (1979) had shown that over 80% of nick translated DNA fragments released from intact cells were able to hybridize with total cellular RNA *in vitro*.

Within a given cell type, DHC domains are categorized as either *constitutive* or *inducible* (Gross and Garrand, 1988). Between cell types, DHC domains can further be classified as *tissue-specific*, or if they appear transiently during ontogeny: *developmental* DHC domains. Some of the classical examples of *constitutive* DHC domains are the heat-shock protein (*hsp 26* and *70*) promoters in *Drosophila* (Wu, 1980; Cartwright and Elgin, 1986). These domains are termed *constitutive* because their organization and maintenance as nucleosome-free domains are independent of heat shock-induced gene expression. Instead, they serve to potentiate transcriptional activation upon binding of heat shock transcription factor (HSTF). Thus, the presence of *constitutive* DHC domains is necessary but not sufficient for committing a gene to be transcriptionally active. In contrast to the nucleosome-free state of *constitutive* DHC domains, the DHC domains at the promoter regions of inducible, tissue-specific and developmental genes are packaged by positioned nucleosomes
(McGhee et al., 1981; Richard-Foy and Hager, 1987; Montecino et al., 1996). Generation of these domains requires chromatin remodelling, which results in perturbation of the nucleosomal array, and concomitant transcriptional activation of the linked gene.

Several factors act independently or in concert in creating and maintaining these domains. First of all, hypersensitivity of a site is often determined by its sequence composition or by the chromatin conformation the sequence adopts. For example, sequences such as polypurine/polypyramidine tracts, and CT repeats are often parts of DHC domains in eukaryotic promoters (Evans et al., 1984; Struhl, 1986; Xu and Goodridge, 1996). The ability of these sequences to adopt a non-B form of DNA in vitro, which disfavors nucleosome formation, has been suggested as a mechanism for maintenance of constitutive DHC sites (Struhl, 1985; Xu and Goodridge, 1998).

It has been previously suggested that DHC domains function in providing access to DNA for relevant enzyme complexes (Eissenberg et al., 1988; Wallrath et al., 1994). In fact, the binding of trans-acting proteins to cis-acting elements is itself another determinant of generation of DHC domains (Emerson and Felsenfeld, 1984; Becker et al., 1991; Tsukiyama et al., 1994; Bhattacharyya et al., 1997). This is especially important for inducible and tissue-specific DHC sites, in that their liberation from assembled nucleosomes requires the initial binding of a positive regulator to its site(s) within the nucleosome. Interestingly, accessibility to binding sites may be facilitated by histone acetylation (Lee et al., 1993; Vettese-Dadey et al., 1996; section 1.1.3).
1.2.3) Position effect variegation, locus control region, heterochromatin.

The formation of positioned nucleosome(s) over regulatory elements to render them inaccessible to transcription factors is the first step in a chromatin compaction process, which leads to transcriptional repression. In fact, on a larger scale, highly condensed segments of chromatin known as heterochromatin, are readily observed in nuclei at interphase. The consequence of this packaging process is that the formation of heterochromatin not only represses transcription of genes therein, but of genes at its borders. This has major implications in transgenic research, since stably expressed transgenes would have variable levels of expression, depending on where they were integrated into the host genome (Grosveld et al., 1987; Stief et al., 1989).

This variability in gene expression, termed position effect variegation (PEV), can be relieved by inclusion of locus control regions (LCR) to the transgene construct, which exerts a dominant transcriptional activation function over a chromatin domain (10-10kB) (Fraser and Grosveld, 1998). The exact mechanisms of transcriptional activation mediated by LCR and its regulation remains to be determined. Unlike enhancers, the effects of LCR are generally seen only in stably transfected animals. Hence, this enhancing effect is probably associated with higher order chromatin structure. LCR has a high density of binding sites for transcription factors, suggesting that LCR can form a loop with the regulatory element of its linked gene. Such an interaction is postulated to be responsible for the unraveling of chromatin fibers (Felsenfeld, 1992; Jenuwein et al., 1993; Fraser and Grosveld (1998) into a more "open" chromatin configuration.
1.2.4) Topology of genes within chromosome territories

According to the model of chromosome territories proposed by Cremer and colleagues (1993, 1996), for the enzymatic machinery of transcription to gain access to its destined gene, a given chromosomal loop domain has to position its expressible sequences to the surface of the chromosome territory. Support of this model came from results of dual-color FISH of chromosome territories and selective genes residing therein. In contrast to non-specific, non-coding sequences, both active transcribed and facultative activable genes are localized preferentially at the periphery of their cognate chromosome territories (Kurz et al., 1996). Similarly, the transcriptionally-active viral genome of HPV-18 also integrates at the territorial surface of chromosome 8 in HeLa cells (Zirbel et al., 1993), as do the over-expressed and transcribed erbB2 sequences in breast tumor cell lines (Park and De Boni, 1998). In fact, in the latter study, the positioning of the erbB2 gene has been shown to occur at that surface of the chromosome territory closest to the nuclear envelope (Park and De Boni, 1998), a region frequently implicated as a compartment for active gene expression (Blobel, 1985; de Graaf, 1990; Park and De Boni, 1999). Although a direct demonstration of transcriptional activation by repositioning genes to the territorial surface has not yet been shown, the notion that genes are dynamically positioned within a chromosome territory for regulation of gene expression is attractive and deserves further investigation.

It remains unclear as to what functional significance exists for inactive genes to be positioned onto the territorial surface along with active genes. However, it is apparent that control of gene activity occurs in both the contexts of supramolecular organization of chromatin (see above), and of regulation of cis-acting elements of genes (van Driel et al.,
Hence, any competent gene located at the surface of a chromosome territory would still require the binding of appropriate trans-acting factors for activation.

Taken together, the above indicates that histone modifications, nucleosomal positioning, and interactions between cis-acting elements would all have to come into play to maintain and render specific genes accessible to the transcriptional machinery (see sections 1.1.3, 1.2.1, 1.2.3). In fact, morphometric analysis of both the active and inactive human X chromosomes at interphase reveal that although both have similar volumes, the active chromosome territories exhibit a larger and more irregular surface area compared to the smoother surface of the permanently transcriptionally-repressed inactive territory (Eils et al., 1996). This increase in surface area is interpreted as a manifestation of the underlying process which decondenses that subset of chromosomal domains which contains transcribing genes of the active chromosome and enhances their exposure to the transcriptional machinery.

1.3) ARCHITECTURE OF THE INTERPHASE NUCLEUS

The current view of the interphase nucleus holds that it is a complex organelle with its components topologically organized into compartments to effect their function (Moen et al., 1995; Carmo-Fonseca et al., 1996; Lamond and Earnshaw, 1998; Park and De Boni, 1999). Specifically, in addition to the compaction of chromatin into chromosome territories (Cremer et al., 1988; Lichter et al., 1988; Bischoff et al., 1993; Park and De Boni, 1998), molecular factors involved in replication, transcription, and RNA-processing are assembled into morphological entities. They execute their function by association with an insoluble
nuclear structure, known as the nuclear matrix (van Driel et al., 1995). Moreover, the observed spatial association of molecular factors involved in sequential steps of (pre)-mRNA metabolism has led to the proposal that nuclear compartmentalization serves to facilitate temporal and spatial coupling of nuclear processes (van Driel et al., 1991; De Boni, 1994). This three dimensional (3-D) spatial positioning of nuclear components is not only cell-cycle and tissue type specific, but may also change dynamically upon changes in cell function. Therefore, the spatial organization of the interphase nucleus may represent a fundamental, supramolecular control mechanism in regulating gene expression.

1.3.1) The nuclear matrix

The nuclear matrix is operationally defined as the residual structure of nuclei after extensive DNAse digestion and extraction of most of the chromatin and soluble protein components (Berezney and Coffey, 1974; Nelson et al., 1986). The nuclear matrix consists of the nuclear lamina-pore complex at the nuclear periphery and an internal, three dimensional, filamentous network with associated granular material. The nuclear lamina is composed of lamin A, B and C, interfilament-like proteins which form a fibrillar reticular structure that lines the nucleoplasmic surface of the nuclear envelope. This network provides the framework for attachment of nuclear membrane, nuclear pore complexes and chromatin (Gerace and Burke, 1988; Nigg, 1989).
In contrast, despite twenty-five years of ongoing research of nuclear matrix (Berezney and Coffey, 1974), the structural protein(s) which presumably polymerize into filaments of the internal matrix are not yet unambiguously identified. This is due to the fact that different preparation methods, using markedly non-physiological conditions, have been employed for nuclear matrix isolation, resulting in protein compositions and ultrastructural changes that are not only variable, but could well represent isolation artifacts (Kauffmann and Shaper, 1984; Mirkovitch et al., 1984; Nickerson et al., 1995, de Jong 1996).

Nevertheless, it is generally agreed that a nuclear scaffold exists to provide the architectural framework for chromatin as well as sites of assembly of proteins or enzymes involved in nuclear processes. In fact, numerous studies have shown that despite the harsh treatment, represented by nuclease digestion and salt extraction, the number and distribution of specific nuclear components remains unaltered, as judged by comparing the distribution of probes specific for these components before and after matrix preparation (Nickerson et al., 1995).

The nuclear matrix has been implicated as the site for DNA replication and transcription, presumably at the interchromosomal domain (ICD) compartment (see section 1.1.6, Cremer et al., 1993; Zirber et al., 1993; Cremer et al, 1996). Several studies, employing different matrix preparation protocols, have demonstrated that newly replicated DNA sequences and certain replication factors remain associated with the nuclear matrix (Foster and Collins, 1985; Tubo and Berezney, 1987a,b; Nakayasu and Berezney, 1989; Hozak et al., 1994). Moreover, in pulse-chase experiments, nascent DNA migrates from matrix bound-replication foci into a surrounding halo (Dijkwel et al., 1986). These
results have led to the current model which states that in vivo, replication occurs by coordinating the DNA template to reel through an immobilized replication "factory", rather than having the latter free-floating, in order to track along the template (Hozak, 1994).

