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Analysis of Madm, a novel adaptor protein that associates with Myeloid Leukemia Factor 1

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Declaration

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university.

To the best of my knowledge and belief this thesis contains no material previously published by any other person except where due acknowledgment has been made.

Signature: .....

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Publications arising from this work

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Summary

Myeloid Leukemia Factor 1 (*Mlf1*) is the murine homolog of *MLF1*, which was identified as a fusion gene with Nucleophosmin (*NPM*) resulting from the (3;5)(q25.1;q34) translocation associated with acute myeloid leukemia and myelodysplastic syndrome (Yoneda-Kato *et al.*, 1996). Mlf1 was independently isolated using cDNA representational difference to identify genes up-regulated when an erythroleukemic cell line underwent a lineage switch to display a monoblastoid phenotype (Williams *et al.*, 1999). Mlf1 has been shown to enhance myeloid differentiation and suppress erythroid differentiation; however, its mechanism of action is unknown. A yeast two hybrid screen was employed to identify Mlf1-interacting proteins. This screen isolated a number of known protein, as well as several novel molecules, that bound Mlf1. One of these was 14-3-3ζ, a member of a family of molecules that bind phosphoserine motifs and regulate the subcellular localization of partner proteins. Mlf1 contains a classic RSXSXP sequence for 14-3-3 binding and associated with 14-3-3ζ via this phosphorylated motif (Lim *et al.*, 2002). The aim of this thesis was to characterise a novel Mlf1-interacting protein that had some homology to protein kinases and was named Mlf1 Adaptor Molecule (Madm). Adaptor proteins are molecules that possess no enzymatic or transcriptional activity, but instead mediate protein-protein interactions.

Madm is encoded by a gene consisting of 18 exons and promoter analysis suggested Madm expression might be widespread; indeed Northern blotting of adult tissues and *in situ* hybridization of embryos demonstrated ubiquitous Madm expression. Significantly, the Madm protein sequence is highly conserved across diverse species. Madm formed dimers and although it contains a kinase-like domain, the protein lacks several critical residues required for catalytic activity, including an ATP-binding site. Purification of recombinant Madm revealed that the protein was not a kinase; however, studies in mammalian cells showed that Madm associated with a kinase and that Madm was phosphorylated on serine residues *in vivo* and *in vitro*. Madm also contains a nuclear localization sequence and nuclear export sequence and was shown to localise to both cytoplasm and nucleus by subcellular fractionation and confocal

microscopy. The presence of two nuclear receptor binding motifs (consensus LXXLL) suggests that Madm may have a functional role in the nucleus.

Madm co-immunoprecipitated with Mlf1 and co-localized in the cytoplasm. In addition, the Madm-associated kinase phosphorylated Mlf1 on serine residues, including the RSXSXP motif. In contrast to wild-type Mlf1, the oncogenic fusion protein NPM-MLF1 did not bind 14-3-3ζ and localized exclusively in the nucleus. Although Madm co-immunoprecipitated with NPM-MLF1 the binding mechanism was altered. As Mlf1 is able to reprogram erythroleukemic cells to display a monoblastoid phenotype and potentiate myeloid maturation (Williams *et al.*, 1999), the effects of Madm on myeloid differentiation was investigated. However, unlike Mlf1, ectopic expression of Madm in M1 myeloid cells suppressed cytokine-induced differentiation.

In summary, the data presented in this thesis reports on the cloning and characterization of a novel adaptor protein that is involved in the phosphorylation of the proto-oncoprotein Mlf1. Phosphorylation of Mlf1 is likely to affect its interaction with other proteins, such as 14-3-3ζ. Complex formation, therefore, may well alter the localization of Mlf1 and Madm, and influence hematopoietic differentiation.

Abbreviations

The following abbreviations are used throughout this thesis:

AML	Acute Myeloid Leukemia
ATP	Adenosine Triphosphate
BSA	Bovine Serum Albumin
CHAPS	3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate
cm	Centimeters
DABCO	1,4-Diazobicyclo-[2,2,2]-octane
ddH_2O	Double Distilled Water
DEPC	Diethyl Pyrocarbonate
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic Acid
EGTA	Ethyleneglycoldiaminetetraacetic Acid
Epo	Erythropoietin
FAB	French, America, British (classification)
FCS	Fetal Calf Serum
h	Hour
Hls	Hemopoietic Lineage Switch
IL	Interleukin
IPTG	Isopropyl-β-Thiogalactopyranoside
Kip	Kinase inhibitor protein
LIF	Leukemia Inhibitory Factor
MDS	Myelodysplastic Syndrome
min	Minutes
mL	Millilitres
MLF	Myeloid Leukemia Factor
MOPS	3-[N-Morpholino] Propane Sulphonic Acid
MT-PBS	Mouse Tonicity Phosphate Buffer Saline
NPM	Nucleophosmin
Pax	Paired box
PBS	Phosphate Bufferd Saline
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PIN	Protein inhibitor of neuronal nitric oxide synthase
PMSF	Phenylmethylsulphonyl Fluoride
PVDF	Polyvinylidene Difluoride
RNA	Ribonucleic acid
s	Sec
SDS	Sodium dodecyl sulphate
SH2	Src Homology Domain 2
TBS-T	Tris-Buffered Saline - Tween 20
V	Volts
X-Gal	5'-bromo-4-chloro-3-indoyl-β-D-galactopyranoside

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Chapter 1: LITERATURE REVIEW

1.1 Hemopoiesis

1.1.1 Primitive and definitive hemopoiesis

The hemopoietic system is established early in embryonic development and functions throughout fetal and adult life to provide a continuous supply of mature blood cells to the embryo, fetus and adult. Development of the hemopoietic system begins in the yolk sac and is known as primitive hemopoiesis. This is replaced by definitive hemopoiesis, which arises in the aorta-gonadmesonephros region of the embryo. These cells then populate the fetal liver, followed progressively by the spleen and thymus. Late in gestation, hemopoietic precursors migrate to the bone marrow which becomes the principle site of hemopoiesis after birth.

Both fetal and adult hematopoietic systems can develop multiple lineages from a common precursor known as the multipotential stem cell (Lemischka *et al.*, 1986, Metcalf, 1999). A characteristic that distinguishes stem cells from other cells in the hematopoietic system is their ability to provide long term repopulation following transplantation into recipient organisms (Keller, 1992, Morrison *et al.*, 1995).

At approximately Day 7.5 of gestation in the mouse blood islands appear in the developing yolk sac (Russell, 1979). This consists of a population of erythroid cells surrounded by a layer of angioblasts that will eventually form the vasculature. The erythroid cells in the blood islands differ from those found in the fetal liver and bone marrow in that they are large, nucleated and produce the embryonic forms of globin; in contrast, definitive erythroid cells are small, enucleate and produce the adult forms of globin (Barker, 1968). The fact that development of the primitive erythroid lineage is restricted to the yolk sac suggests that it represents a distinct lineage, separate from other hemopoietic lineages. However, studies using embryonic stem (ES) cells have identified a precursor able to give rise to both primitive and definitive hemopoietic cells in response to vascular endothelial growth factor (Keller *et al.*, 1998, Kennedy *et al.*, 1997). Gene targeting studies also support the common origin

theory, where transcription factors SCL and rbtn1 are necessary for the development of both primitive and definitive hemopoietic cells (Robb *et al.*, 1996, Warren *et al.*, 1994).

Throughout life hemopoiesis is maintained by a population of multipotential hemopoietic stem cells that have the capacity to self renew (Metcalf, 1999). Under appropriate stimulation these pluripotent stem cells are capable of producing precursors that become irreversibly committed to one of the blood lineages (Metcalf, 1989). Under the influence of growth factors or cytokines, these precursors change from multipotential to bipotential cells, eventually becoming unipotential progenitors that give rise to terminally differentiated cells of a particular type; viz. erythrocyte, macrophage, platelet, eosinophil, neutrophil, basophil, mast cell, NK cell, T or B cell.

1.1.2 Lineage commitment and plasticity

A key unresolved issue in hemopoiesis is whether normal cells are actually committed to a particular lineage, or if they are able to reverse the maturation pathway given different stimuli. Furthermore it is unknown if lineage commitment occurs simultaneously with the initiation of maturation and the cessation of the cells ability to self-renew. There is ongoing debate as to whether lineage commitment is stochastic or determined by extracellular signals. In the first model, stem cells randomly express a pattern of genes that results in maturation of a particular cell type, and extracellular factors or hormones simply act as viability factors (Ogawa, 1993). In contrast, the second model suggests that growth factors determine the commitment to a particular lineage (Metcalf, 1989). It is feasible that a combination of gene expression and environment determine hemopoietic lineage commitment, since it has been shown that extrinsic factors, such as the colony stimulating factors, can enhance the probability of commitment to a particular lineage (Chen *et al.*, 2002).

The intracellular mechanisms that determine the commitment of cells to a specific lineage are gradually being elucidated. As mentioned earlier, gene targeting studies have identified certain transcription factors required for the development of various blood types. SCL has been shown to be crucial for the formation of all blood types (Robb *et al.*, 1996), while PU.1 is a key regulator of lymphoid cells and macrophages (McKercher *et al.*, 1996). GATA-1 is

essential for the development of erythrocytes (Orkin, 1992) (Pevny *et al.*, 1991), NF-E2 for platelets (Shivdasani *et al.*, 1995) and recently Pax5 has been demonstrated to be critical for B cell development (Nutt *et al.*, 1999b).

However, there have been numerous reports where researchers have been able to reprogram cells enabling them to mature along different hemopoietic pathways (Graf, 2002). Klinken *et al.* (1988) demonstrated that Eμ-*myc* B lymphoma cells acquired the phenotype of macrophages in response to over-expression of the *raf* and *myc* oncogenes. Similar lineage switches have been observed upon alterations in the concentration of key hemopoietic transcription factors. Constitutive expression of GATA-1 in transformed myelomonocytic cells reprogrammed cells to resemble either eosinophils or thromboblasts (Kulessa *et al.*, 1995), whereas in monoblastoid M1 cells it resulted in development of both erythroid cells and megakaryocytes (Yamaguchi *et al.*, 1998). Erythroid precursors or megakaryocytes have also been observed in cultures of M1 cells expressing the NF-E2 erythroid transcription factor (Sayer *et al.*, 2000). A striking example of hemopoietic lineage switching was observed in the Pax5 knockout mouse (Nutt *et al.*, 1999a). Pax5 is required for B cell maturation and when deleted differentiation is blocked at the pro-B cell stage. Interestingly, stimulation of Pax5-null cells with appropriate cytokines enabled them to differentiate into functional macrophages, osteoclasts, dendritic cells, granulocytes and natural killer cells (Nutt *et al.*, 1999a, Rolink *et al.*, 1999).

Spontaneous lineage switching *in vitro* has also been observed when the J2E erythroleukemic cell line produced myeloid precursors (Keil *et al.*, 1995). The J2E cell line was produced by infection of hemopoietic precursors from murine fetal livers with the v-*raf* / v-*myc* containing J2 retrovirus (Klinken *et al.*, 1988b). Following severe overgrowth of J2E lines, adherent cells appeared in the cultures. Clones derived from these variant cells showed they had lost the characteristic markers of erythroid cells and acquired a monocytoid phenotype. Furthermore numerous karyotypic abnormalities were found in these myeloid variants suggesting that genetic alterations resulted in activation of genes required for myeloid maturation. Several other examples of erythroid to myeloid lineage switching have been observed. Over-expression of PU.1 in murine erythroleukemic (MEL) cells can lead to a monoblastoid phenotype (Yamada *et al.*, 2001), while K562 erythroleukemic cells can be

induced to become macrophages when treated with hexamethyl bisacetamide (Green *et al.*, 1993).

These studies illustrate that a transformed cell, apparently committed to one lineage, can lose phenotypic markers of that lineage and acquire those of a different pathway, furthermore they illustrate that a significant degree of plasticity exists within the hemopoietic system. Lineage switching also occurs *in vivo*. There are numerous examples where leukemic patients achieve remission, but later re-present with a leukemia of a different nature (Pane *et al.*, 1996, Roman *et al.*, 1995, Gagnon *et al.*, 1989). In these cases it is thought that secondary leukemias result from a lineage switch induced by chromosomal alterations induced by chemotherapy or radiotherapy. An unfortunate consequence of these *in vivo* lineage switches is that the secondary leukemia is often aggressive and patient survival is low.

1.1.3 Myelopoiesis and myeloproliferative disorders

In bone marrow, interleukins IL-1, IL-3 and/or IL-6 induce differentiation of hemopoietic stem cells to a primitive myeloid cell that is referred to as granulocyte-erythrocyte-megakaryocyte-macrophage colony-forming unit (GEMM-CFU) (Metcalf, 2001). In the presence of IL-1 and/or IL-3 this precursor is committed to becoming a granulocyte-macrophage colony-forming unit (GM-CFU), a progenitor of both macrophages and granulocytes (Valledor *et al.*, 1998). As these two lineages are closely linked throughout hemopoiesis, they are commonly referred to as the myelomonocytic lineage (Hamilton, 1993, Lopez *et al.*, 1992). At the GEMM-CFU stage of maturation, cytokines IL-3 and granulocyte-macrophage colony-stimulating factor (GM-CSF) induce proliferation of these myeloid precursors, whereas macrophage colony-stimulating factor (M-CSF) induces not only their proliferation but also differentiation to the promonocyte. Maturation of these monocytic precursors and the subsequent generation of monocytes also requires M-CSF. Monocytes are generally smaller than their immediate precursors and have a well-developed lysosomal system and enhanced phagocytic capability (Ogawa, 1993, Lopez *et al.*, 1992).

Monocytes leave the bone marrow and travel through peripheral blood vessels. Once they reach a tissue they terminally differentiate into macrophages, which involves further

development of the lysosomal system associated with an increased the amount of hydrolytic enzymes, as well as the number and size of mitochondria. Despite a common origin, the function of macrophages depends on the tissue in which they reside: in most tissues macrophages phagocytose microorganisms and dead cells, whereas in the spleen they phagocytose erythrocytes and in the erythroblast centers of bone marrow they transfer iron to erythroblasts (Rama *et al.*, 1988). Tissue macrophages are unique to other terminally differentiated cells in that they retain proliferative capability, and do not rely on circulating monocytes to maintain their population (Ogawa, 1993, Lopez *et al.*, 1992). Dendritic cells, microglia, osteoclasts, Küpffer cells and Langerhans cells are tissue specific and are all thought to be derived from the monocyte/macrophage lineage. Differentiation of myeloid cells can be monitored by measuring expression of lysozyme or by staining for the Cd11b (Mac-1) antigen. This cell surface protein is present on all types of macrophages (tissue and blood), but not on erythroid cells, B or T cells, and levels increase with maturation (Springer *et al.*, 1979).

1.2 Myeloproliferative diseases and Leukemia

1.2.1 Myelodysplastic syndrome

Myelodysplastic syndromes (MDS) are a group of hemopoietic disorders that are defined by the abnormal morphological appearance of multiple blood lineages in the bone marrow and by the occurrence of cytopenias in the peripheral blood. MDS is commonly associated with a propensity towards leukemic transformation (Mufti and Galton, 1986). It is well established that MDS arises through the step-wise accumulation of genomic lesions within hemopoietic stem cells. However, the precise mechanisms underlying the pathogenesis of the disease and its evolution to leukemia is poorly understood. An unusual aspect of MDS is the presence of peripheral cytopenias, despite patients having normal or hypercellular bone marrow. Previously, it has been suggested that this may be due to increased apoptosis of marrow progenitors in the early phases of the disease (Yoshida, 1993). It is conceivable that the increased apoptosis is caused by intrinsic defects within hemopoietic progenitors that lead to abnormalities in cell signaling or cell cycling (Parker and Mufti, 1998).

1.2.2 *Leukemic transformation*

As mentioned above, MDS has a propensity to progress to acute myeloid leukemia (AML). Analysis of murine models has indicated that leukemic transformation arises through the development of genetic lesions that promote proliferation and/or inhibit apoptosis. Genetic mutations in cell cycle regulatory elements, which disrupt orderly progression through the cell cycle, are common in many human cancers. Cyclin D1, which regulates phosphorylation of the retinoblastoma protein (Rb), is over-expressed in many human neoplasms as a result of gene translocation or amplification (Jiang *et al.*, 1993). Alternatively, inactivation of Rb is also observed frequently in solid tumors (Jiang *et al.*, 1993, Harbour *et al.*, 1988). The target of Rb is the E2F transcription factor and while aberrant expression of E2F has not been reported in human cancers, gene amplification and translocations affecting this gene have been detected in several human leukemic cell lines (Saito *et al.*, 1995).

There has been increasing interest in the cyclin-dependent kinase inhibitors (CDKIs) as potential tumor suppressor genes (reviewed by (Hirama and Koeffler, 1995)). Inactivation of the $p15^{INK4B}$ and $p16^{INK4A}$ genes by methylation, or deletion, has been observed in hematological tumors, most commonly in lymphoid malignancies (Hatta *et al.*, 1995). Although inactivation of these genes is seen in myeloid cell lines, it is rarely observed in primary myeloid leukemias and MDS (Nakamaki *et al.*, 1995). Gene inactivation of other CDKIs, such as p18, p19, $p21^{WAF1}$ and $p27^{KIP1}$ are not often detected in human cancer and have not yet been reported in MDS.

Another important molecule in leukemic progression is the protein p53. Originally cloned in 1979 (DeLeo *et al.*, 1979), p53 was the first tumor suppressor gene identified. Mutations in this gene have since been identified in at least 50% of malignancies, and in many other cancers the protein is inactivated indirectly by alterations to genes whose products interact with p53 (e.g. MDM2), or act downstream of p53 (e.g. $p21^{waf1}$) (Vogelstein *et al.*, 2000). p53 is normally inactive and only becomes activated when cells are stressed or damaged. Once active, p53 acts to inhibit cell growth or induce apoptosis. While many biochemical functions have been ascribed to active p53, the most widely documented is its ability to bind DNA and activate the transcription of adjacent genes (el-Deiry, 1998). Importantly, almost all the

mutations identified in the p53 gene reduce the ability of p53 protein to activate transcription. p53 gene mutations have been documented in 5 to 15% patients with *de novo* MDS or AML (Jonveaux *et al.*, 1991, Sugimoto *et al.*, 1993, Wattel *et al.*, 1994) and up to 38% cases of therapy-related MDS/AML (Ben-Yehuda *et al.*, 1996). p53 mutations in MDS mainly involve loss of the wild-type p53 allele and are often associated with cytogenetic abnormalities involving chromosomes 5 and 7 (Sugimoto *et al.*, 1993). Mutations to the p53 gene are usually restricted to certain subtypes of AML and appear to predict leukemic development, furthermore, it has been suggested that disruption of p53-mediated apoptosis may be responsible for progression of MDS to leukemia (Kitagawa *et al.*, 1994).

1.2.3 Acute Myeloid Leukemia

AML is a rapidly progressing leukemia characterized by a block in normal myeloid differentiation and is the most common adult acute leukemia. It accounts for approximately 85% of adult acute leukemia and has a mortality rate of 60 – 80% of diagnosed patients (Lowenberg *et al.*, 1999). AML can occur at any age, but has a peak incidence in the sixth decade of life. Symptoms of the disease include fatigue, weight loss, fevers, weakness, pallor, bone pains, bleeding gums, nosebleeds, easy bruising, enlarged lymph nodes and joint pains. Treatment options include chemotherapy and/or bone marrow transplantation (Lowenberg *et al.*, 1999).

