MITOCHONDRIAL LOCALIZATION OF HIGH MOBILITY GROUP A1 PROTEINS

By

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To the Faculty of Washington State University:

The members of the committee appointed to examine the dissertation of Gregory Allan Dement find it satisfactory and recommend that it be accepted.

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Chair

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Abstract

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High mobility group A1 (HMGA1) proteins classically act within the nucleus of mammalian cells as architectural transcription factors that regulate the expression of numerous genes. However, HMGA1 proteins were found in both the cytoplasm and mitochondria of wild-type murine NIH3T3 cells and transgenic human MCF-7 breast cancer epithelial cells expressing a hemaglutinnin tagged-HMGA1a fusion protein. Initial synchronization studies revealed a dynamic, cell cycle-dependent translocation of HMGA1 proteins from the nucleus into the cytoplasm and mitochondria of NIH3T3 cells. Furthermore, preliminary functionality studies utilizing a modified "chromatin" immunoprecipitation protocol revealed that HMGA1 retains its DNA binding capabilities within the mitochondria and associates with the regulatory D-loop region *in vivo*. As an effect of HMGA1 over-expression mitochondrial DNA (mtDNA) levels in transgenic MCF-7 cells were reduced by approximately 2-fold relative to wild-type while the mitochondrial NADH Dehydrogenase 2 gene transcript was up-regulated approximately 4-fold . Corresponding to reduced mtDNA levels, flow cytometric analysis revealed that overall mitochondrial mass was reduced in these cells as well. In order to determine the effects of these alterations to mtDNA replication studies, cell survival studies were

performed and showed an increased sensitivity of the HMGA1 over-expressing cells to treatment with the inhibitor of glycolysis, 2-deoxy-D-glucose, indicating an overall deficiency in cellular oxidative phosphorylation. These findings may implicate HMGA1 proteins in the mechanism by which tumor cells display a Warburg glycolytic phenotype. HMGA1 in the mitochondria may also negatively affect the ability of the organelle to repair reactive oxygen species induced DNA damage contributing to overall tumor progression. Together, these observations reveal the novel mitochondrial localization of the HMGA1 proteins and present several possible mechanisms by which the translocated form of the protein may participate in mitochondria associated processes related to normal and abnormal cell function.

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CHAPTER 1

INTRODUCTION

Mitochondrial Localization of High Mobility Group A1 Proteins

Contained in this dissertation is a presentation of findings describing a novel subcellular localization pattern and subsequent mitochondrial function for the high mobility group A1 (HMGA1) family of non-histone chromatin proteins. In Chapters 2 and 3, following a general overview in Chapter 1 of the HMGA1 proteins and their role in a variety of biological processes, are detailed descriptions of the molecular biological and biochemical assays utilized and the data they generated. Briefly, indirect immunofluorescence, electron microscopic immunolocalization, and Western Blot studies were performed with a polyclonal antibody probe to endogenous HMGA1 proteins in cell cycle stage synchronized and non-synchronized NIH3T3 murine fibroblast cells. In order to confirm the data obtained from these studies, MCF-7 breast cancer epithelial cells, with and without a hemaglutinnin (HA) tagged HMGA1 transgene, were subjected to similar assays utilizing both a polyclonal antibody to HMGA1 and a monoclonal antibody to the HA tag. Furthermore, a non-probe approach involving transfection of an HMGA1-Green Fluorescent Protein (GFP) fusion construct into NIH3T3 cells was used as an independent method for determining protein localization in live cells.

With confirmed mitochondrial localization of HMGA1, functional studies were initiated to determine the biological role of the proteins in the context of mitochondrial compartmentalization. Modified "chromatin" immunoprecipitation was utilized to determine the ability of HMGA1 to associate with mitochondrial DNA and potentially maintain its function as a transcription factor. Real-time PCR was subsequently performed to determine the effect of HMGA1 over-expression on both transcription and replication of the mitochondrial genome. Broader mitochondrial functionality studies

included determining the effect of altered HMGA1 levels on mitochondrial mass and the ability of cells as a whole to survive following treatment with the inhibitor of glycolysis, 2-deoxyglucose. Finally, because concurrent studies in the lab showed that HMGA1 proteins are involved in the inhibition of the DNA nucleotide excision repair (NER) pathway within the nucleus, gene-specific DNA repair studies within the mitochondria were also completed by a unique quantitative PCR method.

The remainder of this first chapter provides an overview of the HMGA1 proteins and their unique features which allow them to function dynamically in a variety of normal and disease related biological processes.

Overview

Amongst a plethora of specific protein factors acting within the nucleus and cytoplasm of mammalian cells, HMGA1 proteins have, in recent years, emerged as more flexible players in a variety of normal and abnormal cellular processes. Unique features of these small proteins make them capable of diversity in action that is similar to that of more familiar factors such as p53 and c-Myc, which are known to participate, under tight regulation, in a wide variety of biological events. Because of their unusual characteristics and dynamic biological activity, the HMGA1 proteins have become the focus of intense research. Their potential as a diagnostic marker or even therapeutic target for the detection or treatment, respectively, of multiple cancer types has provided much of the motivation for the increased interest. The genesis of such interest came with studies that revealed the over-expression of the HMGA1 proteins in cancerous or immortalized cell

lines, namely, HeLa (Lund et al., 1983) and African green monkey cells (Varshavsky et al., 1983), which is in contrast to their very low level of expression in normal tissue.

Since the discovery of the HMGA1 proteins and their hallmark distinguishing characteristics in the 1980's, basic research coming out of cancer biology and chromatin laboratories has produced a better understanding of their structural characteristics and overall role in normal biological processes including embryonic development and the immune response. It was discovered that the HMGA1 proteins displayed high levels of phosphorylations (Ostvald et al., 1985), were able to bind and alter DNA structure at adenine and thymine (AT)-rich sequences without the need for ATP hydrolysis (Lehn et al., 1988; Solomon et al., 1986), and lacked secondary structure while free in solution (Evans et al., 1992; Evans et al., 1995; Huth et al., 1997, Lehn et al., 1988). Furthermore, the proteins were set apart by their small size (10.6-12 kDA), elevated level of basic, acidic, and proline amino acid residues, acid (5%) solubility, and rapid mobility during electrophoretic separations (Lund et al., 1983; reviewed in Reeves, 2001). These unique characteristics distinguish the HMGA1 proteins from most other nuclear and cytoplasmic proteins, including other members of the HMG protein families, which are abundantly expressed in normal tissue (Johns, 1982).

As a point of distinction, the HMG proteins as a group are composed of three different families named HMGA, HMGB, and HMGN (Bustin, 2001), the first of which contains the HMGA1 isoforms, HMGA1a (107 amino acids (aa); approx. 11.7 kDa) and HMGA1b (96 aa; approx. 10.6 kDa) (Johnson et al., 1989; Friedmann et al., 1993), that are the focus of the research presented here. An additional isoform of the *HMGA1* gene family not covered here is named HMGA1c (179 aa; approx. 19.7 kDa). The varying

isoforms are a product of alternative mRNA splicing, resulting in a deletion of 11 amino acids in the HMGA1b isoform when compared to HMGA1a (Johnson et al., 1989; Reeves and Nissen, 1995). To date, significant differences regarding actual biological function between these two isoforms have not been definitively identified and thus they will be referred to in the remainder of this document collectively as HMGA1, which for the purposes here, excludes the HMGA1c form. As a general statement, HMGA1 has proven to be a critical protein player in multiple molecular/cellular processes, responding to many stimuli and participation in a variety of downstream functions. Such functions include: positive and negative gene regulation; modulation of macromolecular and chromatin structure; cell growth, differentiation, and embryonic development; carcinogenesis and others, as reviewed in Reeves, 2001, where the proteins are appropriately referred to as "hubs of nuclear function" (**Fig.1.1.**). However, as will be presented in the remainder of this dissertation, the function of the HMGA1 proteins is even more dynamic than originally thought, and in fact, is not restricted to nuclear function alone. A detailed description of the structure, molecular modes of action and overall cellular functions of the HMGA1 proteins follows in the remainder of this introductory chapter.

HMGA1: Structure and Molecular Function

HMGA1 proteins are original members of a relatively new class of gene regulatory proteins called architectural transcription factors (Grosschedl et al., 1994) for their ability to recognize structural rather than sequence specific benchmarks of B-form DNA and alter that structure in a variety of ways upon binding (Reeves and Nissen,

1990). This feature allows the proteins to associate with many different distorted or unusual DNA structures. For example, HMGA1 is capable of binding to restricted regions of DNA on the surface of nucleosome core particles and inducing localized changes in the rotational setting of the nucleosomal DNA (Reeves and Wolffe, 1996). It has been proposed that HMGA1 proteins are indeed intimately involved in the process of chromatin remodeling via their unique ability to bind to different regions of nucleosomes and induce these conformational changes in the DNA (Reeves et al., 2000; Reeves and Wolffe, 1996; Reeves and Nissen, 1993). The changes are thought to allow transcription factors access to enhancer and promoter elements by relaxing what would normally be restrictive DNA/core particle interactions. Such a mode of action provides a partial explanation of how HMGA1 is capable of gene expression activation.

The chromatin relaxation model of activation extends to the independent process of actual transcriptional initiation during which HMGA1 mediates the formation of enhanceosome structures over the promoters of inducible genes (**Fig.1.2**) (reviewed in Reeves and Beckerbauer, 2001). While the possibility of their role in initial chromatin remodeling of inhibitory positioned nucleosomes is intriguing and under investigation, the HMGA1 proteins have been definitively shown to interact directly with both enhancer and promoter elements of inducible genes such as IFN-β (Falvo, et al., 1995) and IL-2Rα (John et al., 1995; John et al., 1996) to assist in transcriptional initiation. Additionally, HMGA1 acts like a 'molecular glue' during this process by association with other protein factors necessary for the completion of the enhanceosome structure and the recruitment of basal transcriptional machinery. As a general rule, HMGA1 proteins function in most scenarios by binding to AT-rich DNA, introducing structural changes in the substrate,

and mediating protein-protein interactions. These events are made possible through the action of what is referred to as AT-hook DNA binding motifs.

All members of the HMGA family of proteins are unified by the presence of AT-hooks that preferentially bind to stretches of DNA within the narrow minor groove. Each protein has a set of three functional motifs that have an invariant peptide core palindromic sequence Pro-Arg-Gly-Arg-Pro (i.e. P-R-G-R-P) flanked by conserved positively charged residues (Reeves and Nissen, 1990). While free in solution, these DNA binding regions, along with the rest of the protein, as mentioned previously, lack detectable secondary structure as determined by either NMR or CD analyses (Evans et al., 1995; Huth et al., 1997; Lehn et al., 1988). Intriguingly however, upon binding within the minor groove of B-form DNA, the motif takes on a planar, crescent-shaped configuration (see **Fig 1.3** for details) due to flexibility restrictions resulting from the *trans* positioned proline residues on either side of the central glycine (Evans et al., 1995). The conformational change from non-structured to structured allows for optimal contacts between the peptide motif and AT-rich minor groove and is a major factor contributing to flexible substrate recognition (Huth et al., 1997). Disordered-to-ordered conformational changes in the AT-hook peptide motifs during substrate binding are accompanied by structural changes in the substrate as well (Lehn et al., 1988). Such changes are a primary mechanism by which HMGA1 proteins affect gene activation.

While full-length HMGA1 proteins can bend, straighten, unwind and induce loop formation in linear DNA substrates *in vitro* (Chase et al., 1999; Reeves and Wolffe, 1996; Slama-Schwok et al., 2000), it is interesting to note that studies show a lack of similar conformational changes when a single AT-hook motif is bound to the same substrate

(Huth et al., 1997). Further investigation into this occurrence revealed that multiple AT-hooks must bind to multiple AT-stretches of a given DNA substrate in order to induce significant conformational changes (Li et al., 2000). While the exact mechanism by which the proteins are able to recognize and alter specific substrates is unknown, it is likely that the spacing of the AT-hook motifs on a single HMGA1 protein is important. Essentially, the number and spacing of the motifs serves as a "bar code reader" to identify binding targets, and yet allow for association with many different types of platforms due to the relatively non-sequence specific binding requirement (Radic et al., 1992; Reeves et al., 1987; Reeves and Nissen, 1990). Thus, when considering the potential specific sites or, more broadly, genes that HMGA1 proteins will associate with, the spacing and number of AT-rich segments within a promoter or enhancer element are important. However, computer based algorithms to predict potential binding sites based on this criterion have proven difficult to develop, a likely result of the existence of many variables involved in the dynamic ability of HMGA1 to flexibly interact with both proteins and DNA. Demonstrating this point, the proposed mechanisms for HMGA1 interaction with, and conformational alteration of, a given DNA substrate include: (i) neutralization of negative charges on the DNA backbone; (ii) the binding of each of three AT-hooks from an individual HMGA1 protein to distant regions of a DNA molecule; (iii) the cooperative binding of multiple HMGA1 molecules to localized regions of the DNA; and (iv) the non-covalent cross-linking of different DNA molecules by a single HMGA1 protein (reviewed in Reeves, 2001). Additionally, HMGA1 proteins are capable of simultaneously interacting with protein partners and altering their conformation/function to assist in the DNA interactions/alterations required for enhanceosome formation and

gene activation. In contrast to such gene activation events, HMGA1 is also involved in gene repressor complex formation by similar molecular interactions. Regardless of the complexities surrounding the binding characteristics and substrate specificities of HMGA1 proteins, their general requirement for AT-rich DNA sequences, coupled with their ability to interact with a variety of protein partners, allows for promiscuous interaction with many different substrate types. It is this flexibility that is a major contributor in the ability of HMGA1 proteins to act in a variety of cellular processes involving DNA interactions, the list of which is growing rapidly and is restricted only to DNA elements containing AT-rich regions separated by a varying number (n = 0-5) of nucleotides (Radic et al., 1992; Reeves et al., 1987; Reeves and Nissen, 1990). Importantly, the activities of the proteins have been shown to be specifically regulated by various intra- and extracellular stimuli that ultimately lead to differential post-translational modifications and subsequent alterations in molecular function.

It is now known that HMGA1 function is regulated by a variety of *in vivo* post-translational modifications including phosphorylation, acetylation, and methylation, among others (Banks et al., 2000; Edberg et al., 2004; Reeves and Beckerbauer, 2001). These differential modifications are a result of both cell-cycle dependent and various environmental stimuli that serve to trigger specific signaling pathways. Downstream effects of the secondary biochemical modifications include changes in the ability of the HMGA1 protein to interact with a variety of protein and DNA substrates. As covered latter in the introduction of Chapter 2, HMGA1 modification mediated by specific factors results in specific changes to HMGA1 function and plays a significant role in subnuclear and possibly subcellular protein localization patterns. Currently, there is ample evidence

supporting dynamic, post-translational modification regulated, subnuclear localization patterns for the HMGA1 proteins. Such controlled changes in nuclear localization provide a basis for understanding the novel subcellular localization patterns of the HMGA1 proteins presented in this dissertation.

HMGA1: Nuclear Localization and Function

To date, localization studies, in conjunction with molecular biological and biochemical data, have supported an exclusive nuclear role for the HMGA1 proteins. Within the nucleus, it has become evident that the proteins are quite mobile and can be regulated in a variety of ways to function within distinct subnuclear regions that correspond to specific cellular functions. In fact, localization studies have been instrumental in revealing novel functions for the HMGA1 proteins as well as confirming their previously characterized roles.

Shortly following their discovery in HeLa cells, the HMGA1 proteins were actually designated as alpha proteins because of their ability to bind *in vitro* to AT-rich alpha-satellite DNA from chromatin preparations made from green monkey kidney cells (Varshavsky et al., 1983; Strauss and Varshavsky, 1984). Subsequently, another early localization study, utilizing immunofluorescence, confirmed the role of the proteins in chromatin structure by showing their specific association with G/Q- and C- bands of mammalian metaphase chromosomes (Disney et al., 1989). Specific association of HMGA1 with defined regions of metaphase chromosomes, as observed, suggested that the proteins were in some way involved with the changes in chromatin structure that occur during the chromosome condensation cycle and cell division (Disney et al., 1989;

Reeves, 1992). Support for this suggestion came from co-localization results that demonstrated the presence of HMGA1, along with both histone H1 and topoisomerase II, at scaffold/matrix attachment regions (SARs/MARs) (Saitoh and Laemmli, 1994). These AT-rich sequences are components of metaphase chromosome structure and are thought to be involved in the regulation of chromosome dynamics and gene expression due to their location at the base of loops of gene-containing DNA. In fact, HMGA1 proteins were shown to antagonize histone H1 binding, thus inhibiting the general H1-mediated repression of gene accession and making transcriptional initiation possible (Käs et al., 1993). This localization characteristic confirms a model proposed for the mechanism of HMGA1 gene activation that involves a global alteration to large regions of chromatin structure. That is, the proteins act as "anti-repressors" by competitive SAR binding and displacement of inhibitory proteins such as histone H1 (Zhao et al., 1993).

More recent immunocytochemical studies, based on the hypothesis that the specific interaction of HMGA1 with AT-rich DNA might result in chromatin context specific functions, have revealed sub-populations of HMGA1 proteins within the nucleus (Amirand et al., 1998). Focusing on subnuclear localization patterns, the authors identified three areas of HMGA1 staining that corresponded to heterochromatin, decondensed chromatin, and the general nucleoplasm. These findings were consistent with data showing that HMGA1 is involved in the binding of AT-rich satellite repeats clustered at centromeres and telomeres, and of randomly dispersed satellite DNA sequences, SARs (Zhao et al., 1993), and gene regulatory elements (Falvo, et al., 1995; John et al., 1995; John et al., 1996). Similar double immunolabeling results showed co-localization of HMGA1 with key nuclear components such as NuMA, SC-35, and

$TAF_{II}70$, proteins known to play a role in transcription related events (Tabellini et al. 2001). In general, these localization based studies confirm the role of the HMGA1 proteins as dynamic regulators of gene transcription at the level of large chromatin reorganization and more restricted localized effects at the nucleosomal level as covered in the previous section.

An even more recent study regarding HMGA1 localization within the nucleus was focused on chromatin on/off rates and the effect of post-translational modifications on their mobility and function. Briefly, it was determined, using HMGA1-GFP fusion proteins and fluorescence recovery after photobleaching (FRAP) assays, that HMGA1 is a highly mobile component of euchromatin, heterochromatin, and mitotic chromosomes. Furthermore, it was shown that the kinetic properties of HMGA1a proteins are controlled by the number of functional AT-hooks present and are regulated by differential patterns of phosphorylation. That is, increasing phosphorylation levels were correlated to increased residence times on heterochromatin and chromosomes, as compared to euchromatic regions (Harrer et al., 2004). Overall, this study supports previous work suggesting that the HMGA1 proteins are indeed major players in both local and global changes in chromatin structure. It is important to restate that these structural changes mediated by HMGA1 are carefully regulated events that serve to specifically up or down regulate the expression of genes that code for proteins involved in a variety of biological processes including abnormal cellular events such as transformation and cancerous progression. Also, while these functions have been well described in the context of the nucleus and chromatin structure, the binding requirements of HMGA1 proteins are such that any gene element with enrichment in AT content is a potential target for regulation.

As will be described in Chapters 2 and 3, the genomes of mammalian mitochondria are quite high in AT content and thus possess high potential for binding and transcriptional regulation by HMGA1 proteins.

HMGA1: Biological Functions and Cancer Progression

HMGA1 proteins are involved in the normal biological processes of embryonic development as evidenced by their maximal expression during this period (Chiappetta et al., 1996). Considerable data supports the original idea that the proteins are involved in the control of cell growth and differentiation in the embryo (Bustin and Reeves, 1996). That is, premature gene transcription was shown to be inducible in early mouse embryos by the microinjection of HMGA1 alone (Beaujean et al, 2000). Also, HMGA1 has been implicated in controlling the growth and differentiation of adipocytes (Melillo et al., 2001). Furthermore, HMGA1 expression levels are rapidly induced upon the treatment of resting normal cells with factors that activate metabolism and growth, indicating that the proteins play a role in normal cell proliferation (Friedmann et al., 1993; Johnson et al., 1990; Ogram and Reeves, 1995; Holth et al., 1997). Inducible transcription of multiple immune related genes has also been demonstrated, revealing the role of HMGA1 in cytokine stimulated immune cell activation (reviewed in Reeves and Beckerbauer, 2001).

Beyond these normal cellular functions, much work regarding the HMGA1 proteins has been in the context of transformed cells and the overall process of carcinogenesis and metastatic potential. Again, HMGA1 expression levels in normal, non-proliferating, somatic cells are low to undetectable (Lundberg et al., 1989; Bustin

and Reeves, 1996). This is in contrast to the over-expression of the proteins in neoplastically transformed cells where increased HMGA1 levels are correlated with increased degrees of malignancy and metastatic potential (reviewed in Tallini and Dal Cin, 1999). It is important to emphasize that in normal cells high levels of HMGA1 are quite toxic and effectively induce apoptosis due to a deregulation in cell cycle progression from the S to the G2/M phase. Several unique post-translational modifications are observed during the apoptotic process, emphasizing the importance of these regulatory adducts to the function of HMGA1 proteins (Diana et al., 2001). Ultimately, cells that survive this process of apoptosis induction are altered in a way that allows for their aberrant growth and overall cancer phenotype. The phenomenon of HMGA1 protein over-expression has been demonstrated in a wide variety of clinical cancer types including those of the breast, prostate, colon, cervix, lung and neural tissues (reviewed in Reeves and Beckerbauer, 2001). Because of this, as mentioned briefly above, the HMGA1 proteins have been regarded as potential markers of neoplastic transformation and increasing metastatic potential, and as a therapeutic target for cancer treatment. Consideration of the therapeutic potential of HMGA1 inhibition in these various cancer types brings up the question of causation in regard to the actual role of the proteins in the process of cancer progression.

Evidence suggests that over-expression of the HMGA1 proteins in cells is indeed a causative, rather than effectual, factor in the process of carcinogenesis and progression to a more metastatic phenotype. Such evidence comes from studies utilizing a tissue culture model and artificial over-expression of the proteins in different immortalized cell types. It was shown that when HMGA1 proteins were introduced, the cells became

capable of anchorage-independent growth *in vitro* and were able to form highly malignant and metastatic tumors when injected into nude mice (Wood et al., 2000; Reeves et al., 2001). From a biochemical standpoint, significant progress in understanding the mechanism by which HMGA1 proteins promote tumorigenesis came with an experimental model utilizing transgenic mammalian epithelial cells expressing full length protein under the control of a tetracycline-responsive promoter (Reeves et al., 2001). With this transgenic model, HMGA1 protein expression could be induced by the removal of tetracycline from the growth media and effectively turned off in the presence of the drug. Results from these studies showed that the epithelial cells only had the capacity to form metastatic tumors in nude mice when the transgene was on and HMGA1 was being over-expressed. Furthermore, studies in transgenic mice in which HMGA1 over-expression is targeted to lymphoid cells directly demonstrate that HMGA1 is an oncogene that *in vivo* can induce highly penetrant, aggressive leukemias and other lymphoid malignancies (Xu et al., 2004).

In order to look more closely at the actual mechanism of carcinogenesis mediated by the over-expression of HMGA1 in the mammary epithelial cell model, researchers employed gene expression profiling. Findings showed that over-expression of the HMGA1 proteins resulted in the induced expression of distinctive host cell genes known to take part in tumor initiation, apoptosis, DNA repair, tissue invasion, migration, and colonization (Reeves et al., 2001). As covered in detail above, the role of the HMGA1 proteins as regulators of transcription are consistent with the observed alterations in gene expression profiles as a result of their abnormally high expression. In fact, subsequent studies to verify the effect of HMGA1 on specific genes have supported the hypothesis

that the proteins promote progression of cells to a more metastatic state by up-regulating the expression of tumor promoting genes and down-regulating the expression of genes the prevent tumor progression (reviewed in Reeves,2001). For example, recent studies, covered in greater detail in Appendix 1, have shown that HMGA1 is directly involved in the up-regulation of Kit ligand expression, a protein factor involved in activation of the cancer progression associated Ras/Erk signaling pathway (Treff et al., 2004). Important studies, presented in Appendix 2, have also revealed that HMGA1 over-expression significantly inhibits the ability of the cell to efficiently accomplish nucleotide excision repair (NER) via down-regulation of repair factors, an observation which could be an indicator of a major mechanism by which HMGA1 promotes cancerous progression (Adair et al., 2005). As will be presented in Chapters 2 and 3 of this dissertation, another potential mechanism by which HMGA1 proteins promote tumorigenesis may involve their newly discovered mitochondrial localization. A review of mitochondrial biology and the role of these organelles in cancer etiology reveal multiple potential mechanisms by which HMGA1 may be affecting their function and subsequent alterations to overall cellular homeostasis.