Similar results were obtained regarding RNA synthesis (Jackson et al. 1981, 1993; Wansink et al., 1993; Patturajan et al., 1998). As mentioned above, the nuclear matrix may serve as an axial scaffold for binding of matrix associated regions (MARs) at the ends of chromosomal loops. In addition, many genes or gene clusters are specifically flanked by boundaries of MARs (Mirkovitch et al., 1984; Jarman and Higgs, 1988; Phi-Van et al., 1990). MARs are implicated to juxtapose "looped-out" genes to those nuclear matrix components which house the stationary transcription complex for subsequent transcriptional activation (Boulikas, 1995). Therefore, the interplay between template and transcription "factories" is analogous to that proposed in DNA replication (Jackson et al. 1981, 1993; Wansink et al., 1993; Patturajan et al., 1998). In addition, nuclear processes downstream of transcription, i.e., pre-mRNA processing and nucleocytoplasmic RNA transport also occurred in association with the nuclear matrix (see below).

The nuclear matrix is considered to be a dynamic structure, with its protein composition reflecting the cell type and state of differentiation (Fey and Penman, 1988; Stuurman et al., 1989; Dworetzky et al., 1990). While the composition of nuclear matrix proteins may however change depending on the isolation procedure, it is known that certain tumors, such as human infiltrating ductal carcinoma and human colon adenocarcinoma, have nuclear matrix proteins not present in corresponding normal tissues (Keesee et al., 1994). Progressive changes in nuclear matrix protein pattern have also been documented during rat
osteoblast differentiation (Dworetzky, et al., 1990). Given the pivotal role of the nuclear matrix in the organization of the interphase nucleus, such changes certainly might be considered to influence gene expression and the cell phenotype during development and malignancy.

1.3.2) The nucleolus

The nucleolus, the most easily identifiable object nuclear structure, is the sub-nuclear organelle responsible for synthesis and maturation of ribosomal RNA (rRNA) bound for export to the endoplasmic reticulum (Scheer and Benavente, 1990). Ultrastructural examinations have revealed that the nucleolus consists of one or more fibrillar centers (FCs), each bounded by dense fibrillar components (DFCs), and altogether surrounded by the granular component (GC) (Hozak, 1996). FCs contain ribosomal DNA (rDNA) as well as RNA polymerase I, topoisomerase I and other proteins (Scheer and Rose, 1984). In human and mouse cells, tandem repeats of ribosomal genes are found at loci in a number of chromosomes called nuclear-organizing regions (NORs) (Henderson et al., 1972, 1974). It is the agglomeration of these NORs that contribute to the DNA content of the fibrillar center. The periphery of FCs, together with the DFC, are the first areas to accumulate labeled tritiated uridine 5'-triphosphate or 5-bromouridine 5'-triphosphate (BR-uridine) and are considered as the sites of rRNA synthesis (Wachtler et al., 1989; Dundr and Raska, 1993; Hozak et al., 1994). The GC contains ribonucleoproteins and is postulated to represent a location of post-transcriptional rRNA maturation (Hilliker and Appels, 1989). Similar to synthesis and processing of (pre-) mRNA (see below), such nucleolar organization may
represent a linear production line for ribosomes (Moen et al., 1995) and exemplifies how nuclear processes may occur at discrete compartments.

1.3.3) Spatial rearrangement of chromatin domains

Several lines of evidence indicate that in addition to the topology of genes within a chromosome territory, the three dimensional, spatial positions of specific chromatin domains within the nuclear space, may play a crucial role in the control of gene expression (De Boni, 1994). Since the early work by Rabl (1885), who suggested that interphase chromosomes may retain the polarized arrangement of their chromosomal domains as seen in telophase, many studies have shown that the topology of specific chromatin is not random. Specifically, using the technique of in situ hybridization to label repetitive, nontranscribing centromeric satellite DNA (sDNA) (Manuelidis, 1982; 1984b; 1985a, 1985b, Joseph et al., 1989; Masumoto et al., 1989a, 1989b) and telomeric DNA (tDNA) sequences (Mathog et al., 1984; Hochstrasser et al., 1986; Katsumata and Lo, 1988; Billia and De Boni, 1991), such domains were found to be reproducibly fixed at specific intranuclear regions, in a cell-type specific manner (Manuelidis, 1984a, 1984b; 1985a, 1985b; Manuelidis and Borden, 1988; Billia and De Boni, 1991).

Similar results were obtained by using antibodies specific to kinetochore proteins (Haaf et al., 1990; Haaf and Schmid, 1989; 1991; Holowacz and De Boni, 1991), which are know to remain associated with centromeric satellite DNA during interphase (Matsumoto et al., 1989b). Given that these studies often employed different, adult, G0 arrested neuronal
cell types from the central nervous system (Manuelidis, 1984a; 1984b; 1985a; 1985b; Manuelidis and Borden, 1988), which in turn are derived from one type of progenitor cells during embryogenesis, the observed cell-type specific patterns of specific chromatin domains has been suggested to result from rearrangement of these domains during cell differentiation and that their spatial positions may be responsible for the manifestation of different cell phenotypes (Park and De Boni, 1999).

These nonrandom, intranuclear patterns of chromatin domains are not static. In fact, numerous studies have indicated that these tissue-specific patterns are altered upon changes in the functional state of the cell, such as in pathology, during development or upon induction by physiological stimuli (De Boni, 1994). For example, pioneering work by Borden and Manuelidis (1988) has shown that within the human cerebral cortical neurons at epileptic foci, the 3-D topology of centromeric domains of X chromosome is different from that observed in the same neuronal type in adjacent tissue, outside the epileptic focus. Likewise, the post-natal maturation of Purkinje neurons is operationally divided into four stages, each characterized by a set of morphological features. Interestingly, the transition to each stage occurs concurrent with a redistribution of centromeric domains within the Purkinje neuronal nuclei (Martou and De Boni, manuscript in prep.). A similar phenomenon was also observed during redifferentiation of dorsal root ganglion in vitro (Park and De Boni, 1991; Choh and De Boni, 1996).

A dynamic redistribution of specific chromatin domains was also observed in neurons exposed to the neurotransmitter GABA (Holowacz and De Boni, 1991), in hepatocytes of male Xenopus laevis, in response to de novo activation of vitellogenin genes by estrogen
(Janevski et al., 1995), and in hippocampal neurons exhibiting long term potentiation (Billia et al., 1992). Specifically, in the presence of these physiological stimuli, which are known to induce or to be associated with changes in gene expression (Greenberg et al., 1986; Cole et al., 1989; Dragunow et al., 1989a, 1989b), a concomitant rearrangement of centromeric domains, manifested by changes in the degree of their clustering, was observed.

These findings provide evidence that the observed rearrangement of chromatin domains may reflect a cell function-dependent organization of chromatin during interphase, and that such changes in organization play a role in regulating gene expression. It has been proposed that this dynamic repositioning serves to transpose transcriptionally active genes to transcriptionally competent compartments (De Boni, 1994; Park and De Boni, 1997; 1998). A unifying theory that bridges the structure-function relationship between specific chromatin domain patterns and regulation of gene expression in different cell types remains to be proposed (De Boni, 1994). In addition, it must be considered that the examination of the distribution of specific chromatin domains is routinely performed in a “before-and-after” context, employing cells fixed at different stages of function. Together with the fact that centromeric and telomeric domains are non-transcribing sequences, the question remains whether the dynamic repositioning of these domains directly controls, or is merely associative with, changes in gene expression (Park and De Boni, 1999).

With the advent of green fluorescent protein (GFP) technology, these drawbacks may be circumvented (Misteli and Spector, 1997; Zhu et al., 1998; Sullivan and Shelby, 1999). Specifically, it is now possible to employ a fusion protein consisting of human centromeric binding protein CENP-B coupled to GFP, that can be expressed and targeted to centromeres
in live cells, allowing the investigation of the dynamics of centromeric movement \textit{in vivo} (Shelby et al., 1996). Likewise, by constructing a vector containing tandem arrays of lac operators inserted into a gene and targeting such construct to its cognate genomic loci by homologous recombination, the genomic loci can then be subsequently supravivitally labeled, via the insert, by the lac repressor proteins fused to GFP (Robinett et al., 1996). It is envisioned that in the future, when multi-color fluorescent protein labeling becomes possible, these \textit{in vivo} approaches, in conjunction with time-lapse confocal microscopy, will provide a direct functional link between stimuli, altered gene expression and the associated dynamic reorganization of nuclear topology (Misteli and Spector, 1998)

1.3.4) Spatial coupling of transcription and splicing of (pre-)mRNA

The localization of molecular factors involved in pre-mRNA metabolism, splicing in particular, has been the subject of intense research for the past few decades. Spliceosomes are nuclear RNA-protein complexes which function in the removal of introns from pre-mRNA. They are composed of splicing factors known as small ribonucleoproteins (snRNPs) (Kramer, 1996), non-snRNP splicing factors such as SC-35 and SR proteins (Fu and Maniatis, 1990; Manley and Tacke, 1996), as well as a number of other non-splicing factors (Query et al., 1994).

Using antibodies specific for snRNPs or for non-snRNPs factors (Spector et al., 1983), these nuclear antigens are presented as discrete speckles or foci, in addition to exhibiting a diffuse nucleoplasmic labeling throughout the nucleus (Lamond and Carmo-
Some of these splicing factors, including snRNPs, SR proteins, as well as poly (A)+ RNA (Huang et al., 1994) are highly enriched in ultrastructural entities known as interchromatin granule clusters (IGCs); the equivalent of the speckles detected by light microscopy (Fakan et al., 1984; Spector et al., 1990; Carmo-Fonseca et al., 1991; Wansink et al., 1994). Likewise, the incorporation of tritiated uridine triphosphate in pulse-labeling experiments revealed that nascent pre-mRNA forms ultrastructural domains known as perichromatin fibrils (Pfs). Pfs, representative of transcription sites, (Fakan and Puvion, 1980) are frequently, though not invariably, observed to emanate from densely stained chromatin into IGC (Spector, 1993; Wansink et al., 1994).