Whilst AML is thought of as a single disease, there are in fact seven subtypes of AML. Each subtype can be defined by specific cell characteristics, depending on which cell type is the dominant cell at the time of diagnosis (Bennett *et al.*, 1989). The French-American-British (FAB) subtypes are summarized in the table below:

DESIGNATION	CELL SUBTYPE
M1	Myeloblastic, without maturation
M2	Myeloblastic, with maturation
M3	Promyelocytic
M4	Myelomonocytic
M5	Monocytic
M6	Erythroleukemia
M7	Megakaryocytic

While the exact causes of AML are not completely known, certain factors may contribute to the development of this illness. Exposure to radiation and toxic chemicals that cause damage to DNA has been shown to have a direct relationship to the incidence of AML (Noshchenko *et al.*, 2002). Certain genetic disorders are associated with increased risk of AML. Children with Down's syndrome have a 20 fold increased incidence of the disease (Samouhos, 1983), and other hereditary disorders such as Fanconi's anemia, Klinefelter's syndrome and Bloom's syndrome have all been associated with acute leukemias (Alter, 2003, Keung *et al.*, 2002, Poppe *et al.*, 2001), although less than 1% of acute leukemias have a familial history (Rowley, 2000). Instead the most common cause of AML is chromosomal abnormalities with 60 – 80% of patients carrying somatically-acquired genetic changes (Rowley, 2000). There are three main cytogenetic changes: deletions, translocations and inversions (Rabbitts, 1994). Deletions often result in the loss of a tumor-suppressor gene and while some translocations and inversions are consistently found in certain tumor types, others are specific and only observed in one tumor from a single patient (Rowley, 2000).

1.2.4 Translocations in leukemia

The role of chromosomal translocations in leukemias has extensively studied. The identification of chromosomal translocations, and the genes that are present at the breakpoints, has increased our understanding of the genetic changes in leukemic cells. Genes involved in chromosomal translocations have been directly implicated in disease progression and often encode receptor tyrosine kinases or transcription factors (Rabbitts, 1994). Cloning of the 8;14 translocation involved in Burkitt's lymphoma showed that translocation of the *myc* gene to the immunoglobulin heavy chain locus led to abnormal expression of c-myc (Dalla-Favera *et al.*, 1982, Shen-Ong *et al.*, 1982, Taub *et al.*, 1982, Adams and Cory, 1985). Since then many other genes involved in cancer-associated translocations have been cloned (reviewed in (Rabbitts, 1994)). While several translocations involve the inappropriate expression of genes, the protein products of translocations can also contribute to neoplastic transformation in different ways. For example, the product of the *BCL2* gene, activated in the t(14;18) in follicular lymphoma, inhibits apoptotic cell death (reviewed in (Cory and Adams, 2002)). Interestingly, many of the genes involved in

leukemia-associated translocations encode transcription factors that often have been shown to be critical in normal hemopoiesis. The identification of such genes has provided targets for the development of therapeutic agents, such as the use of all-*trans* retinoic acids in acute promyelocytic leukemia (Chen *et al.*, 1991) and the development of Imatinib (STI571), a specific inhibitor of the Bcr-Abl tyrosine kinase produced by the t(9;22) associated with chronic myeloid leukemia (Carroll *et al.*, 1997, Druker *et al.*, 2001).

Other cytogenetic abnormalities associated with AML generally involve chromosome deletion or addition (e.g. del5q, -7, +8, -Y, del20q) (Rowley, 2000). The most common molecular abnormalities found in MDS are *ras* mutations, followed by *p15* gene hypermethylation, *FLT3* duplications, and *p53* mutations (Fenaux, 2001). However, none of these abnormalities are specific for MDS. Translocations are rarely found in AML; however, the rare cases of balanced translocations have enabled the identification of genes whose rearrangements appear to play a role in the pathogenesis of some cases of MDS. These genes include *MDS1-EVI1* in t(3;3) or t(3;21), *TEL* in t(5;12), *HIP1* in t(5;7), *MLF1* in t(3;5), and *MEL1* in t(1;3). Cytogenetic abnormalities are generally associated with a poor prognosis in AML (Fenaux, 2001).

1.3 NPM-MLF1 and MLF1

Translocation (3;5)(q25.1;q34), defined by Raimondi et al. (Raimondi *et al.*, 1989), is a rare chromosomal aberration found in AML and MDS. With about 1% of patients affected, this mutation has been characterized as a non-random karyotypic abnormality in AML. t(3;5) has been observed in all subtypes of AML with the exception of M3 (promyelocytic). Interestingly, a higher than expected incidence of the translocation in AML M6 subtype (erythroleukemia) has been reported (Raimondi *et al.*, 1989). The breakpoints in three pediatric cases of AML M2 with t(3;5) were cloned and shown to involve the novel oncogene Myeloid Leukemia Factor 1 (*MLF1*) on 3q25.1 and the Nucleophosmin (*NPM*) gene on 5q34, generating a chimeric *NPM-MLF1* transcript (Yoneda-Kato *et al.*, 1996).

The *NPM* gene is also involved in other neoplasia-associated genetic abnormalities. In 1994, Morris *et al.* (Morris *et al.*, 1994b) showed that *NPM* was one of the genes involved in the

(2;5) translocation encountered frequently in patients with anaplastic large cell lymphoma. This genetic aberration juxtaposes the *NPM* gene to a portion of the *ALK* receptor tyrosine kinase gene on chromosome 2. The resultant NPM-ALK hybrid protein is believed to contribute to neoplastic transformation by dimerization, via motifs in NPM, which inactivate the kinase activity of ALK (Bischof *et al.*, 1997, Mason *et al.*, 1998). Additionally, in cases of acute promyelocytic leukemia carrying the (5;7)(q33;q12) translocation, the same N-terminal portion of NPM present in the NPM-ALK protein fuses to part of the *RARα* gene product (Redner *et al.*, 1996). In contrast to the NPM-ALK and NPM-RARα fusion proteins which contain amino acids 1 – 117 of NPM, the NPM-MLF1 fusion protein contains 175 amino acids of the N-terminal portion of NPM. This extra region of NPM contains a nuclear localization signal and a cluster of acidic residues capable of interacting with other proteins (Xie *et al.*, 1997).

NPM (also known as protein B23, NO38, numatrin) is a major nonribosomal nucleolar phosphoprotein (Schmidt-Zachmann *et al.*, 1987). Its protein levels are significantly elevated in tumor and proliferating cells, compared with normal resting cells (Feuerstein *et al.*, 1988b, Feuerstein *et al.*, 1988a). NPM contains a potential phosphorylation site for protein kinase C and a potential metal binding domain. The protein has the capacity of oligomerization and can bind single-stranded nucleic acid with high affinity (Dumbar *et al.*, 1989). The localization of NPM is not strictly confined to the nucleus, as the protein can shuttle between the nucleus and cytoplasm (Borer *et al.*, 1989). The expression of NPM occurs in both myeloid and lymphoid cells; however, little is known about the regulation of NPM expression during myeloid and lymphoid terminal differentiation. Since it is involved in three different translocations associated with leukemias, it is suggested that NPM is a promiscuous partner in translocations associated with hemopoietic tumors (Kalantry *et al.*, 1997). Interestingly, NPM has been shown to interact directly with p53 to increase its stability and activity (Colombo *et al.*, 2002). Since the oncogenic NPM fusion proteins retain the NPM dimerization domain but lack the p53-binding region, it was suggested that these chimeric products may have a dominant-negative affect on wild-type NPM and affect p53 activity (Colombo *et al.*, 2002). Therefore, alterations in NPM function may lead to p53 inactivation which could contribute to the leukemic phenotype.

MLF1 is a recently cloned molecule (Yoneda-Kato *et al.*, 1996) and little is known about its cellular function. The murine homolog was originally isolated as *Hls7* (Williams *et al.*, 1999) but will be referred to hereafter as *Mlf1* (Hitzler *et al.*, 1999). In addition, a related molecule named Myelodysplasia/Myeloid Leukemia Factor 2 (MLF2) has been isolated (Kueffer *et al.*, 1996). *Mlf1* mRNA is detected in several tissues, but is most abundant in skeletal muscle, testis and heart (Hitzler *et al.*, 1999). In hemopoietic cells expression is variable with transcripts detected in some leukemic cells lines (K526, HEL and KG-1) and not others (e.g. HL60 and U937). It is noteworthy that *Mlf1* mRNA was found principally in immature hemopoietic cell types (Williams *et al.*, 1999) and in human CD34-positive bone marrow cells (Matsumoto *et al.*, 2000). Furthermore, expression decreases during the differentiation of M1 monoblastoid and 707 erythroleukemia cell lines, supporting the notion that it is expressed in less differentiated hemopoietic cells (Williams *et al.*, 1999).

Mlf1 is a 35 kDa protein which is predominantly cytoplasmic; however, it has been reported to localize to specific foci within the nucleus (Williams *et al.*, 1999). Over-expression of Mlf1 in J2E erythroleukemic cells altered the morphology and differentiation capability of these cells. J2E cells expressing Mlf1 lost the ability to differentiate in response to the hormone erythropoietin (Epo), despite retaining the necessary signaling molecules and transcription factors for erythroid maturation. Strikingly, Mlf1 changed the J2E cells morphology from proerythroblastoid to monocytoid (Williams *et al.*, 1999). In contrast to the inhibitory effect of Mlf1 on erythroid differentiation, over-expression of Mlf1 enhanced differentiation of M1 monoblastoid cells. Ectopic expression of Mlf1 in normal hemopoietic progenitor cells, resulted in a decrease in the number of erythroid progenitor cells, while increasing the number of myeloid progenitors (Williams *et al.*, 1999). These observations indicate that Mlf1 can play an important role in regulating normal hemopoiesis.

Elevated levels of MLF1 correlate with malignant progression (Matsumoto *et al.*, 2000). In a cohort of patients with AML or MDS, patients with high levels of MLF1 expression had significantly lower rates of survival. In addition, the clinicopathological features found in the high-level expressing patients were comparable to those patients harboring t(3;5) (Matsumoto *et al.*, 2000). MLF1 has also been shown to have a role in cell cycle progression by affecting the CDKI, $p27^{kip1}$. Expression of NPM-MLF1 in K562 cells resulted in decreased

levels of $p27^{kip1}$ protein (Matsumoto *et al.*, 2000). Furthermore J2E cells expressing Mlf1 have decreased $p27^{kip1}$ protein levels and up-regulation of $p27^{kip1}$ following Epo stimulation is impaired (L. Winteringham *et al.*, submitted). Collectively, current data suggest that MLF1 plays a role in the early stages of hemopoiesis and malfunction in MLF1 expression, either by translocation or up-regulation, could disrupt normal hemopoietic cell maturation and lead to malignant transformation.

Recently the cloning of a *Drosophila* homolog of *MLF1* (*dMLF*) has been reported (Ohno *et al.*, 2000). dMLF was identified in a yeast two-hybrid screen as a molecule that bound to the DREF transcription factor. DREF regulates proliferation-related genes in *Drosophila*, such as DNA polymerase α, cyclin A, PCNA, D-raf and the dE2F gene (Hirose *et al.*, 1993, Ohno *et al.*, 1996, Ryu *et al.*, 1997, Sawado *et al.*, 1998). In addition, it has been suggested that DREF is a target of differentiation signals (Hirose *et al.*, 1994) and that it may be involved in cross talk between growth- and differentiation-signaling pathways (Ohno *et al.*, 2000). While dMLF is predominantly cytoplasmic, it could possibly translocate to the nucleus to participate in DREF-mediated transcription (Ohno *et al.*, 2000).

A yeast two-hybrid screen of Mlf1 was performed by Dr. Evan Ingley (Laboratory for Cancer Medicine, Western Australian Institute for Medical Research) and a number of interacting molecules were identified. These molecules included a member of the 14-3-3 family of proteins viz. 14-3-3ζ. Indeed detailed analysis of the Mlf1 protein sequence revealed a characteristic RSXSXP binding motif for 14-3-3 proteins (Williams *et al.*, 1999, Hitzler *et al.*, 1999). Other molecules that associated with Mlf1 in this screen included eukaryotic translation initiation factor 3 (eIF3, p42 subunit), protein inhibitor of neuronal nitric-oxide synthase (PIN, also known as dynein light chain, LC8), calcyclin binding protein, nucleolin and three novel proteins.

1.4 14-3-3 proteins

1.4.1 Scaffolding and adaptor molecules

Eukaryotic signal transduction often involves the reversible assembly of large multiprotein complexes (Pawson and Scott, 1997). These complexes can transmit signals required to

control intracellular ion concentration, cytoskeletal structure, gene expression, cell cycle progression and apoptosis. Recently cross-talk has been described between signaling cascades that were previously considered independent. A class of molecules, named adaptor proteins, has emerged that contribute to cross-talk (Pawson and Scott, 1997). These proteins are usually devoid of catalytic activity, but contain at least two domains that mediate protein-protein interactions. Adaptors can connect a signaling enzyme to a particular organelle, or site within the cell, e.g. anchoring the enzyme in a particular site may bring a substrate into proximity. Alternatively, adaptors may bind several enzymes consecutively to provide an efficient way of initiating signal transduction (Pawson and Scott, 1997). Identification of the interactions between adaptor proteins and their binding partners has led to a better understanding of individual signaling pathways and their responses to various stimuli.

Appreciation for adaptor proteins began with the identification of modules within proteins, such as the Src-homology 2 (SH2) domain (Sadowski *et al.*, 1986). This domain, located at the amino terminus of the Src non-receptor tyrosine kinase, was also present in other signaling molecules. Biochemical analyzes revealed that SH2 domains were involved in protein-protein interactions (Moran *et al.*, 1990). Subsequently, several other protein-binding modules were identified, and with this came the identification of the conserved motifs that they bind, e.g. SH2 domains associate with phosphorylated tyrosine residues, with surrounding amino acids providing substrate specificity for SH2-containing proteins (Mayer *et al.*, 1991, Hidaka *et al.*, 1991). These modules have been proven to be essential for protein function. Mutagenesis of the SH2 and SH3 domains of *v-src* was able to block its ability to transform cells, while having little effect on the tyrosine kinase activity of the molecule (Wilkerson *et al.*, 1985).

Much attention has been focussed on complexes regulated by tyrosine phosphorylation, while phosphorylation of proteins on serine and threonine residues had been thought of as an allosteric modification rather than one that may mediate protein-protein interaction (Yaffe and Smerdon, 2001). Recently however, a variety of signaling molecules and protein domains have been described that specifically interact with short motifs containing phosphoserine/threonine (pSer/Thr), recruiting proteins that contain such motifs into

signaling complexes. These pSer/Thr-binding modules include WW domains, forkhead-associated (FHA) domains, WD40 repeats, LRR modules in F-box proteins and 14-3-3 proteins.

14-3-3 proteins belong to a family of 28 – 33 kDa polypeptides, of which there are seven isoforms in mammals (β, γ, ε, η, σ, τ and ζ). They are expressed at high levels in all eukaryotic cells and are highly conserved in amino acid sequence from yeast to mammals. 14-3-3 proteins consist of nine α-helices and assemble to form homo- and heterodimers (Jones *et al.*, 1995). The 14-3-3 isoforms interact with different cellular proteins, e.g. ζ binds and activates c-Raf kinase (Freed *et al.*, 1994), whereas τ binds Bcr and Bcr-Abl kinases (Reuther *et al.*, 1994). The finding that Bcr and Raf exist in a complex mediated by 14-3-3 (Braselmann and McCormick, 1995) demonstrated that a 14-3-3 dimer can simultaneously bind two proteins. This observation indicated that 14-3-3 proteins can act as adaptors that may bring together diverse signaling pathways to regulate a variety of cellular processes.

1.4.2 14-3-3 target binding

Early studies suggested that 14-3-3 interactions involved binding to phosphoserine residues (Michaud *et al.*, 1995). Further investigation using synthetic phosphopeptides identified a specific motif optimal for association with 14-3-3 proteins (Muslin *et al.*, 1996). This motif is defined as RSXpSXP, where pS represents phosphoserine and X denotes any amino acid. Amino acid substitution demonstrated that the arginine, serine and proline surrounding the phosphoserine were required for high affinity interaction with 14-3-3. Subsequent studies using peptide libraries identified two distinct motifs for optimal 14-3-3 binding: RSXpSXP and RXXXpSXP (Yaffe *et al.*, 1997). This mechanism of 14-3-3 binding to specific phosphoserine-containing motifs is analogous to SH2 domains that bind phosphotyrosine in a sequence-specific manner.

While many of the 14-3-3 interacting proteins identified to date contain the RSXpSXP or RXXXpSXP motif, there are several well characterized proteins that interact with 14-3-3 but do not contain either motif, including IRS-1, Cbl and Wee1 (Craparo *et al.*, 1997, Liu *et al.*, 1997, Honda *et al.*, 1997). This suggests some variation in the sequence is tolerable for 14-3-3 binding. Furthermore, recent studies suggest that binding at two different sites is

necessary for stable association with 14-3-3, such as for Raf (Li *et al.*, 1995, Rommel *et al.*, 1996), where more than one phosphorylation site is required for binding.

Importantly, 14-3-3 has also been shown to bind a variety of non-phosphorylated proteins efficiently, such as Exoenzyme S (Masters *et al.*, 1999) and Cdc25B (Mils *et al.*, 2000) as well as to non-phosphorylated synthetic peptides (Wang *et al.*, 1999). Interestingly, the amino acids of 14-3-3 that mediate phosphopeptide binding also mediate binding to these non-phosphorylated proteins (Wang *et al.*, 1999). Selection of non-phosphorylated 14-3-3 binding peptides using phage display libraries defined a sequence related to the phosphoserine motif, viz. $RSX_{(1-4)}E$. In addition, 14-3-3 proteins have been seen to bind the non-phosphorylated zinc finger domain of Raf, which is sequence rich in cysteine and histidine residues (Petosa *et al.*, 1998).

1.4.3 Modes of regulation through 14-3-3

Over 50 different proteins have been shown to bind 14-3-3. While many of these are involved in regulating cell cycle progression or activation of the Erk1/2 subfamily of MAP kinases, several 14-3-3 binding proteins are effectors of apoptosis, including the kinase ASK1 (Zhang, 1999 #78), the forkhead transcription factor FKHRL1 (Brunet, 1999 #30) and the Bcl2 family member BAD (Zha, 1996 #21). Other 14-3-3 binding proteins include several transcriptional activators and co-activators (Kanai, 2000 #265), human telomerase (Seimiya, 2000 #226) and histone deacetylases 4 and 5 (Grozinger, 2000 #58).

There are a variety of mechanisms by which 14-3-3 proteins regulate target protein function. 14-3-3 binding can alter the ability of a protein to interact with other molecules, e.g. the regulation of BAD by 14-3-3. In response to various signals, BAD is phosphorylated on Ser-112, -136 and -155 by various kinases including Akt and PKA creating 14-3-3 binding sites (Zha *et al.*, 1996, Datta *et al.*, 2000, Tan *et al.*, 2000). 14-3-3 binding interferes with the ability of BAD to inhibit Bcl2, resulting in the inhibition of apoptosis and the promotion of cell survival. Other examples where 14-3-3 binding interferes with target protein interaction include 14-3-3 inhibiting Cbl association with downstream effector molecules (Liu *et al.*, 1999) and 14-3-3 preventing IRS-1 from binding phosphatidylinositol 3-kinase (Kosaki *et al.*, 1998).

14-3-3 binding can also modify the cytoplasmic/nuclear localization of a protein by increasing nuclear export, decreasing nuclear import or both. Proteins shown to be regulated in this way include Cdc25c (Dalal *et al.*, 1999, Kumagi and Dunphy, 1999, Lopez-Girona *et al.*, 1999), telomerase (Seimiya *et al.*, 2000), histone deacetylase (Grozinger and Schreiber, 2000), and FKHRL1 (Brunet *et al.*, 1999). Binding of a 14-3-3 dimer to Cdc25c masks a nuclear localization signal which lies in close proximity to the 14-3-3 binding site, thereby preventing the importin-mediated nuclear import of Cdc25c (Yang *et al.*, 1999). Conversely, 14-3-3 promotes the nuclear localization of hTERT, the catalytic subunit of telomerase. The interaction of 14-3-3 with hTERT is not dependent on phosphoserine, but does involve a serine/threonine cluster. Seimiya *et al.* (2000) concluded that 14-3-3 binding obscures a nuclear export signal, resulting in nuclear localization of the protein.