Mitochondria: General Function and Role in Cancer Biology

Functioning in a semi-autonomous manner, mammalian mitochondria replicate, transcribe, and translate their own 16,569 base pair genome (**Fig 1.4**). The supercoiled, double-stranded circular mitochondrial (mt)DNA contains 37 genes coding for 13 polypeptides essential for oxidative phosphorylation, 22 tRNAs and 2 rRNAs required for translation (Anderson et al., 1981; Taanman, 1999). Completion of oxidative

phosphorylation requires protein products of all 13 mitochondrial genes and 74 nuclear genes which, together, form 5 multi-subunit enzymatic complexes of the electron transport chain (reviewed in Penta et al., 2001). Taking place within the inner mitochondrial membrane, this system is responsible for 90% of cellular adenosine triphosphate (ATP) production, the by product of which is high levels of potentially harmful reactive oxygen species (ROS) within the cell. Due to the proximity of mtDNA to such concentrated levels of ROS, the genome is vulnerable to the accumulation of mutations at a much higher rate than the nuclear genome. Because the mitochondrial genome lacks introns, increased rates of mutations within the organelle translate into coding sequence errors and subsequent abnormalities in protein production. Strong correlations, in fact, exist between the occurrence of mtDNA mutations and multiple cellular disorders, including cancer. Within the context of cancer, altered mitochondrial morphology as well as changes in mitochondrial enzyme patterns and membrane transport systems has been observed (Zafar et al., 1982).

The role of mitochondria in carcinogenesis was first considered following observation, in 1956, that rodent tumors exhibited reduced respiration-coupled oxidative metabolism and increased glycolysis (Warburg et al., 1956). To date, these observations have not been explained but new studies suggest that changes to mitochondrial gene expression profiles observed in cancer cells may be involved in this Warburg phenotype. That is, deficiencies in the ability of a cell to perform oxidative phosphorylation, resulting in a switch to a glycolytic phenotype, may be a result of abnormalities at the mitochondrial genome level. Studies have demonstrated the occurrence of specific alterations to both overall cellular levels of mtDNA and mitochondrial gene expression

levels. In general, tumor cells have increased levels of mtDNA transcripts and either increases or decreases in levels of cellular mtDNA (Glaichenhaus et al., 1986; Pedersen, 1978; Yamamoto et al, 1989). Because HMGA1 is an oncogenic transcription factor and localizes to the matrix of the mitochondria (Chapter 2), an overview of mtDNA transcription/replication is relevant to data presented and hypotheses tested in the following chapters of this dissertation.

Expression and replication of mtDNA in humans have been studied in some detail and important factors involved in the processes have been characterized. However, the manner in which these processes are regulated remains incomplete. Briefly, mtDNA contains an untranslated regulatory region referred to as the D-loop where both transcription and replication are initiated and controlled. Within this region are the origin of heavy-strand (H-strand) replication (O_H) and the transcription promoters for the light (LSP) and heavy (HSP) mtDNA strands (Shadel and Clayton, 1997). A separate distinct origin of replication (O_L) for the light strand (L-strand) exists within a cluster of five tRNA genes two-thirds of the way around the circular genome. Replication of the genome begins with the initiation of H-strand synthesis on the D-loop template sequence. This is followed by initiation of L-strand synthesis which occurs only after H-strand synthesis is two-thirds complete (**Fig 1.5**). Transcription from both D-loop positioned promoters results in the production of polycistronic messages that are subsequently processed to yield mature DNA for translation. Also requiring RNA processing is the actual priming of replication that occurs via the formation of an LSP initiated transcript that creates an RNA-DNA hybrid at O_H, allowing for the docking of the mitochondrial DNA polymerase (pol γ) and DNA synthesis (Chang and Clayton, 1985; Lee and

Clayton, 1998). Therefore, protein factors involved in transcriptional initiation are also important in mtDNA replication.

Currently, only three mammalian proteins are characterized as being directly involved in mitochondrial transcription. They are the human mitochondrial RNA polymerase (Tiranti et al., 1997), the high-mobility-group box protein, mtTFA (Fisher and Clayton, 1988; Parisi and Clayton, 1991), and the transcription factor mtTFB (McCulloch et al., 2002). The nucleus-encoded DNA binding protein, mtTFA, is an approximately 25 kDa transcription factor with the ability to interact with DNA in a manner that is similar to that of HMGA1. That is, mtTFA activates transcription by binding upstream of promoter start sites and displays inherent flexibility in DNA sequence specificity. Upon binding, the protein is capable of condensing, unwinding, and bending DNA at specific sequences of both HSP and LSP (Fisher et al., 1992). Such observations are intriguing when considering the similar function of the HMGA1 proteins as described within the nucleus and may indicate that mitochondrial HMGA1 functions in a similar manner and at similar sequences. This is due in part to the observation that the AT-hook motif of HMGA1 proteins is known to bind with a higher affinity to DNA regions also recognized by HMG box proteins (Hill et al., 1999; Hill and Reeves, 1997). As will be described in greater detail in Chapter 2, the mitochondrial genome is also quite AT-rich (Bibb et al., 1981) and contains many potential HMGA1 binding sites. The functional ramifications of HMGA1 localization to mitochondria in regard to observed D-loop DNA binding (Chapter 2) are studied and discussed in Chapter 3.

Figures

Figure 1.1. Diagram illustrating the highly connected nature of the HMGA1 proteins as integrating 'hubs' of various biological activities. The upper portion of the diagram shows various extra- and intracellular signaling pathways that are known to either regulate transcription of the *HMGA1* genes, control production of HMGA1 proteins and/or modulate secondary biochemical modifications of pre-existing HMGA1 protein. The lower portion of the diagram lists various normal and pathological biological processes in which participation of the HMGA1 proteins has been proposed (Reeves, 2001).

Figure 1.1.

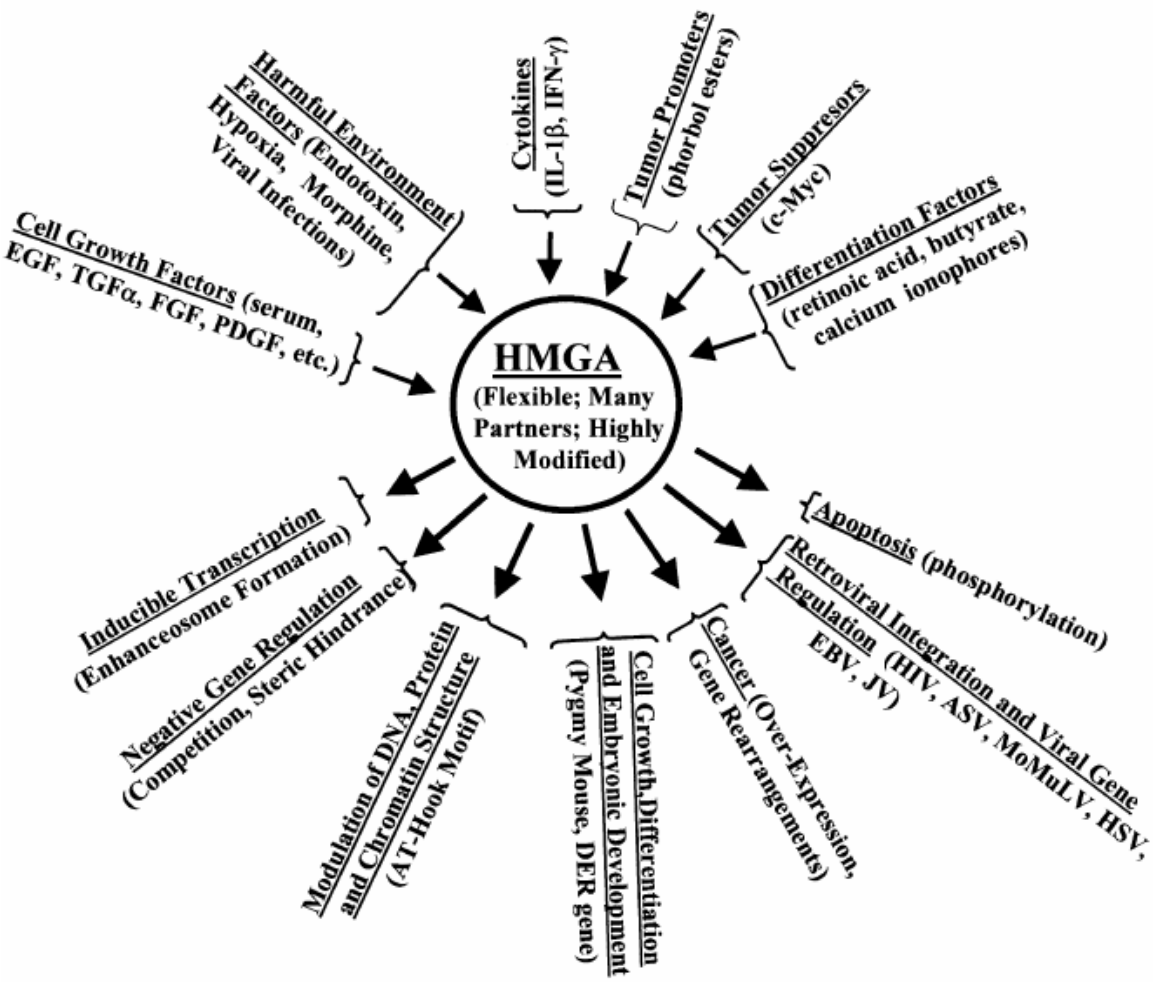

HMGA
(Flexible; Many Partners; Highly Modified)
Harmful Environment Factors (Endotoxin, Hypoxia, Morphine, Viral Infections)
Cytokines (IL-1β, IFN-γ)
Tumor Promoters (phorbol esters)
Tumor Suppresors (c-Myc)
Differentiation Factors (retinoic acid, butyrate, calcium ionophores)
Cell Growth Factors (serum, EGF, TGFα, FGF, PDGF, etc.)
Inducible Transcription (Enhanceosome Formation)
Negative Gene Regulation (Competition, Steric Hindrance)
Modulation of DNA, Protein and Chromatin Structure (AT-Hook Motif)
Cell Growth,Differentiation and Embryonic Development (Pygmy Mouse, DER gene)
Cancer (Over-Expression, Gene Rearrangements)
Retroviral Integration and Viral Gene Regulation (HIV, ASV, MoMuLV, HSV, EBV, JV)
Apoptosis (phosphorylation)

Figure 1.2. Proposed model for participation of HMGA1 (a.k.a. HMGI/Y) proteins in two steps of transcriptional activation of the human IL-2Rα gene promoter: chromatin remodeling and enhanceosome formation (Reeves and Beckerbauer, 2001).

Figure 1.2.

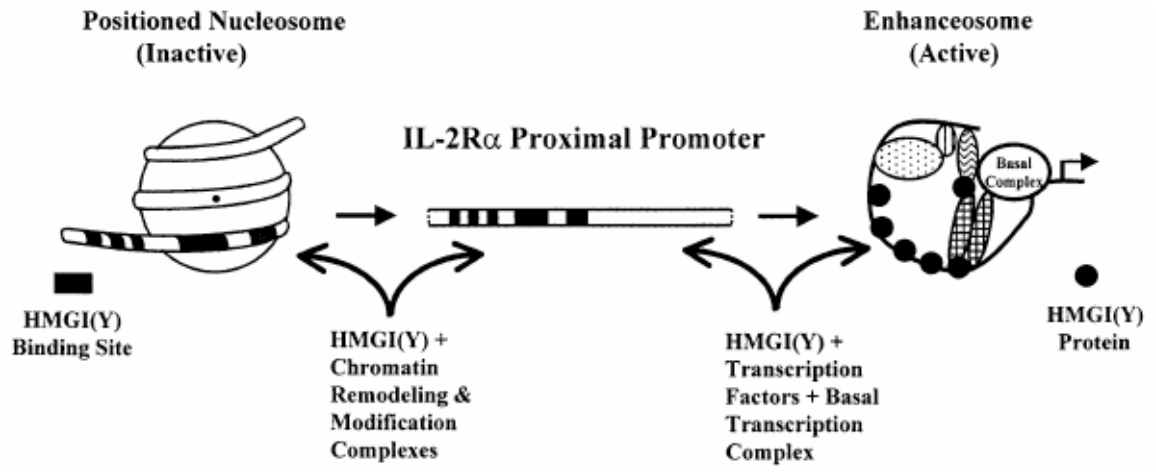

Positioned Nucleosome
(Inactive)
Enhanceosome
(Active)
IL-2Rα Proximal Promoter
Basal Complex
HMGI(Y) Binding Site
HMGI(Y) + Chromatin Remodeling & Modification Complexes
HMGI(Y) + Transcription Factors + Basal Transcription Complex
HMGI(Y) Protein

Figure 1.3. Structure of the second and third DNA-binding domains of the HMGA1a protein in complex with a synthetic duplex oligonucleotide substrate (Huth et al., 1997. (A) Representation of an unstructured AT-hook peptide that is free in solution. (B-E) Various views of the second AT-hook peptide bound to the minor groove of a synthetic duplex with a central sequence of 5'-AAATTT-3'. (B) The best-fit structure of the peptide backbone of the second AT hook as it looks when bound to B-form DNA. (C,D) Co-complex of the second AT-hook with B-form DNA showing the final set of 35 NMR simulated annealing structures for the peptide backbone (frayed `rope' structure) superimposed on the DNA (ribbon structure). (C) Side view looking into the DNA minor groove. (D) Polar view looking down the long axis of the DNA. (E) Polar view of complex showing the side chains of the arginine residues of the AT hook motif projecting into the minor groove and making hydrophobic contacts with adenine bases. (F) Side view of a ball-and-stick model of the third AT hook peptide bound to the minor groove (Reeves and Becerbauer, 2001).

Figure 1.3.

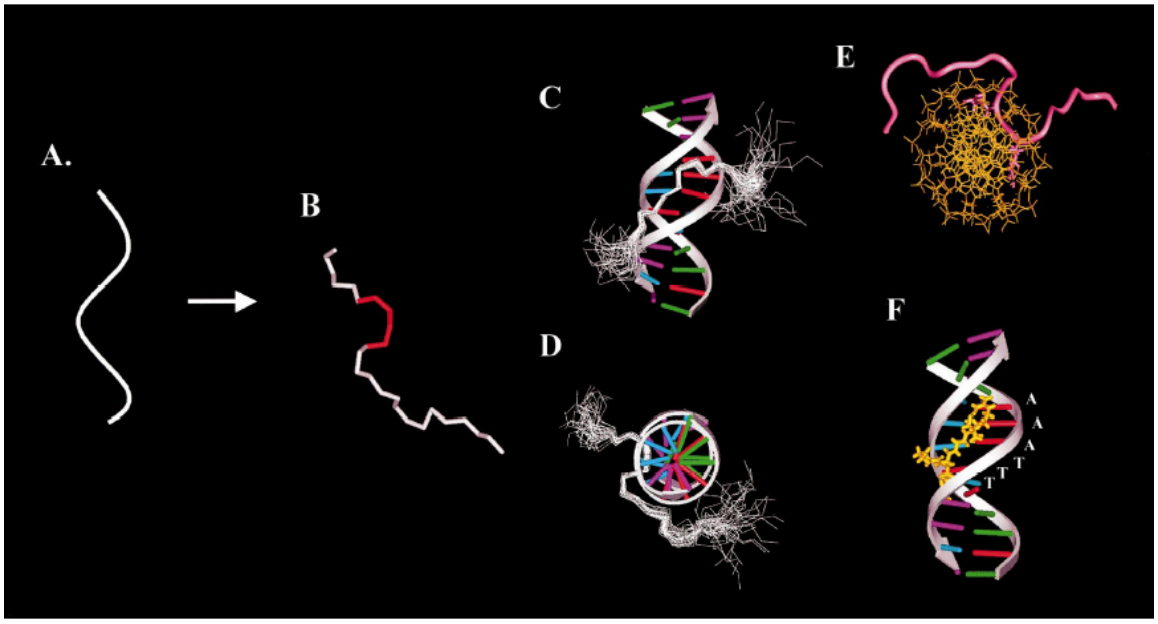


Figure 1.4. Detailed map of the mitochondrial genome (mitomap.org).

Figure 1.4.

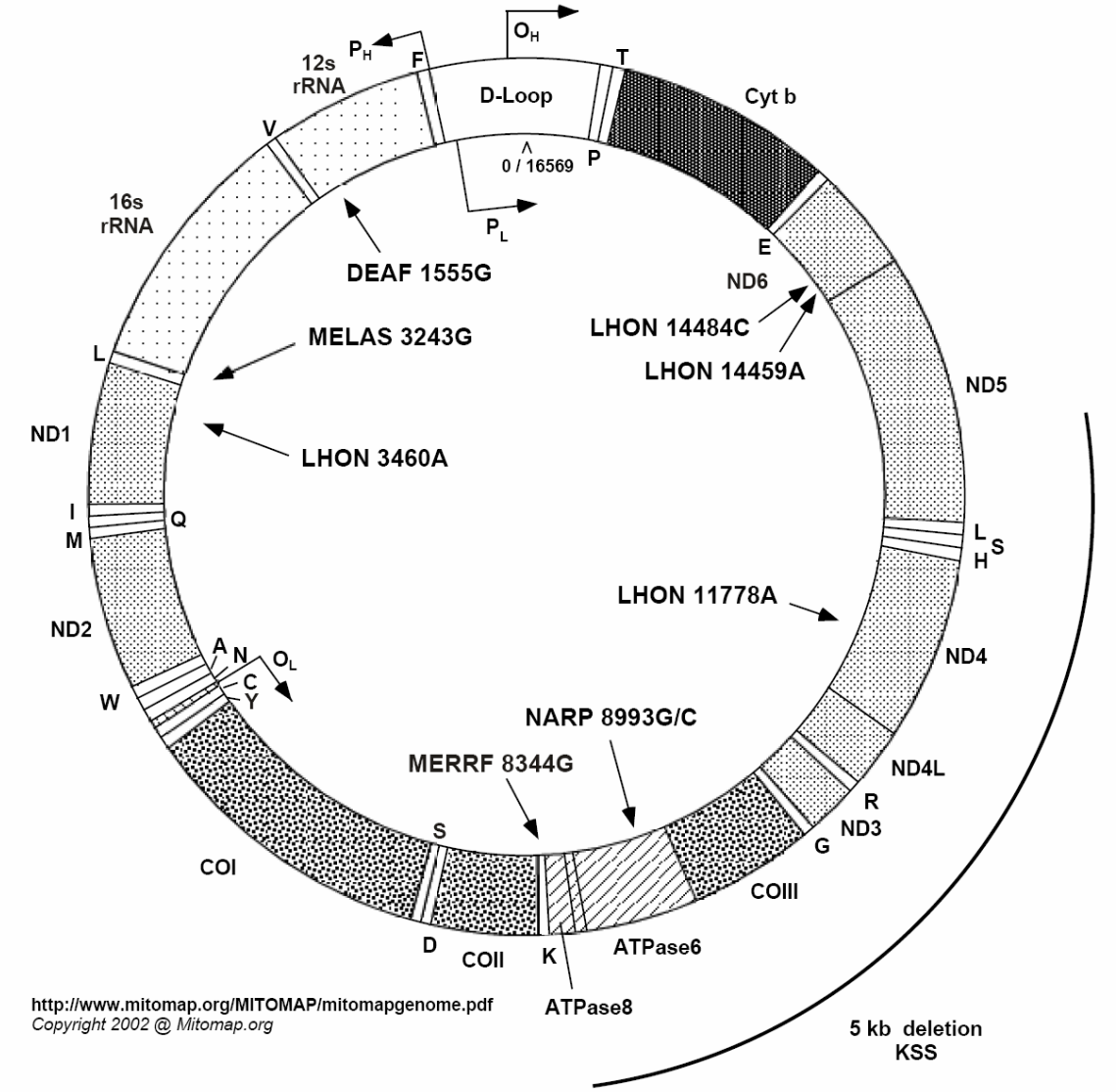
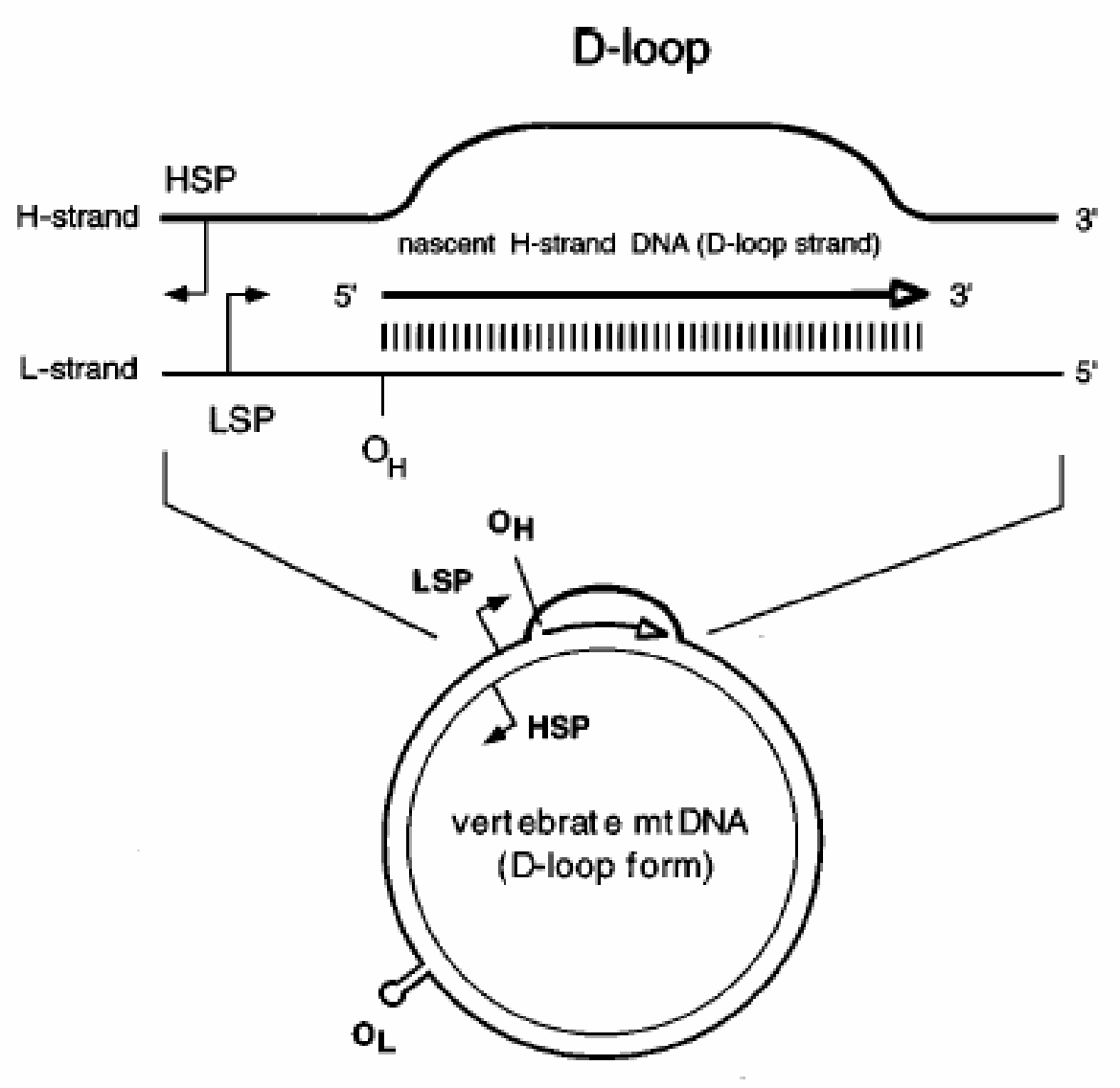

O_H
P_H
12s rRNA
F
D-Loop
T
Cyt b
V
0 / 16569
P
16s rRNA
P_L
DEAF 1555G
E
ND6
LHON 14484C
MELAS 3243G
LHON 14459A
L
ND5
ND1
LHON 3460A
I
Q
M
L
H
S
LHON 11778A
ND2
A
N
O_L
ND4
C
Y
W
NARP 8993G/C
MERRF 8344G
ND4L
R
ND3
S
G
COI
COIII
D
COII
K
ATPase6
ATPase8
http://www.mitomap.org/MITOMAP/mitomapgenome.pdf
Copyright 2002 @ Mitomap.org
5 kb deletion
KSS
Complex I genes (NADH dehydrogenase)
Complex III genes (ubiquinol : cytochrome c oxidoreductase)
Transfer RNA genes
Complex IV genes (cytochrome c oxidase)
Complex V genes (ATP synthase)
Ribosomal RNA genes

Figure 1.5. Schematic representation of replication initiation of the vertebrate mitochonodrial genome (Shadel and Clayton, 1997). The circular D-loop form of mtDNA is shown with the D-loop regulatory region. The mtDNA H-strand and L-strand are depicted as heavy and thin lines, respectively. The nascent H-strand (D-loop strand, thick arrows) is shown bound (dashed lines) to the parental L-strand and displacing the parental H-strand (bubble) to form the D-loop. The origins of H-strand (O_H) and L-strand (O_L) synthesis are labeled. The heavy-strand promoter (HSP) and light-strand promoter (LSP) are depicted as bent arrows in the D-loop regulatory region. Note that LSP transcripts function both in gene expression (usually encoding eight genes) and DNA replication, where they are processed to provide RNA primers at O_H.