This spatial relationship is supported by studies which demonstrate that splicing factors/IGCs are colocalized or associated with nascent RNA from several induced or actively transcribing genes but not with inactive genes in a number of cell types (Lawrence et al., 1989; Huang and Spector, 1991; Xing et al., 1993; 1995; Huang and Spector, 1996b). Poly-A+ RNA, representative of mRNA bound for export, is also found to be co-localized with IGCs/speckles (Carter et al., 1991; 1993; Visa et al., 1993; Huang et al., 1994). Furthermore, splicing factors were shown to associate with transfected intron-containing genes and not with transfected intron-lacking genes. As a result, this body of evidence has led to the proposal that transcription and splicing are spatially coupled by directing nascent transcripts to IGCs/speckles. The distribution of the latter and their proximity to transcription sites is therefore a consequence of their functional role as a splicing compartment (Moen et al., 1995; Clemson and Lawrence, 1996).
In contrast, results from a number of studies counteract this view which states that IGCs/speckles represent competent compartments for splicing (Wankink et al., 1994; Zhang et al., 1994; Puvion and Puvion-Dutilleul, 1996; Huang and Spector, 1996b; Misteli and Spector, 1997). First of all, it is frequently pointed that most, if not all RNA transcripts, are only associated with or mapped to the periphery of speckles, rather than “dead-on”. Second, actively transcribing cells exhibit a more widespread nucleoplasmic distribution of splicing factors and numerous small speckled labeling. Conversely, in cells where transcription and splicing are reduced due to terminal differentiation, drugs, or heat shock, fewer but larger IGCs/ speckles were observed (Antoniou et al., 1993; O’Keefe et al., 1994; Zeng et al., 1997). In fact, in human diploid fibroblasts, digital imaging microscopy revealed that speckles (SC35 in this case) may only represent a minority (20-30%) of the nuclear signals, with the rest attributable to a diffusible pool (Fay et al., 1997).

On the other hand, Zhang et al. (1994) have observed that splicing of the β-actin pre-mRNA occurs at the sites of transcription, although speckles were not detected at those loci. Likewise, although speckles do contain poly(A^+) RNA, suggesting that they contained fully spliced transcripts (Carter et al., 1991; 1993), it was argued that some or all of the speckle-associated poly(A) RNA could be structural, rather than mRNA bound for export (Clemson and Lawrence, 1996; Lamond and Earnshaw, 1998). Last but not least, the full complement of splicing factors that constitute and define a functional spliceosome in vitro may not necessarily always co-localize at speckles in situ (Zamore and Green, 1991; Sahlas et al., 1993).
In short, the function of speckles as splicing compartments remains dubious. It is possible, however, that splicing, even in the absence of a speckled domain juxtaposed to nascent transcripts, can still be a co-transcriptional event. In support of this, it was observed that the coupling of the two processes is effected by the interaction between nascent transcripts and a diffusible pool of splicing factors (Beyer and Osheim, 1988; Bauren and Wieslander, 1994; Wuarin and Schibler, 1994).

In an alternative model, it was proposed that the majority of speckles may function as storage or assembly sites for splicing factors, which in turn depart from the depot for the transcription site via a recruitment process (Jimenez-Garcia and Spector, 1993; Pombo et al., 1994; Huang and Spector, 1996a; Dirks et al., 1997). According to this view, depending on the efficiencies of the various reactions in the (pre-)mRNA pathway, the actual spliceosomes may appose to the transcription site as speckles if the rate of transcription surpasses the processing capacity of the splicing factors (Xing et al., 1993, 1995). Conversely, if transcription is the rate-limiting step (Huang and Spector, 1996a,b), spliceosomes may become too diffuse to be detectable by light microscopy because their splicing action is relatively more efficient (Zhang et al., 1994). In short, the dynamics of splicing factors localization is therefore complex and may involve trafficking between nuclear domains (Misteli an Spector, 1998).

In fact, such a dynamic event has been vividly demonstrated by tracking the localization of the alternative splicing factor (ASF) fused to GFP in viral-infected cells (Misteli et al., 1997). Specifically, upon transcriptional activation of the viral genome, time-lapse microscopy revealed that splicing factors protrude from neighboring speckles and
colocalize at the viral transcription site. Likewise, in non-infected cells, it was frequently observed that "particles" associate or disassociate from speckled domains, implicating recruitment and dismissal of splicing factor to and from transcription sites.

The mechanism underlying the dynamics of splicing factor, at least for ASF proteins, is beginning to unfold (Misteli and Spector, 1998). ASF proteins belong to the superfamily of arginine-serine (RS) splicing factor (Fu, 1995), which contain sites for phosphorylation, known as the RS domain. Protein phosphorylation and dephosphorylation have been implicated in not only regulating the activity of ASF protein, but also in modulating the intranuclear distribution of splicing factors (Colwill et al., 1996; Misteli and Spector, 1996). This is supported by the fact that perturbation of both kinase and phosphatase activities can alter the degree of punctateness and size of the speckles (Misteli and Spector, 1996) in vivo. It remains to be established whether the spliceosomes are assembled at the transcription sites, or are at least partially constructed at the IGCs/speckles.

1.3.5) **Nucleo-cytoplasmic mRNA transport via the nuclear pore complexes**

The nuclear pore complexes (NPCs) are large supramolecular structures (120MDa) which punctuate the nuclear envelope at regions where the inner and outer nuclear membrane fuse. Nuclear pores are constructed from two sets of annuli, each one sitting on the surface of the cytoplasmic and nuclear leaflets respectively, and exhibit an 8-fold symmetry about the central axis perpendicular to the plane of the nuclear membrane. "Spokes" or strand-like components radiate from the annuli into a central pore which may act as an active transporter
NPCs provide communication between the nucleus and the cytoplasm and regulate the traffic of macromolecules between these two compartments. Small molecules and ions move through the pore by diffusion whereas large molecules (>60kDa) are actively transported across the nuclear envelope by an ATP dependent process (Bastos et al., 1995).

The nuclear pore complex is attached to the nuclear lamina fibrous meshwork via the nucleoplasmic ring (Aebi et al., 1986; Whytock and Stewart, 1988). In addition, fibrous material has been reported to emanate from the nucleoplasmic aspect of the pore, forming an octagonal cage/basket-like structure which extends into the nucleoplasm (Scheer et al., 1988). This cage is actually part of a recently documented, highly ordered filamentous network that projects from the NPCs to the nuclear interior and associates with chromatin fiber (Arlucea et al., 1998). Similarly, using EDTA regressive staining, a technique that accentuates ribonucleoprotein structures, the nuclear matrix material can be observed to radiate from the nuclear interior towards the nuclear pore complexes in the form of channels (Monneron and Bernhard, 1969; Franke and Falk, 1970). Therefore, the nuclear lamina may function to align and maintain the contact between NPCs and the nuclear matrix. Given the substantial body of evidence indicating the role of the nuclear matrix in co-transcriptional splicing, such a topological arrangement may further denote a spatial coupling of the nuclear processes of transcription, splicing and nucleocytoplasmic export of mRNA.

In the "gene-gating model" proposed by Blobel (1985), actively transcribing genes of the genome are envisioned to attach (their regulatory elements) to nuclear pore complexes; here termed "gene gating organelles" which are anchored to a particular spatial locale on the
nuclear envelope. The space subjacent to the nuclear pore complexes and extending into the nuclear interior in the form of channels is proposed to serve as sites for transcription and pre-mRNA processing. Transcripts would then be vectorially transported for exit via their designated nuclear pore complexes.

The observation that specific viral transcripts are concentrated in a curvilinear track in nuclei of Epstein-Barr virus (EBV)-infected cells (Lawrence et al., 1989) is a vivid illustration of these transcripts moving from their site of synthesis along a defined pathway to the nuclear envelope. Since the EBV-transcript signal extends beyond the dimension of the gene and remains associated with the nuclear matrix after isolation of the latter, it was suggested that the matrix provides a structural basis for pre-mRNA processing and RNA transport (Xing and Lawrence, 1991). Similarly, within the exceedingly flat nucleus of 3T3 fibroblasts, transcripts of the endogenous c-fos gene, were shown to take the most direct path from the transcription site to the nuclear envelope, in the form of an elongated signal (Huang and Spector, 1991). Tracks have also been observed for a few other viral transcripts and for endogenous RNAs, albeit these tracks were often less pronounced than those of the EBV transcript (Lawrence et al., 1989; Xing et al., 1993; Dirks et al., 1995).

However, RNA tracks are not a general phenomenon for nuclear transcripts, because a number of transcripts are described which are concentrated in foci rather than along tracks (Xing et al., 1993). Also, because very few EBV transcripts are actually present in the cytoplasm (Dambaugh et al., 1986), it has been argued that the observed tracks may only represent RNA accumulation sites rather than areas of active RNA export. Furthermore, some transcript tracks often stop at a considerable distance from the nuclear envelope (Xing...
and Lawrence, 1993). It remains enigmatic as to how transport of RNA transcripts is achieved in this case, and an alternative model which relies on diffusion may provide the explanation (Zachar et al., 1993). However, it is interesting to note in some mammalian cells, there exist long, dynamic tubular channels, derived from the nuclear envelope and that extend and may transverse across the entire nucleus (Fricker et al., 1997). In insects, such intranuclear channels are also evident in the polytene nuclei of salivary gland cells of *Drosophila melanogaster* (Park and De Boni, 1992). These intranuclear channels, which contain local fenestrations suggestive of the presence of nuclear pore complexes, may therefore provide an alternative route for transcripts to exit the nucleus without reaching the "conventional" nuclear surface.
METHODS

2.1) CELL CULTURE

To permit microscopic observation of subcellular detail at high resolution, cells were routinely cultured on glass coverslips (22x22 mm).