In addition to regulating the subcellular localization of a protein, 14-3-3 binding can also alter the catalytic activity of a protein. For instance, phosphorylation of tryptophan dehydroxylase by calmodulin kinase II does not alter the catalytic activity of this enzyme, whereas subsequent binding of 14-3-3 increases its activity several fold (Ichimura *et al.*, 1988). Similarly, association of 14-3-3 with p53 increases its DNA binding (Waterman *et al.*, 1998) and interaction with serotonin *N*-acetyl transferase enhances its activity (Obsil *et al.*, 2001). In contrast, the binding of 14-3-3 to ASK-1 inhibits kinase activity (Zhang *et al.*, 1999), while association with DAF-16 decreases its ability to attach to DNA (Cahill *et al.*, 2001). 14-3-3 binding is also able to protect proteins from dephosphorylation, such as Raf, histone and BAD (Thorson *et al.*, 1998, Chen and Wagner, 1994, Chiang *et al.*, 2001). As mentioned earlier, 14-3-3 can serve as a phosphorylation-dependent adaptor between two proteins, e.g. for Raf and Bcr (Braselmann and McCormick, 1995), Raf and A20 (Vincenz and Dixit, 1996) and Raf and PKC (Van Der Hoeven *et al.*, 2000).

1.4.4 14-3-3 proteins in cancer

14-3-3 proteins have been implicated in several different types of cancers. This is mainly due to the role of 14-3-3 in negative regulation of the cell cycle via binding to CDK2 (Laronga *et al.*, 2000). However, the anti-apoptotic role of 14-3-3 in binding to BAD and FHKRL1 indicates it can act as a pro-survival factor that may be exploited in cancers. Moreover, 14-3-

3 family members were found to associate with the Bcr-Abl fusion protein, generated by a t(9;22) associated with chronic myelogenous leukemias and it was speculated that binding to 14-3-3 created a link between the Bcr-Abl signaling pathway and cell cycle regulation (Reuther *et al.*, 1994). Recent studies suggest that Bcr-Abl also binds 14-3-3 to inhibit the pro-apoptotic activities of BAD (Salomoni *et al.*, 2000). Therefore, 14-3-3 is involved in Bcr-Abl activated anti-apoptotic and proliferative pathways which suggests it may be a useful therapeutic target for cancer treatment.

Recently, there have been several reports on the aberrant regulation of 14-3-3 in a number of tumor types. Inactivation of 14-3-3σ gene expression by hypermethylation has be described as a early event in breast cancer (Umbricht *et al.*, 2001), hepatocellular carcinoma (Iwata *et al.*, 2000), gastric carcinoma (Kang *et al.*, 2002), vulval squamous neoplasia (Gasco *et al.*, 2002) and small cell lung cancers (Osada *et al.*, 2002). In small cell lung cancers, homozygous deletion of 14-3-3ε has been observed; cells containing this mutation also display impairment at the G_2/M checkpoint of the cell cycle that normally prevents cells with damaged DNA from entering mitosis. Importantly, G_2 is regulated by CDK2, a 14-3-3-partner protein (Laronga *et al.*, 2000), and re-introduction of 14-3-3ε restored G_2/M checkpoint responses (Konishi *et al.*, 2002).

The role of 14-3-3 at the G_2/M checkpoint has also been reported in breast cancers. Yarden *et al.* (2002) showed that the tumor suppressor gene, BRCA1, is essential for activation of the Chk1 kinase that regulates DNA damage-induced G_2/M arrest. Cells that have lost BRCA1 display abnormal responses to DNA damage (Yarden *et al.*, 2002), while Chk1 phosphorylates several 14-3-3 binding proteins, namely Cdc25c (Peng *et al.*, 1997) and Wee1 (Lee *et al.*, 2001). Therefore, BRCA1 affects cell cycle progression through the regulation of 14-3-3 and binding of it to target proteins.

1.4.5 Kinases and 14-3-3 regulation

While many binding partners for 14-3-3 have been identified, the kinases responsible for phosphorylating the 14-3-3 binding site are not well characterized. Only five kinases have been identified that specifically phosphorylate proteins to promote 14-3-3 binding. One of these is Akt, an important component of pro-survival signaling pathways. Many survival

factors that suppress apoptosis do so by activation of Akt, which in turn phosphorylates components of the apoptotic machinery, including BAD. This leads to inactivation of these proteins by binding of 14-3-3 (Datta *et al.*, 2000, Brunet *et al.*, 1999)

Akt also acts as a pro-survival factor by regulating transcription. Exposure of cells to survival factors stimulates translocation of Akt to the nucleus where it has been shown to phosphorylate FKHRL1, at Thr-32 and Ser-253 (Brunet *et al.*, 1999). Unphosphorylated FKHRL1 localizes primarily in the nucleus, whereas Akt-phosphorylated FKHRL1 is predominantly cytoplasmic. Subsequently, it was revealed that cytoplasmic retention of FKHRL1 was mediated by binding to 14-3-3 (Brunet *et al.*, 1999).

A few other kinases, viz. Chk1 and C-TAK1, have also been shown to promote 14-3-3 binding to target molecules. As mentioned above, Chk1 phosphorylates CDC25C to promote 14-3-3 binding and inhibits nuclear import (Peng *et al.*, 1997). CDC25C is predominantly phosphorylated on Ser-316 (Ogg *et al.*, 1994) and this mediates 14-3-3 binding (Peng *et al.*, 1997). In *Schizosaccharomyces pombe*, the kinase Cds1 has also been shown to phosphorylate CDC25C on Ser-216 (Zeng *et al.*, 1998). Other studies have isolated a novel serine kinase that bound residues 200-256 of CDC25C (Ogg *et al.*, 1994). This protein kinase was cloned and named C-TAK1 for CDC twenty-five C associated protein kinase. C-TAK1 is a ubiquitously expressed protein which is quite distinct from Chk1. Co-expression of CDC25C and C-TAK1 resulted in enhanced phosphorylation of CDC25C on Ser-216. In addition, a physical interaction between the two proteins was observed suggesting that this kinase was more likely to promote 14-3-3 binding to CDC25C than Chk1 (Peng *et al.*, 1998).

Raf-1 is a serine/threonine protein kinase that plays a critical role in mitogenic signal transduction. Raf-1 activation requires 14-3-3 binding, which is regulated through phosphorylation of Ser-259 and Ser-621; however, the kinases that phosphorylate these sites remain unknown. One study that utilized recombinant glutathione-S-transferase (GST)-Raf-1 fusion proteins as substrates for in-gel kinase assays suggested that a 90 kDa kinase may phosphorylate Raf-1 on Ser-259, whereas a Raf-1 fragment containing Ser-621 was phosphorylated by kinases with molecular weights of 85, 60, 50 and 48 kDa (Kinuya *et al.*,

2000). These results suggest that although Ser-259 and Ser-621 lie in the same consensus motif for 14-3-3 binding, they are phosphorylated by different protein kinases.

1.5 Protein kinases

Of the molecules found to interact with Mlf1 in a yeast two-hybrid screen, three were novel proteins. One of these proteins (M2) had a unique sequence with no recognizable domains or motifs. Another interacting molecule (M58) had closest homology to heterogeneous nuclear ribonucleoprotein U, also known as scaffold attachment factor A, a nuclear matrix protein (Romig *et al.*, 1992). M58 contains a SAP motif (named after SAF-A/B, Acinus and PIAS proteins), which is a putative DNA-binding domain found in many nuclear proteins (Aravind and Koonin, 2000). It also contains an SPRY domain, which to date has no known function, and a P loop for binding ATP. The presence of two bipartite nuclear localization signals suggested a role for M58 in the nucleus. Interestingly, a NAP (nucleosome assembly protein) motif, involved in regulating gene expression by histone modification (Rodriguez *et al.*, 1997), indicated M58 may provide a link between Mlf1 and transcriptional control. The third Mlf1-interacting protein, M44, had homology to protein kinases. As protein kinases and phosphatases coordinate many aspects of cellular function, it was speculated that M44 may be involved in a signal transduction pathway that regulated Mlf1 function. Based on sequence analysis alone, M44 could not be classified as either a serine/threonine or tyrosine kinase; however, due to the interaction between Mlf1 and 14-3-3, it was hypothesized that M44 may regulate serine phosphorylation of Mlf1.

Eukaryotic protein kinases make up one of the largest superfamilies of homologous proteins and genes. In 1987, it was predicted that the mammalian genome contains about 1000 protein kinase genes (Hunter, 1987). Subsequent analysis of the completed human genome sequence indicated there are approximately 500 kinase genes in total (Wain *et al.*, 2002). Kinases use the γ-phosphate of ATP (or GTP) to phosphorylate amino acids containing alcohol groups (on serine or threonine) and/or phenolic groups (on tyrosine) which act as the phosphate acceptors (Hanks and Hunter, 1995). There is also a smaller subgroup of eukaryotic kinases with different catalytic activity. Most of these are related to the prokaryotic histidine protein kinase family, which phosphorylates histidine residues, then transfer the

phospho-group to an aspartate residue (Alex and Simon, 1994). However, a small number of eukaryotic kinases do not qualify as superfamily members, including the Bcr protein involved in the (9;22) translocation (Maru and Witte, 1991) and the A6 kinase (Beeler *et al.*, 1994). Both molecules have kinase domains unrelated to other known eukaryotic or prokaryotic kinase.

The role of tyrosine kinases in signaling initiated by cytokines has been studied extensively. Cytokine receptors lacking intrinsic kinase activity transmit their signal via receptor-associated JAK tyrosine kinases, which activate STAT transcription factors to modulate gene expression (Leonard, 2001). Cytokine receptors with intrinsic tyrosine kinase activity were thought to transduce their signals independent of serine/threonine kinases (McCubrey *et al.*, 2000). However, the involvement of serine/threonine kinases in cytokine-induced signal transduction has since been shown to include the Ras/Raf/MEK/ERK (MAPK) cascade, which results in activation of downstream kinases and transcription factors (Schaeffer and Weber, 1999). Serine/threonine phosphorylation is also involved in regulating anti-apoptotic pathways by controlling the Bcl-2 protein. Importantly, serine/threonine phosphorylation has been implicated in the eitology of cancers (Frank *et al.*, 1997) and novel chemotherapeutic drugs similar to Imatinib are now being targeted at activated serine/threonine kinases (Druker *et al.*, 1996).

1.5.1 Kinase domain structure

The protein kinase domain (or catalytic domain) consists of about 250 – 300 amino acids and has three definable roles: 1) binding and orientation of the ATP/GTP phosphate donor in a complex with divalent cation (usually Mg^{2+} or Mn^{2+}); 2) binding and orientation of the protein/peptide substrate; and 3) transfer of the γ-phosphate from ATP/GTP to the acceptor hydroxyl residue (Ser, Thr, Tyr) of the substrate (Hanks and Hunter, 1995).

By comparing alignments of the catalytic domains of protein kinases, eleven major conserved subdomains (I to XI) have been identified, separated by regions of lower conservation, gaps or inserts (Hanks and Hunter, 1995). The fact that the subdomains display such conservation suggests they must be important for catalytic function, either

directly as part of the active site or indirectly by contributing to the formation of the active site.

Within the subdomains, fifteen amino acids are recognized as being almost invariant throughout the kinase superfamily (conserved in over 95% of 370 sequences) (Hanks and Hunter, 1995). Hence, these amino acids are thought to play essential roles in enzyme function. Using residue numbering for cyclic AMP-dependent protein kinase catalytic subunit alpha (PKA-Cα) these almost invariant residues correspond to: Gly^{50}, Gly^{52} and Val^{57} in subdomain I, Ala^{70} and Lys^{72} in subdomain II, Glu^{91} in subdomain III, Asp^{166} and Asn^{171} in subdomain VI, a Asp^{184}-Phe^{185}-Gly^{186} motif in subdomain VII, Glu^{208} in subdomain VIII, Asp^{220} and Gly^{225} in subdomain XI and Arg^{208} in subdomain XI (Hanks and Hunter, 1995). The majority of these highly conserved amino acids are directly involved in ATP binding and phosphotransfer. With the three-dimensional structure of PKA-Cα solved, precise roles have been assigned for the highly conserved amino acids (Knighton *et al.*, 1991, Zheng *et al.*, 1991). In general the kinase domain folds into a bi-lobed structure. The amino-terminal lobe including subdomains I – IV is involved in anchoring and orienting the adenosine molecule. The larger carboxyl-terminal lobe (subdomains VI – XI) is involved in binding the substrate and mediating phosphotransfer.

The consensus Gly-X-Gly-X-X-Gly motif found in many nucleotide-binding proteins, including protein kinases, is found in subdomain I and involves Gly^{50} and Gly^{52} of PKA-Cα. Based on three-dimensional structures, the Gly-X-Gly-X-X-Gly forms an elbow around the adenosine molecule with the first glycine in contact with the ribose moiety and the second glycine near the terminal pyrophosphate (Sternberg and Taylor, 1984). In subdomain II there is an invariant lysine (Lys^{72} in PKA-Cα) that helps anchor and orient ATP by interacting with the α- and β-phosphates (Zheng *et al.*, 1991). Substitution of alternate amino acids at this site results in loss of protein kinase activity. The highly conserved residues in subdomain VI (Asp^{166} and Asn^{171}) and in subdomain VII (the Asp^{184}-Phe^{185}-Gly^{186} motif) have also been implicated in ATP binding. These amino acids make up a feature found in several bacterial phosphotransferases that use ATP as a phosphate donor (Brenner, 1987).

Subdomain VI contains residues that are specifically conserved for either serine/threonine or tyrosine kinases (Hanks and Hunter, 1995). They are thought to be important in recognition of the correct hydroxy amino acid. Specificity for serine/threonine or tyrosine kinases lies between the conserved Asp and Asn in subdomain VI. A strong indicator of serine/threonine specificity is a consensus sequence of Asp-Leu-Lys-Pro-Glu-Asn, whereas for tyrosine kinases the consensus sequence is either Asp-Leu-Arg-Ala-Ala-Asn or Asp-Leu-Ala-Ala-Arg-Asn (Hanks *et al.*, 1988).

Subdomain VIII contains a conserved Ala-Pro-Glu triplet (Hanks and Hunter, 1995). Mutagenesis studies have shown that each residue in the Ala-Pro-Glu motif is required for the activity of Src kinases (Bryant and Parsons, 1983). Furthermore, sites of autophosphorylation for many kinases lie within 20 amino acids of the Ala-Pro-Glu consensus. For several kinases autophosphorylation near this site results in increased catalytic activity (Hanks *et al.*, 1988).

1.6 Project aims

This study has two broad aims. First to characterize the novel protein M44 that bound Mlf1 in a yeast-two hybrid screen. Second to examine the interaction of M44 and Mlf1. The protein was named Madm for Mlf1 adaptor molecule and the specific aims of this study were to:

1. Analyze the sequence and structure of Madm. This included (i) cloning and characterization of the murine Madm gene and generation of DNA constructs for generating Madm -/- mice, (ii) analysis of the Madm mRNA expression profile and (iii) examination of the amino acid sequence of Madm to identify potential functional domains or motifs.

2. Characterize the biochemical nature of Madm, including (i) analysis of the properties of the Madm protein, (ii) its subcellular localization, (iii) expression and purification of recombinant Madm, (iv) analysis of any enzymatic activity and (v) examination of its phosphorylation status.

3. Characterize the interaction between Madm and Mlf1. This included (i) examining the interaction between the two proteins in both yeast and mammalian cell systems,

(ii) analysis of subcellular localization of both molecules, (iii) investigation of Madm-mediated phosphorylation of Mlf1 and (iv) identification of Mlf1 phosphorylation sites. The interaction of Madm and the NPM-MLF1 fusion protein was also examined.

4. Analyze the role of Madm in myeloid maturation, in particular how it may affect terminal differentiation.

Chapter 2: MATERIALS AND METHODS

2.1 Cell Biology

2.1.1 Cell Lines

Cell lines used:

MEL707 (murine erythroleukemia) (Dube *et al.*, 1975)

F4N (murine erythroleukemia) (Ostertag *et al.*, 1972)

J2E (murine erythroleukemia) (Klinken *et al.*, 1988b)

J2E-NR (murine erythroleukemia) (Klinken and Nicola, 1990)

M1 (murine monoblastoid) (Lotem *et al.*, 1989)

J2E-m1 (murine monoblastoid) (Keil *et al.*, 1995)

J2E-m2 (murine monoblastoid) (Keil *et al.*, 1995)

J2E-m3 (murine monoblastoid) (Keil *et al.*, 1995)

J2E-NRm2 (murine monoblastoid) (Keil *et al.*, 1995)

J2E-NRm3(murine monoblastoid) (Keil *et al.*, 1995)

1Bra/Raf (murine monoblastoid) (Klinken *et al.*, 1988a)

2Mes/Raf (murine monoblastoid) (Klinken *et al.*, 1988a)

3Mes/Raf (murine monoblastoid) (Klinken *et al.*, 1988a)

W265 (murine myeloid) (Walker *et al.*, 1982)

P815 (murine mast cell) (Ralph and Nakoinz, 1977)

3Mes (murine B cell) (Walker *et al.*, 1982)

1-Bra (murine B cell) (Walker *et al.*, 1982)

70Z-3 (murine pre-B cell) (Paige *et al.*, 1978)

EL4 (murine T lymphoblast) (Ralph and Nakoinz, 1977)

COS7 (simian fibroblast) (Gluzman, 1981)

PA317 (murine fibroblast) (Miller and Buttimore, 1986)

NIH/3T3 (murine fibroblast) (Jainchill *et al.*, 1969)

293T (human kidney fibroblast) (Graham *et al.*, 1977)

Hela (human cervical adenocarcinoma) (Puck and Marcus, 1955)

2.1.2 *Mammalian cell culture conditions*

Suspension cell lines

Suspension cells were routinely cultured at 37°C/5% CO_2 in Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen, Carlsbad, USA) containing 5% fetal calf serum (FCS) (JRH Biosciences, Lenexa, USA), 100 IU/mL penicillin and 100 µg/mL streptomycin. Cells were maintained at a density between 0.5 x 10^5 and 2 x 10^6 cells/mL.

Cell viability was determined by exclusion of 0.4% eosin in phosphate buffered saline (PBS), pH 7.3 (140 mM NaCl/40 mM Na_2HPO_4/4 mM NaH_2PO_4). Cell number was determined using a hemocytometer where:

Number of cells/mL = number of cells counted / number of squares counted x 10^4 x dilution factor

Induction of M1 cells

M1 monoblastoid cells were induced to differentiate with Leukemia Inhibitory Factor (LIF) (Chemicon, Temecula, USA) at various concentrations up to 1 ng/mL for maximal induction. Viability was monitored over the course of the induction and differentiation was determined by expression of the cell surface marker, Cd11b (Section 2.1.6).

Adherent cell lines

Adherent cells were cultured at 37°C/5% CO_2 in DMEM containing 10% FCS, 100 IU/mL penicillin and 100 µg/mL streptomycin. Cultures were passaged when 80 - 90% confluency was reached. Trypsin-EDTA (0.05% trypsin, 0.53 mM EDTA) (Invitrogen, Carlsbad, USA) was used to detach cells from the culture dish. Cell viability was assessed by exclusion of 0.5% Trypan Blue in PBS.

Cryopreservation of cells

Cells were cryopreserved at a density of at least 5 x 10^6 cells/mL in DMEM containing 40% FCS and 20% Dimethyl sulfoxide (DMSO) (Sigma, St. Louis, USA). Cells were initially placed at -80°C, then transferred to liquid nitrogen for long term storage.

2.1.3 Cytopreparation of cells

Cells (1 x 10^4) were collected onto clean glass slides by centrifugation using a Cytospin centrifuge (Thermo Shandon, Pittsburg, USA). The slides were air dried for 5 minutes, fixed in ice-cold methanol for 5 min and allowed to dry, then slides were stained with Hematoxylin and Eosin.

2.1.4 Transfection of adherent cells

Cells at 80 - 90% confluency were transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, USA). Typically, 10 μg plasmid DNA diluted in 1 mL antibiotic-free DMEM was mixed with 1 mL antibiotic-free DMEM containing 25 μL Lipofectamine 2000 and incubated at room temperature for 20 min to allow formation of DNA-lipid complexes. Following incubation, 8 mL DMEM containing 10% FCS was added to the DNA-lipid solution, then added to a 10 cm petri dish of cells.