Figure 1.5.


D-loop
HSP
H-strand
3'
nascent H-strand DNA (D-loop strand)
5'
3'
L-strand
5'
LSP
O_H
O_H
LSP
HSP
vertebrate mtDNA
(D-loop form)
O_L

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CHAPTER 2

Dynamic Mitochondrial Localization of Nuclear Transcription Factor HMGA1

Abstract

It has been well established that high mobility group A1 (HMGA1) proteins act within the nucleus of mammalian cells as architectural transcription factors that regulate the expression of numerous genes. Here, however, we report on the unexpected cytoplasmic/mitochondrial localization of the HMGA1 proteins within multiple cell types. Indirect immunofluorescence, electron microscopic immunolocalization, and western blot studies revealed that, in addition to the nucleus, HMGA1 proteins can also be found in both the cytoplasm and mitochondria of randomly dividing populations of wild-type murine NIH3T3 cells and transgenic human MCF-7 breast cancer epithelial cells expressing a hemaglutinnin tagged-HMGA1a fusion protein. While the molecular mechanisms underlying these novel subcellular localization patterns have not yet been determined, initial synchronization studies revealed a dynamic, cell cycle-dependent translocation of HMGA1 proteins from the nucleus into the cytoplasm and mitochondria of NIH3T3 cells. Furthermore, preliminary functionality studies utilizing a modified "chromatin" immunoprecipitation protocol revealed that HMGA1 retains its DNA binding capabilities within the mitochondria and associates with the regulatory D-loop region *in vivo*. We discuss potential new biological roles for the classically nuclear HMGA1 proteins with regard to the observed nucleocytoplasmic translocation, mitochondrial internalization, and regulatory D-loop DNA binding.

Key Words: High Mobility Group; NIH3T3; MCF-7; Synchronization; Mitochondria; D-loop; oncoprotein.

Introduction

Exclusive intranuclear localization of the high mobility group A1 (HMGA1) proteins has been suggested (Amirand et al., 1998). Consistencies of this conclusion with known HMGA1 function(s) are substantial but do not exclude alternative cytoplasmic roles for this important, classically nuclear, family of proteins. As a whole, the HMGA1 group of non-histone chromatin proteins represents a class of 'architectural' transcription factors that possess copies of an 'AT hook' DNA-binding motif (Reeves and Nissen, 1990). This peptide motif, which is conserved from bacteria to humans and is present in multiple non-HMGA1 gene regulatory proteins (Aravind and Landsman, 1998), preferentially associates with the minor groove of AT-rich B-form DNA (Reeves et al., 1987; Solomon et al., 1986) in a structurally specific manner (Huth et al., 1997; Reeves and Nissen, 1990). These DNA binding characteristics, along with the ability to physically interact directly with a range of transcription factors (Reeves, 2001), allows HMGA1 to play a dynamic role in a variety of important nuclear events.

Results from multiple cellular localization studies support this central nuclear function for the HMGA1 proteins. Our laboratory has previously demonstrated the specific association of these proteins with the AT-rich G/Q- and C- bands of human and mouse chromosomes (Disney et al., 1989). This finding lead to the hypothesis that HMGA1 proteins are most likely involved in the processes of cell division and chromatin organization (Reeves and Nissen, 1995). *In vitro* and *in vivo* experiments, involving the use of distamycin A and Hoechst 33258 as competitors of HMGA1 binding, support this idea (Radic et al., 1992; Wegner and Grummt, 1990). Additional support comes from co-localization results that demonstrate the presence of HMGA1, along with both histone H1

and topoisomerase II, at scaffold/matrix attachment regions (SARs/MARs), sequences important for metaphase chromosome structure (Saitoh and Laemmli, 1994). Such data correlate well with biochemical studies implicating HMGA1 in transcriptional regulation, chromatin organization, and the formation of repressor complexes (Reeves and Beckerbauer, 2001).

Unique physical and biochemical properties give HMGA1 proteins the ability to function in the aforementioned array of nuclear processes. These properties do not, however, exclude the possibility of a non-nuclear role for the proteins. For example, each protein has three separate AT hook consensus peptides that typically associate with 5 to 6 tandem AT base pairs (Reeves and Nissen, 1990). Any region of DNA known to be high in AT content is thus a potential candidate for containing HMGA1 binding sites, regardless of specific sequence. This allows the proteins to directly interact with a number of platforms including satellite repeats (Strauss and Varshavsky, 1984), SARs/MARs (Zhao et al., 1993), and multiple gene promoter elements (Reeves and Beckerbauer, 2001). Functional versatility and mobility is increased by the relative small size (10.6-12 kDa) (Lund et al., 1983) and inherent flexibility (Lehn et al., 1988) of the HMGA1 proteins. As free molecules, the proteins have little if any secondary structure (Evans et al., 1992; Evans et al., 1995; Huth et al., 1997). This characteristic is thought to play a significant role in the ability of HMGA1 to interact with multiple proteins and DNA elements as well as to induce conformational changes in these substrates (Reeves and Beckerbauer, 2001). Such interactions are also enhanced by the large number of positively charged lysines and arginines that flank the core AT hook peptide (Huth et al., 1997; Lund et al., 1983).

A final distinguishing factor allowing HMGA1 to function in diverse settings is its array of modified states. Multiple studies have shown that HMGA1 function is regulated by *in vivo* post-translational phosphorylation, acetylation, and methylation, among others (Banks et al., 2000; Edberg et al., 2004; Reeves and Beckerbauer, 2001). These modifications result from both internal and external signaling events that affect important biological events such as cellular activation and proliferation, apoptosis, and cell cycle progression. More specifically, a recent study utilizing fluorescence recovery after photobleaching (FRAP) analysis demonstrated that reversible phosphorylation regulates the chromatin binding characteristics of HMGA1 proteins *in vivo* (Harrer et al., 2004). Thus, as a general conclusion, secondary biochemical modifications contribute to the ability of the cell to control both the function and distribution of these highly mobile and dynamic proteins. Of particular interest for the current study, is the specific phosphorylation of two HMGA1 threonine residues by cdc2 kinase during the G2/M phase of the cell cycle (Reeves et al., 1991). The result is a 20-fold decrease in the DNA binding affinity of the modified protein, indicating the cell stage specific mobilization of HMGA1 for an as yet undetermined function (Nissen et al., 1991).

Here we report on the apparent cell cycle dependent translocation of the HMGA1 proteins from the nucleus to the cytoplasm and mitochondria in NIH3T3 cells and transgenic MCF-7 cells over-expressing an HA tagged HMGA1a fusion protein. Both cell types analyzed reveal preferential nuclear or chromosomal localization of HMGA1 proteins, with occasional cases of cytoplasmic translocation in randomly proliferating cell populations. However, in a majority of synchronized NIH3T3 cells, during the late S and G2 stages, these proteins are also found in abundance within both the cytoplasm and

mitochondria. In order to determine a functional role for the specific mitochondrial localization and internalization of HMGA1, a modified chromatin immunoprecipitation (ChIP) protocol was performed. Interestingly, the results showed that HMGA1 binds, *in vivo* within both the parental (HMGA1 non-over-expressing) and transgenic (HMGA1 over-expressing) MCF-7 cells, to the control region of the mitochondrial genome referred to as the D-loop. These findings not only demonstrate the remarkable dynamic mobility of the HMGA1 proteins inside living cells but also represent the first report of a bona fide nuclear chromatin protein specifically binding to the mitochondrial D-loop. This raises the intriguing possibility that HMGA1 proteins not only contribute to transcription and/or replication control in mitochondria but also function to coordinate the activities of these organelles with those of the nucleus.

Materials and Methods

Cell Culture and Synchronization

Briefly, attached monolayer NIH3T3 cells (American Type Culture Collection) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% calf serum, penicillin (100 ug/ml), and streptomycin (50 ug/ml). Partial synchrony was achieved by seeding cells into 100 mm culture dishes, growing cells to confluence, and maintaining the cells at confluence for up to 4 days. Confluent cultures were trypsinized, washed, and seeded onto 100 mm dishes ($1X10^5$ cells) for DNA content analysis or 60 mm dishes ($1X10^4$ cells) with sterile glass coverslips for immunofluorescence. Cells were then harvested at various time points, fixed in cold absolute ethanol, and stained with 0.01 mg/ml propidium iodide (PI) for DNA content

analysis by flow cytometry using a Becton Dickinson FACS Calibur Cytometer and Cell Quest Software.

The tetracycline-regulated M/tet (referred to as parental) and M/tet/HA-I (referred to as transgenic) human breast epithelial MCF-7 cells were developed as described previously (Reeves et al., 2001). Briefly, the parental M/tet (MCF-7/tet-off) cell line was purchased from Clontech, Palo Alto, CA. This cell line was stably transfected with a plasmid vector (Clontech) containing the tetracycline response element (pTRE) driving the expression of a hemaglutinnin (HA)-tagged HMGA1a transgene to generate the M/tet/HA-I line. M/tet cells were cultured in DMEM supplemented with 10% fetal bovine serum, penicillin (100 ug/ml), streptomycin (100 ug/ml), and G418 (100 ug/ml) to maintain selection of the tetracycline transactivator protein gene. M/tet/HA-I cells were maintained and selected in the same media as for the M/tet, and supplemented with hygromycin (100 ug/ml) for selection of clones containing the HA-HMGA1a expression vector. Partial synchronization of the M/tet/HA-I cells was accomplished by treatment of adherent cells at 50% confluence with 4 mM hydroxyurea (HU) for 24 hours to inhibit DNA synthesis and arrest the cell cycle at the early S phase. Recovery was accomplished by the addition of complete growth media after three washes with 1X PBS to remove HU. Cells were then harvested at various time points up to 24 hours, fixed in cold absolute ethanol, and stained with 0.01 mg/ml propidium iodide (PI) for DNA content analysis as described above. For immunofluorescence analysis of synchronized cells, cultures were grown on sterile glass coverslips before treatment with HU.

Antibodies, Fractionation and Western Blot Analysis

Production and characterization of the rabbit polyclonal antibody against reverse-phase HPLC purified murine HMGA1a protein used in this study has been previously described (Disney et al., 1989). The IgG fraction of the HMGA1a antiserum was purified using Staphylacoccus aureus protein A column (Miller and Stone, 1978) and, in some cases, subsequently purified by affinity chromatography on an HMGA1a-sepharose column (Harlow and Lane, 1988). Titer and specificity of this antibody was determined by enzyme-linked immunosorbent assays (ELISA) and western blot analysis. These techniques showed no cross reactivity to mixed histone preparations, proteins from human or murine cells, or HMG proteins other than HMGA1a and HMGA1b (Disney et al., 1989). Mouse monoclonal antibody (clone 12CA5) to an epitope derived from the hemaglutinnin protein of human influenza virus was a generous gift of J.J. Chen (Chen et al., 1998).

Fractionation of transgenic MCF-7 cells was performed with a Mitochondrial Fractionation Kit (Active Motif, Carlsbad, CA) following the recommendations of the distributor. Briefly, cells were harvested and homogenized on ice with 50 strokes using a loose fitting 2 ml Dounce homogenizer. This was followed by differential centrifugation using a table top microcentrifuge. Protein yield was determined by the Biorad Protein Quantification Assay (Biorad, Hercules, CA), and an equal amount of protein (25 ug) from each fraction was loaded onto a 15% SDS-polyacrylamide gel. Following transfer of the proteins to a PVDF membrane, samples were probed with either affinity purified anti-HMGA1 (MR19) or anti-HA tag antibodies together with anti-Histone H1 (Stressgen Biotechnology, Victoria, BC, Canada) as a nuclear control. Additional control antibodies

used were anti-phosphoMEK1/2 (Cell Signaling Technology) as a cytosolic marker and anti-65 kDa mitochondrial protein (Chemicon – mAb 1273) as a mitochondrial marker.

Indirect Immunofluorescence

Cells grown on glass coverslips were washed in BRB80 buffer to remove serum proteins, and fixed with a solution of 0.3% glutaraldehyde or 4% paraformaldehyde (PFA). Fixed cells were permeabilized with 1% Triton X-100, followed by the reduction of unreacted aldehydes by the addition of 1 mg/ml sodium borohydride. Coverslips were then blocked with 8% bovine serum albumin (BSA) in a solution of PBS and 0.1% Triton X-100 (PBST). The primary antibody (anti-HA tag (1:1000) or anti-HMGA1 (MR19) (1:500)) was diluted in PBST with 1% BSA and added to the cells for 1 hour at room temperature. Following three washes with PBST, anti-mouse or anti-rabbit IgG conjugated with Oregon Green diluted 1:200 in PBST with 1% BSA was added and the cells were incubated for 1 hour at room temperature. After washing with PBS and water, the coverslips were mounted on slides with Vectashield (Vector Laboratories, Burlingame, CA) mounting media containing propidium iodide to stain DNA. Cells were observed using a Nikon Eclipse TE300 confocal laser (Biorad MRC 1024) scanning microscope and images were collected using Biorad Laser Sharp acquisition software.

Preparation of NIH3T3 Cells for Electron Microscopy

Culture plates with monolayers of cells were flooded with 20 ml of chemical fixative containing 1% paraformaldehyde and 1% glutaraldehyde in fetal calf serum buffered with 30 mM PIPES (pH 7.2). The cells were fixed for 15 minutes at room

temperature and rinsed with fetal calf serum twice for 10 minutes. Using a rubber spatula and fresh fetal calf serum, aliquots of cells were harvested and transferred to a 1.5 ml Eppendorf tubes and pelleted by centrifugation. The packed cells were dehydrated using an ethanol series (30, 50, 70, 80, 90, 100%; 10 minutes each) and infiltrated with L.R. White resin in a stepwise fashion (1:4, 1:2, 1:1, 2:1 resin: ethanol, pure resin; 12-24 hours each step). The infiltrated pellets were transferred to gelatin capsules (size 00) having a prepolymerization pad of resin at the bottom and then polymerized at 60^{o}C for 18 hours. The pellets were cut out of the polymerization blocks, and remounted on a plastic rod such that the pellet could be cut in cross-sections. This ensured sampling of all cells in the population regardless of slight differences in density, and thus differential sedimentation during centrifugation. Thin sections (~ 60 nm) were cut on a diamond knife using a Sorvall MT5000 ultramicrotome and transferred to uncoated, 200 mesh nickel grids.

Immunogold Staining:

Grids containing sections were immersed in TBS+BSA (50 mM Tris, 500 mM NaCl, 0.1% Tween, 1% BSA, pH 7.2). They were then transferred to specific or preimmune antibody diluted 1:25, 1:50, or 1:100 in TBS+BSA and incubated for 1 to 3 hours at room temperature while shaking. A dilution of 1:25 and incubation for 2 hours gave the best combination of adequate labeling and low background. The antibodies used were anti-HMGA1a whole serum or purified IgG's, anti-HMGA1a synthetic peptide (Johnson et al., 1989) whole serum, whole preimmune serum and mouse antihistone monoclonal antibodies in ascites fluid. After incubation in the above antibody solutions,

the grids were washed 5 times by immersion in TBS+BSA (10 minutes each while shaking), and then transferred to Protein A-gold (15 nm, Janssen Pharmaceuticals, Piscataway, N.J., USA) diluted 1:50 with TBS+BSA, and incubated for 1 hour. For labeling with murine antihistone antibody, gold-tagged goat anti-mouse antibody was used in place of protein A-gold (same size and source as above). The grids were then washed 4 times with TBS+BSA, 3 times with TBST, and 3 times with distilled water (5-10 minutes each wash). The grids were post-stained with 1% aqueous alkaline lead citrate and 2% aqueous uranyl acetate and examined and photographed using a Hitachi 300 transmission electron microscope operated at 75 KV accelerating potential. To quantitatively assess the number of immunogold particles within mitochondria, compared to other areas of the cell, a pixel selection method employing Adobe Photoshop (ver. 8.0; Adobe Systems Inc., San Jose, CA) was employed. Briefly, the total cell field for a given cross section was selected and the immunogold particles counted. For each count, the number of pixels within the selected region was recorded. This procedure was repeated for each of the mitochondria within the corresponding cell cross section. Count results for three cell cross sections showed 21.33±3.59 mitochondrial particles per unit area compared to 7.37±0.78 total cell particles per unit area. Area was determined by generating a histogram of the selected region that reports the total number of selected pixels. The unit of area was arbitrarily set at 50,000 pixels for a direct comparison of mitochondrial versus total cell particles present within a given area.

Modified Chromatin Immunoprecipitation (ChIP):

Immunoprecipitation of croslinked DNA/protein complexes was performed as described previously (Treff et al., 2004) with several minor changes. Briefly, transgenic MCF-7 cells over-expressing HMGA1 and parental MCF-7 cells not over-expressing HMGA1 were crosslinked with formaldehyde at a final concentration of 1%. Crosslinked cells were then fractionated as described above to collect mitochondria. Mitochondria were then subjected to the complete ChIP protocol with increased sonication to achieve complete fragmentation of the DNA. Additionally, salmon sperm DNA was not added to the Sepharose A protein beads as the high level of mitochondria in the salmon sperm cells resulted in mitochondrial DNA contamination in all control and experimental samples during protocol optimization. Immunoprecipitation of HMGA1/DNA complexes was carried out with polyclonal anti-HMGA1 antibody (MR19) which recognizes endogenous protein. Following Rnase and Proteinase K treatment, PCR amplification of phenol extracted and ethanol precipitated DNA was carried out with two separate primer sets (D-loop Primer Set 1: upper: 5'-CCCCTCACCCACTAGGATAC-3', lower: 5'-ACGTGTGGGCTATTTAGGC-3'; and D-loop Primer Set 2: upper: 5'-GCATTGCGAGACGCTGGAGC-3', lower: 5'-TCTGGTTAGGCTGGTGTTAG-3') specific for regions of the D-loop sequence identified as containing potential binding sites for HMGA1. Binding site potential was determined by a Seqweb Pattern Search with the following sequence pattern entry: (wwwww (N) {0,5} wwwww) where "w" is either an adenine or thymine and "N {0,5}" is zero to five of any nucleotide. Though this pattern does not provide definitive

identification of HMGA1 binding sites, it is effective for determining an increased probability of binding.

Results

Localization of HMGA1 in Non-synchronized Cell Populations

Immunofluorescence analysis of endogenous HMGA1 in randomly dividing suspension cultures of human K562 cells and murine Friend erythroleukemia cells (data not shown) confirmed the primarily nuclear localization patterns that have been previously observed (Disney et al., 1989). Somewhat surprisingly, in the same asynchronously growing populations of both cell types, we also observed cytoplasmic localization of the HMGA1 proteins (data not shown). Because both the K562 and Friend cells grow in suspension, these analyses involved cytocentrifugation of live cells onto microscope slides prior to fixation and immunolocalization analysis. As a result, cellular compartments were sometimes difficult to distinguish by immunofluorescence, making the observation of non-nuclear HMGA1 localization a possible artifact.

In order to avoid cytocentrifugation steps and facilitate a more accurate analysis of intracellular protein localization, mouse NIH3T3 cells, which display adherent growth characteristics, were cultured and prepared for indirect immunofluorescence analysis. When attached asynchronous populations of NIH3T3 cells were examined, it was found that most of the cells, like the K562 and Friend cells, exhibited a preferential nuclear or chromosomal pattern of HMGA1 localization (data not shown).

Nevertheless, some of the NIH3T3 cells from actively proliferating populations exhibited noticeable cytoplasmic fluorescence. Specifically, it appeared that such

fluorescence was more intense at structures similar to mitochondria. In different cells within the same population, the immunofluorescence was localized either exclusively in the nucleus (**Fig. 2.1A**), both in the nucleus and mitochondria (**Fig. 2.1, B.1**) or, less intense in the nucleus, with a strong signal in the cytoplasm and mitochondria (**Fig. 2.1, B.2**). Overall, patterns of HMGA1 subcellular localization differed from cell to cell in randomly growing populations, suggesting that cell cycle stage might influence HMGA1 distribution.

Localization of HMGA1 in Synchronized NIH3T3 Cells

To test the idea of cell cycle dependent HMGA1 localization control, NIH3T3 cells were partially synchronized by seeding from contact inhibited cultures. Analysis of cell cycle stage by flow cytometry confirmed that the cells could be reproducibly induced to undergo a cycle of partially synchronous replication. From these studies, the approximate length of the NIH3T3 cell cycle was estimated to be 20 hours with the G1 phase occupying about 10 hours (0-10 hours post-seeding), the S phase 6 hours (10-16 hours post-seeding), and the G2/M phase about 4 hours (16-20 hours post-seeding) (**Fig. 2.2A**).

With a reproducible and accurate method of partial synchronization, it was possible to analyze populations of NIH3T3 cells by indirect immunofluorescence at various time points corresponding to particular cell cycle stages (**Fig. 2.2B, C**). As mentioned previously, randomly growing populations displayed fluorescence primarily over the nucleus with few examples of cytoplasmic and mitochondrial staining. Cells

treated with no primary antibody were observed to display negligible, if any, fluorescent signal in these random populations (data not shown).

In contrast to mixed populations of cells, a regular pattern of HMGA1 localization was observed in partially synchronized cells. Briefly, at 12 hours post-synchronization (**Fig. 2.2B**, 12hr), fluorescence was distributed throughout the nucleus with only low level cytoplasmic staining and minor localization over mitochondria. Localization patterns for this population of cells was demonstrative of cells analyzed from 0-12 hours and thus represented the G1/early-S phase of the NIH3T3 cycle. Mid-S phase, which occurred at approximately 14 hours (**Fig. 2.2B**, 14hr) post-seeding, was characterized by a similar pattern of intense nuclear staining with some cells displaying slightly increased cytoplasmic fluorescence. By the late S/early G2 stage, which occurred at approximately 16-17 hours (**Fig. 2.2B**, 16-17hr), a much more intense pattern of cytoplasmic/mitochondrial immunofluorescent staining was observed. To ensure that cells analyzed by immunocytochemistry at these specific time points were synchronized properly and represented a specific cell cycle stage, DNA content analysis was performed on parallel samples. Results from these analyses showed that the cells observed at each time point represented highly synchronized cell populations (**Fig. 2.2C**).

Mitochondrial Immunolocalization Studies in NIH3T3 Cells

Mitochondrial localization of HMGA1 was confirmed by immunofluorescence co-localization (**Fig. 2.3**) studies in cell populations16-18 hours post-synchronization. Previously mentioned findings suggested that a peak of mitochondrial localization occurred in these late S/G2 cells. Indirect immunofluorescence with anti-HMGA1 (**Fig**

2.3A) combined with prior live cell Mito Tracker Red staining (**Fig 2.3B**) of late S/G2 NIH3T3 cells revealed co-localization (**Fig 2.3C**) of HMGA1 with mitochondria. From these results it was clear that HMGA1 was also found diffuse in the cytoplasm but concentrated in or around mitochondria.

In order to further confirm this co-localization and look more closely at the sub-mitochondrial localization of HMGA1, electron microscopy was performed on thin sections of NIH3T3 cells 16-18 hours post-synchronization (**Figs. 2.4-2.6**). Results of control experiments utilizing pre-immune or normal non-immune sera showed low background and no specific labeling of any cellular structures (**Fig. 2.4A**). The general pattern of labeling seen with anti-HMGA1 antibody (**Fig. 2.4B**) revealed diffuse cytoplasmic localization with concentration over the nucleus and mitochondria. Most of this specific antibody labeling was greatly reduced or competed away by the addition of increasing amounts of pure HMGA1 protein to the antibody prior to labeling (**Fig. 2.4C**). In such competition experiments, the residual gold label observed was randomly distributed over the entire field, including on the grid outside of the cells themselves, and was thus likely a result of protein precipitation with subsequent physical trapping of the immunogold particles in these non-specific aggregates (data not shown). Unlike the studies with anti-HMGA1 antibody, control immunolocalization experiments with anti-histone antibody showed strictly nuclear labeling (**Fig. 2.4D**).

A more detailed analysis of the HMGA1 labeling observed in late-S/G2 cells revealed significant localization within the mitochondria and nucleus (**Fig. 2.5**). Within the nucleus, there often appeared to be greater labeling of heterochromatin and the inner periphery of the nuclear membrane than in the interior nucleoplasm (**Fig. 2.5A**; data not

shown). Low magnification photographs (**Figs. 2.5B** and **C**) showed mitochondrial and nuclear labeling that was noticeably higher than the amounts observed in the general cytoplasm. A higher magnification photograph of a separate population of mitochondria also showed heavy labeling over the mitochondria (**Fig 2.5D**). Numerous mitochondria (**Figs. 2.5B** and **C**) observed in the low magnification photographs, as well as the counting of immunogold particles, gave additional support to the conclusion that mitochondrial labeling was not due to random statistical fluctuation. Employing the quantitative counting method outlined in Materials and Methods, it was determined that in late S/G2 cells (**Fig. 2.5**) there was an approximately 3-fold increase in particles per unit area within the mitochondria versus the entire cell field (mitochondrial particles: 21.33±3.95 per unit area; total cell particles: 7.37±0.78 per unit area). High magnification views of the distribution of HMGA1 labeling within the mitochondria allowed for a more detailed description of possible submitochondrial protein localization patterns (**Figs. 2.6A-D**). It appeared that labeling was primarily along and between the mitochondrial cristae, and more generally, within the matrix. Further analysis is required, however, to precisely define the distribution of HMGA1 proteins within mitochondria. Nevertheless, these studies clearly demonstrated that HMGA1 proteins are localized to the nucleus and mitochondria in NIH3T3 cells during the late-S and G2 phases of the cell cycle.