2.1.1) Preparation of coverslips

To ensure adhesion of the growth substrate (see below) and the removal of organic contaminants on glass coverslips, they were placed vertically on an acid resistant porcelain rack (Fisher Scientific) and etched by 50% (v/v) aqueous nitric acid in a hot water bath for 2 hours. Then, using a clamp specifically designed to hold the porcelain rack, the coverslips were removed from the acid, and repeated washed with distilled water to remove traces of the acid. Each coverslip was dehydrated and stored in absolute ethanol.

Coverslips cleaned in the preceding manner were air-dried, both sides sterilized by a 10 min exposure to ultraviolet light in a laminar flow hood, and were transferred to sterile polystyrene dishes with tight-fitting lids to prevent evaporation of medium (Falcon #1006). The coverslips were then coated with approximately 0.1mL of aqueous poly-D-lysine hydrobromide solution (0.1mg/mL, 30min; ICN #150175), washed (0.5mL per wash, 3x5 min) and kept in sterile Type I distilled water prior to use.
2.1.2) 3T3 fibroblasts

3T3 fibroblasts (ATCC, Rockville, MD) were maintained in 25 cm² culture flasks (Falcon #3013) in a medium consisting of 89% of Minimum Essential Medium with Hank’s salts containing L-glutamine (MEM; Gibco BRL #1575-032), 10% Fetal Bovine Serum (FBS, heat inactivated; Gibco BRL #16140-064), 50 units/ml penicillin and 50 mg/ml streptomycin (Gibco BRL #15145-014), and supplemented with D-glucose (BDH) to a final concentration of 5 mg/ml.

Cell cultures were subcultured upon reaching confluence. The medium was aspirated, followed by incubation in 2 mL of 0.25% trypsin (Gibco BRL # 15050-065, 37 °C, 6 min) and inactivation of the trypsin with FBS (1 mL). The cells were then dislodged gently by trituration, transferred with a flamed-polished Pasteur pipette to a 10 mL polystyrene test tube (Falcon #2057), and sedimented by centrifugation (3 min, 500 g). After the supernatant was aspirated, the cell pellet was resuspended in 2 mL of prewarmed culture medium. 2 drops of cell suspension were added to flasks preconditioned with 5 mL of prewarmed medium, followed by mechanical agitation to disperse the cells uniformly. Although the use of Hank’s salts permitted growth of these cells in a humidified environment at 37°C without exogenous CO₂, it was empirically determined that optimal growing environment required low concentration of CO₂ (<1%). The cultures were fed with prewarmed medium every 2 days and were consistently subcultured every 5 days.

For experiments, the cell suspension derived above was further diluted ten- or fifteen-fold with prewarmed medium. Sterile, freshly prepared poly-D-lysine coated coverslips (see Section 2.1.1) were preconditioned with Hank’s Balanced Salt solution (0.5
mL; Gibco BRL), followed by seeding of approximately 200 μL of the diluted cell suspension. The cultures were maintained by quantitative exchange of the culture medium at day 2 after seeding and were used after 3 days in vitro.

2.1.3) Long term storage of 3T3 cell line

For freezing and storage of cells, culture media was removed from flasks and the cells were detached from the growth surface by treatment with 0.25% (v/v) trypsin for 6 min at 37°C. The trypsin was inactivated by addition of 1 mL of fetal bovine serum, and the contents of the flasks were transferred into a 10 mL polystyrene test tube. Cells were monodispersed with a flame polished Pasteur pipette and centrifuged for 3 min (500 g). The supernatant was discarded by aspiration and the cell pellet resuspended in 10 mL of prewarmed cell medium. Cells were sedimented again, resuspended in 0.5 mL of medium, and transferred to a 1.5 mL Eppendorf centrifuge tube (with screw caps). Following addition of 0.5 mL of storage medium (20% dimethylsulfoxide (DMSO), 80% cell medium), the Eppendorf tube was briefly but rigorous vortexed to homogeneously mix the cells with DMSO. After equilibrating for 3 min, the Eppendorf tube was acutely submerged into a container with liquid nitrogen, and transferred to a liquid nitrogen tank for long-term storage.

2.2) OBSERVATION OF LIVE CELLS BY PHASE CONTRAST MICROSCOPY

Cells to be employed for the assessment of the effects of and extent of hypotonic expansion were seeded onto large (45x50 mm), poly-D-lysine coated coverslips which formed the base of Bionique chambers (Bionique, Saranac Lake, N.Y.). These chambers, essentially of Rose chamber design (Gabridge, 1981), permit the observation of live cells at
high resolution with an oil immersion objective by use of an inverted microscope. Prior to assessment, chambers with cells were transferred to the incubator stage (37°C) of a Nikon inverted microscope and monitored by phase contrast microscopy throughout expansion and fixation. Representative images of cells were recorded on 35 mm photographic film.

2.3) HYPOTONIC EXPANSION

Prior to use, the temperature of all KCl solutions and fixatives dissolved in KCl solutions are equilibrated to ambient temperature (approximately 25°C) and their pH adjusted to between 7.0 to 7.6. Preliminary experiments showed that fixation of hypotonically expanded cells with 4% formaldehyde, which is hypertonic, resulted in their collapse and a poor preservation of their nuclear morphology (Fig. 3D,D'). Thus, a “modified“ fixation protocol was developed. For this, the culture medium was aspirated, and the cultures were immediately exposed to isotonic (300 mOsm, control) or hypotonic (50 and 100 mOsm) KCl solutions (2 min), followed by fixation in a series of increasing concentrations of fixative, ranging from 0.2%, 0.4%, 0.8%, 2% (5 min each) to 4% formaldehyde (40 min), dissolved in KCl solutions of the same osmolarity used during expansion. Additional cultures were fixed in 4% (w/v) formaldehyde in phosphate buffered saline (PBS, pH 7.2, 60 min) to permit comparisons of the effects of fixation by isotonic PBS-buffer and isotonic KCl. This was followed by washes in PBS, (3 x 5 min). Cells were then permeabilized (Triton X-100, 0.25%, PBS, 15 min), washed (PBS, 3 x 5 min), and RNAse A digested (Boehringer Mannheim, 100 µg/ml, 1h, 37°C) to restrict subsequent labeling by ethidium bromide (1 µg/ml, PBS, 15 min) to nuclear DNA. Then, as in all the fluorescent microscopy work
described below, coverslips were mounted onto glass slides in freshly prepared anti-fading reagent (para-phenylene diamine, 1mg/mL, 50% (v/v) glycerol in PBS).

2.4) \textit{IN SITU} NICK TRANSLATION AND IMMUNOCYTOCHEMISTRY

Prior to \textit{in situ} nick translation, hypotonically expanded, fixed cultures and controls were permeabilized (Triton X-100, 0.5%, PBS, 45 min), and washed (PBS). Next, they were post-fixed in cold (-20° C), absolute methanol (5 min) to enhance preservation of nucleosomal structures (Hutchison and Weintraub, 1985), followed by washing (PBS, 3 x 5 min). Cultures were then rinsed in nick translation buffer (50 mM Tris-HCl (pH 7.8), 5 mM MgCl₂, 10 mM β-mercaptoethanol, 10 mg/mL Bovine Serum Albumin (BSA)), and incubated in nick-translation reaction mixture (40 mM each of dATP/ dCTP/ dGTP/ dTTP/ biotin-16-dUTP, all from Boehringer Mannheim; 10 U/ml DNAse I, Pharmacia; 10 U/ml DNA polymerase I, Promega) in nick translation buffer for 1 h. The labeling reaction was terminated by washing in 20 mM EDTA (5min) and in PBS (3x5min). Following incubation of cultures in 4% BSA (200μL, PBS, 30min) to reduce non-specific binding of antibodies, incorporated biotin was labeled by mouse anti-biotin monoclonal antibody (Boehringer Mannheim, 1:100 dilution, 4% BSA in PBS, RT, 1hr) and then by a secondary FITC-conjugated sheep anti-mouse antibody (Boehringer Mannheim, 1:100 dilution, 4% BSA in PBS, RT, 1hr). After the nuclear DNA was counterstained by ethidium bromide (1μg/mL, PBS, 15min), the cultures were mounted in anti-fade medium (1mg/mL p-phenylene diamine in 50% glycerol, PBS). Controls for the labeling procedure in which either DNAse I, DNA polymerase or primary antibodies were omitted were routinely carried out in parallel with each experiment.
2.5) DUAL LABELING OF DHC DOMAINS WITH SMALL RIBONUCLEOPROTEINS (snRNP) OR KINETOCHORES

Procedures of in situ nick translation reaction, including the addition of primary and secondary antibodies to detect incorporated biotin, were carried out as described in the section above, with the omission of the RNAse A digestion. Cultures were then again blocked by 4% BSA (PBS, 200μL, 30 min) prior to incubation in antibody either to snRNPs or kinetochores. For labeling of snRNPs, the cells were incubated in anti-Sm human autoimmune serum (ANA reference serum #5, Center for Disease Control (CDC), Atlanta, GA, 1:100, 4% BSA in PBS, RT, 1 hr). Following washing (PBS) and a second blocking step (4% BSA, PBS), the cells were incubated in TRITC-conjugated goat anti-human IgG (H+L, Jackson ImmunoResearch, 1:100, 4% BSA in PBS, RT, 60 min). Kinetochore labeling was achieved by using human anti-centromeric autoimmune serum (cross-referenced with CDC Reference Serum #8, 1:1000 in PBS, 37°C, 2 h, gift of Dr. L. Rubin), followed by TRITC-conjugated goat anti-human IgG (same as above). In these cases, nuclear DNA was not counterstained with ethidium bromide. Controls included the omission of the respective primary antibodies.