2.1.5 Amphotrophic viral infection of cells

The MSCV-neo 2.2 retroviral vector (Hawley *et al.*, 1994) was used to over-express cDNAs in J2E and M1 cell lines. The PA317 packaging cell line was transfected (Section 2.1.4) with MSCV constructs that had been linearized by digestion with *Sca*I. Transfected cells were selected in Geneticin (Sigma, St. Louis, USA) and culture supernatants containing amphotrophic viral particles were harvested and debris removed by passage through a 0.4 μm filter (Millipore, Billerica, USA). Viral supernatants were then added to J2E or M1 cells before culture in methyl cellulose containing Geneticin (1 mg/mL for J2E cells and 400 μg/mL for M1 cells). Individual clones were isolated for analysis of unique viral integration sites by Southern blot analysis and expression of viral RNA by Northern blotting (Section 2.2).

2.1.6 Flow cytometric analysis of Cd11b

To assess Cd11b expression, 1 x 10^6 differentiating M1 cells were collected, washed in PBS containing 2% FCS and 10 mM sodium azide and incubated with anti-Cd11b antibody (Springer *et al.*, 1979) for 30 min on ice. Following washes, fluorescein isothiocyanate (FITC)-conjugated sheep anti-rat secondary antibody (Silenus, Boronia, Australia) was

added for 30 min on ice in the dark. Cells were washed and resuspended in buffer containing 20 µg/mL propidium iodide to assess cell viability, before analysis on a Beckman-Coulter Epics XL/MCL flow cytometer (Fullerton, USA).

2.1.7 Confocal microscopy

Cells (1 x 10^5) were seeded onto coverslips in 12 well trays, transfected with 0.5 µg plasmid DNA using Lipofectamine 2000 (Section 2.1.4), then fixed and permeabilized in 50% acetone/50% methanol for 5 min at 4°C. For staining, coverslips were blocked for 30 min with 3% BSA in Tris-buffered saline-Tween-20 (TBS-T: 20 mM Tris.HCl pH 7.5, 150 mM NaCl, 0.05% Tween-20). The appropriate primary antibody was added for 1 h. After washing (3 x 5 min, 3% BSA-TBS-T), fluorescently-labeled secondary antibodies were applied for 60 min in the dark. The secondary antibodies used were either AlexaFluor™ 488 (green) anti-mouse or anti-rabbit IgG or AlexaFluor™ 546 (red) anti-mouse or anti-rabbit IgG was used (Molecular Probes, Eugene, USA). Following washes, coverslips were mounted in 50 mM Tris.HCl pH 8, 50% glycerol, 2.5% 1,4-diazobicyclo-[2.2.2]-octane (DABCO) (Fluka, Buchs, Switzerland) containing 0.00005% Hoechst 33258 (CalBiochem, San Diego, USA). For visualization of green-fluorescent protein (GFP) fusion proteins, cells were fixed and mounted as described above. Fluorescence was visualized on a Bio-Rad MRC 1024 UV Laser Scanning Confocal Microscope (Hercules, USA).

2.1.8 Cell synchronization analysis

For cell cycle synchronization, cells were arrested at the G_1/S phase border by the addition of 2 mM thymidine to the culture medium. Following incubation overnight, thymidine was removed by washing the cells three times with PBS, after which fresh media was applied. Cells were harvested at specified time points following release from the thymidine block.

Flow cytometry was used to determine the phase of the cell cycle when cells were harvested. Fresh, or ethanol-fixed, cells (1 x 10^6) were washed in PBS and incubated in staining buffer (PBS containing 0.1% Nonidet P-40, 30 mM EDTA, 30 mM EGTA, 5 µg/mL propidium iodide and 0.1 mg/mL RNase A) for 30 min on ice, before analysis on a Beckman-Coulter Epics XL/MCL flow cytometer.

2.2 Molecular biology

2.2.1 Preparation and digestion of genomic DNA

Genomic DNA was prepared using the method of Wang *et al.* (Wang *et al.*, 1994). Briefly, 1 x 10^7 cells were resuspended in lysis buffer (1% Triton X-100, 0.32 M Sucrose, 5 mM $MgCl_2$, 10 mM Tris.HCl pH 7.5). Nuclei were pelleted by brief centrifugation (20 s) at 10 000 x g, and washed in 1 mL lysis buffer. Nuclei were disrupted by addition of a solution containing 1% SDS, 5 mM EDTA, 10 mM Tris.HCl (pH 8). RNA was removed by incubation with 25 µg/mL RNase A at 37°C for 10 min, before proteinase K was added to a final concentration of 85 µg/mL and samples incubated at 37°C for 60 min. Following incubation, samples were mixed with 0.3 mL NaI solution (7.6 M NaI, 20 mM EDTA, 40 mM Tris.HCl pH 8) and genomic DNA precipitated by addition of 0.5 mL isopropanol. DNA was collected by centrifugation at 10 000 x g for 10 min and washed initially with 40% isopropanol, then 70% ethanol. DNA pellets were dried and resuspended in TE buffer (10 mM Tris.HCl pH 8, 1 mM EDTA).

For restriction enzyme digestion, 10 µg genomic DNA was incubated with 75 U enzyme in a total reaction volume of 300 µL at 37°C overnight. DNA was precipitated by addition of 0.1 volumes of 3 M sodium acetate and 2.5 volumes of 100% ethanol at -80°C for 30 min. DNA was pelleted by centrifugation at 10 000 x g for 30 min at 4°C, washed with 70% ethanol and resuspended in 20 µL TE buffer.

2.2.2 Genomic DNA electrophoresis and Southern blotting

Following restriction enzyme digestion of genomic DNA (Section 2.2.1), DNA loading dye (0.05% bromophenol blue, 0.05% xylene cyanol, 30% glycerol) was added to each sample. DNA was electrophoresed in a 0.7% agarose gel in TAE buffer (40 mM Tris, 20 mM EDTA, 0.11% glacial acetic acid) at 80 V. After electrophoresis, gels were treated with denaturing solution (1.5 M NaCl, 0.5 M NaOH) for 40 min, washed twice with ddH_2O then soaked with two changes of neutralizing solution (0.5 M Tris.HCl pH 7.4, 1.5 M NaCl) for 1 h. DNA was transferred to Hybond N+ (Amersham, Bucks, UK) in 10 x SSC (1.5 M NaCl, 150 mM trisodium citrate) by capillary action overnight and membranes were rinsed in 2 x SSC (300

mM NaCl, 30 mM trisodium citrate). DNA was fixed to the membrane by UV cross linking using a Stratalinker (Stratagene, La Jolla, USA).

2.2.3 *Preparation of RNA*

Total cellular RNA was extracted using the method of Chomczynski and Sacchi (Chomczynski and Sacchi, 1987). Briefly, 0.4 g tissue, or 1 x 10^7 cells, were resuspended in 4 mL Solution D (4 M guanidinium thiocyanate, 25 mM sodium citrate pH 7, 0.1 M N-lauroylsarcosine, 100 mM 2-mercaptoethanol in diethylpyrocarbonate (DEPC)-treated water) and homogenized for 30 s. To these homogenates, 0.4 mL 2 M sodium acetate pH 4, 4 mL DEPC-treated water-saturated phenol and 0.8 mL chloroform isoamylalcohol (49:1 v/v) were added sequentially. Preparations were vortexed, incubated on ice for 15 min and centrifuged at 4000 x g for 20 min at 4°C to separate organic and aqueous phases. RNA was precipitated from the aqueous phase by the addition of 2.5 volumes 100% ethanol, followed by storage at –80°C. An aliquot of precipitated RNA was pelleted by centrifugation (10 000 x g for 20 min at 4°C), washed with 70% ethanol and resuspended in RNA sample buffer (1.75% formaldehyde (37%), 50% formamide in 1 x MOPS buffer (20 mM 3-[N-morpholino] propane sulphonic acid (MOPS), 5 mM sodium acetate, 1 mM EDTA, pH 7)).

2.2.4 *RNA electrophoresis and Northern blotting*

Prior to electrophoresis, RNA (10 μg) in RNA sample buffer was denatured at 65°C, quenched on ice and 2 μL loading dye added (0.05% bromophenol blue, 0.05% xylene cyanol, 30% glycerol). Samples were loaded onto formaldehyde gels (1.2% w/v agarose, 1.8% v/v formaldehyde (37%), 1 μg/mL ethidium bromide in 1 x MOPS buffer). Following electrophoresis (80 V for 2 h) gels were destained in ddH_2O, before visualization of RNA using an UV transilluminator. RNA was transferred to Hybond N+ (Amersham, Bucks, UK) in 10 x SSC by capillary action overnight and membranes were rinsed in 2 x SSC. RNA was fixed to the membrane by UV cross linking using a Stratalinker (Stratagene, La Jolla, USA).

2.2.5 *Hybridization*

DNA probes were excised from plasmids by restriction enzyme digestion, separated on agarose gels and purified using a gel purification system (Invitrogen, Carlsbad, USA), then

100 ng of probe was labeled with [α-^{32}P]dCTP (Amersham, Bucks, UK) using a random prime labeling kit (Amersham, Bucks, UK) according to the manufacturer's instructions. Labeled probes were denatured at 100°C for 5 min and immediately quenched on ice.

Membranes were pre-hybridized at 65°C in RapidHyb (Amersham, Bucks, UK) for 30 min, prior to the addition of radiolabeled probe for 2 h. Membranes were washed in 2 x SSC/ 0.1% SDS for 15 min at 65°C, followed by 1 x SSC/0.1% SDS for 15 min at 65°C and finally 0.1 x SSC/0.1% SDS for 15 min at 42°C. Hybridized DNA or RNA was detected using a Molecular Dynamics 445SI Phosphorimager (Amersham, Bucks, UK).

2.2.6 *Polymerase chain reaction*

Typically a polymerase chain reaction (PCR) would contain 100 ng DNA template, 2 mM $MgCl_2$, 50 µM dNTP, 10 pmol of each primer and 0.5 U *Taq* polymerase (Invitrogen, Carlsbad, USA) in a volume of 20 µL in the buffer supplied by the manufacturer. DNA was denatured by heating reactions to 95°C for 45 s, primers were annealed at approximately 5°C above their melting temperature and DNA extended at 72°C for 1 min per kilobase of DNA. Generally 30 cycles were completed.

When PCR was performed to amplify DNA to use for protein expression, a DNA polymerase with 3' – 5' exonuclease (proofreading) activity was used. These were performed using either *Pfu* polymerase (Promega, Madison, USA) or *Pfx* polymerase (Invitrogen, Carlsbad, USA). *Pfu* reactions were done in 1 x reaction buffer (supplied by the manufacturer) containing 250 µM dNTP, 10 pmol of each primer, 50 ng DNA template and 1.5 U *Pfu* polymerase. Cycling was identical to that described for *Taq* polymerase. Reactions for *Pfx* polymerase were performed in 1 x amplification buffer and 1 x enhancer buffer (supplied by the manufacturer) and contained 1 mM $MgSO_4$, 200 µM dNTP, 10 pmol of each primer, 50 ng DNA template and 1 U *Pfx* polymerase. Reactions were cycled as described for *Taq* polymerase, but with the extension temperature lowered to 68°C for maximal activity of the *Pfx* enzyme.

2.2.7 *Site-directed mutagenesis*

For site-directed mutagenesis, the desired mutation was inserted into sense and antisense primers complementary to the region of interest, with the mutation in the center of the primer

containing 16 bp either side of the mutation. For amino acid substitutions, the codon most preferred in mammalian cells was selected as the mutation. Two individual PCR products were generated using *Pfu* polymerase (Section 2.2.6); the first product was amplified using the 5' primer for the fragment of interest and the mutated antisense primer, while the second product was amplified using the 3' primer for the fragment of interest and the mutated sense primer. These PCR products were separated in agarose gels and purified using a gel purification system (Invitrogen, Carlsbad, USA). Purified PCR products were digested with *Dpn*I (2 U) in 80 µL reactions at 37°C for 1 h. Since *Dpn*I only digests methylated DNA, this removed the original DNA template (plasmid DNA purified from bacteria) without digesting the PCR products. Both PCR products (40 ng) were then mixed and used as a template for a second PCR using *Pfu* polymerase as described, and the 5' and 3' primers that were used in the two original PCRs. The full length PCR product was subsequently gel-purified for cloning. This technique was also modified to delete large segments from within DNA fragments. In this instance, primers were synthesized that covered 18 nucleotides 5' and 18 nucleotides 3' of the desired region for deletion.

2.2.8 Cloning

Vector and insert DNA were digested with appropriate restriction enzymes and purified by agarose gel electrophoresis followed by gel extraction. For cloning of PCR products, restriction sites were introduced into the ends of primers. Primers were also designed for cloning into vectors that contained protein tags for the purpose of protein purification such that reading frame was retained. After PCR amplification, products were gel-purified, digested with appropriate restriction enzymes to cut the ends of the primers and further purified by using a PCR purification system (Invitrogen, Carlsbad, USA).

Fragments were ligated using T4 DNA Ligase (Promega, Madison, USA) in 20 µL reactions containing about 100 ng DNA, with vector and insert DNA mixed in a ratio of 1:3 and 10 U enzyme made up in the buffer provided by the manufacturer. Ligation reactions were incubated at room temperature for 1 – 3 h or at 16°C overnight and 5 µL was used to transform 50 µL XL2-Blue *Escherichia coli* (Stratagene, La Jolla, USA) made competent using the method of Inoue *et al.* (Inoue *et al.*, 1990). Bacteria were incubated on ice for 30

min and heat-shocked at 42°C for 30 s before being returned to ice for 2 min. Bacteria were allowed to recover by incubation in 0.5 mL SOC medium (20 g/L bacto-tryptone, 5 g/L yeast extract, 8 mM NaCl, 2.5 mM KCl pH 7, 20 mM glucose) at 37°C for 1 h. Transformed bacteria were plated onto Lauria Bertani (LB) agar plates (10 g/L bacto-tryptone, 5 g/L yeast extract, 170 mM NaCl, 15 g/L agar) containing appropriate antibiotics for selection of positive transformants.

2.2.9 Sequencing

Sequencing reactions were prepared using a dye terminator sequencing kit (Applied Biosystems, Foster City, USA) according to manufacturers instructions and sequence information was collected using 373A automated sequencer (Applied Biosystems, Foster City, USA). DNA sequences were edited using SeqEd software (Applied Biosystems, Foster City, USA).

2.2.10 Library screening

A murine strain 129 genomic library that was constructed by a partial *Sau*3A digest and cloned into *Bam*HI sites of Lambda DASH™ II (Stratagene, La Jolla, USA) was screened for clones containing the *Madm* gene. Overnight cultures of XL1-Blue *E. coli* (Stratagene, La Jolla, USA) grown in LB broth containing 20 mM maltose and 10 mM $MgSO_4$. The library titre was calculated to be 6 x 10^8 plaque forming units (pfu)/mL. For the primary screen, 5 x 10^5 pfu were used to inoculate 6 mL XL1-Blue overnight culture. Infected bacteria were divided into 10 tubes. Added to each tube was 7 mL LB-agarose (10 g/L bacto-tryptone, 5 g/L yeast extract, 170 mM NaCl, 7.2 g/L agarose) containing 10 mM $MgSO_4$ that had been prewarmed to 45°C. Tubes were mixed by inversion and poured evenly over prewarmed (37°C) 15 cm diameter LB agar plates (10 g/L bacto-tryptone, 5 g/L yeast extract, 170 mM NaCl, 15 g/L agar) containing 10 mM $MgSO_4$. Plates were incubated at 37°C until the plaques reached a diameter not exceeding 1.5 mm or were just beginning to make contact with each other (9 –10 h). Plates were chilled at 4°C for to allow the agarose to harden. Numbered Hybond N+ filters were laid onto LB-agarose, incubated for 2 min then removed and soaked in denaturing solution (1.5 M NaCl, 0.5 M NaOH) and neutralizing solution (1.5 M NaCl, 0.5 M Tris.HCl pH 7.4) for 5 min each. DNA was fixed to membranes by UV cross linking using a

Stratalinker (Stratagene, La Jolla, USA). Membranes were hybridized as described in Section 2.2.5 and exposed to X-OMAT autoradiography film (Kodak, New Haven, USA). Films were aligned with membranes and agar plugs of positive plaques were removed into 1 mL lambda dilution buffer (100 mM NaCl, 35 mM Tris.HCl pH 7.5, 10 mM $MgSO_4$, 2% gelatin). After overnight elution, phage were replated to obtain 100 – 500 plaques on a 10 cm plate. Plaques were re-screened twice and a single, well-isolated plaque was eluted into 500 µL lambda dilution buffer for DNA purification.

2.2.11 Isolation of bacteriophage DNA

To amplify phage, the eluted plaque (about 250 µL) from library screening was used to infect 1 mL overnight culture of XL1-Blue grown in LB broth containing 20 mM maltose and 10 mM $MgSO_4$. Infected bacteria were placed in five tubes and mixed with 3 mL LB-agarose then poured onto LB agar plates containing 10 mM $MgSO_4$. Plates were incubated at 37°C for 9 – 10 h or until plaques became confluent. The agarose layers were scraped into 10 mL lambda dilution buffer and phage were eluted at room temperature for 30 min with vigorous shaking. Agarose was removed by centrifugation at 10 000 x g for 10 min at 4°C. Phage DNA was purified using Wizard® Lambda Preps DNA Purification System (Promega, Madison, USA) according to the manufacturer's instruction. Insert DNA was separated from vector by digestion with *Not*I. Insert DNA was digested into smaller fragments with appropriate restriction enzymes and cloned into pBluescript KS+ or pZero-1 (Section 2.2.8) for sequencing (Section 2.2.9).

2.2.12 Yeast transformation

Yeast transformation was based on the method originally devised by Fields and Song (Fields and Song, 1989). L40 yeast strain was grown overnight in YPAD (10 g/L yeast extract, 20 g/L bacto-peptone, 0.1 g/L adenine, 2% glucose). Cultures were diluted to OD_{600nm} of 0.4 and grown for an additional 2 h at 30°C. Yeast cells were harvested by centrifugation at 1000 x g for 10 min at room temperature. Pellets were resuspended and harvested successively in 0.8 volumes and 0.1 volumes of sterile water. Yeast were made competent using lithium acetate, alkaline cations and polyethylene-glycol (PEG). Following the final wash, pellets were resuspended in a buffer consisting of 100 mM lithium acetate, 5 mM Tris.HCL pH 8 and

0.5 mM EDTA, then incubated at room temperature for 10 min. Plasmid DNA (1 μg) and denatured salmon sperm DNA (10 μg) were added to 100 μL yeast suspension. The yeast/DNA mixture was mixed thoroughly with 700 μL transformation buffer (100 mM lithium acetate, 40% PEG-4000, 10 mM Tris.HCl pH 8, 1 mM EDTA) and incubated at 30°C for 30 min. Following incubation, 88 μL DMSO was added and yeast were heat shocked at 42°C for 7 min. Yeast were collected by centrifuging briefly at 10 000 x g and pellets were washed once with 1 mL TE buffer before plating on selective medium. Yeast were grown on minimal agar (0.17% (w/v) yeast nitrogen base, 2% glucose, 40 mM ammonium sulfate, 15 g/L agar) containing 0.1 g/L adenine and 0.05 g/L histidine, however for selection of the pBTM116 and pVP16 vectors the media lacked tryptophan and leucine respectively.

2.2.13 β-Galactosidase reporter assay

Yeast colonies grown on minimal agar containing 0.1 g/L adenine and 0.05 g/L histidine were lifted onto a nitrocellulose filter and dried. Filters were cooled by floating above liquid nitrogen for 30 s and yeast were permeabilized by immersion in liquid nitrogen for 10 s. Membranes were thawed for 5 min before placing on filter paper pre-soaked with Z buffer (100 mM sodium phosphate pH 7, 10 mM potassium chloride, 1 mM $MgSO_4$, 0.75 mg/mL X-Gal, 40 mM β-mercaptoethanol). Membranes were incubated at 30°C and monitored for blue color formation after 1 h.