Localization of HA-HMGA1a Fusion Protein in Non-Synchronized MCF-7 Cells

Although results utilizing the anti-HMGA1 polyclonal antibody were compelling, as an independent method for assessing the subcellular distribution of HMGA1 proteins,

we went further and analyzed the localization patterns of a transgenic hemaglutinnin (HA) 'tagged' HMGA1a protein in a genetically engineered line of MCF-7 breast cancer epithelial cells over-expressing this protein. A monoclonal anti-HA tag antibody was used in a series of indirect immunofluorescence experiments to confirm results obtained for the NIH3T3 cell line. In this way, undetectable cross reactivity of the polyclonal anti-HMGA1 as a possible explanation for the observed localization patterns could be ruled out, if in fact similar patterns occurred with the alternative monoclonal antibody probe. Initial anti-HA antibody control experiments done with the parental MCF-7 cell line lacking the HA-HMGA1a transgene showed only very low background signal and confirmed the high specificity of the monoclonal antibody (data not shown).

Transgenic cells in a randomly proliferating population analyzed with the anti-HA or anti-HMGA1 antibody showed primarily nuclear localization of the fusion protein with some examples of cytoplasmic and mitochondrial staining in groups of cells (**Figs. 2.7A, B**). As an additional control, cells treated with the secondary antibody alone showed little if any background fluorescence (**Fig. 2.7C**). Overall, examples of cytoplasmic localization of the transgenic HA-HMGA1a protein were present in populations of asynchronously growing MCF-7 cells and appeared strikingly similar to patterns observed in populations of exponentially growing NIH3T3 cells. Cells always exhibited primarily nuclear localization of HA-HMGA1 with isolated examples of cytoplasmic and mitochondrial distribution in the cell population.

In order to further demonstrate these HMGA1 protein localization patterns by independent experimental methods, transgenic MCF-7 cells were fractionated and the subcellular fractions analyzed by western blot (**Fig. 2.8**). Using both anti-HA and anti-

HMGA1 antibodies in combination with nuclear, cytoplasmic, and mitochondrial control antibodies, it was found that HMGA1 was present in both the cytoplasm and mitochondria. Histone H1 was not detectable in these fractions showing that premature nuclear lysis during the fractionation process was not responsible for the release of HMGA1 into the cytoplasmic and mitochondrial fractions. An antibody to a 65 kDa outermembrane mitochondrial protein showed proper enrichment of the organelle while an anti-posphoMEK1/2 antibody served as a control against contamination of the mitochondrial fraction with soluble cytoplasmic proteins. Similarly, the purity of the mitochondrial fraction was further confirmed by it's lack of reactivity to an antibody against caspase-3 in Hs578T cells, a line of cancerous human mammary epithelial cells that express this strictly cytosolic enzyme (unpublished data). These biochemical results confirmed the phenomenon of cytoplasmic and mitochondrial localization of HMGA1 proteins observed within the NIH3T3 cells, suggesting that the observed redistribution may be a common theme amongst different mammalian cell types.

HMGA1 Binds to the Regulatory D-loop Region of Mitochondrial DNA

With evidence of mitochondrial internalization and an established role for HMGA1 in transcriptional control within the nucleus, it was hypothesized that the proteins may also bind to the AT-rich sequences in the regulatory D-loop region of mitochondrial DNA (**Fig. 2.9A)**. To test this hypothesis, a "chromatin" immunoprecipitation protocol (ChIP), modified to accommodate the histone free and circular mitochondrial genome, was performed. Transgenic MCF-7 cells over-expressing HA-HMGA1 were crosslinked with formaldehyde and fractionated to collect

mitochondria. Sonication of mitochondrial lysates was then optimized to achieve approximately 500 bp protein/DNA complex fragments prior to pull down with a polyclonal anti-HMGA1 antibody, pre-immune IgG's (non-specific), or with no antibody. Following protein digestion and DNA isolation, PCR was performed using primer sets specific for two different regions of the mitochondrial D-loop. Primer design was based on sequence analysis to identify potential AT-rich HMGA1 binding sites (**Fig. 2.9A**).

Modified ChIP analyses of the regions flanked by primer set 1 within the D-loop region detected the expected diagnostic PCR amplified band in the samples containing total cellular DNA, input DNA before antibody pull down and, most importantly, in samples specifically immunoprecipitated with anti-HMGA1 antibody (**Fig. 2.9B**). In marked contrast to these results, ChIP assays performed with primer set 2 which flanked multiple potential HMGA1 binding sites in the D-loop region (**Fig. 2.9A**) did not result in any detectable DNA amplification in the anti-HMGA1 pull down sample (**Fig. 2.9B**). As expected, negative control samples without added antibody, with non-specific antibody, or with pre-immune IgG's showed no DNA amplification (**Fig. 2.9B**; data not shown). Additional control ChIP assays utilizing total transgenic MCF-7 cell lysates and primers specific for the hypoxanthine-guanine phosphoribosyltransferase (HPRT) promoter, which lacks significant AT rich sequences and is not known to be regulated by HMGA1, were also recently performed in the laboratory. Results of these control assays showed that when crosslinked DNA protein complexes were probed with the same anti-HMGA1 antibody used in the current study, no HPRT promoter DNA was pulled down (Treff et al., 2004). This further demonstrated that the ChIP protocol employed was effective at specifically identifying only regions of DNA bound by HMGA1 proteins in both the

nucleus and mitochondria of living cells. In order to ensure that over-expression of the HA-tagged HMGA1 protein was not the cause of mitochondrial internalization and DNA binding, the protocol was also performed on parental, non-transgenic, MCF-7 cells using the same antibody to endogenous HMGA1 (**Fig. 2.9B**). Amplification with primer set 1 was positive in this anti-HMGA1 pull down assay, showing that endogenous protein expressed at normal cellular levels was capable of mitochondrial internalization and DNA binding.

Discussion

A number of studies have identified proteins that display changes in subcellular localization as a function of cell cycle stage and/or stress. Several examples are the cell cycle regulators, cyclin B1 and D1(Alt et al., 2000; Pines and Hunter, 1991), the DNA repair protein APE-Ref1 (Tell et al., 2001), several tumor suppressors including BRCA1 (Coene et al., 2005) and p53 (Fabbro and Henderson, 2003), as well as the nuclear orphan receptor, TR3 (Li et al., 2000). Some of these proteins, such as p53 and TR3, that translocate from the nucleus to the mitochondria during the induction of apoptosis, essentially abandon their primary role for a time and take on entirely distinct roles in the cell (Li et al., 2000; Marchenko et al., 2000). Consistent with these studies, we report here on the unexpected translocation of the HMGA1 proteins from the nucleus, where they act as dynamic transcription factors, to the cytoplasm and mitochondria. Such changes appear to be related to cell cycle stage in NIH3T3 cells (**Fig. 2.2)**. Similar *in situ* immunolocalization observations have also been made for HMGA1 redistribution in murine CTLL2 cells synchronized by interleukin-2 starvation (unpublished data),

suggesting that a cell cycle mechanism may exist in different mammalian cell types. At this point, further studies are being performed to look more closely at cell cycle stage dependence. To that end, initial live cell imaging results obtained in NIH3T3 cells transiently expressing an HMGA1-GFP fusion protein indicate that the subcellular translocation of HMGA1 is rapid and reversible, initiating with an outward movement of the fusion protein from the nucleus to the cytoplasm (unpublished observation).

The mechanism by which HMGA1 proteins undergo subcellular translocation is unclear, but several characteristics of these small basic proteins allow for some speculation. Nuclear import of HMGA1 proteins via nuclear pores is likely given the presence of a number of amino acid sequences (Evans et al., 1992; Johnson et al., 1988) that have the potential to act as nuclear localization signals (NLS). While computer based protein sequence analysis shows the lack of a typical nuclear export signal (NES) for HMGA1, there is evidence for the NLS mediated outward movement of proteins from the nucleus (Guiochon-Mantel et al., 1994). Therefore, the nuclear import sequences could also be important for HMGA1 nuclear export. However, it is possible that chaperone proteins may also be involved in the transport process in the absence of a typical NES. As alluded to in the introduction, HMGA1 post-translational biochemical modifications might also have the potential to regulate the process of transport in any of the above situations. We are beginning to explore these possibilities and further describe the mechanism and regulation of HMGA1 subcellular localization changes. Mitochondrial transport pathways used by HMGA1 to gain access to the interior matrix (**Figs. 2.4-2.6**) are difficult to predict at this point. Most nucleus-encoded proteins that translocate to, and function within, the mitochondria contain N-terminal presequences

that are recognized by receptor complexes in the outer (TOM complex) and inner (TIM complex) membranes (Neupert, 1997; Roise and Schatz, 1988; Schatz, 1996). HMGA1, like approximately 30% of nucleus-encoded mitochondrial proteins, does not have such a sequence. In particular, none of the known outer membrane proteins, a number of the inner membrane proteins, and two matrix proteins (2-isopropylmalate synthetase and 3-oxoacyl-CoA thiolase) lack typical N-terminal presequences (Hartl et al., 1989). Such proteins often possess varying internal targeting signals that facilitate organelle entry, while the mechanism of transport for others is unknown (Diekert et al, 1999).

Imported mitochondrial proteins do possess some general characteristics, however, and can be placed into three main categories (Hartl et al., 1989; Hartl and Neupert, 1990; Hoogenraad et al., 2002; Verner and Schatz, 1988). First, they have an internal amino acid sequence composed of a relatively hydrophobic, hydroxyl-containing region, flanked by positively charged residues. This sequence is specifically recognized by the mitochondrial import system. Second, they must be soluble in the cytosol. And third, they have to assume an unfolded conformation that is suited for translocation into or across membranes. In this regard, it is notable that HMGA1 proteins are quite soluble, even in dilute acids (Elton and Reeves, 1985), and have little, if any, folded secondary structure while free in solution (Huth et al., 1997). Furthermore, HMGA1 proteins contain an internal stretch of 11 relatively hydrophobic amino acids flanked by positively charged residues that is quite similar in composition to the mitochondrial-directing sequences of the matrix-localized 2-isopropylmalate synthetase and 3-oxoacyl-CoA thiolase proteins (Hartl et al., 1989). The involvement of this internal region of the HMGA1 proteins in the process of mitochondrial targeting remains to be determined.

Based on the cumulative evidence presented here, HMGA1 proteins internalize into the mitochondria and maintain their typical function as DNA binding proteins. As flexible dynamic proteins that regulate a host of nuclear genes, it is reasonable to assume a similar role within the mitochondria, where the genomic DNA is known to be high in AT content (Bibb et al., 1981). Transcriptional regulation of genes within the mitochondria is primarily mediated by mitochondrial transcription factor A (Tfam), a protein factor that contains a high mobility group (HMG) box DNA binding motif (Clayton, 1992; Parisi and Clayton, 1991). It is of interest to note that the region we identified as bound by HMGA1 by ChIP analyses (**Fig. 2.9)** is the same as that shown to contain binding sites for Tfam (Fisher et al., 1992). While HMGA1 could potentially act in concert with this transcription factor to increase mitochondrial gene expression, it is also possible that a competitive relationship exists. This is primarily due to the fact that the AT-hook motif of HMGA1 proteins is known to bind with a higher affinity to DNA regions also recognized by HMG box proteins and, as a consequence, will out-compete them for, or displace them from, substrate binding (Hill et al., 1999; Hill and Reeves, 1997). Such a competitive role for HMGA1 within the mitochondria may serve the purpose of regulating mitochondrial biogenesis, as this process is intimately coupled to the transcription of endogenous genes (Siedel-Rogol and Shadel 2002). This raises the possibility that HMGA1 proteins play an integral part in the control of mitochondrial metabolism and/or maintenance within dividing cells.

Currently, only a small number of proteins have been demonstrated to bind specifically to the D-loop *in vivo* (i.e., TFAM, TFB1M, and TFB2M (Kelly and Scarpulla, 2004)) and all have been shown to control transcription and/or replication of

mitochondrial genes. Because there are still gaps in the current understanding of how replication and transcription of the mitochondrial genome is controlled, the addition of HMGA1 to the D-loop binding repertoire is extremely important for a complete understanding of how mitochondrial function is regulated. Furthermore, to our knowledge, the findings reported here represent the first example of a known nuclear transcription factor that localizes to, and maintains its primary DNA-binding activities within, the mitochondria. Therefore, it is possible that the HMGA1 proteins are active in coordinating nuclear and mitochondrial processes during different cellular events including but not limited to cell cycle progress. Finally, the results of this research extend a common emerging theme in the literature. That is, an increasing number of proteins (described above) that have previously been regarded as compartmentalized and restricted to a particular function actually are much more dynamic in both their subcellular movements and their biological functions. In the case of HMGA1, such previously unrecognized subcellular localization patterns have the potential to open up new areas of research concerning mechanisms by which these proteins mediate cellular proliferation and cancerous progression, and now, regulate mitochondrial function.

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Figures

Figure 2.1. Intracellular immunolocalization of HMGA1 proteins in proliferating populations of NIH3T3 cells. Adherent mouse NIH3T3 cells were fixed and treated with polyclonal rabbit anti-HMGA1 and goat anti-rabbit IgG's conjugated with Oregon Green. **Panel A**: Cells exhibiting primarily nuclear fluorescence. **A1**: Digital zoom of a select cell showing nuclear fluorescence. **Panel B**: Cells exhibiting increased cytoplasmic and mitochondrial fluorescence. **B1**: Digital zoom of a select cell showing strong nuclear and cytoplasmic fluorescence. **B2**: Digital zoom of a select cell showing decreased nuclear and strong cytoplasmic/mitochondrial fluorescence. 600X total optical magnification.

Figure 2.1

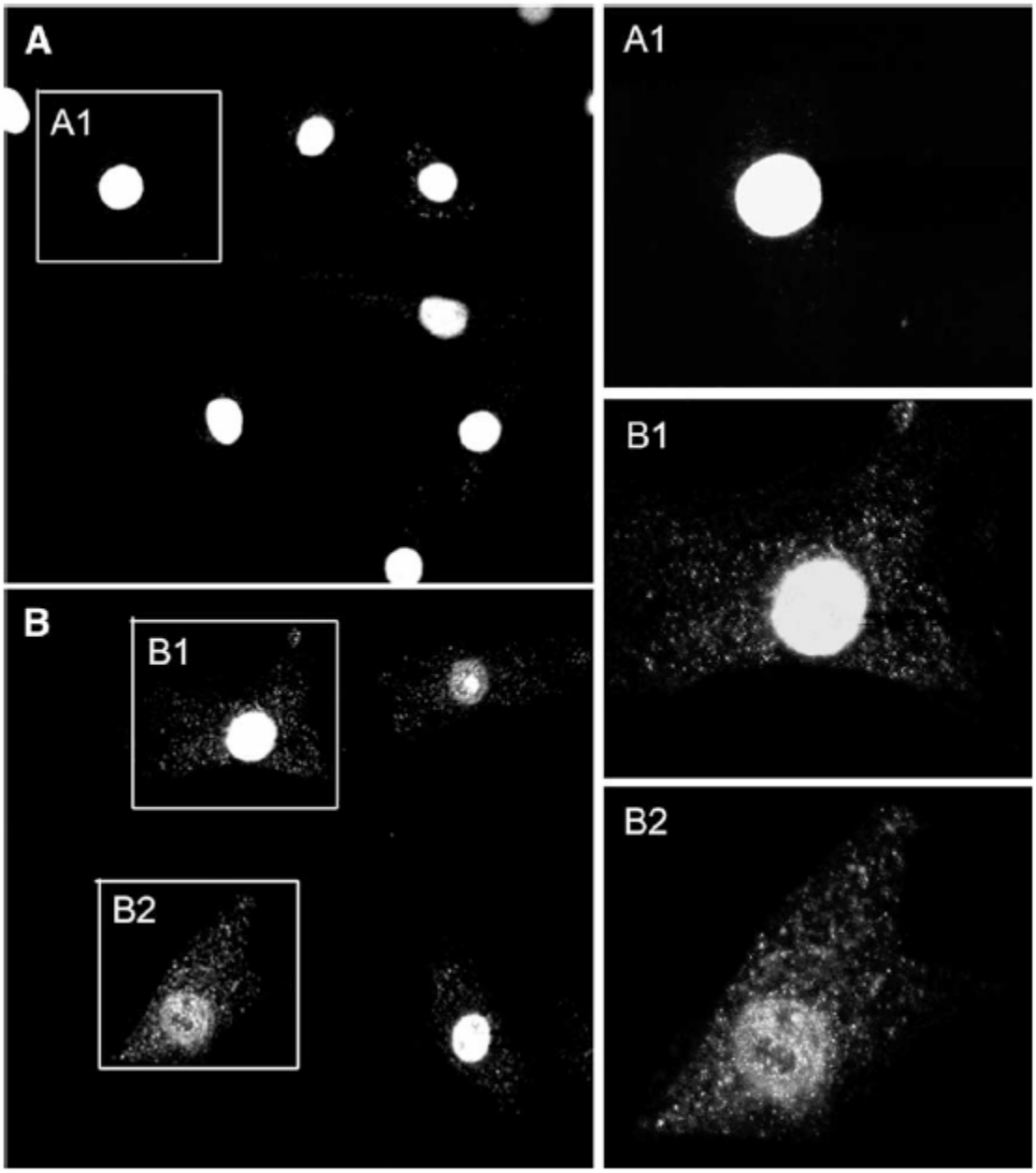


Figure 2.2. Panel A: Flow cytometric DNA content analysis showing partial synchronization of NIH3T3 cultures. Histogram plots of DNA content analysis were generated and used to determine the percent of cells with a particular content of DNA where cells containing 2N DNA represented the G1 stage and those with 4N DNA the G2/M stage. Cells in between these two extremes were considered in the S phase. The Y-axis represents percent gated cells in a particular cell cycle stage and the X-axis represents the time point following contact inhibition release. **Panels B and C:** Immunolocalization of HMGA1 proteins in partially synchronized NIH3T3 cells at different stages of the cell cycle. At selected times post-seeding, cells were fixed and treated with polyclonal rabbit anti-HMGA1 and goat anti-rabbit IgG's conjugated with Oregon Green (white). **Panel B** shows five different cells from populations at the following times post-seeding: 12 hr; 14 hr; 16 hr; 17 hr. See text for descriptions. 600X total magnification. **Panel C** represents flow cytometric DNA content analysis of parallel synchronized cell populations as verification of cell cycle stage. Histogram plots were generated from cells fixed and stained with propidium iodide at different time points following contact inhibition release: 12 hr; 17 hr; 22 hr. Histogram peaks represent numbers of cells (counts; y axis) at a particular fluorescence intensity (DNA content; x axis).

Figure 2.2

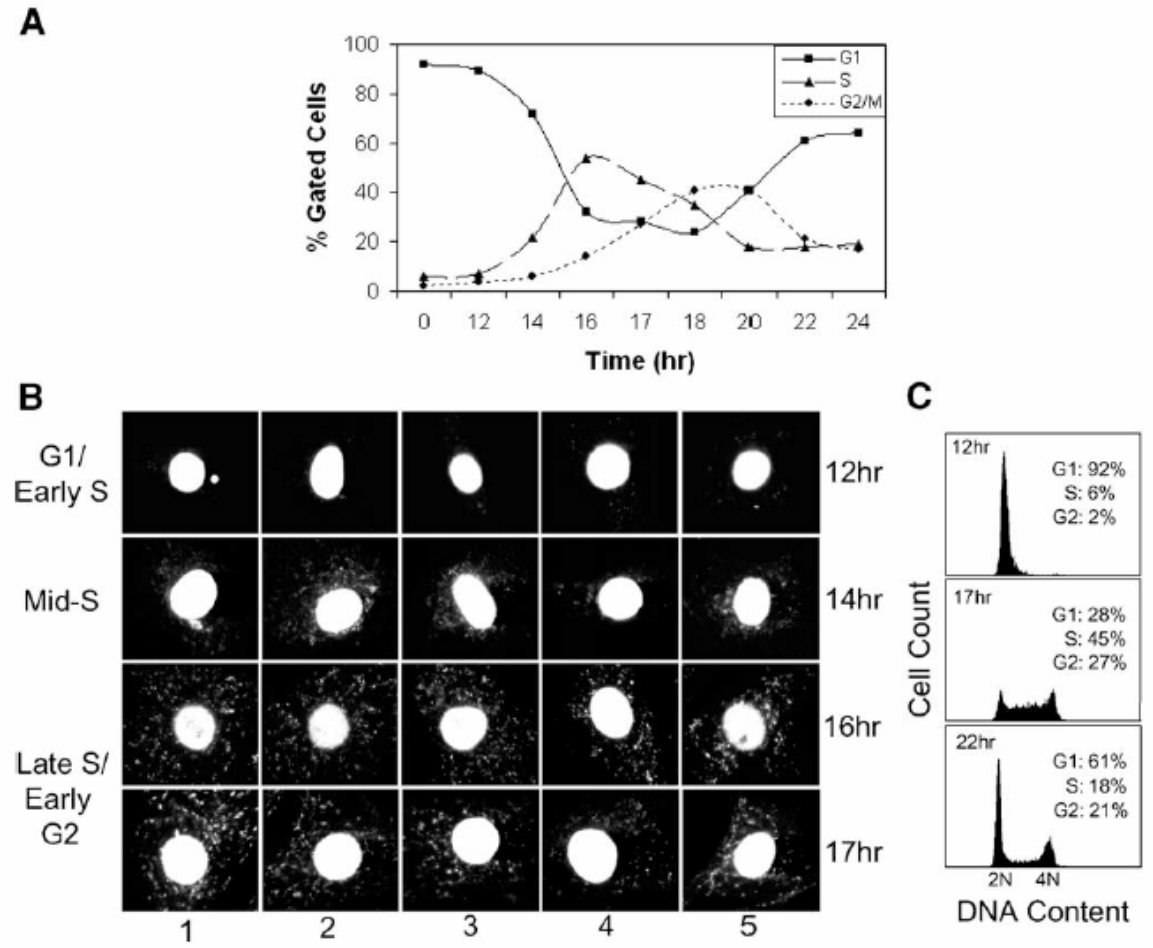

A
100
80
60
40
20
0
% Gated Cells
G1
S
G2/M
0
12
14
16
17
18
20
22
24
Time (hr)
B
G1/
Early S
Mid-S
Late S/
Early
G2
12hr
14hr
16hr
17hr
1
2
3
4
5
C
Cell Count
12hr
G1: 92%
S: 6%
G2: 2%
17hr
G1: 28%
S: 45%
G2: 27%
22hr
G1: 61%
S: 18%
G2: 21%
2N
4N
DNA Content

Figure 2.3: Co-localization of HMGA1 with Mitochondria in NIH3T3 cells 16-18 hours post-synchronization. Cells were treated with Mito Tracker Red followed by fixation and probing with anti-HMGA1 and goat anti-rabbit IgG's conjugated with Oregon Green. **Panel A**: HMGA1 signal (green). **Panel B**: Mitochondrial signal (red). **Panel C**: Merged image of the two channels where co-localization is indicated by a color change (yellow/orange). 600X total magnification.

Figure 2.3

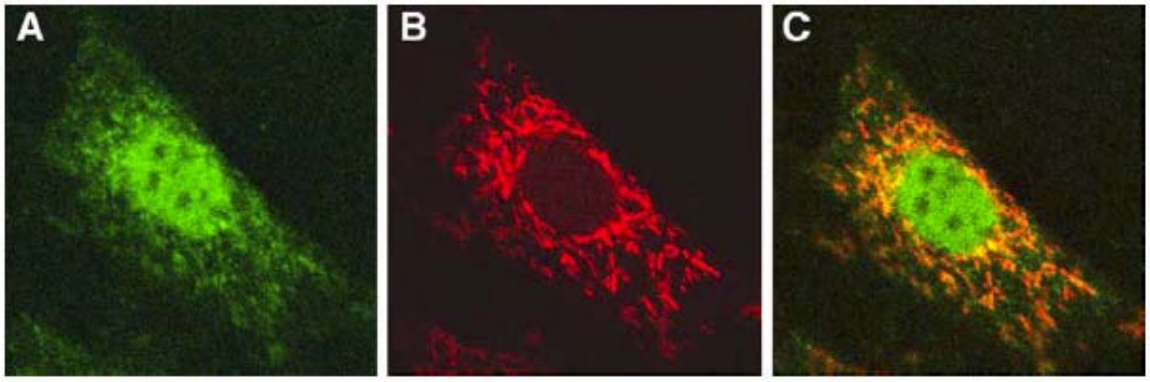


Figure 2.4: Control and specificity experiments for electron microscopic immunogold localization studies of HMGA1 proteins in partially synchronized NIH3T3 cells 16-18 hours post-synchronization. **Panel A:** Control normal, non-immune, serum showing low background and the lack of specific labeling. **Panel B:** Anti-HMGA1 antiserum showing primarily nuclear and mitochondrial labeling with some cytoplasmic signal. **Panel C:** Competition experiment in which pure reverse phase HPLC-purified HMGA1 protein (400 μg/ml) was added to anti-HMGA1 antiserum prior to reaction with NIH3T3 cells. **Panel D:** Nuclear pattern of labeling observed with anti-histone monoclonal antibody. **Key:** N, nucleus; C, cytoplasm; M, mitochondria. Bar, 1 μm.