2.6) IMMUNOCYTOCHEMISTRY OF NUCLEAR PORE COMPLEXES (NPCs)

To test the hypothesis that the distribution of nuclear pore complexes reflects the distribution of DHC domains, cells were immunolabeled for NPCs and observed by confocal microscopy. Fixed cells (2% formaldehyde, PBS, 20 min) were first treated with RNAse A (Boehringer Mannheim, 100 mg/mL, PBS, 37°C, 1 hr) to restrict labeling by ethidium bromide to DNA within the nucleus in the subsequent counterstaining step (see below). Cells
were then washed (PBS, 3x5 min), post-fixed in cold (-20° C), absolute methanol (5 min), and washed again (same as above). Next, cells were incubated with 4% BSA (200µL, PBS, 1hr) to reduce non-specific binding of antibodies, and NPCs were labeled with a mouse anti-NPC monoclonal antibody (Bio/Can Scientific mAb414 #MMS120R, 1:75 dilution, 100µL, 4% BSA in PBS, 1hr). Following washing and a second block (same as above), the incorporated primary antibodies were labeled by a secondary FITC-conjugated sheep anti-mouse antibody (Boehringer Mannheim, 1:200 dilution, 4% BSA in PBS, RT, 1hr). Finally, cells were washed (PBS, 3x5min) and nuclear DNA counterstained by ethidium bromide (1 µg/ml, PBS, 15 min).

2.7) CONFOCAL MICROSCOPY

Cells were examined using a ZEISS LSM410, laser scanning confocal microscope, equipped with a 63x, 1.4-n.a. objective lens and an argon/ krypton laser. FITC, TRITC or ethidium bromide signals were detected by excitation with the 488-nm or 568-nm lines of the laser respectively. In all experiments, a random sample of cells was chosen, with electronic amplification parameters kept constant to acquire comparable images. For evaluation of the distribution of nuclear components, serial optical sectioning, at nominal focal steps of 0.33 µm and a relative pinhole size of 10, was performed. In addition, for morphometric analysis of the changes in nuclear geometry induced by hypotonic expansion, the length of the optical (z) axis was estimated by counting the number of confocal optical sections that contained nuclear DNA labeled by ethidium bromide. Major and minor axes (XY) were measured from confocal sections at the z-axis midplane. Nuclear volumes were calculated based on the assumption that nuclei exhibit an ellipsoidal geometry.
For assessment of NPC labeling, two investigators interactively scanned a random sample of labeled nuclei to decide whether the NPCs were preferentially present at the apical side of the nucleus, or whether NPCs were distributed equally on both apical and basal sides of the nucleus. The assignment of NPC labeling to the first category was made only if such a distribution was readily identifiable and by consensus between the two investigators.

2.8) CELLS ON “L-SHAPED” COVERSLEIPS

Laser confocal microscopes have a superior ability to resolve structures along the optical axis when compared to conventional microscopes. Nevertheless, the resolution along the optical axis remains less than that in the XY-plane. As an alternative to view nuclear detail in what is conventionally the z axis, cells were grown on narrow glass strips (approximately 3mm x 30mm), cut from coverslips with a diamond stylus, which were then formed into a “L-shape” by heating in a flame. These strips were pre-treated with poly-D-lysine, as described above. When confluent, cells were expanded by 50 mOsm KCl (10 sec), followed by fixation in 4% formaldehyde in 50mOsm KCl (1 h) and labeled for DHC domains as above. For microscopic observations, the “L-shape” coverslips were placed sideways, at right angle with respect to the original growth surface, into a Bionique chamber in antifade medium (above) so that intranuclear structures which were originally along the z axis, were now transposed into the XY plane and were viewed at higher resolution (Fig. 2).
Figure 2. The "L-shaped" coverslip is used to observe nuclear detail parallel to growth surface.
2.9) ULTRASTRUCTURAL EXAMINATION OF NUCLEI OF 3T3 FIBROBLASTS

2.9.1) Cell culture

3T3 fibroblasts were grown on glass coverslips coated with poly-D-lysine hydrobromide (ICN) but which, in addition, were previously coated with carbon. The carbon film provided an interface between the cells and the glass, and facilitated, following embedding in Epon, the removal of the cell monolayer from the coverslips for sectioning (see below).

2.9.2) Hypotonic expansion of 3T3 fibroblasts for ultrastructural assessment

Cells were exposed to hypotonic (50 and 100 mOsm) or isotonic (300 mOsm) KCl solutions (60 sec), followed by fixation with 3% aqueous glutaraldehyde (1 h), and washing in PBS (3 x 5 min). Cells were then post-fixed in osmium tetroxide (2%, PBS, 60 min), washed (PBS, 3 X 5 min) and rinsed briefly in H₂O. After dehydration in an ascending ethanol series (30%, 50%, 75%, 2 x 100%, 5 min each), cells were cleared in propylene oxide (15 min), infiltrated (50% Epon in propylene oxide, 60 min), and embedded in 100% Epon (75° C, overnight), with the cell monolayer apposed to the block face. Coverslips were removed from the block by immersion in liquid nitrogen. Cells were sectioned at 70 nm thickness parallel to the block face to assess changes in the morphology of the cells and their nuclei, induced by hypotonic expansion. After staining with uranyl acetate and lead citrate (5 min each) to delineate nuclear DNA and nuclear membrane respectively, cells were examined by a Hitachi H-7000 transmission electron microscope (TEM). In addition, cells embedded in resin were sectioned perpendicular to the block face, stained with Toluidine
Blue and examined by light microscope with a 100 X oil immersion objective. This was carried out to validate the measurements of the “thickness” of nuclei obtained by confocal microscopy, as outlined above.

2.9.3) Lanthanum nitrate staining of nuclear pore complexes (NPCs)

Fibroblasts, grown on a carbon coated coverslips, were fixed in formaldehyde (4% paraformaldehyde, PBS, 60 min), washed in 0.1 M cacodylate buffer (3x5 min) to remove all traces of phosphate, and incubated in a LaNO₃ solution (5.75 mM in cacodylate buffer, 60 min). Bound lanthanum ions were then precipitated by immersing the coverslips in Soerenson’s phosphate buffer (3x5 min), prior to post fixation (3.5% glutaraldehyde, 60 min). Cells were washed in phosphate buffer (3x5 min) and dehydrated in an ascending ethanol series (same as above). Cells were cleared in propylene oxide (5 min), infiltrated (50% Epon in propylene oxide, 15 min) and embedded in 100% Epon with the cell monolayer apposed to the block face. Coverslips were removed from the block by immersion in liquid nitrogen. Cells were sectioned at 70 nm thickness in the plane orthogonal to the block face. A series of 23 consecutive sections, spanning approximately 1.6 μm, were stained with uranyl acetate and lead citrate (5 min each) and examined by Hitachi H-7000 transmission electron microscope.

2.9.4) Ultrastructural analysis of distribution of nuclear pore complexes (NPCs)

Serial sections of nuclei were photographed by electron microscope, and the images digitized by an AGFA scanner (Model ARCUS II) connected to a Mackintosh G3 computer. The positions of individual nuclear pores were quantified by NIH Image 1.61 program, as
follows. The centroid of each nuclear section was calculated, and a line, which traverses through the centroid, was drawn parallel to the plane of the coverslip. The position of each nuclear pore on the nuclear envelope was expressed as an angle, subtended from the nuclear centroid, in relation to this line. Those signals positioned above and below the line were defined as being on the apical and basal aspects of the nucleus, and were assigned positive and negative values respectively. For schematic representation of the distribution of the pores, the angle at all the sections were pooled and plotted on an idealized elliptical template. The number of pores on the apical side of the nucleus was then divided by the number on the basal side to derive an apical-to-basal ratio.
CHAPTER THREE

RESULTS

3.1) TOPOLOGY OF DNASE HYPERSENSITIVE CHROMATIN

In agreement with previously reported data for several other cell types which present with a flat nucleus, i.e. approximately 3 μm in length along the optical axis (Puck et al., 1991; Park and De Boni, 1996), DHC domains were found to exhibit a speckled distribution in the cells employed in the present work. This topology of DHC was invariably observed when nuclei were viewed perpendicular to the substrate surface either by conventional fluorescent microscopy or by confocal microscopy at the nuclear mid-plane (Fig. 3 B). However, detailed analysis of the spatial distributions of DHC domains along the optical axis revealed that a pan-nuclear, speckled distribution was present only in a relatively small subset of the cells examined. In contrast, the majority of cells exhibited a polarization of DHC domains towards the apical pole of the nucleus, i.e. that pole which faced the culture medium (Table 1). Specifically, detailed confocal analyses showed that DHC domains change from a speckled distribution which is evident throughout the optical sections at the apical aspect of the nucleus, to become speckled, peripheral annuli, towards the basal aspect of the nucleus, i.e. that pole which faces the growth substrate (Fig. 3A-D). A residual speckled annulus is observed at the basal aspect of the nucleus (Fig. 3 D). In computer-rendered, 3-D nuclear volumes, reconstructed from serial confocal sections, DHC domains presented as dome-like, subnuclear domains with their “cap” forming the apical pole of the
Figure 3. Representative confocal sections showing the distribution of DHC domains (green) and ethidium bromide stained DNA (red) in an unexpanded (columns 1,2) and an expanded nucleus (columns 3,4, 100 mOsm KCl). DHC domains appear as apical caps (A, F), terminating as annuli towards the base (D, I), forming a dome. This is evident in 3-D volume reconstructions, rotated 90 ° (E, J). The more shallow nature of the dome in the unexpanded nucleus results in annuli which appear wider (C,D) than those in the expanded nucleus (G,H,I). Ethidium bromide labeling (columns 2, 4) shows that DNA is present throughout the nuclear volume.