2.2.14 Whole mount in situ hybridization

Preparation of RNA probe

Plasmid DNA (5 μg) was linearized using 30 U of restriction enzyme in 50 μL of appropriate buffer solution and incubated as 37°C for 2 h. Linearized DNA was purified by phenol/chloroform extraction followed by ethanol precipitation and the extent of digestion analyzed by electrophoresis. For transcription of a RNA digoxygenin (DIG)-labeled probe, 1 μg linearized DNA was placed in RNase-free autoclaved tube with the following reagents: 50 U T7 or T3 RNA polymerase, 1 x transcription buffer (Promega, Madison, USA), 10 mM DTT (Promega, Madison, USA), 50 U RNAsin (Promega, Madison, USA), 1 mM ATP, 1 mM CTP, 1 mM GTP, 0.65 mM UTP, 0.35 mM DIG-11 UTP (Roche, Basel, Switzerland) and made to a

total volume of 25 µL. The reaction was incubated for 2 h at 37°C, after which the DNA was digested with 5 U RNase-free DNase (Promega, Madison, USA) for 15 min and the reaction stopped by the addition of 2 µL 0.5 M EDTA. RNA was precipitated using 0.1 volumes 4M LiCl and 3 volumes 100% ethanol, placed at –80°C for a minimum of 30 min before 20 min centrifugation at 4°C. The resulting pellet was washed in 70% ethanol, air-dried and resuspended in 20µL sterile water.

Pre-treatment and hybridization of embryos

Embryos were dissected in PBS and fixed overnight in 4% paraformaldehyde in PBS. Embryos were washed twice in PTW (0.1% (v/v) Tween-20 in PBS) and dehydrated by sequential washes in PTW/25% methanol, PTW/50% methanol, PTW/75% methanol and two washes in 100% methanol then stored at –20°C until ready for use. Rehydration was performed by taking the embyros through these steps in reverse order. Embryos were bleached with 6% hydrogen peroxide in PTW for 1 h then washed three times in PTW. Treatment with 10 µg/mL Proteinase K in PTW was performed to make the embryos more accessible to probe by incubation for 4 min followed by washing in freshly prepared 2 mM glycine in PTW. Embryos were refixed in fresh 0.2% glutaraldehyde/4% paraformaldehyde/PBS for 20 min followed by two washes in PTW.

After transfer into a smaller tube, embyros were rinsed once in PTW/Hybridization mix (1:1), once in Hybridization mix and pre-hybridized in Hybridization mix (50% (v/v) formamide, 1.3 x SSC, 5 mM EDTA pH 8, 50 µg/mL tRNA, 0.2% Tween-20, 0.5% (w/v) 3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), 100 µg/mL heparin) for 2 h at 65°C. Following removal of the pre-hybridization mix, pre-warmed Hybridization mix containing 1 µg/mL DIG-labeled RNA probe was added to embryos and incubated overnight at 65°C.

Following hybridization embryos were washed twice in Solution 1 (50% formamide, 5 x SSC, 1% SDS), then twice in Solution 2 (50% formamide, 2 x SSC) at 65°C. Embryos were rinsed once in Solution 2/MAB (100 mM maleic acid, 150 mM NaCl) (1:1 mix), rinsed three times in

MAB, washed in MAB for 30 min followed by a wash in MABT (100 mM Maleic acid, 150 mM NaCl, 0.1% (v/v) Tween-20) for 30 min.

Antibody detection and staining

Embryos were blocked in MABT containing 2% blocking reagent (Boehringer, Ingelheim, Germany) and 20% heat-inactivated lamb serum for 2 h. Blocking solution was replaced with a solution of MABT containing 2% blocking reagent (Boehringer, Ingelheim, Germany), 20% heat-inactivated lamb serum and 1/2000 dilution of anti-DIG antibody (Boehringer, Ingelheim, Germany) and incubated overnight at 4°C. Unbound antibody was removed by rinsing three times in 0.1% BSA in PTW, five 45 min washes in 0.1% BSA in PTW and two 20 min washes in PTW at RT. Endogenous alkaline phosphatase activity was blocked by washing embryos in 0.48 mg/mL Levamisole in NTMT (100 mM NaCl, 100 mM Tris.HCl pH 9.5, 50 mM $MgCl_2$, 1% (v/v) Tween-20). Embryos were transferred to glass vials and incubated in the dark with NTMT containing 9 µL/mL nitroblue tetrazolium chloride (NBT) and 7 µl/mL 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) until color developed to the desired intensity. After color developed, embryos were washed three times in PTW then re-fixed in PTW containing 4% paraformaldehyde and 0.1% glutaraldehyde overnight followed by three PTW washes. Azide was added to a concentration of 0.1% for storage.

2.3 Biochemical Techniques

2.3.1 Protein preparation from cell lines

Total protein lysate were prepared by incubating 1 x 10^7 cells in 1 mL lysis buffer (1% Nonidet P-40, 0.1% SDS, 0.5% sodium deoxycholate, 150 mM NaCl, 50 mM Tris.HCl pH 7.5, 10 mM sodium fluoride, 1 mM phenylmethylsulphonyl fluoride (PMSF), 10 µg/mL aprotinin, 10 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 mM benzamidine) on ice for 10 min followed by centrifugation at 10 000 x g for 10 min.

2.3.2 In vitro *transcription and translation*

In vitro RNA transcription and protein translation was performed using the TnT® coupled reticulocyte lysate system (Promega, Madison, USA). Briefly, reactions contained 12.5 µL

TNT® rabbit reticulocyte lysate, 1 μL TNT® reaction buffer, 20 μM amino acid mixture (without methionine), 1 μL Redivue Pro-mix L-[^{35}S] *in vitro* cell labeling mix (Amersham, Bucks, UK), 20 U RNasin® Ribonuclease inhibitor (Promega, Madison, USA), 0.5 μg plasmid DNA template, 10 U T7 RNA polymerase (Promega, Madison, USA) and water to 25 μL. Reactions were incubated at 30°C for 90 min, then stopped by the addition of sample buffer (final concentration: 16 mM Tris.HCl pH 6.8, 0.025% SDS, 5.84 mM 2-mercaptoethanol, 1.5% glycerol). Translated protein was analyzed by SDS-PAGE and Western blotting (Section 2.3.3).

2.3.3 Polyacrylamide gel electrophoresis (PAGE) and Western blotting

Protein was diluted in sample buffer (final concentration: 16 mM Tris.HCl pH 6.8, 0.025% SDS, 5.84 mM 2-mercaptoethanol, 1.5% glycerol), denatured at 100°C for 5 min and resolved on denaturing polyacrylamide gels. Separating gels contained 7 - 15% acrylamide (stock: 29.2% acrylamide-0.8% bis-acrylamide), 375 mM Tris.HCl pH 8.8, 0.1% SDS, 0.1% ammonium persulfate and 0.05% TEMED. Stacking gels contained 5% acrylamide, 125 mM Tris.HCl pH 6.8, 0.1% SDS, 0.1% ammonium persulfate and 0.1% TEMED. Electrophoresis was performed at room temperature (200 volts, 2 h) in protein electrophoresis buffer (25mM Tris, 192 mM glycine, 3.5 mM SDS).

For Western blotting, protein was transferred to nitrocellulose or polyvinylidene fluoride (PVDF) membrane in Western buffer (2.5 mM Tris, 192 mM glycine, 20% methanol) at 35 V, overnight at 4°C. Membranes were blocked for 1 h with 5% skim milk powder in TBST. Primary antibodies diluted in TBST/5% skim milk were added to the membranes for 1 h at room temperature. Blots were washed (3 times for 15 min each) in TBST prior to the addition of Horse Radish Peroxidase-conjugated anti-IgG antibodies (Amersham, Bucks, UK) for 1 h. Membranes were washed as before and developed using the Enhanced Chemiluminescence system (Amersham, Bucks, UK) according to manufacturer's instructions. The membranes were then exposed to hyperfilm (Amersham, Bucks, UK).

2.3.4 *Immunoprecipitation*

Protein lysates (about 400 μg) were incubated with up to 5 μg antibody for 2 h rotating at 4°C. Protein G agarose (Sigma, St. Louis, USA) or Protein A sepharose (Amersham, Bucks, UK) (10 μL of 50% slurry) was added for a further hour. The beads were washed three times in lysis buffer before use in further experiments or beads were boiled in 1 x sample buffer and analyzed by PAGE and Western blotting.

The following antibodies were used:

anti-HA	HA.11 (Covance, Berkeley, USA).
anti-myc	9E10 ascites (Evan *et al.*, 1985).

2.3.5 *Kinase assays and phosphoamino acid analysis*

Cells were lysed in kinase lysis buffer (1% Nonidet P-40, 0.1% SDS, 0.5% sodium deoxycholate, 150 mM NaCl, 50 mM Tris.HCl pH 7.5, 2 mM EDTA, 2 mM EGTA, 10 mM sodium fluoride, 1 mM PMSF, 10 μg/mL aprotinin, 10 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 mM benzamidine) and immunoprecipitation performed as described previously (Section 2.3.4). Immunoprecipitates were washed three times in kinase lysis buffer and twice in 20 mM Tris.HCl pH 7.5, 10 mM $MgCl_2$, 1 mM Na_3VO_4. Beads were resuspended in sterile water (with or without substrate) to a final volume of 20 μL. Kinase buffer was added to a final concentration of 1 x (5 x: 100 mM Tris.HCl pH 7.5, 50 mM $MgCl_2$, 10 mM EGTA, 5 mM DTT, 0.5 mM Na_3VO_4, 8 mM protein kinase A inhibitor (Sigma, St. Louis, USA), 2mCi/mL [γ^{32}P]ATP (Amersham, Bucks, UK) and reactions were incubated at 37°C for 30 min unless otherwise stated. Reactions were stopped by the addition of sample buffer, denatured at 100°C for 5 min, resolved on denaturing polyacrylamide gels and either dried for analysis or transferred to nitrocellulose membrane (Section 2.3.3). Radiolabeled protein was detected using a Molecular Dynamics 445SI Phosphorimager (Amersham, Bucks, UK) and proteins subsequently identified by immunoblotting with specific antibodies (Section 2.3.3).

For [^{32}P]-phosphoamino acid analysis, kinase assays were performed and proteins transferred to PVDF after separation by SDS-PAGE. Radiolabeled Madm or phosphorylated

substrates were excised, hydrolyzed and analyzed by one-dimensional thin-layer chromatography (Hardin and Wolniak, 1998).

2.3.6 In vivo *phosphorylation assays*

Individual wells of a 6-well plate were seeded with COS7 cells, then transfected with empty vector or constructs expressing Madm (Section 2.1.4). Cells were incubated overnight before the culture medium was removed, replaced with 750 µL/well of phosphate-free DMEM/10% FCS containing 100 µCi of [^{32}P]-labeled phosphoric acid, then incubated a further 7 h. The cells were lysed with 500 µL/well of phosphate-buffered RIPA (1% Nonidet P-40, 0.1% SDS, 1% sodium deoxycholate, 150 mM NaCl, 10 mM sodium phosphate buffer pH 7.2, 2 mM EDTA, 10 mM sodium fluoride, 1 mM PMSF, 10 µg/mL aprotinin, 10 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 mM benzamidine) and immunoprecipitation performed as described earlier (Section 2.3.4). Immunoprecipitated proteins were resolved on denaturing polyacrylamide gels and transferred to PVDF membrane (Section 2.3.3). Radiolabeled protein was detected using a Molecular Dynamics 445SI Phosphorimager (Amersham, Bucks, UK) and proteins subsequently identified by immunoblotting with specific antibodies (Section 2.3.3).

2.3.7 *Subcellular fractionation of protein*

To separate nuclear and cytoplasmic compartments, cells were washed with ice cold PBS, scraped and harvested at 1000 x g for 5 min. The cell pellet was resuspended in 0.5 mL hypotonic buffer (20 mM Tris.HCl pH 7.4, 1 mM EDTA, 1 mM DTT, 1 mM PMSF, 10 µg/mL aprotinin, 1 mM benzamidine) and incubated on ice for 20 min. Cells were Dounce homogenized (50 passes) and incubated on ice for a further 20 min. Nuclei were pelleted by centrifugation at 1000 x g for 5 min, washed twice in hypotonic buffer and resuspended in hypotonic buffer containing 1 x sample buffer (Section 2.3.1). The supernatant was centrifuged five times at 1000 x g for 5 min to remove any nuclear contaminants, before centrifugation at 100 000 x g for 30 min to obtain the cytoplasmic fraction. Nuclear and cytoplasmic fractions were subsequently analyzed by PAGE and Western blotting (Section 2.3.3).

2.3.8 Protein expression in bacteria

For expression of protein in *E. coli*, the open reading frame was cloned into either pET28a or pGEX2T expression vectors. Plasmids were introduced into BL21 CodonPlus™ bacteria (Stratagene, La Jolla, USA) then transformed bacteria were inoculated into 20 mL LB (containing 30 μg/mL kanamycin, 50 μg/mL chloramphenicol and 12.5 μg/mL tetracycline) and grown overnight at 37°C. Overnight cultures (20 mL) were used to inoculate 2 L of LB (containing 30 μg/mL kanamycin, 50 μg/mL chloramphenicol and 12.5 μg/mL tetracycline) which was incubated at 37°C until an OD_{600nm} of 0.6 was reached. Cultures were chilled briefly on ice before induction of protein by the addition of isopropyl-β-thiogalactopyranoside (IPTG) to 0.5 mM, for 4 h at room temperature. Bacteria were harvested by centrifugation at 6000 x g for 5 min. Pellets were resuspended in 40 mL (2% culture volume) lysis buffer (300 mM NaCl, 50 mM Tris.HCl pH 8, 1% Triton X-100, 1 mg/mL lysozyme, 1 mM PMSF, 10 μg/mL aprotinin, 1 mM benzamidine) and 20 mL aliquots were sonicated four times for 30 s using a Branson Sonifer (Emerson, St. Louis, USA). Aliquots were pooled before the addition of 50 U/mL DNase I and incubation on ice for 60 min. Insoluble debris was removed by centrifugation at 15000 x g for 20 min at 4°C and the supernatant containing soluble protein used for further purification.

2.3.9 Purification of His-tagged proteins from bacteria

Metal chelate chromatography

Recombinant proteins containing a polyhistidine (6 x His) tag were initially purified using immobilized metal affinity chromatography, where the resins were charged with either nickel (Ni-NTA resin, Qiagen, Valencia, USA) or cobalt ions (TALON® metal affinity resin, Clontech, Palo Alto, USA). Typically, proteins were purified using 200 μL of a 50% slurry of beads at 4°C. The resin was equilibrated by washing three times in lysis buffer (300 mM NaCl, 50 mM Tris.HCl pH 8, 1% Triton X-100) before addition to the cleared bacterial lysate (Section 2.3.8). Protein and resin were incubated on a rotating wheel for 4 – 16 h. Beads were collected by centrifugation (1000 x g for 2 min) and washed three times in wash buffer (1 M NaCl, 50 mM Tris.HCl pH 8). Bound protein was eluted by two washes in 0.5 mL elution buffer (300 mM NaCl, 50 mM sodium phosphate buffer pH 7, 250 mM imidazole). Imidazole

was subsequently removed by dialysis overnight into 20 mM Tris.HCl pH 6.8, following which benzamidine was added to 1 mM and protein stored at 4°C.

Ion exchange chromatography

To remove protein contaminants after metal chelate chromatography, ion exchange chromatography was performed. Purification was performed using a BioLogic LP low-pressure chromatography system (Bio-Rad, Hercules, USA) using a 1 mL High Q anion exchange column (Bio-Rad, Hercules, USA) and all solutions were passed through the column at 1 mL/min. Protein was loaded and the column was washed with 10 volumes of 20 mM Tris.HCl pH 6.8 to remove unbound proteins. A linear gradient combining 20 mM Tris.HCl pH 6.8 (100 – 0%) and 1 M NaCl/20 mM Tris.HCl pH 6.8 (0 – 100%) was set to run over 50 mL. Madm protein eluted at a NaCl concentration around 300 mM. These fractions were collected and Madm concentrated using an Amicon® stirred cell concentration unit (Millipore, Billerica, USA) where proteins were filtered under nitrogen pressure through a regenerated cellulose ultrafiltration membrane with a molecular weight exclusion size of 30 kDa (Millipore, Billerica, USA).

2.3.10 Purification of GST-tagged proteins from bacteria

The purification of GST fusion proteins was achieved by affinity chromatography using agarose beads conjugated with glutathione (Sigma, St. Louis, USA). Before use the glutathione agarose was equilibrated by washing three times with PBS, then made up to a 50% slurry in PBS. Typically, 200 µL beads per 2 L culture was added to soluble protein lysate (Section 2.3.8) and protein was allowed to bind for between 2 and 16 h at 4°C. Unbound protein was removed and saved for further analysis, while the beads were washed three times in 20 mM Tris.HCl pH 8. To elute the GST fusion proteins the beads were washed twice in 500 µL elution buffer (500 mM Tris.HCl pH 9.6, 10 mM reduced glutathione). Excess glutathione was removed by dialysis overnight into storage buffer (20 mM Tris.HCl pH 6.8), following which benzamidine was added to 1 mM and protein stored at 4°C.

2.3.11 Antibody generation

A fusion protein consisting the first 55 amino acids of Madm was expressed in *E. coli* as a GST-fusion protein using the expression vector pGEX 2T (Amersham, Bucks, UK). The fusion protein was induced and purified as described in Sections 2.3.8 and 2.3.10 respectively. Purified protein (10 µg) was separated using SDS-PAGE, then visualized by staining with 0.05% Coomassie Brilliant Blue R-250 prepared in water. The band corresponding to GST-Madm (aa 1-55) was then excised and the gel slice fragmented by repeated passes through a 21 gauge syringe.

A New Zealand white rabbit was immunized with the gel slice resuspended in 1 mL PBS and mixed with 1 mL Freund's complete adjuvant (Sigma, St. Louis, USA) until the mixture thickened. The rabbit was injected subcutaneously at 4 separate sites using 0.5 mL immunization mix per site. After four weeks a booster was administered which consisted of the same amount of protein prepared in Freund's incomplete adjuvant (Sigma, St. Louis, USA). A test bleed (10 mL) was taken six weeks after the initial immunization to monitor the immune response. A final boost was given at eight weeks and the rabbit was exsanguinated at 10 weeks yielding approximately 100 mL whole blood.

After collection the blood was allowed to clot for 60 min at 37°C. Clotted blood was separated from the sides of the tube and allowed to contract overnight at 4°C. Serum was removed from the clot and insoluble material removed by centrifugation at 10000 x g for 10 min. Sodium azide (0.02%) was added to the serum which was then aliquotted and stored at –80°C. The reactivity of the antiserum was determined by immunoblotting of the fusion protein and protein lysates from COS7 cells transiently transfected with Madm.

Chapter 3: ANALYSIS OF THE MADM GENE, mRNA AND PROTEIN SEQUENCE

3.1 Introduction

Mlf1 is a recently described oncoprotein (Yoneda-Kato *et al.*, 1996), which is able to influence hemopoietic lineage commitment, and reprogram erythroleukemic cells to develop a monoblastoid phenotype (Williams *et al.*, 1999). The importance of *Mlf1* in oncogenesis is highlighted (i) by the (3;5) translocation, which exposes the leukemogenic potential of the molecule, and (ii) over-expression of wild-type *Mlf1* in patients with acute myeloid leukemia, which correlates with poor prognosis and reduced survival (Yoneda-Kato *et al.*, 1996, Matsumoto *et al.*, 2000).

Madm is a novel molecule that was found to interact with Mlf1 in a yeast two-hybrid screen. The fragment of *Madm* that was isolated from the screen was a 219 bp cDNA sequence that had no homology to any other gene in the database at the time. The following chapter describes the cloning of the murine Madm cDNA and gene. Since *Mlf1* is a gene that is involved in hemopoietic lineage commitment and differentiation (Williams *et al.*, 1999), it was of interest to determine the expression patterns of Madm in various hemopoietic cell types.

Work described in this Chapter examined the primary sequence of Madm which may provide clues as to its function, activity and how it may be interacting with Mlf1.

3.2 Results

3.2.1 Madm contains multiple domains

Madm was originally identified as a 219 bp cDNA fragment that produced a protein which interacted with Mlf1 in a yeast two-hybrid screen. This fragment was used to screen an EML C.1 cDNA library and a 2153-bp cDNA was isolated. An open reading frame of 1608 bp was identified, encoding a putative 535 amino acid protein with a predicted molecular weight of 60 kDa (Figure 3.1). The probable initiating AUG was in the characteristic context for a translation start site (Kozak, 1987).