Figure 2.4

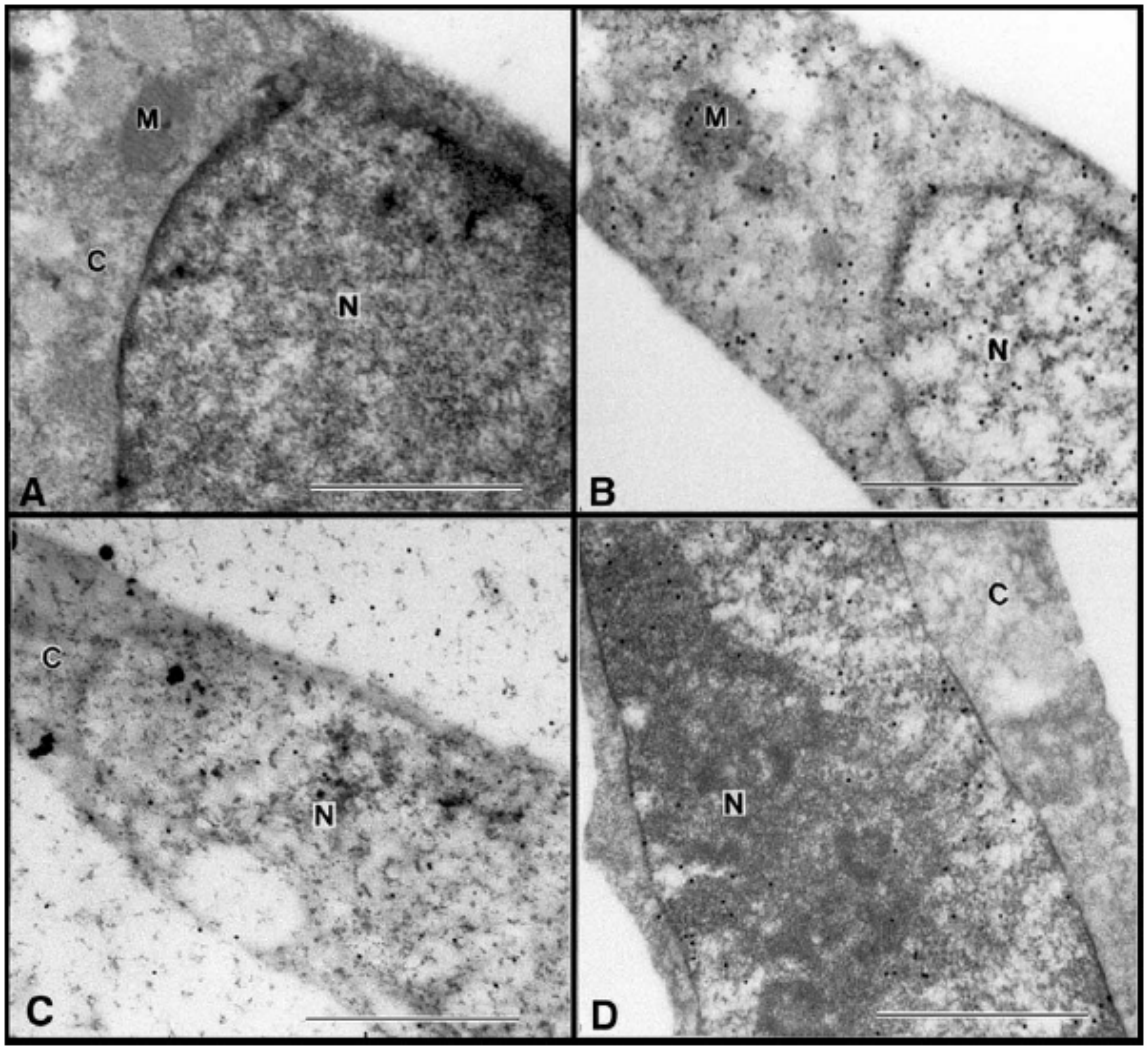


Figure 2.5: General pattern and distribution of anti-HMGA1 immunogold labeling of thin sections of NIH3T3 cells 16-18 hours post-synchronization. **Panels A-D:** Low magnification photographs of different cells showing distribution of immunogold labeling between cell compartments. Labeling of the nucleus and mitochondria is the strongest with less in the general cytoplasm. Note that the majority of the mitochondrial gold labeling appears to be internal. **Key:** as in Fig. 6. Bar, 0.5 μm.

Figure 2.5

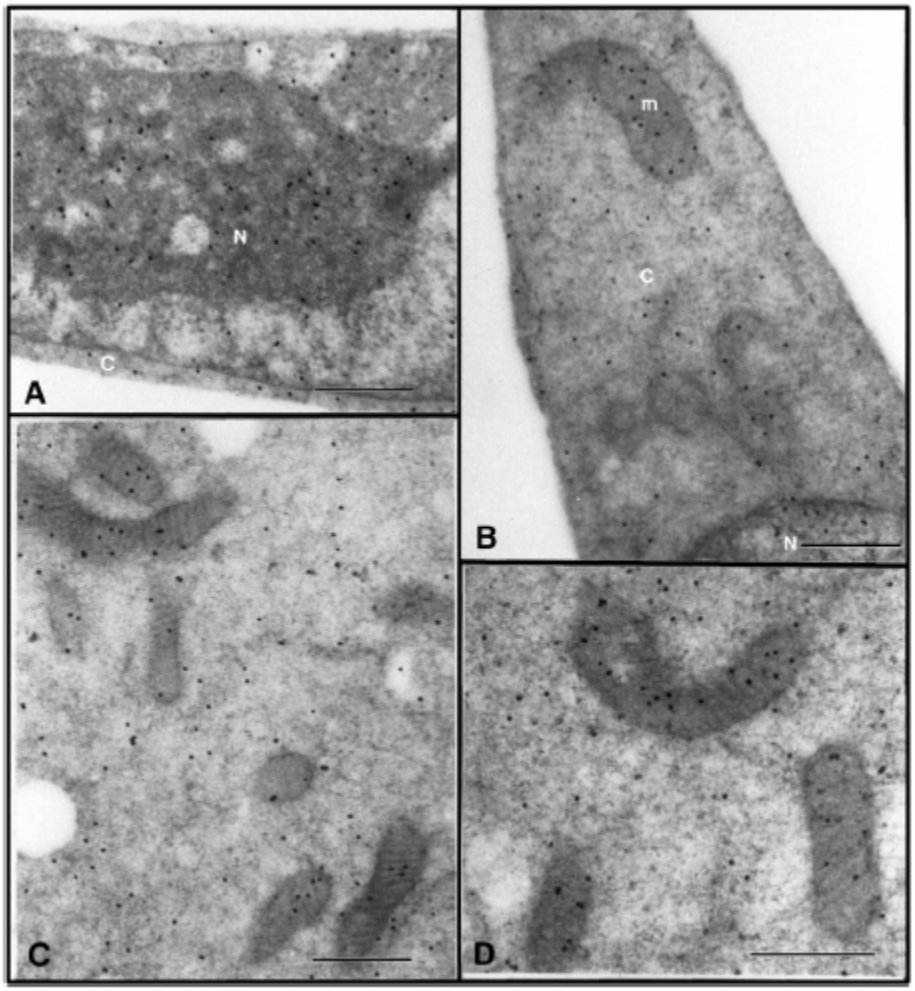


Figure 2.6: High magnification photographs of mitochondrial HMGA1 immunogold labeling in NIH3T3 cells 16-18 hours post-synchronization. See text for discussion. Bar, 0.5 μm (A and D); 0.25 μm (B and C).

Figure 2.6

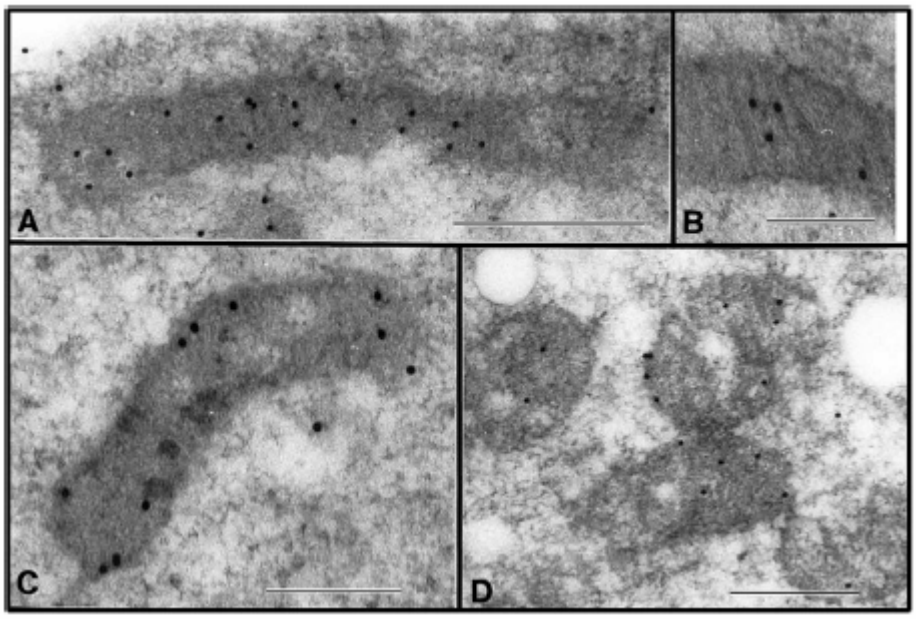


Figure 2.7: Indirect immunofluorescence analysis of transgenic HA-HMGA1a proteins in MCF-7 breast epithelial cells. Cells were labeled with mouse anti-HA tag/anti-mouse Oregon Green (**Panel A**) or rabbit anti-HMGA1/anti-rabbit Oregon Green (**Panel B**). As a negative control samples were treated with the Oregon Green conjugated secondary antibody alone (**Panel C**). 600X total magnification.

Figure 2.7

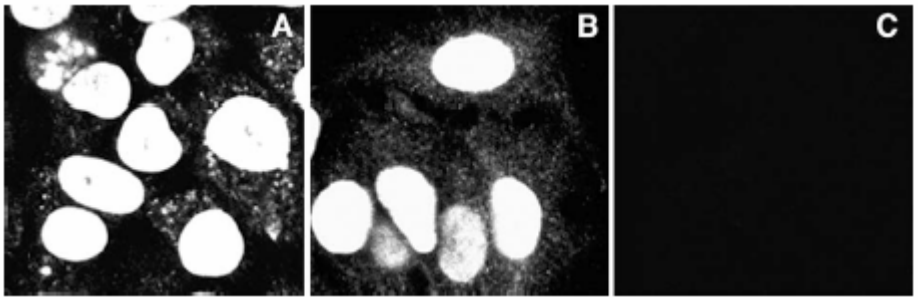


Figure 2.8: Western blot analysis of transgenic MCF-7 subcellular fractions using affinity purified polyclonal anti-HMGA1 and monoclonal anti-HA tag antibodies. Transgenic HMGA1 is shown in the cytoplasm and mitochondria of normally dividing MCF-7 cells by separate probes used on separate sample sets. In both cases, membranes were simultaneously treated with anti-Histone H1 antibody to demonstrate that nuclear contaminants were absent from the cytoplasmic and mitochondrial fractions. Mitochondrial enrichment was verified with an antibody to a 65 kDa mitochondrial outer membrane protein (anti-mito). A lack of cytosolic protein contamination in the mitochondrial fraction was shown by probing with an anti-phosphoMEK1/2 antibody. Note: The 'Nucleus/Whole Cell' fraction is primarily sedimented nuclei but also contains whole cells that were not completely lysed during the homogenization step of the fractionation protocol.

Figure 2.8

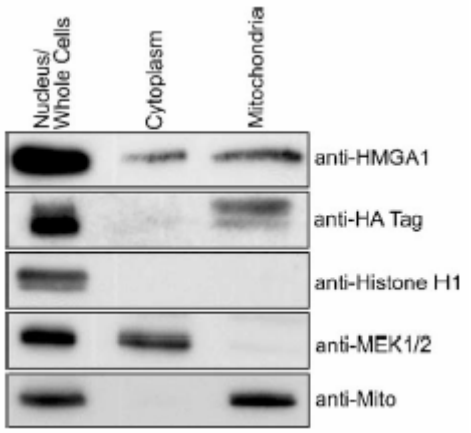

Nucleus/
Whole Cells
Cytoplasm
Mitochondria
anti-HMGA1
anti-HA Tag
anti-Histone H1
anti-MEK1/2
anti-Mito

Figure 2.9: HMGA1 binds *in vivo* to the control D-loop region of the mitochondrial genome as determined by a modified "ChIP" protocol. **Panel A**: Mitochondrial D-loop sequence showing primer sets used for analysis, AT rich regions (dashed boxes), potential HMGA1 binding sites (solid boxes), and the heavy (HSP) and light (LSP) strand promoter sequences. **Panel B**: PCR sample analysis showing amplification of the desired product size in the anti-HMGA1 pull down sample with primer set 1 but not 2. Positive results are shown for both the transgenic HA-tagged HMGA1 over-expressing MCF-7 cells and the parental MCF-7 cells expressing normal levels of HMGA1. DNA sample lane descriptions are as follows. 'Whole cell DNA': samples isolated from total Jurkat cell lysates as an independent positive control. 'Input': samples isolated from mitochondrial lysates prior to antibody pull down as an internal positive control. 'No antibody', 'non-specific', and 'anti-HMGA1': samples isolated after pull down with no antibody, preimmune IgG's, or polyclonal anti-HMGA1, respectively.

Figure 2.9

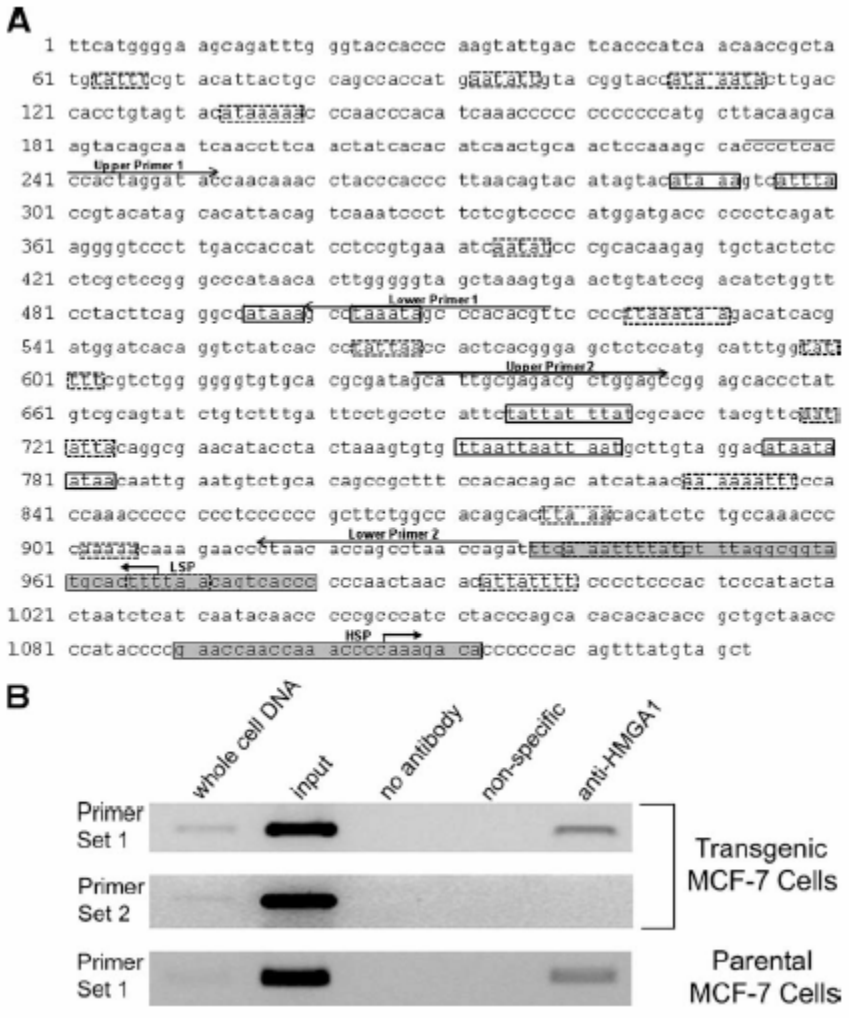

A
Upper Primer 1
Lower Primer 1
Upper Primer 2
Lower Primer 2
LSP
HSP
B
whole cell DNA
input
no antibody
non-specific
anti-HMGA1
Primer Set 1
Primer Set 2
Primer Set 1
Transgenic MCF-7 Cells
Parental MCF-7 Cells

Figure 2.1S. Bar graph showing a 3-fold increase in the number of mitochondrial immunogold particles per unit area versus total cell particles per unit area. Total cell and mitochondrial regions counted were selected in Adobe Photoshop and the number of pixels within the selected area recorded. The X-axis represents pixels*1000 where 50 was used as an arbitrary unit of area. The Y-axis represents the number of immunogold particles.

Figure 2.1S.

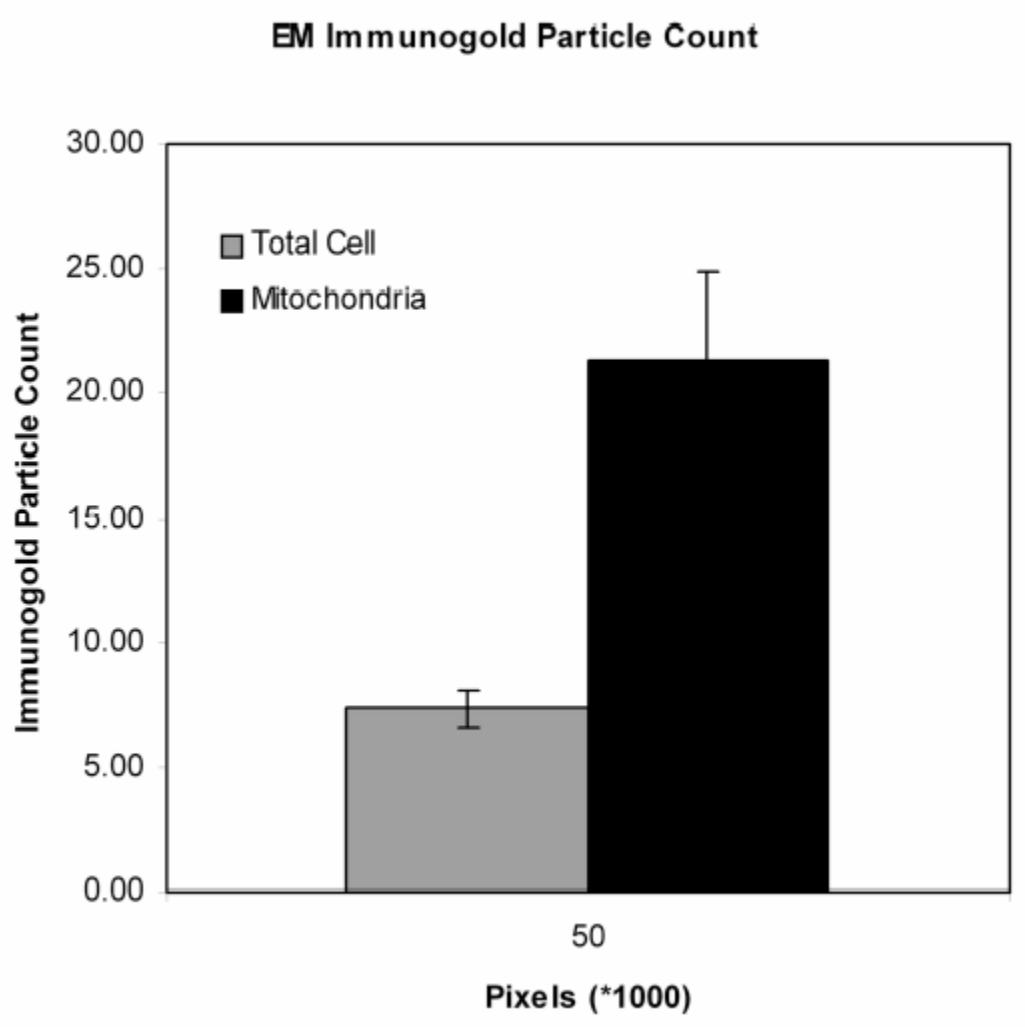
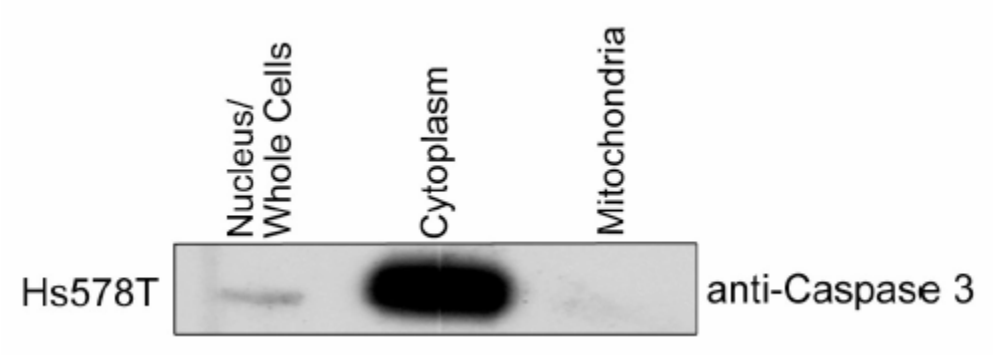

EM Immunogold Particle Count
30.00
25.00
20.00
15.00
10.00
5.00
0.00
Immunogold Particle Count
Total Cell
Mitochondria
50
Pixels (*1000)

Figure 2.2S. Western blot analysis of Hs578T subcellular fractions using a monoclonal anti-caspase 3 antibody. Caspase 3 was found at low levels in the nucleus/whole cells and was abundant within the cytoplasm. Caspase 3 was not detected in the mitochondria indicating a lack of cytosolic protein contamination in the mitochondrial fraction. Note: The 'Nucleus/Whole Cell' fraction is primarily sedimented nuclei but also contains whole cells that were not completely lysed during the homogenization step of the fractionation protocol.

Figure 2.2S.


Nucleus/
Whole Cells
Cytoplasm
Mitochondria
Hs578T
anti-Caspase 3

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CHAPTER 3

HMGA1 Proteins Act as Transcriptional Regulators within the Mitochondria and Alter Overall Organelle Related Cellular Function

Abstract

Recent novel localization findings from our lab placed HMGA1 proteins into the complex milieu of the mitochondria and raised the intriguing possibility that these classically nuclear transcriptional regulators may play similar roles within the mitochondria. Normally functioning to either up- or down-regulate genes within the nucleus containing promoter and enhancer regions that are high in AT content, the HMGA1 proteins were not surprisingly found to be capable of binding to the highly AT rich mitochondrial genome at a particular control region referred to as the D-loop. In order to elucidate the specific functional role that HMGA1 may be playing in the mitochondria at this D-loop region, we employed several experimental methods to analyze mitochondrial gene transcription and replication, as well as overall organelle related cellular function. Utilizing real-time PCR, it was determined that in transgenic MCF-7 cells (HA7C) over-expressing an HA-tagged HMGA1 fusion protein, mitochondrial DNA (mtDNA) levels were reduced by approximately 2-fold relative to wild-type MCF-7 cells while the mitochondrial NADH Dehydrogenase 2 gene transcript was up-regulated approximately 4-fold. Corresponding to reduced mtDNA levels, flow cytometric analysis revealed that overall mitochondrial mass was reduced in these cells as well. Revealing a possible effect of the organelle changes, cell survival studies showed an increased sensitivity of the HA7C cells to treatment with an inhibitor of glycolysis, 2-deoxy-D-glucose, indicating an overall deficiency in cellular oxidative phosphorylation. Finally, preliminary studies utilizing a unique quantitative PCR method revealed that HMGA1 in the mitochondria may negatively effect the ability of the organelle to repair reactive oxygen species (ROS) induced DNA damage. Together, these observations are

consistent with previously described cancer phenotypes and reveal several possible mechanisms by which mitochondrial HMGA1 may affect tumor progression or other normal or disease related mitochondrial processes.

Key Words: High Mobility Group; MCF-7; Mitochondria; D-loop; transcription factor; NADH Dehydrogenase subunit 2; glycolysis; oxidative phosphorylation; reactive oxygen species (ROS); mtDNA damage.