Magnification: A to I', Bar = 5 μm; E, J, Bar = 2.5μm.
<table>
<thead>
<tr>
<th>Condition</th>
<th>Fraction with apical dome (mean ± sem)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n = 969)</td>
<td>86.13 ± 11.44 %</td>
</tr>
<tr>
<td>300 mOsm KCl (n=1199)</td>
<td>73.50 ± 1.81 %</td>
</tr>
<tr>
<td>100 mOsm KCl (n=1010)</td>
<td>98.70 ± 0.56 %</td>
</tr>
<tr>
<td>50 mOsm KCl (n=1152)</td>
<td>98.85 ± 0.58 %</td>
</tr>
</tbody>
</table>

Table 1. Fraction of nuclei exhibiting DHC domains as apical domes as a function of treatment. Fraction does not change between conditions (Kruskal-Wallis test, p>0.05). (At least three experiments for each condition, n identifies number of nuclei).
nucleus. Examination of such volumes in unexpanded cells, showed that the interior of the basal aspects of nuclei was devoid of DHC domains and was exclusively occupied by DNA solely labeled by ethidium bromide (Fig. 3 E). Specificity of all labeling reactions was evident from the observation that controls (see methods) were invariably negative for intranuclear labeling.

3.2) CHANGES IN NUCLEAR GEOMETRY AND MORPHOLOGY: EFFECTS OF HYPOTONIC EXPANSION AND FIXATION.

3.2.1) Observations in live cells

The sideviews of the 3-D reconstructions of the nuclear volume, as described above, were computer-rendered by extrapolation of nuclear detail along the optical axis, the very axis which even by confocal microscopy exhibits the lowest spatial resolution. Therefore, cells and their nuclei were expanded by exposure to hypotonic solutions to enhance resolution along this axis.

Observations by phase contrast microscopy, using Bionique chambers, of untreated, live 3T3 fibroblasts, revealed nuclei with phase-dark aggregates which collectively represent both nucleoli and heterochromatin. During exposure to hypotonic solutions, especially at 50 mOsm, these aggregates became less refractive or even became non-discernable. These changes occurred without evidence of rupturing of cells and nuclei or formation of intranuclear vacuoles (Fig. 4 A’, B’, C’). The hypotonic expansion was in fact reversible. Specifically, the nuclear organization of heterochromatin aggregates, which was lost in cells
Figure 4. Time lapse, phase-contrast photomicrographs of live cells showing osmotically induced changes in nuclear morphology and reversibility of chromatin decondensation. Cells shown are in isotonic medium (A,B,C), in 100 mOsm KCl (A') or 50 mOsm KCl (B',C') for 2 min, respectively. The altered morphology of nuclei and of chromatin by hypotonic expansion is retained after fixation (A'',B'') and is reversible within 30 s (C'') following replacement of hypotonic KCl by isotonic medium. Bar = 5 μm.
exposed to hypotonic, 50 mOsm KCl for 2 min, was restored when the cells were re-exposed to isotonic culture medium (Fig. 4 C’’). Moreover, such cells remained viable for three days following re-exposure to isotonic medium, as assessed by continued observation of the same cells by phase contrast microscopy.

3.2.2) Fixed Cells: Changes in nuclear geometry by hypotonic expansion and fixation

An empirical fixation protocol was developed which allowed the optimal preservation of the morphology of nuclei in intact cells after exposure to hypotonic solutions. It was found that a progressive increase of the formaldehyde concentration during the fixation step prevented the crenation of plasma and nuclear membranes (Fig. 4 A’’, B’’), which was commonly observed when osmotically swollen cells were directly placed into an essentially hypertonic fixative such as 4% formaldehyde (Fig. 5 B, B’). Following fixation by this modified protocol and counterstaining of nuclear DNA by ethidium bromide, changes in the nuclear geometry were quantified from serial optical sections by confocal microscopy. Three dimensional reconstructions of nuclear volumes rendered from optical sections (Fig. 5 A, C, D) revealed that both unexpanded and expanded nuclei exhibited a geometry approximating that of an ellipsoid. This was evident when nuclei were viewed parallel to the substrate surface (Fig. 5 A’, C’, D’).

Measurements showed that no change in nuclear volume occurred when cells were exposed to 300 mOsm KCl. In contrast, cells exposed to 50 and 100 mOsm KCl, respectively, exhibited an increase in nuclear volume. Nuclei were expanded approximately two-fold along the optical axis, accommodated by some reductions in the major and minor axes (Fig. 6). Similar to the results observed by phase contrast microscopy, chromatin
Figure 5. Nuclear morphology as a function of fixation protocol. Expanded cells were fixed either by gradually increasing the formaldehyde concentration (see methods), or directly by 4% fixative. Three dimensional volumes were reconstructed from nuclei stained by ethidium bromide. Note retention of nuclear integrity in unexpanded (a, a') and expanded (c to d') nuclei viewed at 0° (a, c, d) and at 90° rotation (a', c', d'), upon fixation by modified protocol, as contrast to partial collapse, associated with artifactual groove, in nuclei fixed by 4% formaldehyde (b, b') Bar = 5 μm.
Figure 6. Changes in nuclear dimensions (mean ± sem) as a function of osmolarity. Note expansion along the optical axis (C) which, while partially accommodated by reductions in the major and minor axes (A, B), increases the calculated nuclear volume (D). Asterisks indicate differences from control (ANOVA, Tukey Test, p≤0.05, n: control, 41 nuclei; 300 mOsm KCl, 20 nuclei; 100 mOsm KCl, 45 nuclei; 50 mOsm KCl, 30 nuclei).
aggregates were decondensed and appeared more homogenous throughout the nucleus (Fig. 5 C, D).

3.2.3) **Assessment of ultrastructure**

Electron microscopic assessment confirmed the observations made by phase contrast microscopy. Nuclei of cells from control cultures and from cultures exposed to 300 mOsm KCl, exhibited typical, electron dense aggregates of chromatin (Fig. 7 A’, B’). In nuclei of cells which were hypotonically expanded, these aggregates became diffuse and difficult to discern (Fig. 7 C’, D’, arrows), while the integrity of the nuclear and cell membranes remained uncompromised. Measurements of nuclear dimensions of resin-embedded cells cut at right angles to the growth surface confirmed the results obtained by confocal microscopy. They showed that nuclei in control cultures and in cultures exposed to 300 mOsm KCl exhibited a “thickness” of 2.7 ± 0.06 μm (n=118 nuclei) and 3.2 ± 0.08 μm (n=102 nuclei), respectively, not different from that estimated by confocal microscopy (Fig. 6).

3.3) **TOPOLOGY OF DHC DOMAINS AND OTHER NUCLEAR COMPONENTS IN HYPOTONICALLY EXPANDED NUCLEI**

3.3.1) **Observations of nuclei in the plane orthogonal to the substrate surface**

Assessment of the distribution of DHC domains by confocal microscopy, in expanded nuclei confirmed the observations obtained from unexpanded nuclei, at a higher resolution along the optical axis (Fig. 3). In the majority of cells DHC domains were again apposed to the nuclear periphery, while in a small fraction of cells DHC domains were detected
Figure 7. Electron micrographs of nuclei from cells in control (A), 300 mOsm KCl (B), 100 mOsm KCl (C) and 50 mOsm KCl (D) conditions. Note that the electron dense aggregates in unexpanded cells (A', B') are dispersed following hypotonic expansion (C', D', arrowheads). Bar: A to C, 5 μm; A' to D', 2 μm.
throughout the nuclear volume (Table 1). Similar to the majority of nuclei before hypotonic expansion, the polarization of DHC domains in the form of an apical dome was maintained. DHC domains at the basal aspects of nuclei formed annuli, which converged to form a cap at the apical pole (Fig. 3). In addition, the data revealed that this topology is not an artifact resulting from changes in chromatin structure induced by the hypotonic expansion *per se*, since DNA, counterstained by ethidium bromide, persisted throughout the entire nuclear volume (Figs. 3, 5).

3.3.2) Observations of nuclei in the plane parallel to the substrate surface

When cells and intranuclear detail were viewed in the plane parallel to the growth substrate by use of the “L-shaped” coverslips (see methods), and thus at the superior resolution of the XY-plane of the microscope, DHC remained resolvable as an apical dome at that aspect of the nucleus which faced the culture medium. DNA, counterstained by ethidium bromide, remained detectable throughout the nuclear volume (Fig. 8 A, B). Single confocal sections, together with nuclear volumes rendered from cells observed at this "parallel view" (Fig. 8 A’, B’) facilitated the examination of subtle features of the topology of DHC domains. Previously, when interpreting the distribution of DHC domains from sections obtained in the view orthogonal to the growth substrate, it was considered that the DHC annuli at the basal aspect of nuclei might be artifactual; that they might represent "bleed-through" of signals from DHC annuli present at the nuclear midplane or below (Fig. 3 C, D, H, I). However, the use of the “L-shaped” coverslips showed that annuli of DHC...
Figure 8. Confocal sections and corresponding, computer-rendered volume reconstructions of nuclei in cells viewed parallel to the growth substrate by use of "L-shape" coverslips. DHC domains (green) are co-labeled (yellow) with DNA counterstained by ethidium bromide (red, A and B, single sections; A' and B', volume reconstructions) or with snRNPs (red, C and D, single sections; C' and D', volume reconstructions) in either unexpanded (A,A',C,C') or expanded (B, B', D,D') nuclei. Note increased resolution, compared to that obtainable in views orthogonal to the growth substrate (Fig. 3 E, J) along the former optical axis, here viewed in the XY plane. Bar = 5 μm.
domains clearly extend towards basal edge of the nuclei (Fig. 8 A, A', B, B', see arrowheads). This view also permitted more accurate measurements of the thickness of nuclei and the extent of expansion achieved by exposure to hypotonic solutions. The results showed that unexpanded (n=10) and expanded nuclei (n=10) displayed a thickness of $2.9 \pm 0.14 \mu m$ (mean $\pm$ SEM) and $5.27 \pm 0.22 \mu m$, respectively. This compared favorably with the thickness estimated as $2.25 \pm 0.04 \mu m$ and $5.82 \pm 0.14 \mu m$ for unexpanded (n=41 nuclei) and expanded nuclei (n=30 nuclei) respectively, derived from orthogonal views (Fig. 6 and methods).