A comparison of the Madm amino acid sequence using the Swiss-Prot database revealed similarities with a variety of protein kinases, and a consensus kinase domain (Schultz *et al.*, 1998) was identified. However, the conserved ATP-binding motif (GXGXXG), found in conventional protein kinases (Hanks and Hunter, 1995), was not present in this protein (Figure 3.1). Madm also contained many potential sites for serine/threonine phosphorylation, including one potential site for cAMP- and cGMP-dependent protein kinase phosphorylation, eight potential protein kinase C phosphorylation sites and 12 possible casein kinase II (CK2) phosphorylation sites. This indicates that phosphorylation is likely to play an important role in Madm regulation and function.

Intriguingly, a bipartite nuclear localization signal (NLS) was present within the catalytic domain, as well as a potential nuclear export signal (NES) (Figure 3.1), suggesting that the protein may shuttle between the nucleus and cytoplasm. It is important to note that several of the potential phosphorylation sites on Madm mentioned above are in the vicinity of both the NLS and NES, suggesting that phosphorylation may play a role in regulating Madm subcellular localization.

Sequence comparisons of Madm shown in Figure 3.2, revealed that it had 51% amino acid identity with an uncharacterized protein from *Drosophila melanogaster* (AF145690) and 36% identity with a predicted protein from *Caenorhabditis elegans* (CAA99887) demonstrating evolutionary conservation. The *D. melanogaster* protein is 94 amino acids longer than the murine molecule and fairly divergent at both the amino- and carboxyl-termini; however, there

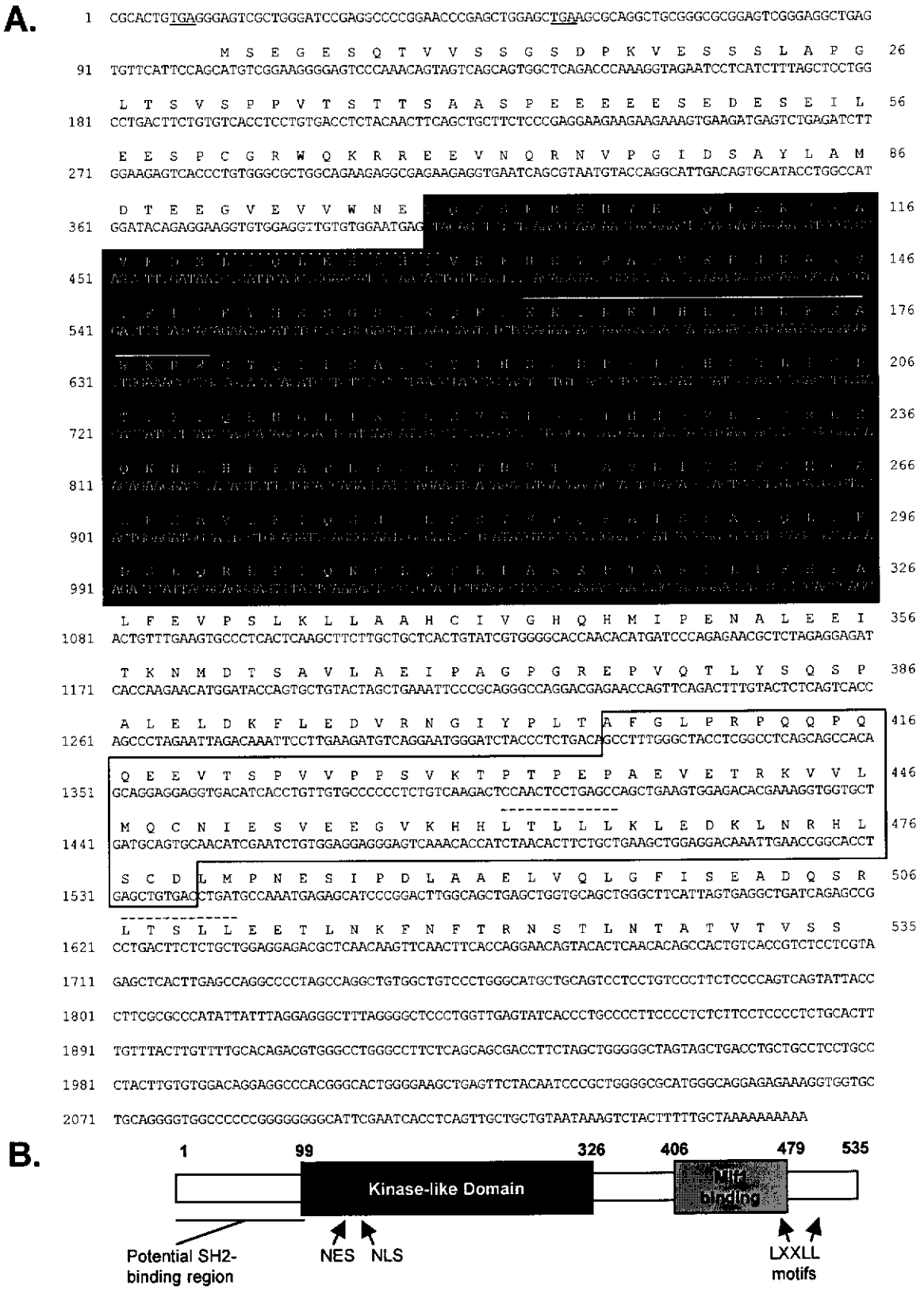


Figure 3.1. *Madm* cDNA and protein sequence. (A) The nucleotide sequence of murine *Madm* cDNA is shown with the predicted amino acids indicated above. Nucleotides are numbered on the left and amino acids on the right. The in-frame termination codons in the 5' untranslated region are underlined. The putative kinase domain is in black and the nuclear export sequence (NES, amino acids 121-129) and nuclear localisation sequence (NLS, amino acids 163-181) are indicated by dots or lined above, respectively. The boxed sequence indicates the region identified in the Mlf1 yeast two-hybrid screen. Dashed lines indicate the two putative nuclear receptor-binding motifs (consensus, LXXLL). (B) Schematic representation of the Madm protein shows the potential SH2-binding region, potential NES and NLS, the kinase-like domain and the Mlf1-binding domain.

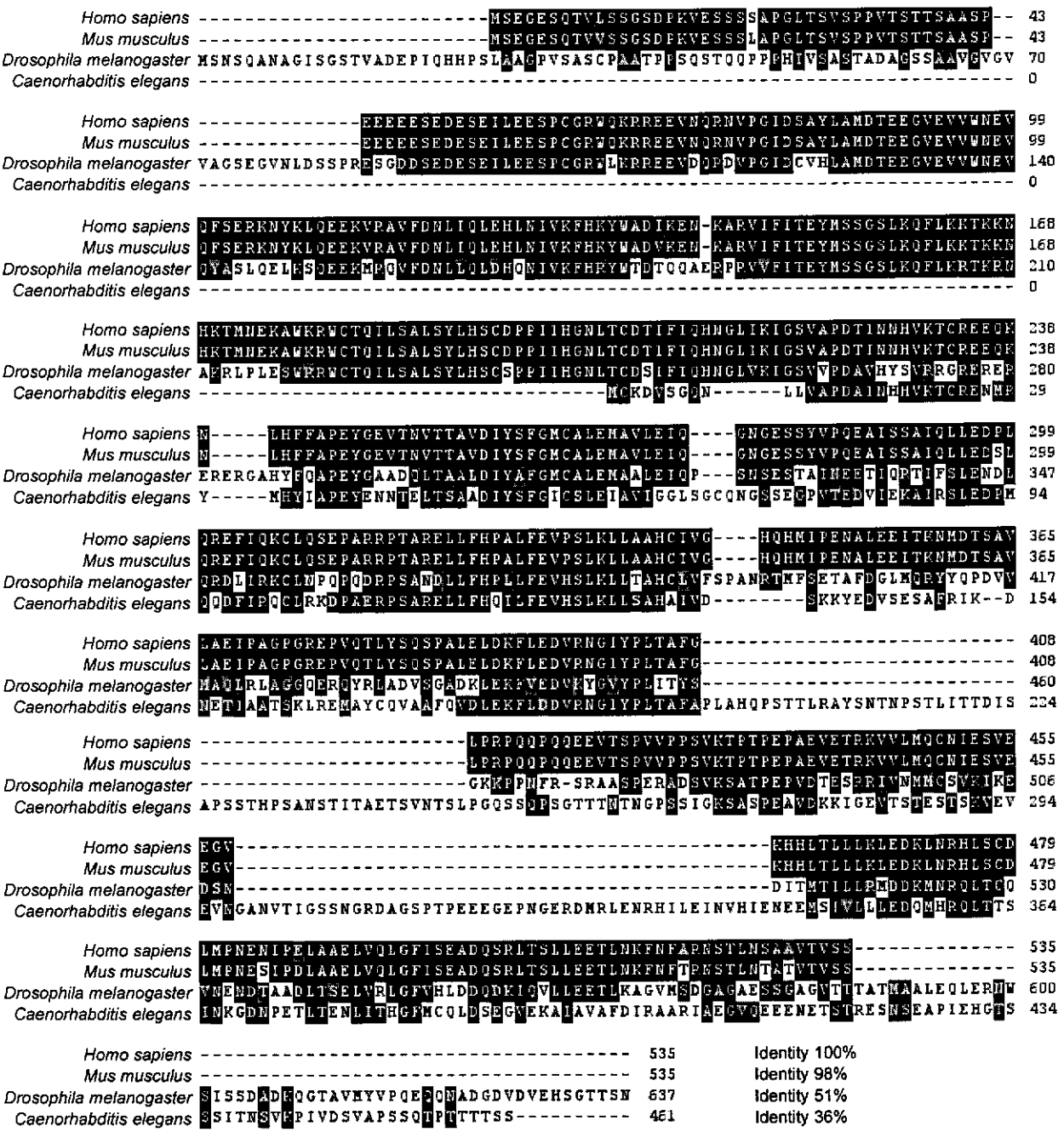


Figure 3.2. Alignment of the Madm protein sequence from different species. The GenBank accession numbers are: *H.sapiens*, NM_013392, *M. musculus*, AF302138; *D. melanogaster*, AF145690; *C. elegans*, CAA99887

is high conservation within the core region of the protein. In contrast, the *C. elegans* protein is 304 amino acids shorter than the mouse and human Madm proteins and shows less identity to the other species. During this study, a human homologue of Madm was submitted to GenBank and named NRBP for Nuclear Receptor Binding Protein (NM_013392) due to the presence of two putative nuclear receptor binding motifs (LXXLL) (Hooper *et al.*, 2000). The human clone contains an open reading frame of identical size to Madm, and shares 98% amino acid identity (Figure 3.2). The mouse Madm kinase domain was highly conserved with 99% and 60% for the human and *Drosophila* proteins, respectively. However, Hooper *et al.* (Hooper *et al.*, 2000) considered the human homologue of Madm unlikely to possess functional kinase activity because it lacked 7 of the 15 highly conserved kinase catalytic domain amino acid residues, including a classic ATP binding site.

In addition to the kinase-like domain, NLS, NES and the LXXLL motifs, Madm also contains a region rich in glutamic acid and serine residues at the amino terminus (Hooper *et al.*, 2000). Regions rich in these amino acids have been shown to confer binding to SH2 modules in a phosphotyrosine-independent manner (Joung *et al.*, 1996). As mentioned earlier (Section 1.4.1) SH2 domains are conserved protein modules that mediate assembly of multi-component signaling complexes; this suggests that the SH2-binding sequences in the amino terminus of Madm may be involved in a signal transduction network.

3.2.2 Structure of the Madm gene

A murine genomic library was screened for clones containing the *Madm* gene, using a radiolabeled cDNA probe consisting of the Madm open reading frame. A positive clone (m2.2) was identified and plaque purified (Figure 3.3). The exons 3 to 18 were fully sequenced and intron/exon boundaries were determined (Table 3.1). The first two exons were not present in clone m2.2, so the library was rescreened using a 5' cDNA probe corresponding to bp 1 – 433 of the Madm mRNA. A clone (m1.4) was isolated that overlapped m2.2 and contained exons 1 to 14. Exons 1 and 2 were also sequenced, including approximately 1 kb upstream of the putative start of the mRNA, and the intron/exon boundaries were identified (Table 3.1). Of the donor splice sites, seven did not conform to the normal consensus sequence and similarly seven acceptor splice sites did not fit the

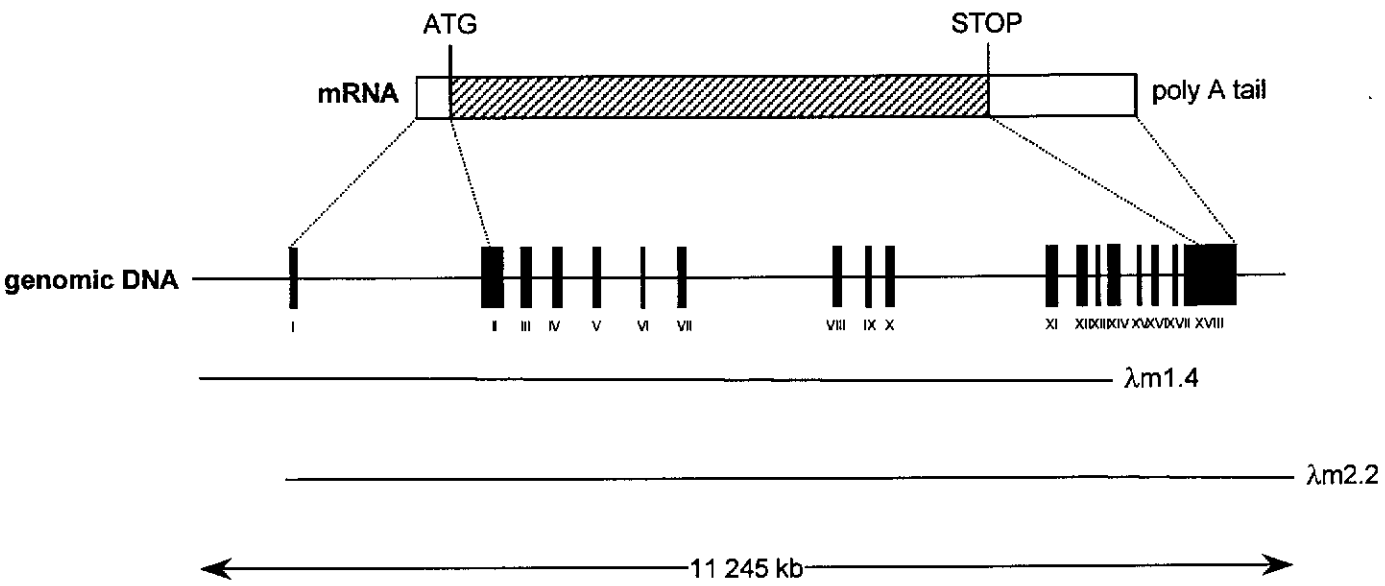


Figure 3.3. Organization of the murine *Madm* gene. The coding sequence is indicated by the shaded box, while the 5' and 3' untranslated regions are shown by open boxes. For the genomic sequence, the exons are represented as black boxes and numbered with Roman numerals and the introns by single lines. The complete genomic copy of mouse *Madm* was isolated as two overlapping phage clones as shown below.

3' acceptor sequence				5' donor sequence			
Intron	Exon	Exon number	Exon size (bp)	Exon	Intron	Intron number	Intron size (bp)
	CGCAGCTGTG	1	85	GAGTCGGGAG	gtgagctccg	1	1894
acaaatgcag	GCTGAGTGTT	2	229	GCGAGAAGAG	gtgaggtggt	2	174
tgcttctcag	GTGAATCAGC	3	123	ACTGCAGGAG	gtaggtgatg	3	172
cctgctccag	GAAAAGGTCC	4	102	CAAGGCTAGG	gtaagatttt	4	320
Gcttccctag	GTGATTTTCA	5	90	GAATGAAAAG	gtacatagag	5	409
ctttttccag	GCTTGGAAAC	6	41	CTGCCCTAAG	gtaggtaata	6	347
gcccccacag	CTACCTGCAC	7	94	GATTGGCTCT	ggtgagggga	7	1499
cctattccta	GTGGCTCCTG	8	85	GAGTATGGAG	gtgaggtagc	8	258
ttttctttag	AAGTCACAAA	9	59	TGCACTGGAG	gtgaggagac	9	155
ctcactatag	ATGGCAGTGC	10	99	ATTACAGAGG	gtaagactca	10	1550
ctcttaacag	GAGTTTATTC	11	133	GGGCACCAAC	gtgagtcacc	11	209
tacccaccag	ACATGATCCC	12	106	TTCAGACTTT	gtgagtaacc	12	95
tttcttccag	GTACTCTCAG	13	51	AAGATGTCAG	gtgagatagg	13	89
ctcccaccag	GAATGGGATC	14	136	GACACGAAAG	gtggggactc	14	178
tcctgttcag	GTGGTGCTGA	15	54	CAAACACCAT	gtaagctcag	15	83
ctttttctag	CTAACACTTC	16	64	CTAATGCCAA	gtaagcctct	16	147
tctctgtcag	ATGAGAGCAT	17	56	CATTAGTGAG	gtgagggtcc	17	77
gcttccccag	GCTGATCAGA	18	536	ACTTTTTGCT			

Table 3.1. Nucleotide sequences of the intron-exon boundaries of the murine *Madm* gene.

consensus. However, comparison of the human gene sequence (available at NCBI) showed that all, but one, splice sites were preserved between mouse and human.

The gene is ~11 kb in length with 18 exons and 17 introns (Figure 3.3). The exons ranged in size from 41 bp (exon 6) to 536 bp (exon 18), and the introns ranged in size from 83 bp (intron 15) to 1894 bp (intron 1). Most of the introns (1, 2, 3, 4, 5, 8, 9, 10, 14, 15, 17) were type 0, which interrupts between codons. Of the other introns, three were type 1 (7, 11, 16) that interrupt after the first nucleotide of a codon and the remaining introns (6, 12 and 13) were type 2 interrupting after the second nucleotide of the codon. Exon 1 (85 bp) contains exclusively 5' untranslated sequence, with exon 2 containing 19 bp of 5' untranslated region, as well as the initating methionine. Exon 18 is 536 bp in length and contains the stop codon along with the entire 3' untranslated sequence.

3.2.3 Chromosomal localization the Madm gene

The chromosomal localization of the *Madm* gene was determined using the EUCIB *Mus musculus/Mus spretus* backcross facility available at http://www.informatics.jax.org/. *Madm* mapped to chromosome 5 and lies within the first intron of the protein phosphatase 1G, magnesium-dependent, gamma isoform (*Ppm1G*) gene. The *Ppm1G/Madm* locus lies between the markers D5mit149 and D5Cph17 and was estimated to localize 19 cM from the centromere (Figure 3.4). The human gene structure was also determined using the NCBI Map Viewer services (http://www.ncbi.nlm.nih.gov/cgi-bin/Entrez/map_search). Like the murine gene, human *MADM* (or *NRBP*) mapped within the first intron of the *PPM1G* gene. The gene is located on chromosome 2p23.3 which is syntenic with mouse chromosome 5 and contains an intron/exon structure identical to the murine gene. Translocations within this region of chromosome 2 are frequently associated with anaplastic large cell lymphoma, a high grade non-Hodgkin lymphoma and are known to involve *ALK* (Morris *et al.*, 1994b, Rosenwald *et al.*, 1999, Touriol *et al.*, 2000). On the human chromosome, *MADM* is transcribed towards the centromere, which is in the same direction as *PPM1G*. There are several well characterized genes that are found on 2p23, including several metabolic enzymes, phosphatases, kinases, salt channel proteins and transcription factors (Figure 3.5).