Introduction

Mammalian high mobility group A1 (HMGA1) proteins have been established as dynamic regulators of transcription within the nucleus where they alter global and local chromatin structure to induce or inhibit gene expression in response to multiple external and internal cell stimuli (reviewed in Reeves, 2001). Such stimuli usually affect HMGA1 activity in part by causing their post-translational modification which results in changes to the proteins ability to interact with different DNA and protein substrates (Banks et al., 2000; Edberg et al., 2004; Reeves and Beckerbauer, 2001). Because of their small size (10.6-12 kDa) (Lund et al., 1983), inherent flexibility (Lehn et al., 1988), and high levels of post-translational modifications, the proteins are uniquely equipped to adapt and function during a variety of biological processes and with multiple distinct substrate types (Huth et al., 1997) in a highly regulated manner. Additionally, HMGA1 proteins are unified by their possession of a versatile DNA binding motif called the AT-hook (Reeves and Nissen, 1990). Each isoform resulting from alternative splicing of the *HMGA1* gene (Johnson et al., 1989; Reeves and Nissen, 1995) contains three separate AT-hooks that allow the proteins to bind in a structurally specific manner within the minor groove of AT-rich B-form DNA (Reeves and Nissen, 1990). This characteristic non-sequence specific DNA binding is a major factor contributing to the proteins ability to bind to multiple different gene substrates. Finally, the ability of HMGA1 to mediate protein-protein interactions adds a level of regulated diversity to their activities displayed by their numerous protein partners and multiple specific gene substrates (reviewed in Reeves and Beckerbauer, 2001). Recently the number and type of molecular and cellular functions for the HMGA1 proteins has expanded as a result of these unique characteristics.

Two recent studies out of our lab have revealed previously unrecognized functions for the HMGA1 proteins. First, localization data showed that HMGA1 undergoes nucleocytoplasmic translocation and accumulates in the mitochondria where it is able to bind to AT-rich DNA within the control D-loop region of the circular mitochondrial genome (Dement et al., 2005, Chapter 2). Evidence suggests that the protein moves in a cell-cycle dependent manner and that the movement is directional, from the nucleus to the cytoplasm and back again. Correlative studies interestingly have shown the specific post-translational phosphorylation of the HMGA1 proteins by cdc2 kinase during the late G2 stage of the cell cycle, the precise stage at which the nucleocytoplasmic translocation was shown to occur (Reeves et al., 1991). This phosphorylation was further shown to reduce the capacity of the proteins to bind to DNA 20-fold, indicating mobilization of the protein and a possible mechanism of translocation initiation. Regardless of translocation mechanism, the mitochondrial localization of the HMGA1 proteins opened new areas of research regarding possible organelle specific functions for the proteins in respect to normal and abnormal cellular functions, including cancer.

Specifically, as mentioned previously, the mitochondrial genome is quite AT-rich (Bibb et al., 1981) and because HMGA1 was found associated with the control D-loop region, the proteins likely play a role in expression and potentially replication of the mtDNA as these processes are intimately coupled (Siedel-Rogol and Shadel 2002). Furthermore, because HMGA1 proteins are major contributors to the process of carcinogenesis, the many abnormalities being discovered in mitochondrial genome stability and overall organelle, and organelle related, function associated with cancer

phenotypes are intriguing. Particularly, it has been shown that mtDNA levels in multiple different cancer types are significantly altered as are the mitochondrial gene expression profiles. For example, rat fibroblast cells immortalized by viral and cellular oncogenes displayed activation of the mitochondrial genes for COXI, COXII, and 16S rRNA (Glaichennhaus et al., 1986). Activation of genes for ATP6, ND5, cytochrome b, COXI, and COXII was also observed in papillary thyroid carcinomas as compared to normal thyroid tissue (Haugen et al., 2003). In fact, a general theme in the literature is the up-regulation of mitochondrial gene expression in the context of a cancer phenotype, while both increases and decreases in the levels of tumor cell mtDNA have been reported (Torroni et al., 1990). However some studies do show reduction in gene activity in certain cancer types such as glioblastomas (Dmitrenko et al., 2005). Many of these changes in mtDNA expression and replication are thought to reflect mitochondrial genomic adaptation to perturbations in cellular energy requirements (Penta et al., 2001). However, changes to mitochondrial genomic expression and replication may precede overall cellular metabolic abnormalities associated with cancer.

In fact, the role of mitochondria in carcinogenesis was first considered following observation, in 1956, that rodent tumors exhibited reduced respiration-coupled oxidative metabolism and increased glycolysis (Warburg et al., 1956). To date, these observations have not been explained but the above studies suggest that changes to mitochondrial gene expression profiles observed in cancer cells may be involved in this Warburg phenotype. That is, deficiencies in the ability of a cell to perform oxidative phosphorylation, resulting in a switch to a glycolytic phenotype, may be a result of abnormalities at the mitochondrial genome level. Furthermore, such changes may result from changes in

expression levels of oncoproteins like HMGA1, that are over-expressed in cancers and likely act as transcriptional regulators within the mitochondria. Another characteristic of mitochondria within normal and cancer cells is the high level of mtDNA mutations thought to be related to high level of reactive oxygen species (ROS) generated during oxidative metabolism

In normal cells, it has been reported that the mutation rate of mtDNA is as much as two orders of magnitude greater than that of nuclear DNA (Khrapko et al., 1997; Marcelino and Thilly, 1999; Wang et al., 1997). Due to the lack of introns within mtDNA, mutations occurring at this high rate likely occur in coding sequences. The result is possible alterations to the expression levels of proteins involved in the electron transport chain and increased ROS production leading to additional mutations and further increases in ROS production. Such a progression of events has the potential to promote carcinogenesis through the generation of persistent oxidative stress (Toyukuni et al., 1995). This is consistent with studies showing specific mtDNA mutations within multiple cancer types including breast (Bianchi et al., 1995), colorectal (Polyak, et al., 1998), colon (Alonso, et al., 1997), kidney (Horton et al., 1996), and gastric (Tamura et al., 1999) tissues. In fact, nearly 100 sites not related to other mitochondrial diseases have been shown to be mutated in tumors (Fliss et al., 2000). While the increased occurrence of mutations in the mitochondria of cancer cells may be due to persistent oxidative stress alone, it is possible that a deficiency in the ability of the organelle to repair the damaged DNA is a contributing factor.

A second recent study from our lab, mentioned above, revealed that the nucleotide excision DNA repair pathway (NER) within the nucleus is inhibited in breast cancer

epithelial MCF-7 cells over-expressing the HMGA1 proteins (Adair et al., 2005). Regarding a potential function for the HMGA1 proteins within the mitochondria, this study suggests that a similar inhibition may exist in the presence of the ROS rich organellar environment. Though UV- induced cyclobutane pyrimidine dimers are not know to be repaired in the mitochondria via the NER pathway (Clayton et al., 1974), recent studies support that DNA repair occurs in the mitochondria. These studies have shown that base excision repair (BER) occurs in the mitochondria as simple monofunctional alkylating agents are repaired efficiently from mtDNA (Pirsel and Bohr, 1993) and several DNA repair enzymes have been identified in the organelle. These include AP endonucleases (reviewed in Croteau and Bohr, 1997), uracil DNA glycosylase (Anderson and Friedberg, 1980), hOGG1 (Takao et al., 1998) and others.

In the current study, we explore the various possible functions that HMGA1 proteins may participate in within the mitochondria. It was hypothesized that the proteins affect transcription/replication of the mtDNA and subsequently contribute to alterations in the overall cellular mitochondrial content and metabolic function. Additionally, high levels of mutations within mtDNA in cancer cells suggested that the over-expression of HMGA1 within this context may lead to a deficiency in the ability of the mitochondria to repair its DNA. Utilizing real-time PCR, it was determined that in transgenic MCF-7 cells (HA7C) over-expressing an HA-tagged HMGA1 fusion protein, mitochondrial DNA (mtDNA) levels were reduced by approximately 2-fold relative to wild-type MCF-7 cells while the mitochondrial NADH Dehydrogenase 2 gene transcript was up-regulated approximately 4-fold . Corresponding to reduced mtDNA levels, flow cytometric analysis revealed that overall mitochondrial mass was reduced in these cells as

well. Revealing a possible effect of the organelle changes, cell survival studies showed an increased sensitivity of the HA7C cells to treatment with the inhibitor of glycolysis, 2-deoxy-D-glucose, indicating an overall deficiency in cellular oxidative phosphorylation. Finally, preliminary studies utilizing a unique quantitative PCR method revealed that HMGA1 in the mitochondria may negatively effect the ability of the organelle to repair reactive oxygen species induced DNA damage. Results and potential future work are presented and discussed in the remainder of this chapter.

Materials and Methods

Cell Culture and Synchronization

The tetracycline-regulated M/tet (referred to as parental or MCF-7) and M/tet/HA-I (referred to as transgenic or HA7C) human breast epithelial MCF-7 cells were developed as described previously (Reeves et al., 2001). Briefly, the parental M/tet (MCF-7/tet-off) cell line was purchased from Clontech, Palo Alto, CA. This cell line was stably transfected with a plasmid vector (Clontech) containing the tetracycline response element (pTRE) driving the expression of a hemaglutinnin (HA)-tagged HMGA1a transgene to generate the M/tet/HA-I (HA7C) line. MCF-7 cells were cultured in DMEM supplemented with 10% fetal bovine serum, penicillin (100 ug/ml), streptomycin (100 ug/ml), and G418 (100 ug/ml) to maintain selection of the tetracycline transactivator protein gene. HA7C cells were maintained and selected in the same media as for the MCF-7, and supplemented with hygromycin (100 ug/ml) for selection of clones containing the HA-HMGA1a expression vector.

Quantitative real-time RT-PCR analysis

Real-time PCR was performed using Custom TaqMan® Gene Expression Assays for mitochondrial and beta-globin DNA sequences and Assays-on-Demand for human target genes, NADH Dehydrodegenase subunit 2 (ND2) (assay ID: Hs00846374_s1 MTND2) and HPRT1 (assay ID: Hs99999909_ml HPRT1) (Applied Biosystems, Foster City, CA, USA). TRIzol and phenol choloroform extracted total cellular DNA was isolated from MCF-7 and HA7C cells and 100 ng was used as template for real-time PCR for analysis of mitochondrial DNA levels using the beta globin gene as an endogenous control. Assays were performed in triplicate and repeated utilizing DNA isolation with Genomic Tip/20 Columns (QIAGEN). For the analysis of the mitochondrial ND2 gene using HPRT as the endogenous control, TRIzol extracted total RNA was purified using an RNeasy Kit (QIAGEN) and reverse transcribed using 2 μg in a 1st Strand cDNA Synthesis Kit for RT-PCR (AMV) (Roche-cat.no. 1 483 188) following the recommendations of the supplier. Following the reverse transcriptase reaction, 3 μl of the resulting cDNA was used as template for the RT-PCR using TaqMan Universal PCR Master Mix, No AmpErase (Applied Biosystems) as with the mitochondrial DNA assays. Reactions were performed in triplicate. All RT-PCR applications were performed on an ABI PRISM® 7000 Sequence Detection System. Amplification plots were generated from delta Rn values exported from the system software. Fold change was determined by the comparative Ct method as described in User Bulletin #2: Relative Quantitation of Gene Expression (P/N 4303859) (Applied Biosystems). Briefly, threshold values were determined automatically using ABI PRISM 7000 software and resulting Ct values (Ct = fluorescence intensity at threshold where exponential amplification begins) were

generated for each of three sample replicates. As outlined in Tables 3.1 and 3.2 fold change is determined by the following calculation. The ΔCt value is determined by subtracting the average Ct value for the endogenous control sample (e.g. beta globin) from the average Ct for the experimental sample (eg. mtDNA). The standard deviation of the difference is calculated from the standard deviations of the two samples as shown below:

$$s = \sqrt{s_1^2 + s_2^2}$$

where s = standard deviation. The ΔΔCt value is determined by subtraction of the ΔCt of an untreated sample from a treated sample. In this case, the ΔCt for HA7C cells (HMGA1 over-expression is considered treated) minus the ΔCt for wild-type MCF-7 cells gives the ΔΔCt. Because this subtraction is of an arbitrary constant, the standard deviation of ΔΔCT is the same as the standard deviation of the ΔCT value. If the ΔΔCt is negative, there is a fold change increase that is calculated as follows:

$$2^{-\Delta\Delta Ct}$$

If the ΔΔCt is positive, there is fold change decrease that is calculated as follows:

$$-2^{\Delta\Delta Ct}$$

FACS Analysis for Mitochondrial Mass

MCF-7 and HA7C cells were grown in DMEM without phenol red and supplemented as described above. Removal of phenol red was done in order to prevent detection interference during flow cytometry. Cells were stained with 100 nM nonyl acridine orange (NAO) (Invitrogen: Molecular Probes), a metachromatic dye that accumulates in mitochondria regardless of the mitochondrial membrane potential.

Specifically, the dye binds to cardiolipin in all mitochondria, regardless of their energetic state. Cells were grown to 70-80% confluence and incubated at 37°C in 5% CO_2 for 30 min. in the presence of 100 nM NAO. Cells were then washed with PBS, trypsinized, spun at 1000 rpm on a clinical centrifuge for 10 min and resuspended in 1 ml of either pre-warmed PBS or media. Fluorescence activated cell sorting (FACS) analysis was performed on a FACScalibur (Becton Dickinson: Immunocytometry Systems) utilizing an emission wavelength of 488 nm and a 525 nm excitation detector. Data acquisition and analysis was performed using Cell Quest Pro Software.

2-Deoxy-D-Glucose Sensitivity Assay

MCF-7 and HA7C cells were seeded (200 µl) into a 96-well tissue culture plate from a resuspension at a concentration of $1X10^4$ cells/ml. Following 24 hrs to allow adherence, media was replaced with complete growth media containing 0 (untreated), 1, 1.5, 2, 2.5, 3, 3.5, 4, and 4.5 mM 2-deoxy-D-glucose (Sigma), a specific inhibitor of glycolysis. Treatments were performed in triplicate within individual plates. The cells were then incubated at 37°C in 5% CO_2 for 96 hrs without changes to the media. Following drug treatment, DNA content was determined by a Hoechst 33258 (Sigma) staining method. Briefly, cells were washed in serum free media once and fixed by adding 100 µl of 100% ethanol per well and letting sit at room temperature for 10 min. Following a second wash with serum free media, 100 µl of TNE buffer (10 mM Tris, 1 mM EDTA, 2M NaCl, pH 7.4) was added to the cells. Hoechst 33258 stain was then diluted to 20 ug/ml in TNE buffer and 100 ul was added to each well. Cells were incubated at 37°C for 30 min before analysis. Relative DNA content was determined by

reading fluorescence intensities with a 360 nm excitation filter and a 460 nm emission filter on a 96-well plate reader (Wallac Victor-2). Arbitrary fluorescence units were used to plot relative DNA content which was considered as relative cell number using Microsoft Excel.

Mitochondrial DNA Repair Analysis using Q-PCR

The following protocol was adapted from Santos et al., 2002. Cells were seeded ($1X10^6$ cells) in a 100 mm tissue culture plate and allowed to adhere for 16 hr. Following a wash with PBS, cells were treated with 200 uM H_2O_2 in serum free media for 30 min at 37°C in 5% CO_2. At 30 min, treatment media was replaced with complete growth media (DMEM) and cells were harvested at 5, 15, 30, 45, 60, 90, and 180 min by trypsinization, centrifugation, and immediate freezing of the cell pellet at -70°C. Total cellular DNA (nuclear and mitochondrial) was extracted using Qiagen Blood and Cell Culture DNA Mini Kit (cat. no. 13323) containing Genomic Tip/20 columns following the exact recommendations of the supplier. Isolated DNA was pre-quantified using PicoGreen® dsDNA Quantitation Kit (Invitrogen: Molecular Probes cat. no. P-7589) and a 96-well plate reader (CytoFluor 4000-Applied Biosystems: Perseptive Biosystems) with a 485 nm excitation filter and a 535 nm emission filter. Using these values, DNA was diluted to10 ng/ul in TE and re-quantified using PicoGreen in the same manner. A final dilution to 3 ng/ul was performed and used as the working stock for template in subsequent PCR reactions. PCR was performed with the GeneAmp XL Kit (Applied Biosystems) which includes *rTth* DNA polymerase XL and the remaining reagents required for successful amplification of long targets up to 20 kb. PCR conditions were as follows, 1X

Polymerase Buffer, 1.3 mM Mg(OAc), 200 uM dNTPs, 100 ng/ul BSA, 100 pmol of each primer, 15 ng of template DNA, and 1U of rTth XL polymerase. Polymerase was added following temperature ramping to 75°C to accomplish a hot start. Cycle parameter were 94°C for 1 min, 19-32 cycles of (94°C – 15 sec, 64°C – 12 min), and a final 10 min at 72°C. Primer sets used for amplification of 8.9 kb of the mitochondrial genome were Mito 5999 S (5'- TCT AAG CCT CCT TAT TCG AGC CGA-3') and Mito 14841 AS (5'- TTT CAT CAT GCG GAG ATG TTG GAT GG-3') where S and AS represent sense and antisense, respectively. Following PCR amplification, products were quantified again with the PicoGreen reagent and a 96-well plate reader. Each reaction was analyzed in triplicate and concentration of DNA determined by a lambda DNA standard curve (2.5, 1.25, 0.625, 0.313, and 0.156 ng/ul).

Results

Mitochondrial DNA Levels are Reduced in HA7C Cells

In order to determine the effect of HMGA1 over-expression on the levels of mitochondrial DNA within MCF-7 breast cancer epithelial cells, a transgenic cell line (HA7C), expressing an HA-tagged HMGA1 fusion protein was used in comparison to wild-type MCF-7 cells. TRIzol isolated DNA and more highly purified isolates using a Qiagen Genomic Tip/20 Kit were collected from both the over-expressing and non-over-expressing cell lines and used as template to perform quantitative real-time (RT) PCR. Initial non-RT-PCR results on genomic DNA isolated by TRIzol revealed differential amplification on equal amounts of template, indicating polymerase inhibition by a sample contaminant. Nevertheless, following phenol extraction, these samples amplified equally

during screening and were thus used in subsequent RT-PCR along side Qiagen isolated DNA. Triplicate data from individually isolated sample sets revealed an overall down-regulation (-1.7±0.5 fold) in HA7C mtDNA relative to MCF-7 cells, using beta globin as an endogenous DNA control (**Table 3.1, Fig. 3.1**)

Mitchondrial Gene, NADH Dehydrogenase subunit 2, is Activated in HA7C Cells

RNA harvested from the same TRIzol samples used for the isolation of DNA isolated for mtDNA quantification was reverse transcribed to yield total cellular cDNA. This cDNA was subsequently used as template in an RT-PCR application as above, using primers and probes specific to the NADH Dehydrogenase subunit 2 (ND2) and the nuclear gene HPRT1 promoter as an endogenous control. Again, in triplicate, results indicated an overall up-regulation (1.7±0.3 fold) of ND2 gene transcript levels in HA7C cells relative to the wild-type MCF-7 cells (**Table 3.2, Fig. 3.2**). Because the mitochondrial DNA levels in these same cells was shown to be reduced by approximately two-fold, this was factored in into the final calculations of the total fold change for the ND2 gene. Normalizing for decreased mtDNA levels as described above, the total up-regulation of the ND2 gene is approximately 3-fold.

Mitochondrial Mass is Reduced in HA7C Cells

Because mitochondrial DNA levels were shown to be reduced in HMGA1 over-expressing cells, it was assumed that other mitochondrial functions or characteristics would be altered. Though the link between mitochondrial genome levels and organelle mass within cells is poorly understood, it was hypothesized that reduction in mtDNA

levels would translate into a reduced level of total mitochondria in the cell. Using fluorescence activated cell sorting (FACS) analysis, transgenic HA7C and wild-type MCF-7 cells were comparatively studied by staining with the metachromatic dye, nonyl acridine orange (NAO), which stains mitochondria independently of mitochondrial membrane potential. Data analysis showed that a majority of the HMGA1 over-expressing cells had lower mitochondrial mass than the wild-type MCF-7 cells (**Fig. 3.3**)

Increased Sensitivity of HA7C to Treatment with 2-deoxy-D-glucose

Upon establishing that both mitochondrial DNA expression and replication is altered and that overall mitochondrial mass in HMGA1 over-expressing cells is reduced, studies to determine the functional ramifications of such changes were performed. Due to observations that tumor cells have a substantially greater capacity for aerobic glycolysis consistent with a reduction in oxidative phosphorylation (Nakashima et al., 1984), it was hypothesized that HMGA1 may contribute to deficient mitochondrial oxidative metabolism. Thus, treatment with a specific inhibitor of glycolysis, 2-deoxy-D-glucose (2-DG), was hypothesized to preferentially kill HA7C cells over-expressing HMGA1 in comparison to wild-type non-over-expressing MCF-7 cells. Following treatment of both cell types with varying concentrations of 2-DG and an incubation of 96 hrs, cells were fixed and stained with Hoechst 33258 and the DNA content values were used as an indicator of cell number or survival. Results (**Fig. 3.4**) showed that the phenotypically normal cell line, MCF-7 was largely unaffected by the drug treatment up to between 4-5 mM concentrations. In contrast, the highly metastatic transgenic HA7C cells began reducing in number at 1.5 mM 2-DG. At 3 mM 2-DG, HA7C were

maximally sensitive in comparison to MCF-7 cells. Utilizing arbitrary fluorescence units, HA7C cells were calculated to be approximately 200-fold more sensitive to treatment with 3 mM 2-DG than the MCF-7 cells.

Reduced Capacity of HA7C Cells to Repair ROS Generated DNA Damage

As outlined in the introduction, the role of HMGA1 within the mitochondria may relate to their newly discovered role in the inhibition of DNA repair in the nucleus. This is possible primarily because of the fact that increased rates of mutations are found in the mitochondria of cancer cells expressing high levels of HMGA1 proteins. In order to study the rate of DNA repair in mitochondria a quantitative PCR method was used as described by Santos et al. (2002). Following treatment of both wild-type MCF-7 and transgenic HA7C cells with hydrogen peroxide, total genomic DNA was isolated and quantified as described in Methods and Materials. Mitochondrial primers designed to amplify an 8.9 kb amplicon were employed in optimization PCR reactions to ensure that cycle number was resulting in amplification within the linear range. Upon cycle optimization, damaged DNA substrates harvested at different timepoints following removal of hydrogen peroxide were used in the optimized reaction to determine differential rates of repair within the mitochondria. It is noteworthy that during preliminary experiments DNA isolates lost their integrity over time (~2 weeks), thus it was important to perform all experiments on freshly isolated DNA samples. Fresh DNA isolates required cycle number optimization prior to analysis of DNA damaged sample.

Upon amplification of the various samples, it was determined in this preliminary assay (**Fig. 3.5**), that there was an apparent inhibition in the ability of HA7C cells to

repair ROS damaged DNA when compared to MCF-7 cells. Results indicate, as was seen previously (Santos et al., 2002) , that hydrogen peroxide is cleared from the cell over the course of 60 min. followed by repair of the DNA between 90 and 180 min in the MCF-7 but not in the HA7C cells. PCR products were quantified in triplicate and show statistically significant differences between the two cell types.

Discussion

Localization of the HMGA1 proteins within mitochondria is a phenomenon that holds multiple new and exciting potential areas of research in the field. Based on the data presented here, it is clear that HMGA1 is playing a role in the mitochondria that is capable of producing phenotypic changes to the characteristics of the entire cell. Regarding non-disease related mitochondrial function, there is clearly a normal function for the HMGA1 proteins within the organelle, as the proteins can be found associated with the regulatory D-loop region in both normal and highly-metastatic cells (Dement et al., 2005, Chapter 2). Furthermore, the movement appears to be cell cycle dependent, indicating a potential role for HMGA1 in regulating mitochondrial biogenesis during the process of mitotic cell division. Though the focus of the research presented in this manuscript has been on the exploration of a potential joint role for HMGA1 and mitochondria during carcinogenesis, the data may shed light on normal processes mediated by HMGA1 as well as processes related to other mitochondrial diseases.

Because mtDNA levels were shown to be down-regulated by approximately 2-fold in cells over-expressing HMGA1 (**Table 3.1, Fig. 3.1**), and because the proteins were previously shown to associate with the control D-loop region, it is very likely that

they are playing a role in regulating the replication process. This regulation is most likely at the level of transcriptional termination during the formation of RNA primers from the light strand promoter (LSP). During this process, transcripts from LSP are terminated at conserved sequence blocks (CSBs) the first of which (CSB I) is quite high in AT content (Chang and Clayton, 1985). These blocks are essential for the proper termination and primer formation that allows for a transition from RNA to DNA synthesis for the initiation of replication at the heavy strand origin (O_H) by mitochondrial DNA polymerase (pol γ) (Lee and Clayton, 1998). Any alterations to the regions due to binding and induced conformational changes mediated by HMGA1 could serve to inhibit the transition process and disallow initiation of replication. It is noteworthy that recent ChIP analyses performed in our lab showed the specific association of HMGA1 at a region downstream of the LSP start site (Dement et al., 2005, Chapter 2) and in close proximity to the CSB sequences.