3.3.3) Co-labeling of DHC domains with snRNPs and kinetochores

It might be argued that the polarized distribution of DHC might have resulted from a lack of access of the reagents used to detect DHC domains to all subnuclear compartments. Cells were thus co-labeled for DHC domains and nuclear ligands which are distributed throughout the nuclear volume, such as snRNPs and kinetochores, the latter known to remain associated with centromeric domains throughout the cell cycle (Matsumoto et al., 1989). Confocal analyses showed that a lack of access is unlikely to be responsible for the preferential labeling at the nuclear surface. Both snRNPs and kinetochores were clearly detectable throughout nuclei, spanning the space from the apical pole to the basal aspect of nuclei in both unexpanded and in expanded cells, while DHC domains showed a typical, peripheral localization (Fig. 9). Similar results were obtained upon co-labeling of DHC domains and snRNPs in both unexpanded and expanded nuclei in cells grown on the “L-shaped” coverslips, a method which permitted increased resolution along the axis orthogonal to the substrate surface (Fig. 8).
Figure 9. Confocal micrographs demonstrating access of high molecular weight probes to the nuclear interior. Sections at the midplane of unexpanded (A to A”, C-C”) and expanded (B to B”, D to D”) nuclei labeled for DHC (A to D) or snRNPs (A’, B’, red) or kinetochores (C’, D’, red) and corresponding merged images (A” to D”) demonstrate the retention of the peripheral localization of DHC domains and the persistence of labeling of snRNP and kinetochore signals in the interior of the nuclei after expansion. Bar = 5 μm.
3.4) ASSESSMENT OF THE DISTRIBUTION OF THE NUCLEAR PORE COMPLEXES

3.4.1) Confocal microscopy

One of the prevailing concepts of the organization of interphase nuclei is that nuclear processes that are part of the same pathway are spatially coupled (see section 1.3). Given that transcriptionally active sequences, represented as DHC domains, display a dome-like distribution towards the apical aspect of the nucleus, we proposed that a similar distribution of nuclear pore complexes would be required to efficiently couple transcription and nucleocytoplasmic transport of nascent mRNA. Hence, nuclear pore complexes were labeled by immunocytochemistry. As revealed by serial optical sectioning of nuclei by confocal microscopy, nuclear pore complexes appear as punctate fluorescent signals predominantly localized at the nuclear surface (Fig. 10). Analysis of the spatial distribution of the pores along the optical axis revealed that they in fact are preferentially localized towards the apical aspect of nucleus in a polarized distribution similar to the DHC domains. This polarized distribution of NPCs was found in $80.3 \pm 4.2\%$ (mean± sem) of nuclei ($n = 336$ cells) examined, a number which compares favorably with the fraction of nuclei ($86.13 \pm 11.44 \%$, $n = 969$) which exhibit DHC domains in the form of an apical dome (Table 1, Fig. 11).

3.4.2) Ultrastructural assessment of the distribution of nuclear pore complexes (NPCs)

To confirm the results obtained by confocal microscopy which showed predominantly an apical localization of nuclear pore complexes, the distribution of nuclear pores, labeled
Figure 10. Serial confocal sections of a representative nucleus revealing a polarized distribution of nuclear pores. Note the more prominent labeling of pores (green) at the apical aspect of the nucleus (middle, bottom row), at the periphery of the nuclear midplane (right, middle row) compared to the base (top row, right). Bar = 5μm.
Figure 11. Bar graph showing similarity in the fraction of nuclei, as assessed by confocal microscopy, which exhibit DHC \((n=969\) nuclei\) or nuclear pores \((n=336\) nuclei\) as an apical dome (Mann-Whitney Rank Sum Test, \(p > 0.05\)). (Three experiments for each condition, \(n\) identifies number of nuclei).
by lanthanum, was furthered assessed at high resolution by sectioning Epon-embedded 3T3 cells at the plane orthogonal to the substrate surface. Nuclear pores were evident as electron-dense spots on the nuclear envelope. In agreement with routine examination, the pores were present at regions where euchromatin, rather than heterochromatin, contacted the nuclear surface. (Fig. 12 C, arrowheads). This was taken to confirm the specificity of the labeling reaction (Shaklai and Tavassoli, 1982). In total, four nuclei were examined in detail, only one of which exhibited a polarized distribution of nuclear pores towards the apical aspect of nuclei (Fig. 12). In this particular nucleus, this preferential localization, already evident in individual sections (Fig. 12 A, B), was more clearly evident upon superimposing the position of NPCs from a series of consecutive sections onto a diagrammatic representation (Fig. 12 D). Although the lanthanum labeled NPCs were detectable on all aspects of the nuclear surface, their presence at the apical surface (count = 161) exceeded those at the basal side (count = 27) of the nucleus by approximately a factor of 6.
Figure 12. Assessment of distribution of nuclear pores by lanthanum labeling. Electron micrographs of two sections of a representative nucleus sectioned serially (A,B) at the plane orthogonal to the coverslip (gray lines). Detail of this nucleus at higher magnification (C) reveals the presence of a higher number of nuclear pores at the apical aspect, compared to the base (arrowheads). Results of counts of pores on twenty-three serial sections reveal polarization of pores at the apical aspect of this nucleus (D). Magnification: A to B, Bar = 2 μm; C Bar = 1 μm.
CHAPTER FOUR

DISCUSSION

4.1) POLARIZED TOPOLOGY OF DHC DOMAINS

The nuclear periphery, by its proximity to the cytoplasm, has been postulated to play a role in gene expression by facilitating nucleo-cytoplasmic transport of nascent mRNA synthesized in that compartment (Blobel, 1985; de Graaf et al., 1990; Sahlas et al., 1993; Park and De Boni, 1998, 1999). This postulate is supported by the finding that, in some cell types, the nuclear periphery is enriched in DHC domains (Hutchison and Weintraub, 1985; de Graaf et al., 1991; Haag et al., 1994; Puck et al., 1991; Park and De Boni, 1996, 1998). If the periphery of nuclei indeed represents a specialized functional domain, assuming an economy of design for common functional purposes, it may be argued that such a domain should exist in all cell types, irrespective of nuclear geometry. Given that the mean, measured thickness of the DHC domains, which have been detected at the nuclear periphery in spherical nuclei, is approximately 1.7 μm (Park and De Boni, 1996, 1998), the apical and basal domains, when closely apposed in a flat nucleus of a thickness of 3 μm, would not be resolvable as separate entities.

This argument is supported by the results of the work reported here, in which very flat nuclei were induced to undergo acute changes in geometry from a flat ellipsoid to a spheroid. The fact that there exists not only a preferential association of DHC domains with the nuclear periphery, but in addition, a polarization of such domains, supports the postulate that the
nuclear periphery may serve as a specialized functional, nuclear compartment. In addition, the results presented here suggest that DHC domains might be tethered to a component of the nuclear lamina-pore complex (section 1.3.1, see below). As a result, DHC domains, via their association with the nuclear envelope, are postulated to move along with the latter as the nucleus expands along the optical axis, during acute exposure to hypotonic solutions.

A contribution to the exclusive red fluorescent labeling of DNA by ethidium bromide at the basal aspect of nuclei might be considered to result from chromatic aberration along the conventional optical axis (Fig. 3 E, J). By using the "L-shaped" coverslips (see methods), the results from the present work are effective in demonstrating that an exclusive labeling of nuclear DNA by ethidium bromide at the basal aspects of nuclei is in fact not the case. Specifically, in optical sections and volumes rendered from cells viewed parallel to the growth substrate, using the "L-shaped" coverslips, DHC domains are detected at the basal edge of the nuclei (Fig. 8, see arrowheads). Given that any chromatic aberration present would now be shifted in a direction parallel to the growth substrate, this observed co-localization of DHC domains with DNA counterstained by ethidium bromide at the basal aspect of nuclei, may thus be considered to represent their actual, respective distributions (Fig. 8).

The observation that heterochromatin and nucleoli became less discernible under hypotonic conditions may suggest an irreversible disruption in the integrity of nuclear components. However, the restoration of such chromatin domains to the naive appearance when cells were re-exposed to isotonic medium (Fig. 4 C''), along with the observation that such cells remained viable, indicates that the structural, submicroscopic organization of nuclear components was still maintained during hypotonic expansion. This is supported by
the fact that even after hypotonic expansion, a time when the morphological appearance of chromatin was clearly altered (Fig 7 C', D'), preferential labeling of DHC domains remained restricted to the chromatin at the nuclear periphery and at the apical cap (Fig. 3). This indicates that the hypotonic expansion itself did not alter the conformation of chromatin domains, at least not at the level of resolution detectable by light microscopy, nor did it render the bulk chromatin DNAse hypersensitive. The only change observed was that the peripheral DHC domains in expanded nuclei presented with a less speckled appearance than those in unexpanded cells.

Conversely, it must be considered that the increase in the volume of expanded nuclei may have restricted the access, to the interior of nuclei, of the enzymes used for in situ nick translation. This is unlikely in view of the fact that antibodies, with molecular weights higher than or comparable to DNA polymerase and DNAse I respectively, readily labeled both snRNPs and kinetochores throughout the nuclear space, including its basal aspects, of both unexpanded and expanded cells (Fig. 9 B'', D''). Thus, in keeping with previous work (Hutchison and Weintraub, 1985; de Graaf, 1990), limited accessibility is not considered to be the factor responsible for the preferential labeling of DHC at the nuclear periphery.

4.2) SPATIAL RELATIONSHIPS BETWEEN SnRNPs SPECKLES AND DHC DOMAINS: IMPLICATIONS FOR CO-TRANSCRIPTIONAL SPICING

In agreement with the results from a number of studies on HeLa cells, which also exhibit nuclei with a flat geometry (Spector, 1990; Carmo-Fonseca et al., 1991; Huang and Spector, 1992; Zhang et al., 1994), snRNPs, splicing factors of nascent RNA, show a pan-
nuclear speckled distribution in the 3T3 fibroblasts employed here. In contrast, within the spheroidal nuclei of differentiated PC 12 cells and of dorsal root ganglion neurons, snRNPs were predominantly located at the nuclear periphery in the form of a spherical shell apposed to the nuclear envelope (Sahlas et al., 1993; Park and De Boni, 1996). Therefore, the correlation between nuclear geometry and the distribution of snRNPs and of DHC domains, observed in a number of different cell types (see also introduction), further indicates a role of the nuclear geometry in organizing the spatial distribution of nuclear domains (Ferreira et al., 1997; Park and De Boni, 1999).