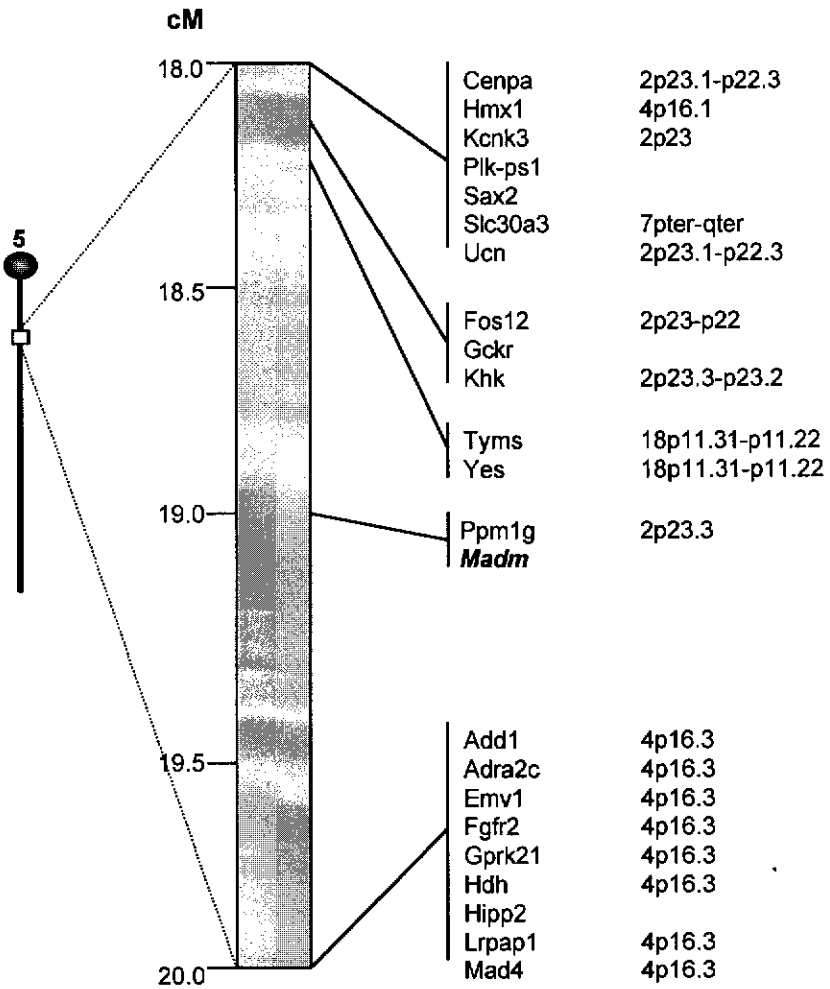


Figure 3.4. Localization of *Madm* to mouse chromosome 5. *Madm* maps to the same locus as *Ppm1g* and is found within intron 1 of *Ppm1g*. Flanking genes and their corresponding localization on the human genome are shown at right. The Ppm1g/Madm locus lies between markers D5mit149 and D5Cph17 according to the EUCIB (BSB) mouse chromosome 5 linkage map.

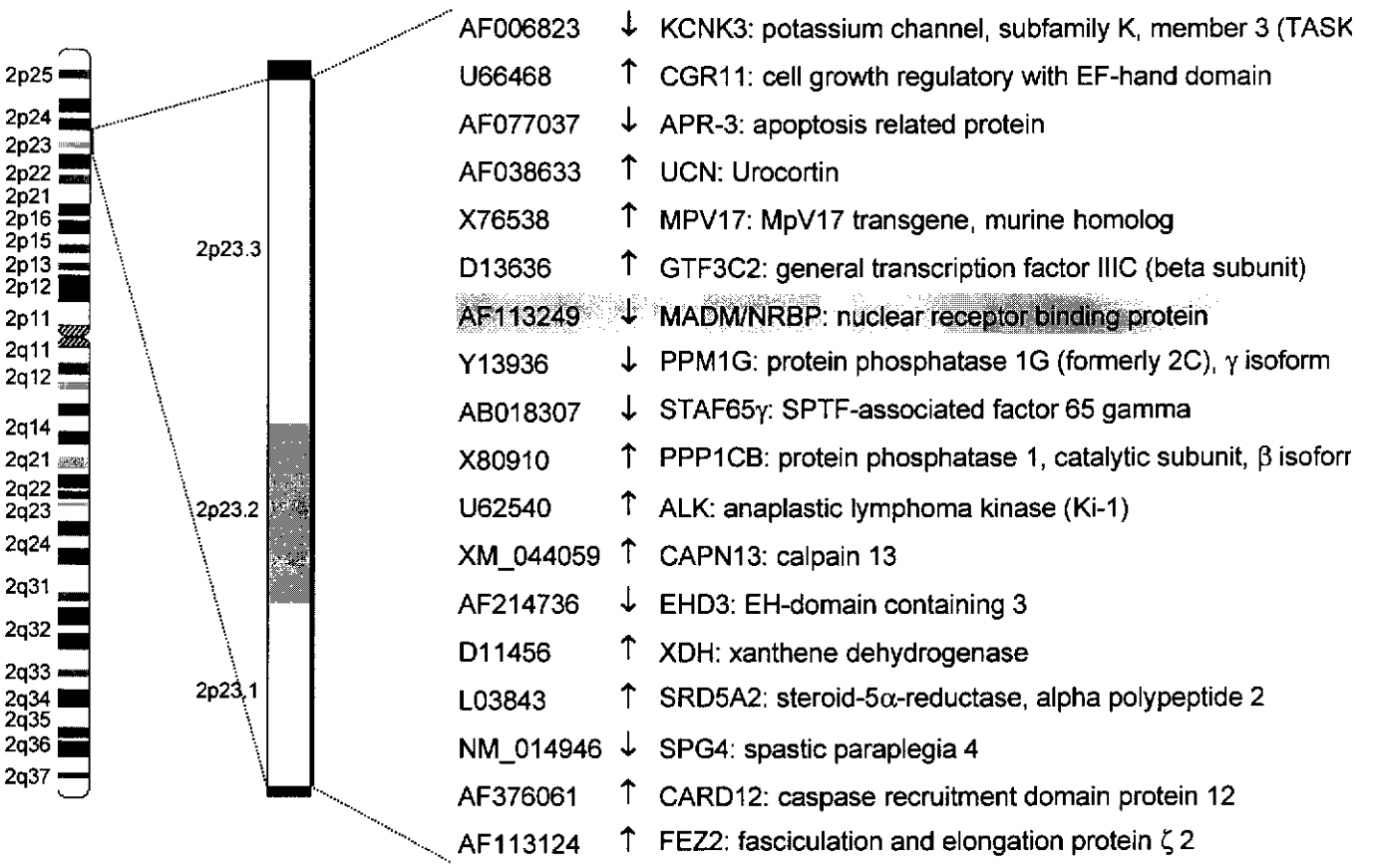


Figure 3.5. Location of *MADM/NRBP* on human chromosome 2. The *MADM/NRBP* gene is located on 2p23.3 and lies within intron 1 of the PPM1G gene. Other characterized genes found on this chromosomal region are shown with accession numbers and direction of transcription indicated by arrows.

3.2.4 Analysis of the Madm promoter

The 5' flanking sequence of the Madm gene was determined by sequencing almost 1 kb upstream of the mRNA sequence found on the λ clone m1.4 (Figure 3.4). Since primer extension experiments were not performed on the Madm cDNA, the exact transcription initiation site is unknown. Computational database searches using the Neural Networks Promoter Prediction program at Berkeley Genome Drosophila Project (http://www.fruitfly.org/seq_tools/ promoter.html) were used to predict the Madm initiation site. This search predicted a transcription initiation site at –29 bp, relative to the start of the Madm mRNA sequence isolated from the cDNA library (Figure 3.6). In the 5' flanking sequence classical CAAT boxes were not observed; however, a TATA box for binding TFIID, a 700-kD complex composed of the TATA box binding protein (TBP) and a set of polymerase-specific TBP-associated factors (Burley and Roeder, 1996), and two GC boxes were present. The sequence was compared with known *cis*-sequence motifs that bind transcription factor proteins using the TFSearch tool at http://molsun1.cbrc.aist.go.jp/research/db/TFSEARCH.html. The results included putative binding sites for CCAAAT/enhancer binding protein (C/EBP), octomer binding factor 1 (Oct-1) and an upstream binding site for GATA-1 (Figure 3.6). Many ubiquitously expressed genes are regulated by C/EBP and Oct-1, suggesting Madm expression may be wide spread. There were also potential binding sites for other transcription factors with lower sequence matches to the predicted transcription factor binding sites. Among these were binding sites for several hemopoietic transcription factors such as myeloid-specific zinc finger protein 1 (MZF-1) for expression in myeloid cells, as well as Oct-2B and Oct-2C for expression in lymphoid cells, pre-B and B cells. Interestingly, comparison of the murine and human promoters revealed that the major regulatory sequences identified in the mouse genome had been retained (Figure 3.7). The human promoter, however, contained more elements such as two additional GATA-1 binding sites, an AP-1 binding site and CREB regulatory sequence.

3.2.5 Expression profile of Madm mRNA

Analysis of the tissue distribution profile of *Madm* mRNA demonstrated that it was indeed ubiquitously expressed. Northern blot analysis of total RNA from 13 normal adult murine

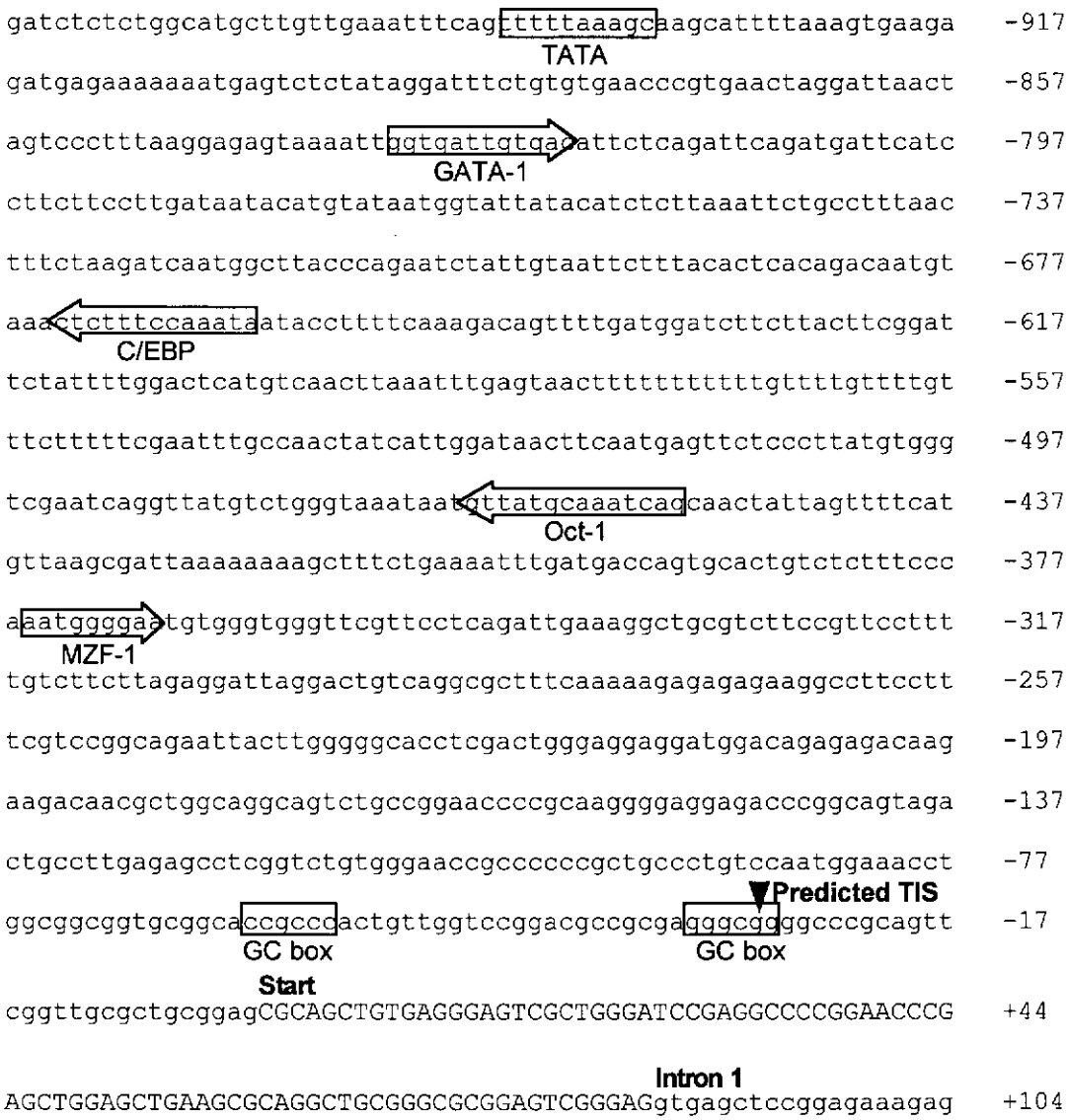


Figure 3.6. The 5' upstream region of the murine *Madm* gene. The nucleotide sequence of the first exon and 5' upstream region (-1 kb) is shown. The first exon is in upper case and intronic sequence in lower case.The filled arrow head indicates the predicted transcriptional start site (TIS). Putative GC boxes and binding sites for transcription factors GATA-1, C/EBP, Oct-1 and MZF-1 are shown.

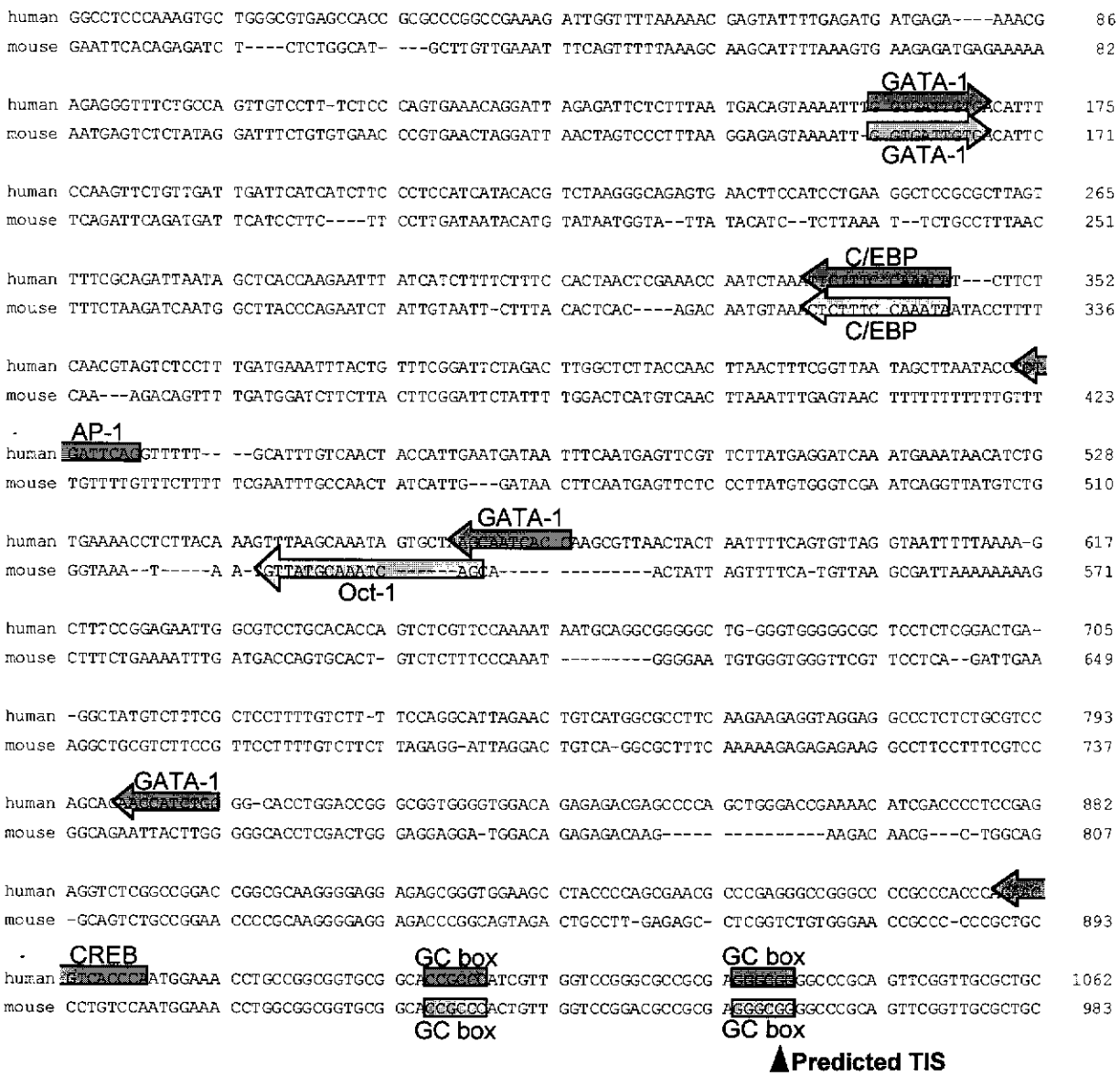


Figure 3.7. Homology between human and murine *Madm* promoters. The nucleotide sequence of the 5' flanking region of the *Madm* gene is shown. Regions that match the consensus binding sites for known transcription factors are highlighted. Sites that are conserved between human and mouse include GATA-1, C/EBP, GC boxes and the predicted transcription initiation site (TIS).

tissues demonstrated constitutive expression of *Madm* as a single 2.2 kb transcript (Figure 3.8A). This analysis was extended to 13 transformed hemopoietic cell lines where *Madm* mRNA was present in all cell lines examined (Figure 3.8B). Moreover, the levels of mRNA were comparable in most samples analyzed (Figure 3.8C); however there was significantly higher levels of *Madm* mRNA expression in testes, spleen and brain.

To examine the expression of *Madm* during development whole mount *in situ* hybridization was performed. Day 10 mouse embryos were prepared and *in situ* hybridization performed using a DIG-labeled *Madm* RNA probe as described in Section 2.2.14. It was evident that *Madm* mRNA was expressed at high levels throughout the developing embryo (Figure 3.8D). These results suggest *Madm* is widely expressed and may have a broader function that is not restricted to its interaction with Mlf1, which has a much more limited tissue expression profile (Williams *et al.*, 1999, Hitzler *et al.*, 1999).