Activation of the ND2 mitochondrial gene by approximately 3-fold (**Table 3.2, Fig. 3.2**) within the HA7C cells over-expressing HMGA1 is consistent with multiple mitochondrial gene expression studies. While there are many examples of up-regulated mitochondrially transcribed genes in different cancer types, ND2 specifically, was shown to be activated in malignant tissue as compared to normal tissue taken from the same colorectal cancer patients (Chester et al., 1990). As the majority of the protein coding genes on the mitochondrial genome are expressed as a single polycistronic mRNA under the control of the heavy strand promoter (HSP), it is interesting that the HMGA1 protein binds to a region of AT-rich DNA upstream of this promoter (Dement et al., 2005, Chapter 2). This is, in fact, the same sequence bound by HMGA1 that may play a role in

inhibition of replication by interference with the LSP primer formation process. This presents the possibility that the HMGA1 proteins have a dual function within the processes of mtDNA expression and replication by binding to a single site. However, a more extensive study of HMGA1 binding sites around the mtDNA is necessary to begin further speculation about the mechanism by which HMGA1 is acting. Furthermore, additional genes need to be analyzed in the context of HMGA1 over-expression to verify that the protein is indeed able to promote transcriptional initiation from the HSP. Regardless of the future work that needs to be done, the present quantitative PCR data combined with previous binding site ChIP studies strongly suggest that HMGA1 proteins maintain their function as transcriptional regulators within the mitochondria.

The consequence of this regulation and subsequent changes in mtDNA expression and replication levels may be the alteration of overall mitochondrial number, morphology, and ultimately function as suggested by the observed reduction in organelle mass within the HMGA1 over-expressing cells (**Fig. 3.3**). Because the exact relationship between mitochondrial mass, mRNA, and mtDNA copy number have yet to be examined, it is difficult to suggest that this observation is a direct result of low mtDNA levels (reviewed in Penta et al., 2001). In fact while multiple cancer types show an increase in mtDNA levels, many also display an approximately 50% reduction in mitochondrial mass (Pedersen et al., 1978), a number consistent with our current findings. Downstream of the reduction in mitochondrial mass is the potential for obvious alterations in the metabolic activity of the cell.

As demonstrated by Warburg in 1956, and confirmed here by results showing the reduced capacity of the highly metastatic HA7C cell line to survive in the presence of a

glycolytic inhibitor, 2-DG (**Fig. 3.4**), cancer cells have abnormal metabolism. That is, the cells display a substantially greater capacity for aerobic glycolysis and an apparent decreased level of oxidative phosphorylation (Warburg, 1956). Although the role of the HMGA1 proteins in this phenotype is currently difficult to interpret, the present data suggest that the alterations to metabolic activity may be a consequence of HMGA1 binding to mtDNA elements that regulate HSP and LSP activity. However, it has been suggested that increased expression of mtDNA-encoded respiratory chain complexes observed in solid tumors may reflect mitochondrial gene adaptations to perturbations in cellular energy requirements (Penta et al., 2001). In order to sort these possibilities out, depletion studies are currently being performed in the lab utilizing tetracycline treatment to inhibit the expression of the HMGA1 transgene that is under the control of a tetracycline responsive promoter. Initial data suggests that the sensitivity to 2-DG treatment may be reversed following down-regulation of HMGA1 transgene (data not shown), indicating that HMGA1 may play a causative role.

Finally, we showed with preliminary Q-PCR results that HMGA1 over-expressing cells display a reduction in their capacity to repair ROS generated DNA damage within the mitochondria (**Fig. 3.5**). We present data showing that the Q-PCR technique can be successfully optimized and DNA damage and repair assessed. Due to promising results, these studies will move forward to verify results and begin looking into a mechanism of DNA repair inhibition within the mitochondria. The current results are consistent with literature demonstrating cancer related increases in mutations within the mitochondria. Mitochondrial localization of the over-expressed oncogenic HMGA1 proteins may be a major contributor to the occurrence of increased mutations observed within tumor tissue.

In order to study these questions further, and confirm a direct role for the HMGA1 proteins within the above studied mitochondrial processes, further studies are necessary and underway. These include repeating the above assays in the context of tet, antisense, or RNAi mediated depletion of HMGA1 within the transgenic cell line to observe reversals of the apparent HMGA1 affects to mitochondrial and cellular function. Also, increasing affects may be observed by directing the protein to the mitochondria by the construction of an HMGA1 fusion vector coding for an HMGA1-mitochondrial targeting sequence (MTS) protein. Recently, we have developed an HMGA1 fusion construct containing the MTS of manganese super oxide dismutase, within a vector designed for stable transfection. Following transfection and proper localization of the HMGA1 fusion protein to the mitochondria, HMGA1 specific affects should be greatly amplified.

Tables and Figures

Table 3.1. Relative Quantitation of Mitochondrial DNA Levels.

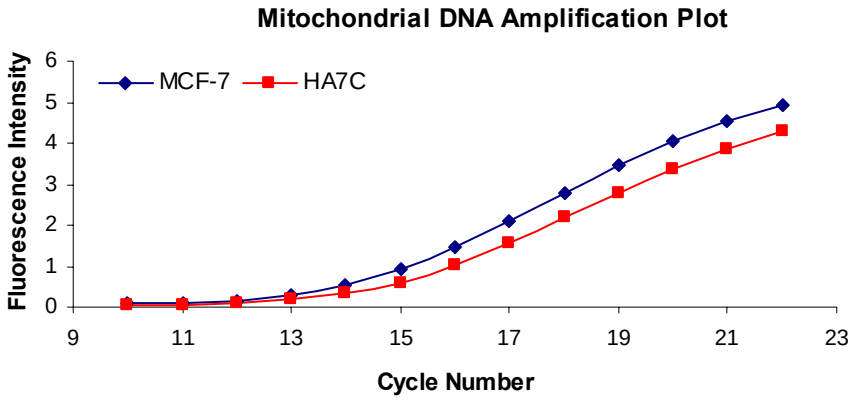
Cell Line	Mitochondria Average Ct	Beta Globin Average Ct	ΔCt: Mito-Beta Globin	ΔΔCt: 7C ON - MCF-7	Mito Rel. to MCF-7
MCF-7	12.75±0.11	23.89±0.13	-11.15±0.17	0.00±0.17	1.0 (0.9 to 1.1)
HA7C	13.50±0.34	23.92±0.25	-10.42±0.42	0.72±0.42	-1.7 (-1.2 to -2.2)

* Fold change values determined by comparative Ct method (see Methods and Materials section)

Figure 3.1. Amplification plots and fold change data demonstrating an approximately 2-fold down regulation in the levels of mitochondrial DNA in HA7C cells relative to MCF-7 cells. **Panel A:** Plot showing increasing levels of amplification during PCR cycles as represented by fluorescence intensity from the FAM reporter on a probe specific for a region of the mitocohondrial D-loop. **Panel B:** Plot showing the same amplification progression by fluorescence readings from a FAM reporter probe specific to the beta globin gene. Cycle numbers included in both plots correspond to those in which exponential amplification is beginning. Using the comparative Ct method, fold change relative to an endogenous control can be determined as demonstrated in Table 3.1.

Figure 3.1.

A


Mitochondrial DNA Amplification Plot
Fluorescence Intensity
MCF-7
HA7C
0
1
2
3
4
5
6
9
11
13
15
17
19
21
23
Cycle Number

B

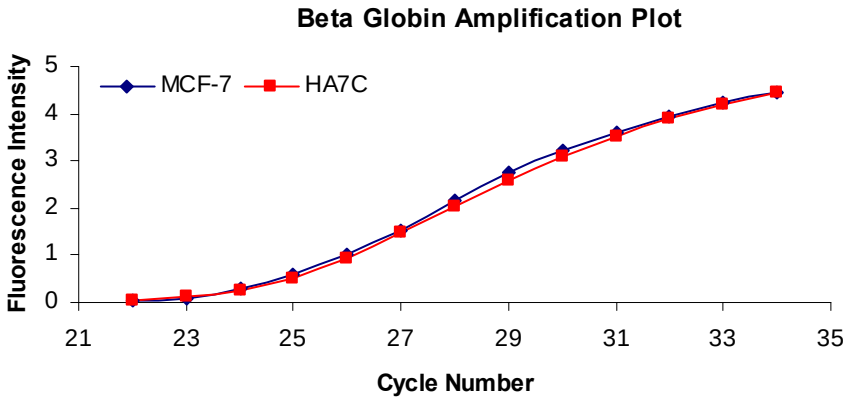

Beta Globin Amplification Plot
Fluorescence Intensity
MCF-7
HA7C
0
1
2
3
4
5
21
23
25
27
29
31
33
35
Cycle Number

Table 3.2. Relative Quantitation of Mitochondrial NADH Dehydrogenase Subunit 2 Gene Transcript Levels.

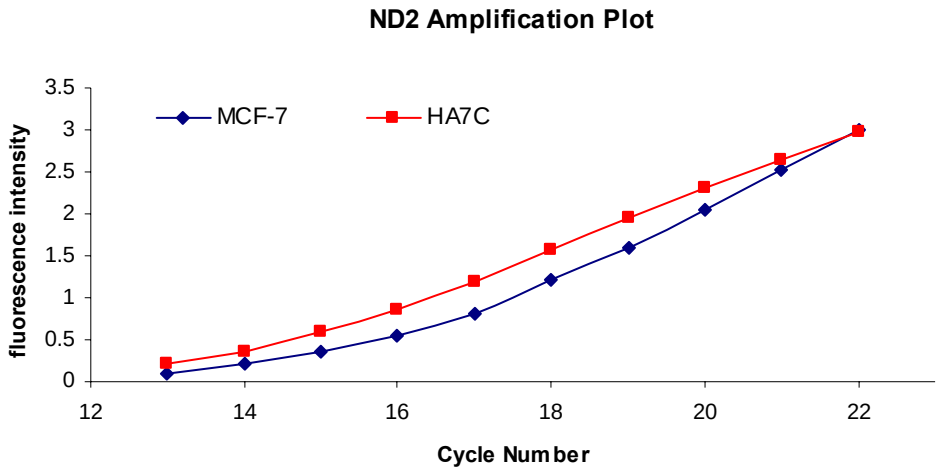
Cell Line	ND2 Average Ct	HPRT Average Ct	ΔCt: ND2-HPRT	ΔΔCt: HA7C - MCF-7	ND2 Rel. to MCF-7
MCF-7	16.02±0.68	20.42±0.10	-4.4±0.69	0.00±0.69	1(0.6 to 1.6)
HA7C	15.58±0.15	20.70±0.11	-10.42±0.19	-0.72±0.19	1.7 (1.4 to 1.9)

* Fold change values determined by comparative Ct method (see Methods and Materials section)

Figure 3.2. Amplification plots and fold change data demonstrating an approximately 2-fold up-regulation in the levels of NADH dehydrogenase subunit 2 transcript in HA7C cells relative to MCF-7 cells. Taking the reduced mitochondrial DNA levels in these cells as demonstrated in Fig. 3.1, the total positive fold change for ND2 is approximately 4-fold. **Panel A:** Plot showing increasing levels of amplification during PCR cycles as represented by fluorescence intensity from the FAM reporter on a probe specific for a region of ND2 cDNA. **Panel B:** Plot showing the same amplification progression by fluorescence readings from a FAM reporter probe specific to HPRT cDNA. Cycle numbers included in both plots correspond to those in which exponential amplification is beginning. Using the comparative Ct method, fold change relative to an endogenous control can be determined as demonstrated in Table 3.2.

Figure 3.2.

A


ND2 Amplification Plot
MCF-7
HA7C
fluorescence intensity
3.5
3
2.5
2
1.5
1
0.5
0
12
14
16
18
20
22
Cycle Number

B

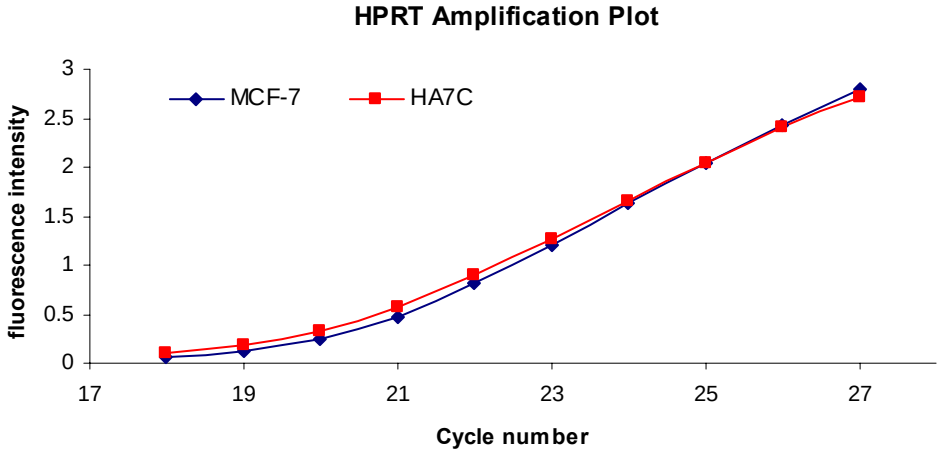

HPRT Amplification Plot
MCF-7
HA7C
fluorescence intensity
3
2.5
2
1.5
1
0.5
0
17
19
21
23
25
27
Cycle number

Figure 3.3. Mitochondrial mass within HA7C cells is reduced relative to MCF-7 cells. Histogram shows nonyl acridine orange (NAO) staining intensity on the logarithmic X axis and cell counts on the Y axis. A total of 10,000 events were analyzed by FACS analysis. The shift in the HA7C peak to a lower staining intensity represents an approximately 20% reduction of mitochondria in the majority of these HMGA1 over-expressing cells as determined by Cell Quest Software.

Figure 3.3.

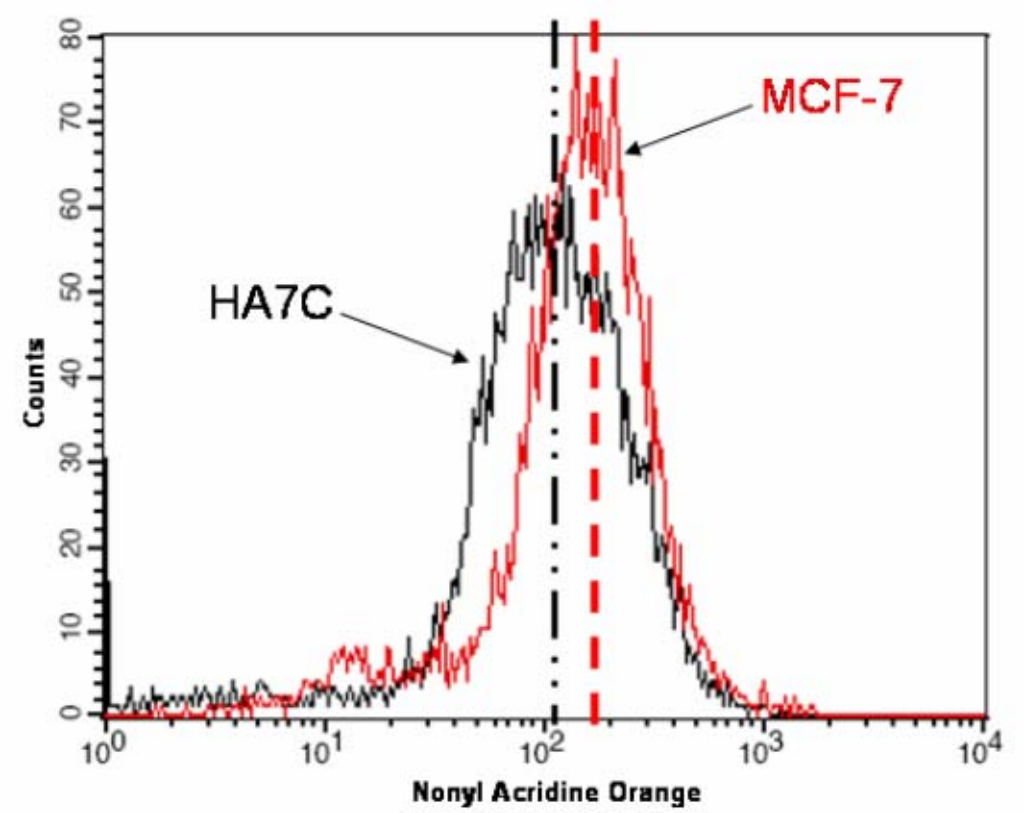

MCF-7
HA7C
Counts
0
10
20
30
40
50
60
70
80
10^0
10^1
10^2
10^3
10^4
Nonyl Acridine Orange

Figure 3.4. Increased sensitivity of HA7C transgenic cells to treatment with the inhibitor of glycolysis, 2-Deoxyglucose (2-DG), as compared to wild-type MCF-7 cells. Relative fluorescence units represent Hoechst 33258 staining of 2-DG treated samples (1-4mM) relative to untreated samples. A value of 1 represents cell numbers equal to that of the non-treated samples. Decreasing values represent a decrease in the number of cells as compared to the cell type specific non-treated control. The greatest difference between the HA7C and MCF-7 cells lines exists at 3 mM 2-DG.

Figure 3.4.

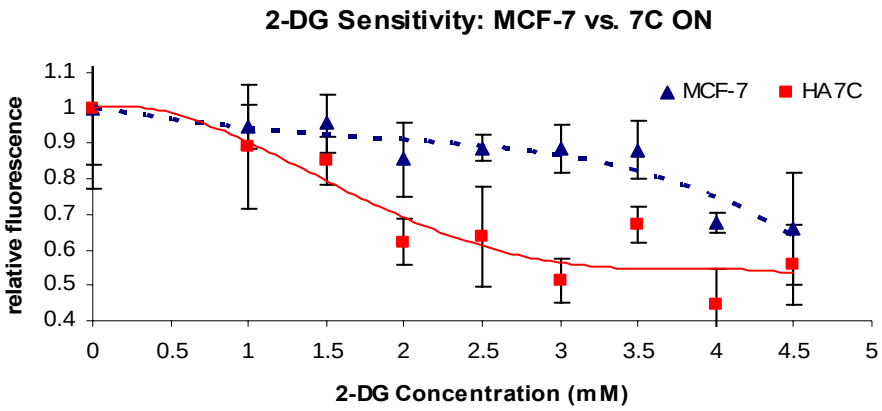
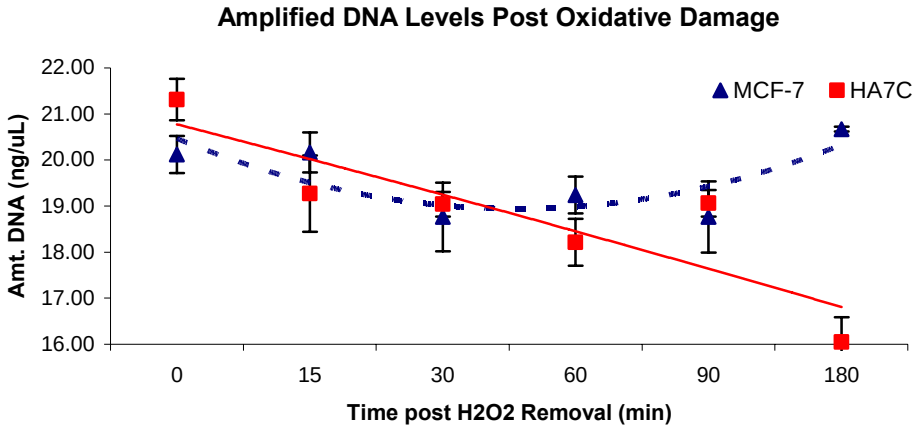

2-DG Sensitivity: MCF-7 vs. 7C ON
MCF-7
HA7C
relative fluorescence
1.1
1
0.9
0.8
0.7
0.6
0.5
0.4
0
0.5
1
1.5
2
2.5
3
3.5
4
4.5
5
2-DG Concentration (mM)

Figure 3.5. HMGA1 over-expressing cells (HA7C) have an apparent reduced ability to repair mitochondrial DNA damaged by treatment with the ROS H_2O_2. Alternatively, these cells may lack the ability to effectively remove accumulating ROS. **Panel A:** Plot showing DNA product amounts following PCR amplification of total cellular DNA at 0, 15, 30, 60, 90, and 180 min post removal of H_2O_2 from the growth media. Reductions in the amount of amplification represent increased levels of DNA damage due to the inhibition of polymerase activity by damage lesions. During the first 60 min, damage is increasing as ROS is cleared from the cell. In the MCF-7 cells, repair begins between 90 and 180 min whereas in HA7C cells, damage increases during all timepoints.

Figure 3.5.


Amplified DNA Levels Post Oxidative Damage
MCF-7
HA7C
Amt. DNA (ng/uL)
22.00
21.00
20.00
19.00
18.00
17.00
16.00
0
15
30
60
90
180
Time post H2O2 Removal (min)

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CHAPTER 4

CONCLUSIONS AND FUTURE WORK

HMGA1 Proteins and Mitochondrial Biology: New Directions for Cancer Research

Historically, aberrant mitochondrial activity has been recognized as a consistent occurrence associated with the process of carcinogenesis and metastatic progression (Bandy and Davison, 1990; Baggetto, 1993; Hoberman, 1975; Caveli and Liang, 1998; Neubert et al., 1981; Richter, 1988; Schumacher et al., 1973; Wilkie et al., 1975; and Shay and Werbin, 1987). Additionally, mitochondria display abnormal genotypic and phenotypic characteristics within the context of multiple non-cancer diseases and aging (MITOMAP.org; Chomyn and Attardi, 2003). Increased mutations, abnormal organelle morphology and membrane potential, changes in mtDNA expression and replication, and altered cellular metabolism are distinct changes to mitochondria and mitochondrial related processes observed in association with multiple cancer types and diseases (Amuthan et al., 2002; Warburg, 1956; Zafar et al., 1982). Findings suggest that correlations between cancer/disease phenotypes and mitochondria result from, or are associated with, the oxidative environment of the organelle. As covered in detail previously, reactive oxygen species (ROS) generated mtDNA mutations have the potential to produce disease and, within the context of cancer, promote persistent oxidative stress and the accumulation of more mutations (reviewed in Penta et al., 2001). However, the work contained in this dissertation provides evidence that altered mitochondrial function within the context of a cancer phenotype may, in part, be a product of aberrant HMGA1 expression. Data suggests that such effects represent abnormalities in the normal mitochondrial role for HMGA1, which may be to regulate mitochondrial activity or biogenesis during the process of cell division. These findings regarding the relationship between HMGA1 proteins and mitochondrial function are compelling and require further experimentation.

In order to follow up on the findings presented here, particularly the novel discovery that HMGA1 is present in the mitochondria, many areas of research need to be pursued. These include but are not limited to the mechanism(s) of HMGA1 nucleocytoplasmic translocation, the transcriptional role of HMGA1 within the mitochondria, HMGA1 mediated changes to mitochondrial function, and finally the possible role of HMGA1 in the inhibition of mtDNA repair and the accumulation of disease and cancer related mutations.

Mechanisms of HMGA1 Translocation

As presented in Chapter 2, HMGA1 was clearly shown to be within the mitochondria of both murine fibroblasts NIH3T3 (**Fig 2.1, 2.3, 2.5, and 2.6**) and human breast epithelial MCF-7 (**Fig. 2.7 and 2.8**) cells. In NIH3T3 cells, synchronization and live cell imaging studies suggested that the proteins move from the nucleus to the cytoplasm in a cell cycle dependent manner (**Fig. 2.2**). As detailed in the Chapter 2 Discussion, HMGA1 proteins lack typical sequences associated with nuclear export or mitochondrial internalization, but display specific characteristics that may allow for these translocation events to occur. Because of the lack of specific export and internalization signals, the mechanism of translocation is likely mediated by chaperone proteins and regulated by post-translational modifications. In fact, it has been shown that HMGA1 DNA binding affinity is reduced 20-fold in response to specific phosphorylation of the protein at two threonine residues by cdc2 kinase during the G2/M phase of the cell cycle (Nissen et al., 1991; Reeves et al., 1991). This event would allow for mobilization of the proteins for interactions with nuclear export machinery and/or export chaperone proteins.

Based on the findings presented above, several lines of experimentation are necessary in order to study the possible mechanisms of HMGA1 translocation.

Involvement of nuclear export machinery in the process of HMGA1 nucleocytoplasmic translocation can be initially evaluated by immunolocalization and GFP live cell imaging studies following treatment of cells with leptomycin B (LMB). Via direct associations, LMB is a specific inhibitor of the CRM1 complex responsible for the active transport of leucine-rich nuclear export signal (NES) containing proteins across the nuclear membrane (Kudo et al., 1998). While HMGA1 does not appear to possess a classical NES based on computer based analysis (data not shown), it does contain nuclear localization signals (NLS) that may act bidirectionally to promote nuclear export (Guiochon-Mantel et al., 1994). Furthermore, the leucine-rich NES has not been well defined as rapid export of proteins carrying NES sequences with only one leucine (Bogerd et al., 1996) or completely atypical sequences (Mancuso et al., 1994; Fridell et al., 1993) has been observed. This indicates that the lack of a canonical NES on the HMGA1 protein does not rule out the possibility of CRM1 mediated export. In order to determine the involvement of the nuclear export machinery in HMGA1 nucleocytoplasmic translocation, partially synchronized NIH3T3 cells can be treated with low concentrations of LMB (20 uM to 200 nM) and analyzed by fixed cell immunofluorescence at 16-18 hrs, post-synchronization, or by live cell GFP-HMGA1 imaging. Because we have recently developed a transgenic line of NIH3T3 cells expressing the GFP-HMGA1 fusion protein, live cell imaging can be performed in the context of cell synchronization and time sensitive LMB treatments without the need for the coordination of transient transfections.