In 3T3 fibroblasts, the pan-nuclear, speckled distribution of snRNPs persists after hypotonic expansion, at a time when DHC domains are shown to migrate with the nuclear membrane of the expanding nucleus. This results in a reduction of overlap between DHC domains and snRNPs (Fig. 9 B”), as previously reported (Park and De Boni, 1996). This may indicate that the association between DHC domains and snRNP speckles observed in unexpanded 3T3 cells, may not necessarily be fixed (Fig, 8 C, C') but rather is one of spatial proximity. This view is consistent with the model proposed by Misteli and Spector (1996, 1998) in which the speckles are in close proximity to, but remain physically independent of transcription sites.

The present data indicates that DHC domains and snRNPs are likely to be physically anchored to different structural components of the nucleus. DHC domains, as suggested earlier, might be anchored to some component of the nuclear lamina. In fact, a direct physical linkage between a DNAse-sensitive network of chromatin with nuclear pore complexes (NPCs) has recently been demonstrated (Arlucea et al., 1998, Engelhardt, 1999). This linkage occurs putatively via the zinc finger, DNA binding protein nup153, a NPC protein.
which faces exclusively to the nucleoplasmic side of the NPC (Sukegawa and Blobel, 1993). Likewise, snRNPs are likely to be dynamically associated with some component in the nuclear interior. Under some conditions though, snRNPs may actually independently and dynamically redistribute to the nuclear periphery, where they co-localize with DHC domains (Sahlas et al., 1993; Garcia-Segura et al., 1993; Park and De Boni, 1996).

4.3) DATA DERIVED BY IN SITU NICK TRANSLATION DIFFERS FROM THAT DERIVED BY DIRECT LABELING OF NASCENT RNA; A CONTROVERSY

Although the results reported here concur with other work which uses the topology of DHC domains as a marker for transcriptionally active sites (de Graaf et al., 1990; Puck et al., 1991; Puck and Krystosek, 1992; Haag et al., 1994; Park and De Boni, 1996; 1998). The results must be contrasted with studies which address the localization of transcription sites by the presence of nascent RNA transcripts. Such studies, which use ribonucleotide analogues as markers, routinely show transcription to occur throughout the nuclear space (Jackson et al., 1993, 1998; Wansink et al., 1993,1996a; de Jong et al., 1996; Fay et al., 1997; Zeng et al., 1997; Cmarko et al., 1999). This controversy remains unresolved (Wansink et al., 1996b). To some extent, this could be a matter of semantics. Some of the cell types employed in the studies which show nascent RNA throughout the nuclear space, such as human diploid fibroblast (Fay et al., 1997) and HeLa cells (Jackson et al., 1993, 1998), display nuclei that are characteristically flat. If transcription sites are associated with the nuclear envelope, the flat geometry of these nuclei might constrain chromatin domains to the extent that transcription sites that are associated with the apical or basal aspects of the nuclear envelope
are interdigitating at the nuclear midplane (Ferreira et al., 1997; Park and De Boni, 1999). This in turn, may lead to the interpretation that transcription sites have a "pan-nuclear" distribution. It must be conceded, however, that this argument is not applicable to the pan-nuclear distribution of nascent transcripts observed in cell types such as hepatocytes, which exhibit a more spheroidal nuclear geometry (Fakan et al., 1976; Fakan and Puvion, 1980).

A combination of factors may contribute to the differences in the data derived by \textit{in situ} nick translation and by direct labeling of nascent RNA. It should be noted that the two techniques differ in their sensitivities to detect transcriptionally active sites. The extent of ribonucleotide analogue incorporation into nascent transcripts depends upon the rate of transcription of a particular gene. In contrast, genes transcribed at different rates are in fact equally susceptible to DNAse I digestion (Garel et al., 1976). Likewise, the presence of DHC within a gene is necessary but not sufficient in committing a gene to be transcriptionally active (Eissenberg et al., 1985). In fact, \textit{in situ} nick translation detects the cis-acting elements of competent genes, irrespective of whether such genes are actively transcribed or not. The extent of deoxyribonucleotide incorporated into the DHC of a given gene by nick translation also depends upon the number and the length of histone-free cis-acting element(s) of that gene. Such features may not necessarily have correlation with the rate of transcription (Feng et al., 1999). Therefore, if transcription is indeed occurring throughout the nuclei of 3T3 fibroblasts, one explanation for the observed peripheral, polarized distribution of DHC domains might be that \textit{in situ} nick translation is effective in
marking genes that contain many cis-acting elements, and that such genes are preferentially localized at the apical aspect of these nuclei.

A combined assessment of the distribution of DHC domains labeled by in situ nick translation, together with the distribution of nascent RNA pulse-labeled by incorporation of ribonucleotide analogues within the same cell, might shed more light in resolving the differences in the data derived by these two techniques. As yet, the biological significance of a polarized distribution of DHC domains in 3T3 fibroblasts in vitro remains obscure. Interesting enough, a preferential localization of snRNPs, SC35 and poly A⁺ RNA towards the basal aspects of nuclei has been documented in other mammalian cultured cells (Carter et al., 1993). Although the mechanism by which the polarized distribution of these nuclear components are maintained remains to be determined, the results of the present work suggests that such polarization might be effected by an association of the component with the nuclear envelope.

4.4) POLARIZATION OF NUCLEAR PORE COMPLEXES (NPCs)

In contrast to the putative non-physical, non-structural association between DHC domains and snRNP speckles (section 4.2), recent evidence supports a direct morphological association between a DNAse-sensitive network of chromatin with NPCs (Arlucea et al., 1998, Engelhardt, 1999). This linkage may be facilitated by the NPC protein nup153, which contains zinc finger motifs, binds DNA, and exclusively faces to the nucleoplasmic side of the NPC (Sukegawa and Blobel, 1993). Together with these novel findings, the work presented here, while preliminary, has demonstrated a correlation between the distribution of
DHC domains and NPCs (Fig. 11). Therefore, the putative association of these two nuclear components might be indicative of a spatial coupling between transcription and nucleocytoplasmic transport. Since the assessment of the labeling of DHC domains and NPCs was conducted in different experiments and on separate cultures, an association can only be implied. However, a direct association of the distribution between the two nuclear components could be ascertained by performing both in situ nick translation of DHC domains and immunolabeling of NPCs within the same cells, an experiment which, while feasible, was outside the scope of the current investigation.

In agreement with the results from a similar study (Kubitscheck and Peters, 1996), the fluorescent signals of NPCs vary in size and mean signal intensity, indicative of clustering of some NPCs that are too close to be resolved as separate entities by confocal microscopy (Kubitscheck and Peters, 1998). Nevertheless, a detailed analysis of one of the nuclei stained by lanthanum ions, a method which permits better resolution of individual NPCs, revealed a 6-fold greater number of pores at the apical side of the nucleus than at the basal side (Fig. 12 D). Therefore, preliminary evidence indicates that the density of NPCs at the apical side of the nucleus is indeed higher than that at the basal side, resulting in a polarized distribution of NPCs as observed by confocal microscopy (Fig. 10).

It remains unclear why the remaining lanthanum stained nuclei (3 out of 4) failed to display an apical localization of NPCs (data not shown). However, given that the extent of nuclear surface surveyed by ultrastructural assay is very limited (23 sections X 70 nm ≈ 1.5μm), it might be considered that the serial sections obtained actually correspond to regions at the lateral aspect of the nucleus. In fact, in keeping with the view that the DHC domains and NPCs are structurally linked, it must be considered that within the nuclei of 3T3
fibroblasts, DHC domains invariably encroached on the basal aspect of the nuclei in the form of a speckled annulus (Fig. 3 C, D). Similar to the observation at the apical nuclear surface, the basal nuclear envelope subjacent to this annulus of DHC domains might therefore require a comparable number of NPCs, a question to be answered by additional experiments. Also, since 3T3 fibroblasts are cells maintained in vitro, it remains enigmatic as to whether a polarized distribution of DHC domains and NPCs would confer any biological relevance to differentiated cells in vivo. Given that the acinar cells of the pancreas and salivary glands display a highly polarized localization of rough endoplasmic reticulum (rER) towards the basolateral end of the cell (Kalinin and Nevorotin, 1975), these cell types might represent two ideal models in vivo to test the hypothesis that a basolateral distribution of rER may be associated with a polarized distribution of NPCs and of DHC domains.

4.5) SUMMARY

The results of the present work, while in agreement with a number of previous studies (Hutchison and Weintraub, 1985; de Graaf, 1990; Puck et al., 1991; Park and De Boni, 1996, 1998) are the first demonstration that DHC domains, which contain transcriptionally active or competent sequences, might be associated with the nuclear periphery, even within flat nuclei such as 3T3 cells in vitro. In addition, in 3T3 fibroblasts, this putative association of DHC domains with the nuclear envelope was predominantly observed at that pole of the nucleus which faced the culture medium, i.e. the distribution of DHC domains was polarized. Such a feature has not been reported for any cell type. Furthermore, preliminary evidence suggests that the spatial, polarized distribution of DHC domains and that of NPCs are linked. Ongoing work in our laboratory is addressing the
question whether such a polarization of DHC and NPCs also exists within polarized cells of
tissue in situ, such as acinar cells of the pancreas and the salivary gland. To date, available
data supports the suggestion that such spatial coupling of DHC domains and NPCs at the
nuclear envelope may be of functional advantage in the activation and transcription of
specific sequences and in nucleo-cytoplasmic transport (Blobel, 1985; Sukegawa and Blobel,
REFERENCES