Beyond the specific involvement of the CRM1 complex in the export process, energy dependence can by evaluated and used to determine the general manner in which translocation occurs. That is, due to the small size of the HMGA1 proteins, passive diffusion following some cellular stimuli that alters the binding of DNA and chromatin binding characteristics of the proteins may be partially responsible for export. Determining the need for ATP during nuclear export will either confirm results showing LMB inhibition, or the lack there of, and determine if passive diffusion is involved. While phosphorylation of HMGA1 during the G2/M phase of the cell cycle has been shown to mobilize the proteins, subsequent passive diffusion is not likely due to evidence that the process is quite slow compared to active transport (Laskey and Dingwall, 1993). This conclusion is based on our live cell imaging data showing the rapid movement of the HMGA1 proteins from the nucleus to the cytoplasm and back again (data not shown). ATP dependency can be determined in a manner similar to the LMB assay above by replacing LMB treatment with specific reagents capable of depleting intracellular ATP such as carbonyl cyanide m-chloro phenyl hydrazone (CCCP) and azide. These reagents deplete the ATP level to ~ 10% within 30 min. of treatment and thus their addition can be performed just prior to known timepoints of HMGA1 translocation to prevent adverse cellular side effects (Engel et al., 1998).

While the above mentioned phosphorylation of the HMGA1 proteins could simply serve to mobilize the proteins for translocation, the modification or others may also be involved in promoting chaperone protein interactions. This is a logical conclusion based on the fact that HMGA1 proteins have been shown to be highly modified between the second and third AT-hook DNA binding motifs where protein

partners typically interact (reviewed in Reeves, 2001). Because protein-protein interactions involved in HMGA1 translocation are potentially complex and have not been studied, anti-HMGA1 pull-down followed by the identification of unknown binding partners by mass spectrometry (MS) would be an efficient method of determining chaperone protein involvement. Interestingly, a recent study utilized a unique affinity chromatographic strategy coupled with 2-D gel electrophoresis and MALDI-TOF or ESI IT MS to identify HMGA1 interacting partners (Sgarra et al., 2005). This method involved the production of recombinant HMGA1 proteins used for derivatization of affinity columns and subsequent exposure of the columns to nuclear extracts of various cell types. Following elution of bound proteins, 2-D gel electrophoresis and MS were used for protein identification. The *in vitro* method resulted in the discovery or confirmation of multiple protein binding partners, including heat shock cognate protein 71, a known chaperone. Modifications of these methods to analyze protein-protein interactions following *in vivo* formaldehyde cross linking at the specific cell cycle stage of HMGA1 nucleocytoplasmic translocation may serve to identify specific translocation associated protein binding partners. Following these studies, confirmatory antibody assays specific for the identified protein partners could be used in reciprocal co-immunoprecipitation and western blot analysis.

Regardless of the precise mechanism of actual translocation, post-translational modifications of the HMGA1 protein are very likely to be involved in the initiation and regulation of the process as is true for other proteins that display similar cell cycle dependent localization patterns. Of particular interest is the controlled localization of cyclins B1 and D1 during specific stages of the cell cycle. In the case of cyclin B1,

during the majority of the cell cycle, accumulation occurs in the cytoplasm and at the nuclear envelope. Immediately prior to mitosis, a dramatic translocation of the protein into the nucleus takes place (Pines and Hunter, 1991). Similarities between the cytoplasmic localization morphologies observed for cyclin B1 and those observed for HMGA1 within NIH3T3 cells (**Fig. 2.1**) are conspicuous, and suggest association of both proteins with mitochondria. In fact, cyclin B1 has been shown to co-localize with insoluble 'cytoplasmic structures' that have yet to be identified, but are most likely not the Golgi apparatus or the endoplasmic reticulum, as determined by double immunofluorescence experiments (Pines and Hunter, 1991). In contrast to cyclin B1 redistribution, cyclin D1 resides in the nucleus throughout the G1 phase, and relocalizes to the cytoplasm for the remainder of interphase (Alt et al., 2000). We show a similar pattern of redistribution, with HMGA1 translocating from the nucleus to the cytoplasm during the late S/G2 phase (**Fig. 2.2**). Such correlations between cell cycle controlled redistribution of HMGA1 and cyclins B1 and D1 raise the possibility of a common mechanism of localization control for these proteins.

Regulation of cyclin D1 redistribution at specific stages of the cell cycle is dependent on the phosphorylation of a single threonine residue. While this has been firmly established, the exact manner in which this modification results in nuclear export has not been resolved (Alt et al., 2000). However, post-translational modifications are thought to be one of the primary factors involved in the induction of protein nucleocytoplasmic translocations (Topham et al., 1998; Alt et al., 2000; Fabbro and Henderson, 2003; Haendeler et al., 2003). Not surprisingly, phosphorylation of cyclin B1 at a specific 'cytoplasmic retention region' has been regarded as a potential mechanism

for release of the protein into the nucleus (Pines and Hunter, 1994). This, coupled with the high level of modifications that occur on the HMGA1 proteins, makes this increasingly common theme of localization control a very possible mechanism for cell cycle dependent HMGA1 redistribution. In order to determine the role of post-translational modifications in this process, mutagenesis of HMGA1-GFP fusion proteins at residues known to be modified can be done in conjunction with live cell imaging and/or fixed cell immunofluorescence with anti-GFP antibodies. Similar studies were recently performed in the context of subnuclear HMGA1 localization and chromatin dynamics (Harrer et al., 2004). Briefly, it was determined, using mutagenic HMGA1-GFP fusion proteins and fluorescence recovery after photobleaching (FRAP) assays, that HMGA1 is a highly mobile component of chromatin and mitotic chromosomes. Furthermore, it was shown that the kinetic properties of HMGA1a proteins are controlled by the number of functional AT-hooks present and are regulated by differential patterns of phosphorylation. That is, increasing phosphorylation levels were correlated to increased residence times on heterochromatin and chromosomes, as compared to euchromatic regions. Because these studies were not concerned with subcellular localization patterns, differential post-translational modifications observed in these studies are potential regulators of cell cycle stage specific HMGA1 mobilization and mitochondrial localization.

Once nuclear export is achieved, HMGA1 proteins are capable of gaining access to the interior matrix of the mitochondria (**Fig 2.6, 2.9**). In order to further confirm internalization, submitochondrial fractionation by ultracentrifugation and subsequent western blot analysis of the various fractions should be performed. Internalization of

HMGA1 into mitochondria is, again, likely a process involving chaperone proteins coupled with transport machinery on the outer and inner mitochondrial membranes as discussed in Chapter 2. While the mitochondrial import system has not been completely characterized, and many internal mitochondrial proteins that are encoded in the nucleus enter by an unknown mechanism, some experiments can be done to begin determining how HMGA1 gains entry. This would primarily be accomplished by isolation of cytoplasmic and mitochondrial fractions from synchronized or mixed populations of cells following formaldehyde crosslinking. As described above, immunoprecipitation of HMGA1 proteins can be followed by MS analysis to identify unknown protein partners. Potential proteins involved in the process of HMGA1 internalization include soluble chaperone proteins and membrane bound transport subunits of the TOM and TIM mitochondrial transport machinery.

Transcriptional Activity of HMGA1 in Mitochondria

Because HMGA1 proteins were shown to be bound to, or indirectly associated with, the regulatory D-loop of the mitochondrial genome (**Fig 2.9**), it was hypothesized that transcription and/or replication of the genome may be affected by differential levels of the protein. Indeed, as determined by real-time PCR analysis, both the levels of mtDNA (**Table 3.1**, **Fig. 3.1**) and mitochondrial gene expression (**Table 3.2**, **Fig. 3.2**) were altered in the presence of HMGA1 over-expression. Paradoxically, while mtDNA levels were decreased by approximately 2-fold when compared to MCF-7 cells expressing normal levels of HMGA1, the gene transcript levels were up-regulated by approximately 3-fold. As discussed in chapter 3, this in not inconsistent with multiple

studies of mitochondrial DNA abnormalities in the context of a cancer phenotype and may be indicative of a dual role for the HMGA1 protein. In order to further study the roles of HMGA1 in the process of transcription and replication of mtDNA, multiple techniques can be utilized to identify mitochondrial HMGA1 protein partners and mtDNA nucleoid complexes, sites of HMGA1 binding around the entire genome, and affects of differential HMGA1 expression on both LSP and HSP controlled gene expression.

A short list of protein factors involved in transcription within the mitochondria have been described and are often found in the context of mtDNA nucleoids. These nucleoid complexes are recognized as an organization of mtDNA and protein factors located at discrete foci within mitochondria. Originally, these complexes were described in yeast (Miyakawa et al., 1984), but more recently, both genetic and cell biological evidence suggest that mtDNA is organized in nucleoid-like structures in mammalian cells as well (Jacobs et al., 2000; Lehtinen et al., 2000). Confirmation for this came with cytological and biochemical assays to determine nucleoid location and protein composition within various mammalian cell types (Garrido et al., 2003). Such studies can be modified to determine if HMGA1 proteins are present within the nucleoid complexes and in association with known mitochondrial transcription factors. Briefly, immunocytochemical analysis can be performed to determine co-localization of HMGA1 and various known mitochondrial protein factors (TFAM, mtSSB, POLG2 etc.) with a Twinkle-GFP fusion protein. Twinkle is a protein that is similar to the phage T7 gene 4 protein and was the first factor found to co-localize with mtDNA in mammalian cells (Spelbrink et al., 2001). Nucleoids can also be identified by bromo deoxy uridine (BrdU)

incorporation into mtDNA of cells lacking cytoplasmic thymidine kinase (TK-). BrdU is a thymidine analog that is incorporated into DNA only after being phosphorylated by a thymidine kinase. In cells lacking cytoplasmic thymidine kinase , but retaining the mitochondrial isozyme, mtDNA incorporation can be easily detected by performing immunocytochemistry with a BrdU specific mAb. This, coupled with co-localization assays using anti-HMGA1 antibodies is an independent method to determine the involvement of HMGA1 in mtDNA transcription that is indicative of mtDNA nucleoid association. Beyond these cytological methods, biochemical means of isolating mtDNA nucleoids and associated proteins exists and is important for confirming immunocytochemical results. Briefly, isopycnic gradient centrifugation isolated mitochondria are lysed and separated into supernatant and pellet fractions that are centrifuged in a sucrose gradient. Gradient fractions can then be analyzed by PCR to confirm the presence of mtDNA or by western blot to identify specific proteins present.

The next logical step in determining the possible roles that HMGA1 plays within mitochondria is to identify the sites of association around the entire mitochondrial genome. By mapping the sites of HMGA1 binding around the genome, specific functions can be extrapolated based on the number and types of DNA elements that HMGA1 interacts with. As has been shown, HMGA1 associates with a specific region of the D-loop that is upstream of HSP and downstream of LSP, close to CSBI (**Fig. 2.9, Ch. 3 Discussion**). As discussed in Chapter 3, this single observed binding site suggests a dual and differential role for the HMGA1 proteins with regard to their affect on HSP or LSP activity. Complete mapping of the HMGA1 binding sites will serve to confirm this possibility and reveal more detailed mechanisms by which HMGA1 is able to directly

alter overall cellular mtDNA levels and/or mitochondrial gene expression. This can be directly accomplished by ChIP analysis with multiple primer sets designed to specific regions around the mitochondrial genome. Because this method would require the optimization of numerous PCR reactions corresponding to the different primer sets, it would be particularly time consuming. However, alternative microarray ChIP methods developed and carried out by NimbleGen Systems Inc. (www.nimblegen.com) exist that would remove the in house optimization and greatly increase accuracy and efficiency. These methods are designed to rapidly identify protein binding sites along long stretches of DNA and would serve to characterize the entire mitochondrial genome in a single assay.

Currently, we have generated limited data regarding analysis of actual mitochondrial gene transcript levels in the context of HMGA1 over-expression. The NADH dehydrogenase subunit 2 (ND2) gene transcript was shown to be increased by approximately 3-fold in cells over-expressing HMGA1 relative to non-over-expressing cells (**Table 3.2, Fig. 3.2**). This observation likely translates into increases in the majority of mitochondrially expressed proteins as the ND2 message is transcribed as part of a large polycistronic message under the control of the mitochondrial HSP. If HMGA1 acts to enhance transcriptional initiation at DNA elements associated with the HSP, other proteins resulting from the translation of the HSP generated mRNA, which are the majority of mitochondrially encoded proteins (**Fig. 1.4**), would likely be increased. In order to look more closely at this, real-time PCR or Northern Blot analysis should be done for the remainder of the HSP and LSP genes. Based on what we know, it is likely that transcripts from both promoters are elevated in the presence of increased HMGA1

levels. This would be consistent with data showing the HMGA1 mediated decrease in mtDNA levels overall (**Table 3.1, Fig. 3.1**), as non-terminated transcription at LSP, resulting in increased expression of L-strand genes, would preclude replication of the genome.

In regard to the observed reduction in mtDNA, it is possible to look for a more dramatic affect of HMGA1 on the replication of the genome. That is, it is possible to reversibly deplete mtDNA within mammalian cells in culture and monitor the rate of mtDNA return. This is accomplished by the addition of low concentration of ethidium bromide (EtBr) to the tissue culture media for an extended number of passages (up to 30). Upon removal of the EtBr, cells recover the mtDNA. Because HMGA1 potentially inhibits mtDNA replication via prevention of LSP transcript termination, we hypothesize that the rate of mtDNA return in the context of EtBr depletion would be severely inhibited within cells over-expressing the protein. This would strongly confirm the role of HMGA1 as a transcriptional regulator within the mitochondria and as a major factor contributing to reductions in the levels of mtDNA within cancer cells. In addition to EtBr, an alternative reagent (ditercalinium chloride) has been identified that acts in a similar manner but appears to function more effectively over a wider range of cell types (Okamaoto et al., 2003) and thus may be useful in these experimental approaches.

HMGA1 Mediated Alteration to Mitochondrial Function

Changes in mtDNA maintenance and expression in HMGA1 over-expressing cells likely translate into alterations in the overall function of mitochondria. This has the potential to affect multiple cellular processes including ROS production, ATP production,

apoptosis, overall metabolic phenotype, and even stimulation of signaling pathways that affect nuclear function. Our results show that overall cellular mitochondrial mass is reduced in the presence of high levels of HMGA1 (**Fig. 3.3**). Furthermore, cells over-expressing HMGA1 are much more sensitive to treatment with 2-DG relative to non-over-expressing cells (**Fig. 3.4**), indicating that HMGA1 is involved in the glycolytic phenotype of cancer cells. This may or may not relate directly to overall reductions in mtDNA and mitochondrial mass. In order to confirm the direct role of HMGA1 in producing these differential effects, it is important to deplete HMGA1 levels in over-expressing cell lines and assay for a return to normal mitochondrial mass and 2-DG sensitivity. As discussed in Chapter 3, initial studies utilizing tetracycline (tet) depletion of the HA-HMGA1 transgene in MCF-7 cells showed reversal of 2-DG sensitivity compared to tet treated wild-type MCF-7 cells. Because pleiotropic effects of tet treatment may be responsible for the observed reversals, it is important to perform similar HMGA1 depletion studies using different techniques such as antisense or RNAi technology.

In addition to characterizing the above observed effects, other mitochondrial specific functions can be studied in the context of HMGA1 over-expression. Changes in mitochondrial membrane potential ($\Delta\psi_m$) are detectable and have been observed following EtBr mediated depletion of cellular mtDNA in C2C12 rhabdomyoblasts (Biswas et al., 1999). Interestingly, the change in $\Delta\psi_m$ observed in this study was accompanied by elevated cytosolic free $[Ca^{2+}]_c$ and activation of calcium responsive transcription factors indicating mitochondria-to-nucleus crosstalk. Because HMGA1 was shown to reduce mtDNA levels, it is possible that the proteins also indirectly affect

$\Delta\psi_m$. Furthermore, such changes may be accompanied by alterations to $[Ca^{2+}]_c$ and subsequent changes in nuclear gene expression profiles consistent with the cancerous phenotype of HMGA1 over-expressing cells. In fact, subsequent mtDNA depletion studies revealed that stress induced changes to $[Ca^{2+}]_c$ originating with altered mitochondrial $\Delta\psi_m$ resulted in the induction of an invasive phenotype in the otherwise non-invasive C2C12 rhabdomyoblasts (Amuthan et al., 2001). The invasive behavior was later attributed to mtDNA depletion induced elevation of steady state $[Ca^{2+}]_c$ and resulting over-expression of tumor invasion markers such as cathepsin L and TGFβ1, and activation of calcium-dependent MAP kinases (ERK1 and ERK2) and calcineurin. Also, the levels of anti-apoptotic proteins Bcl2 and Bcl-X_L were increased, while pro-apoptotic proteins Bid and Bax were reduced (Amuthan et al., 2002). Considering these findings, it is intriguing that HMGA1 over-expressing cells were recently shown to display an increased sensitivity to the activation of the Ras/ERK signaling pathway (Treff et al., 2004). A contributing factor to this finding may very well be HMGA1 mediated reductions in mtDNA levels and subsequent or direct alterations to mitochondrial $\Delta\psi_m$. To study this, cells with and without HMGA1 over-expression can by analyzed for differential $\Delta\psi_m$ using the cationic lipid JC-1 (Invitrogen – Molecular Probes). JC-1 is a dye that exists as a green-fluorescent monomer at low membrane potential. Upon interactions with higher membrane potentials, the dye forms red-fluorescent "J-aggregates" that exhibit a broad excitation and emission maximum at ~590 nm. The ratio of red-to-green fluorescence is independent of morphological factors such as mitochondrial size, shape, and density that may influence single-component fluorescence signals (Smiley et al., 1991; Invitrogen-Molecular Probes – Product Literature).

Other cancer related alterations to mitochondrial function are changes to the efficiency of ATP production by oxidative phosphorylation and resulting ROS production. Total cellular ATP levels can be determined by bioluminescence using a ATP-bioluminescent somatic cell assay kit (Sigma-Aldrich). Mitochondrially generated ATP can be determined by subtracting the amount of ATP synthesized in cells pretreated with CCCP or oligomycin from the total cellular ATP in untreated cells. Because HMGA1 produces sensitivity to treatment of cells with 2-deoxyglucose, ATP produces via oxidative phosphorylation in the mitochondria is likely reduced in HMGA1 over-expressing cells. If results from ATP analysis support this hypothesis, it is likely that HMGA1 proteins play a causative role in the generation of a Warburg glycolytic phenotype observed in cancer cells. This effect would logically be an indirect result of HMGA1 mediated reductions in mtDNA as a loss of mitochondrially generated ATP has been shown within EtBr treated cells displaying a large percent reduction in mtDNA (Amuthan et al., 2002).

In addition to ATP, oxidative phosphorylation in the mitochondria produces ROS that can damage matrix localized mtDNA. Elevated levels of mitochondrial ROS in cancer cells may be partly responsible for an increased occurrence of mtDNA mutations. Although probably indirect, HMGA1 over-expression may lead to increases in total cellular ROS. This would be consistent with findings showing that increased H_2O_2, a common ROS generated in the mitochondria, is capable of inducing MAP kinases in HeLa cells (Nemoto et al., 2000). Again, similar to possible HMGA1 mediated reductions in mtDNA, effects of HMGA1 over-expression on ROS levels may be a partial mechanism by which the proteins activate Ras/ERK signaling pathways (Treff et

al, 2004). Multiple assays exist to monitor differential ROS levels in HMGA1 over-expressing and non-over-expressing cells. Cells can be analyzed by flow cytometry following treatment with the cell permeant nonfluorescent 2'7'-dicholorodihydrofluorescein diacetate (H_2DCFDA). Upon exposure to intracellular ROS, the dye becomes oxidized, resulting in the highly fluorescent 2',7'-dichlorofluorescein (DCF). Increased levels of ROS in cells over-expressing HMGA1 may directly contribute to increased mtDNA mutations in cancer cells.

HMGA1 Mediated Inhibition of Mitochondrial DNA Repair

Initial data suggests that HMGA1 may be involved in reducing the capacity of mitochondria to repair DNA damage resulting from matrix generated ROS (**Fig. 3.5**). This is consistent with the ability of HMGA1 to inhibit nuclear NER (Adair et al., 2005) and possibly base excision repair (BER) (unpublished observations) pathways. Confirmation of the mtDNA repair data by increased timepoints following exposure to different DNA damaging agents is necessary. In addition to repetition of gene-specific repair assays as described in Chapter 3, alternative approaches exist. These include treatment of cells with damaging agents such as menadione which after isolation of DNA and treatment with NaOH results in strand breaks at sites of damage. Using this platform, blot analysis. Importantly, it turns out that the method of mtDNA isolation is critical when evaluating the levels of DNA base lesions present, as oxidation during the process results in artificial increases in detectable damage. For this reason, assays, such as the gene specific repair assay described in Chapter 3, which do not require mtDNA isolation, are preferable. These assays involved isolation of total cellular DNA and

subsequent analysis of DNA lesions. Using this method, isolated genomic DNA can also be treated with specific DNA glycosylases that cut the damaged DNA at specific oxidative base lesions. Strand breaks can then be quantified by Southern blot analysis.

Beyond studying the effect of HMGA1 over-expression on rates of DNA damage repair in the mitochondria, interacting protein partners can be identified to further confirm the role of HMGA1 in the process. In mitochondria, oxidative DNA damage is largely in the form of 8-oxo-guanine (8-oxoG) and thymine glycol (TG) lesions (Ames, 1989; Dizdaroglu, 1993). To date, it appears that these lesions are primarily repaired by short patch BER (reviewed in Bohr, 2002). While little is known regarding the mitochondrial BER pathway compared to the nuclear equivalent, several key protein factors have been determined to be involved. These include a mammalian mitochondrial endonuclease that recognizes AP sites (Tomkinson et al., 1988), a DNA ligase related to DNA ligase III (Lakshmipathy and Campbell, 1999), and a mitochondrial oxidative damage endonuclease (mtODE) (Croteau et al., 1997). Co-immunoprecipitation studies to determine if HMGA1 interacts with any of these proteins would confirm the role of HMGA1 in the mtDNA repair process. These studies would utilize MS analysis to identify unknown protein partners within mitochondrial extracts and would be verified by western blot analysis.

In addition to directly studying DNA repair in mitochondria, it would be interesting to determine the affect of HMGA over-expression on the actual occurrence of mtDNA mutations. Assuming HMGA1 is capable of inhibiting DNA repair in mitochondria, it is likely that subsequent increases in DNA mutations would also be detectable. This would be consistent with studies that have detected an increased

frequency of mutations in mtDNA within cancers of the bladder, head and neck, lung, liver and pancreas when compared to normal tissue (Fliss et al., 2000; Jones et al., 2001; Nishikawa et al., 2001). Of particular interest is the high level of mutations found within the non-coding D-loop region (Fliss et al, 2000). Mutations in mtDNA within cells over-expressing HMGA1 can be determined by over lapping primer PCR amplification and cycle sequencing (Nomoto et al., 2002).

Closing Remarks

Possible future work concerning mitochondrial localization of the HMGA1 proteins presented above represents initial steps toward a greater understanding of both carcinogenesis and mitochondrial biology. New links between aberrant mitochondrial function and the process of transformation and metastatic progression demonstrate the vital importance of filling in gaps in our current understanding of the mitochondria. To date, transcription and replication of mtDNA is not well understood, emphasizing the importance of the addition of HMGA1 to the list of factors involved these processes. Considering the nuclear role of HMGA1, research regarding its function within the mitochondria should be focused on direct transcriptional activity and repair of mtDNA. Alterations to both of these processes are potentially at the center of overall changes to mitochondrial function observed in HMGA1 over-expressing cancer cells. A major step forward in our ability to study these and other processes will come with the creation of new experimental models specific to mitochondrial HMGA1 function. For example, the experiments outlined in this chapter will be greatly strengthened and confirmed by their repetition within a transgenic cell line expressing an HMGA1 protein fused to a

mitochondrial localization signal (MTS) as briefly mentioned in Chapter 3. By directing HMGA1 directly to the mitochondria, alterations to organelle function on all fronts will be much more easily studied. Using this model, the effects of actual HMGA1 directed changes to mitochondrial function should be amplified, while those changes which can be attributed to an overall cancer related phenotype should remain unchanged. Currently, this model is under development in the lab and should yield important data regarding HMGA1 function within the mitochondria and how this is related to cancer.

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