3.2.6 Construct generation for Madm deficient mice

Further characterization of Madm will involve analysis of the physiological and morphological changes in Madm knockout mice. To this end, a targeting vector for the deletion of Madm was constructed (Figure 3.9). The vector backbone was used by Dr Warren Alexander (Walter and Eliza Hall Institute, Victoria, Australia) to generate SOCS2 -/- mice (Metcalf *et al.*, 2000). The construct was designed so that homologous recombination with the endogenous *Madm* locus would delete the entire Madm coding sequence, and place the *lacZ* gene under the transcriptional control of the *Madm* promoter. In addition, the targeting construct contained a selectable PGK*neo* gene to confer neomycin resistance in transfected ES cells. The PGK*neo* gene was flanked by two loxP sites to enable removal of the gene using *cre* recombinase once cells positive for homologous recombination had been identified. The 5' arm of the targeting construct was generated by PCR amplification of bp 17 – 2985 from the *Madm* genomic sequence (see Appendix 1); this fragment was cloned upstream of the *lacZ* gene (Figure 3.9). The 3' arm was a ~4 kb fragment also generated by PCR that corresponded to bp 10791 through to the end of λ clone m2.2. The PCR product was cloned downstream of the PGK*neo* gene and loxP sites (Figure 3.9). Sequencing was

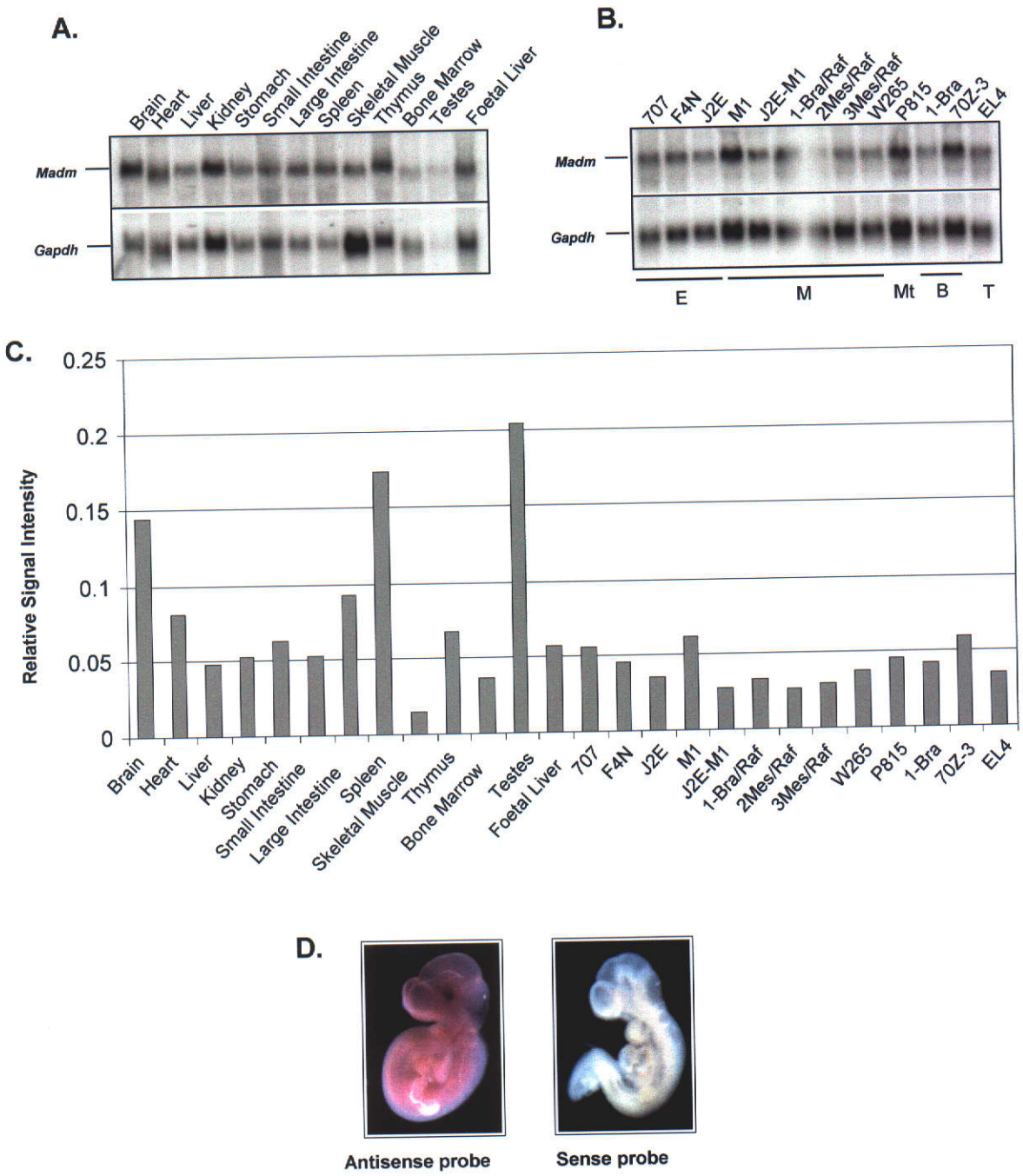


Figure 3.8. *Madm* mRNA expression pattern. (A) Northern blot analysis of total RNA (10 μg/lane) from 13 different murine tissues. The blot was probed with a [^{32}P]-labelled 1608 bp *Madm* fragment and reprobed with a *Gapdh* probe as a loading control. (B) Northern blot analysis of total RNA (10 μg/lane) from 13 different hemopoietic cell lines. Cell lines are grouped and labelled as erythroid (E), myeloid (M), mast (Mt), B cell (B) and T cell (T). The blot was probed as described in (A). (C) Graphical representation of *Madm* mRNA expression levels determined from tissues and cell lines. Expression is represented as the ratio of *Madm* signal intensity relative to *Gapdh*. The numbers on the *y* axis denote arbitrary units. (D) *In situ* hybridization of Madm. Shown are 10 d embryos probed with a DIG-labelled *Madm* RNA probe transcribed in either the antisense or sense direction.

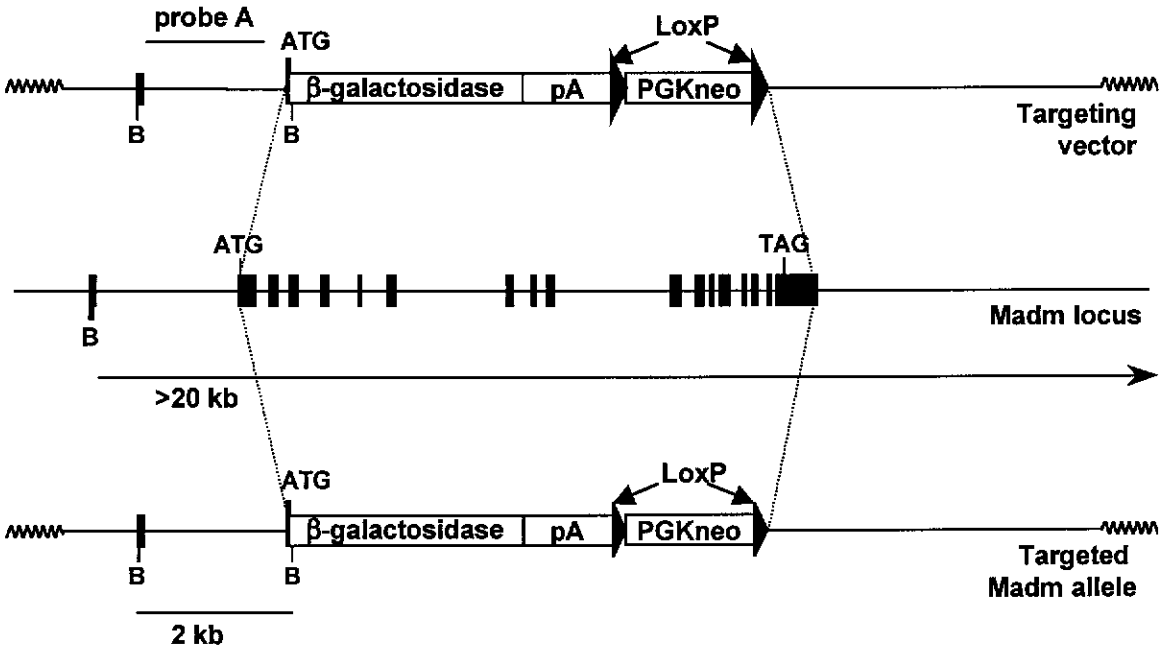


Figure 3.9. Construct for disruption of the *Madm* locus by homologous recombination. The The murine *Madm* genomic locus is shown (B, *Bam*HI), with exons illustrated as closed boxes. In the targeting vector the entire Madm coding region was replaced by a β-gal-PGKneo cassette in which the *β-galactosidase* coding region (followed by the *β-globin* poly A sequence) was fused to the *Madm* initiation codon. Southern blots of *Bam*HI-digested genomic DNA can be hybridized with probe A to distinguish between endogenous (<20 kb) and mutant (~2 kb) Madm alleles. Hybridization with a probe containing the coding region of Madm would also be used to confirm the loss of genetic material in Madm -/- mice.

performed to confirm that the ATG of β-gal was in the correct context for expression via the *Madm* promoter.

3.3 Discussion

In the work described above, a novel cDNA fragment was isolated from a yeast two-hybrid clone that bound to the oncoprotein, Mlf1. A cDNA library was screened using this fragment and a novel transcript encoding a kinase-like molecule, Madm identified. The complete nucleotide sequence revealed an open reading frame encoding a protein of 535 amino acids with a predicted molecule mass of 60 kDa.

To further analyze Madm, a genomic library was screened to isolate the *Madm* gene. Examination of the 5' flanking sequence of the Madm gene revealed a TATA site for binding for transcription factor TFIID (Chen and Hampsey, 2002). The promoter sequence contained other putative transcription factor binding sites, including C/EBP and Oct-1, indicative of ubiquitous expression (Grange *et al.*, 1991, Groenen *et al.*, 1992). It also contained a GATA-1 binding site for expression in erythroid cells (Merika and Orkin, 1993), as well as Oct-2 binding sites for lymphoid-specific expression . The presence of a potential binding site for the MZF1 transcription factor was intriguing. This zinc finger protein is expressed in hemopoietic progenitors committed to the myeloid lineage (Morris *et al.*, 1994a). Since Mlf1 has been shown to enhance myeloid differentiation and induce a lineage switch from erythroid to myeloid cells (Williams *et al.*, 1999), this may suggest that Madm expression is tightly regulated during this stage of hemopoietic development and may participate with Mlf1 to affect differentiation.

Comparison of the human and mouse *Madm* promoters revealed several transcription factor binding sites were conserved (i.e., GATA-1 and C/EBP) and most likely represent some conserved requirement of Madm expression in erythroid cells as well as throughout the body. However, other transcription factor binding sites, including AP-1 and CREB, were only present in the human promoter. Since these transcription factors are associated with ubiquitously expressed genes it suggests that Madm is a gene expressed in numerous tissues and cell types.

The predicted wide-spread expression profile of Madm was confirmed by Northern blot analysis of adult mouse tissues and *in situ* hybridization during embryogenesis. The

expression of *Madm* mRNA in all tissues, in embryos and adults, suggests that Madm is an important molecule. Similarly, other Mlf1-interacting proteins, such as PIN/LC8 and 14-3-3ζ, are also ubiquitously expressed (Jaffrey and Snyder, 1996, Aitken *et al.*, 1992). Mlf1, on the other hand is not ubiquitously expressed, with mRNA detectable in only hemopoietic cells, testis, skeletal muscle, lung and heart (Hitzler *et al.*, 1999). It could, therefore, be suggested that while Madm may play a role in Mlf1 function, its role may not be restricted entirely to Mlf1. Madm may also be important for the function of other proteins, in a similar manner to other Mlf1-interacting proteins, i.e. 14-3-3ζ.

An important role for Madm is further supported by the high conservation of the Madm protein sequence across diverse species. With 36% amino acid identity between *C. elegans* Madm and its human counterpart, and 51% amino acids conserved with the *D. melanogaster* sequence, it is likely that most of the primary amino acid sequence of Madm is required for either its structure and/or function. It is interesting to note that during this study the *Drosophila* homolog of *Mlf1* was cloned (Ohno *et al.*, 2000). Referred to as dMLF, the protein was cloned as a molecule that interacted with the transcription factor DREF. While dMLF is not as conserved as Madm (22% identity and 36% similarity to Mlf1), it does suggest that both these proteins may be involved in the same signaling events in both *Drosophila* and mice. Furthermore, the DREF-binding site on dMLF was localized to a central region of the protein which displays the greatest conservation among species. It is, therefore, possible that binding of transcription factors may be a conserved function of Mlf1. Over-expression of DREF in *Drosophila* produces a rough eye phenotype, which is partially rescued by co-expression with dMLF (Ohno *et al.*, 2000). It would be interesting to determine if the *Drosophila* homolog of Madm was involved in dMLF/DREF functions, and if Madm could enhance or attenuate the rescue of the rough eye phenotype in transgenic flies. It is noteworthy that two other proteins (14-3-3ζ and PIN/LC8) isolated from the Mlf1 yeast two-hybrid screen also displayed high amino acid conservation from *Drosophila* to mouse.

The most noticeable feature of the Madm protein sequence was the kinase-like domain between amino acids 99 and 326. It is possible that the Madm gene may have evolved from a different gene encoding a functional kinase. As mentioned in Section 1.5.1, kinases

contain 11 subdomains required for activity, and within these subdomains lie 15 highly conserved amino acids (Hanks and Hunter, 1995). While this region of Madm shares many characteristics with kinases, several important residues are not present including the amino acids required to bind ATP. Although Madm contains the second glycine from the Gly-X-Gly-X-X-Gly motif in subdomain I and the invariant lysine in subdomain II, the first glycine known to contact the ribose moiety of ATP is absent, suggesting the protein is unable to bind ATP (Hanks and Hunter, 1995). Altogether Madm contains only eight of the highly conserved amino acids leading Hooper *et al.* (2000) to suggest it was unlikely to possess functional kinase activity; however, functional experiments on the Madm protein would be required to rule out any potential enzymatic activity.

Although it has been suggested that Madm may not have intrinsic activity (Hooper *et al.*, 2000), it does contain many potential phosphorylation sites, and its function may be regulated in part by phosphorylation. Interestingly, there are several phosphorylation sites located in the proximity of a bipartite NLS indicating that phosphorylation may regulate Madm subcellular localization. Phosphorylation appears to be the main mechanism controlling the nuclear transport of a number of proteins, including NFκB and the well-characterized simian virus 40 T-antigen (SV40 T-ag) (Jans and Hubner, 1996). One of the ways phosphorylation regulates nuclear import is by masking the NLS, e.g. for the adenomatous polyposis coli (APC) protein. In one study a phosphorylated serine within the NLS of APC was mutated to alanine resulting in increased nuclear localization of the protein (Zhang *et al.*, 2000). In contrast, when the same serine was mutated to negatively charged aspartic acid, nuclear localization was decreased (Zhang *et al.*, 2000). The importance of the Madm NLS will need to be defined to determine if phosphorylation does play a role in Madm subcellular localization. Another indication that Madm may change subcellular localization is the presence within the kinase-like domain of a potential NES, which is characterized by a short stretch of hydrophobic amino acids, primarily leucine residues. Therefore, just as nuclear import may be important for the function of Madm, nuclear export may be of equal significance.

The presence of two putative nuclear receptor-binding motifs in Madm indicate that it may have a role in the nucleus (LTLLL at aa 462-466 and LTSLL at aa 507-511). Secondary

structure prediction software suggests that both LXXLL motifs exist as α-helices, which are required for transcription factor binding in the case of co-activators SRC-1/p160 and CBP/p300 (Heery *et al.*, 1997). Since Mlf1 has been shown to localize to punctate spots within the nucleus (Williams *et al.*, 1999) and bind DNA (J. Stillitano, unpublished observation), it has been speculated that it may be involved in gene transcription. The presence of LXXLL motifs in Madm may indicate a role for the protein in Mlf1-mediated gene transcription. However, a function for Mlf1 in gene transcription is yet to be demonstrated and is only inferred from its localization in nuclear spots (Williams *et al.*, 1999) and binding of the *Drosophila* homolog to the DREF transcription factor (Ohno *et al.*, 2000).

Dissemination of cellular signals is often mediated by protein-protein binding, and one motif that facilitates many of these interactions is the SH2 domain (Cohen *et al.*, 1995). Classically, SH2 domains bind phosphotyrosine residues; however, proteins have recently been shown to bind SH2 domains in a different manner (Pendergast *et al.*, 1991). Hooper *et al.* (2000) noted that human Madm contains a region rich in glutamic acid and serine residues at the amino terminus, and these amino acids have been shown to bind SH2 domains in a phosphotyrosine-independent manner (Malek and Desiderio, 1994, Malek *et al.*, 1996). Such modes of binding have been demonstrated for the Brc-Abl oncoprotein (Pendergast *et al.*, 1991), the serine/threonine kinase $p130^{PITSLRE}$ (Malek and Desiderio, 1994) and the nuclear protein $p150^{TSP}$ (Malek *et al.*, 1996). In each of these cases serine or threonine phosphorylation was required for binding to the SH2 domain. Interestingly, the amino terminus of Madm contains seven potential serine phosphorylation sites, including four CK2 phosphorylation sites. Therefore, Madm may be involved in multi-protein complexes mediated by interaction with SH2 domain-containing proteins.

The conservation of Madm across diverse species also suggests an important function for the molecule. A key experiment in the characterization of a novel gene is to delete it by homologous recombination. However, a common problem with deletion of kinase genes is functional redundancy, where similar proteins can compensate for the deleted gene (Shastry, 1995). This does not appear to be a significant problem in the case of Madm, since complete sequencing of both the human and mouse genomes has failed to identify another protein that has a similar sequence to Madm. Given the ubiquitous expression of Madm in

fetal life, embryonic lethality is one foreseeable problem in the generation of Madm-deficient mice. However, if this occurs, the cause of death may still provide an indication of Madm function. The generation of Madm -/- mice will be extremely valuable in understanding the role of this molecule *in vivo*.

Chapter 4: CHARACTERIZATION OF MADM

4.1 Introduction

Translocation (3;5) appears to be a crucial step in the pathogenesis of a proportion of patients with MDS leading to AML (Yoneda-Kato *et al.*, 1996), and Mlf1 most probably becomes oncogenic when fused to NPM. Its involvement in leukemia has lead to examination of Mlf1 in hemopoietic cells, where elevated *Mlf1* transcripts are detected in immature cell lines (Williams *et al.*, 1999), and in CD34+ human bone marrow cells (Matsumoto *et al.*, 2000). Previous studies have shown that *Mlf1* mRNA decreases during the maturation of M1 monoblastoid and MEL 707 erythroleukemia cell lines, supporting the notion that it is expressed in less differentiated hemopoietic cells (Williams *et al.*, 1999). Conversely, over-expression of Mlf1 in the erythroleukemic J2E cell line has been shown to induce an immature monoblastoid morphology and inhibit differentiation (Williams *et al.*, 1999). Significantly, ectopic expression of Mlf1 in normal hemopoietic progenitor cells decreases the number of erythroid progenitor cells, while increasing the number of myeloid progenitors (Williams *et al.*, 1999).

In an attempt to understand how Mlf1 was regulating hemopoietic lineage commitment and differentiation, a yeast two-hybrid screen was performed to identify interacting molecules. One Mlf1-binding protein was a member of the 14-3-3 family of proteins viz. 14-3-3ζ. Several studies have shown that subcellular localization of some proteins (e.g. BAD and FKHRL1) is regulated by binding 14-3-3. Since Mlf1 localizes to both the cytoplasm and nucleus (Williams *et al.*, 1999, Yoneda-Kato *et al.*, 1996), it was hypothesized that 14-3-3ζ regulated Mlf1 in a similar fashion. 14-3-3 usually binds partner proteins in a phosphoserine-dependent manner (Michaud *et al.*, 1995), hence attention also focused on identifying the kinase that phosphorylates Mlf1. As mentioned in Chapter 3, Madm had homology to protein kinases and contained features of a signaling molecule; therefore, the kinase activity of Madm was examined in this chapter. In addition, other features of the Madm protein, such as the putative NLS and NES, were investigated. Nuclear transport of proteins is a bi-directional, regulated process that occurs through specific structures that span the nuclear envelope

known as the nuclear pore complex. While the presence of an NLS is important for transport into the nucleus, similar specific recognition sequences are required for nuclear export.

4.2 Results

4.2.1 Expression and immunological detection of Madm

For functional characterization of the 535 amino acid Madm protein, the entire protein coding region, including a 10 amino acid HA tag at the amino-terminus or a 10 amino acid myc tag at the carboxyl-terminus, was cloned into the pcDNA3 expression vector. Using this vector, expression of Madm is brought under the control of the human cytomegalovirus immediate early promoter in mammalian cells; it can also be transcribed *in vitro* using T7 polymerase via a T7 promoter.

To determine whether the putative translational start site was functional, the predicted open reading frame of Madm was transcribed and translated *in vitro*. Transcripts were generated using T7 polymerase and translated *in vitro* incorporating L-[^{35}S]-methionine and L-[^{35}S]-cysteine to visualize the protein. As shown in Figure 4.1A, a major protein product of approximately 65 kDa was identified, corresponding to translation from the AUG at nucleotide 104. This molecular weight is slightly greater than that predicted from the primary amino acid sequence (60 kDa), suggesting that Madm is post-translationally modified. Additional less intense and faster migrating bands were detected at 51, 44 and 43 kDa which may correspond to translation initiation from AUG codons at nucleotides 359, 560 and 617 respectively.

To analyze the affects of deleting different regions of Madm, several mutants were made that lacked amino acids N-terminal to the kinase-like domain (1 – 98, MadmΔN98), amino acids C-terminal to the Mlf1-binding region (480 – 535, MadmΔC56) or amino acids C-terminal of the kinase-like domain (406 – 535, MadmΔC129). Translation of these mutants did not yield as much protein as the wild type molecule suggesting they are either not translated as efficiently or not tolerated as well by this system. Two of the mutants, MadmΔC56 and MadmΔC129 had molecular weights higher than those predicted by the amino acid sequence (54 kDa and 45 kDa, respectively) suggesting that although regions in the carboxyl terminus have been deleted, post-translational modifications were still occurring on other parts of the protein. In contrast, MadmΔN98 had a molecular weight comparable to the

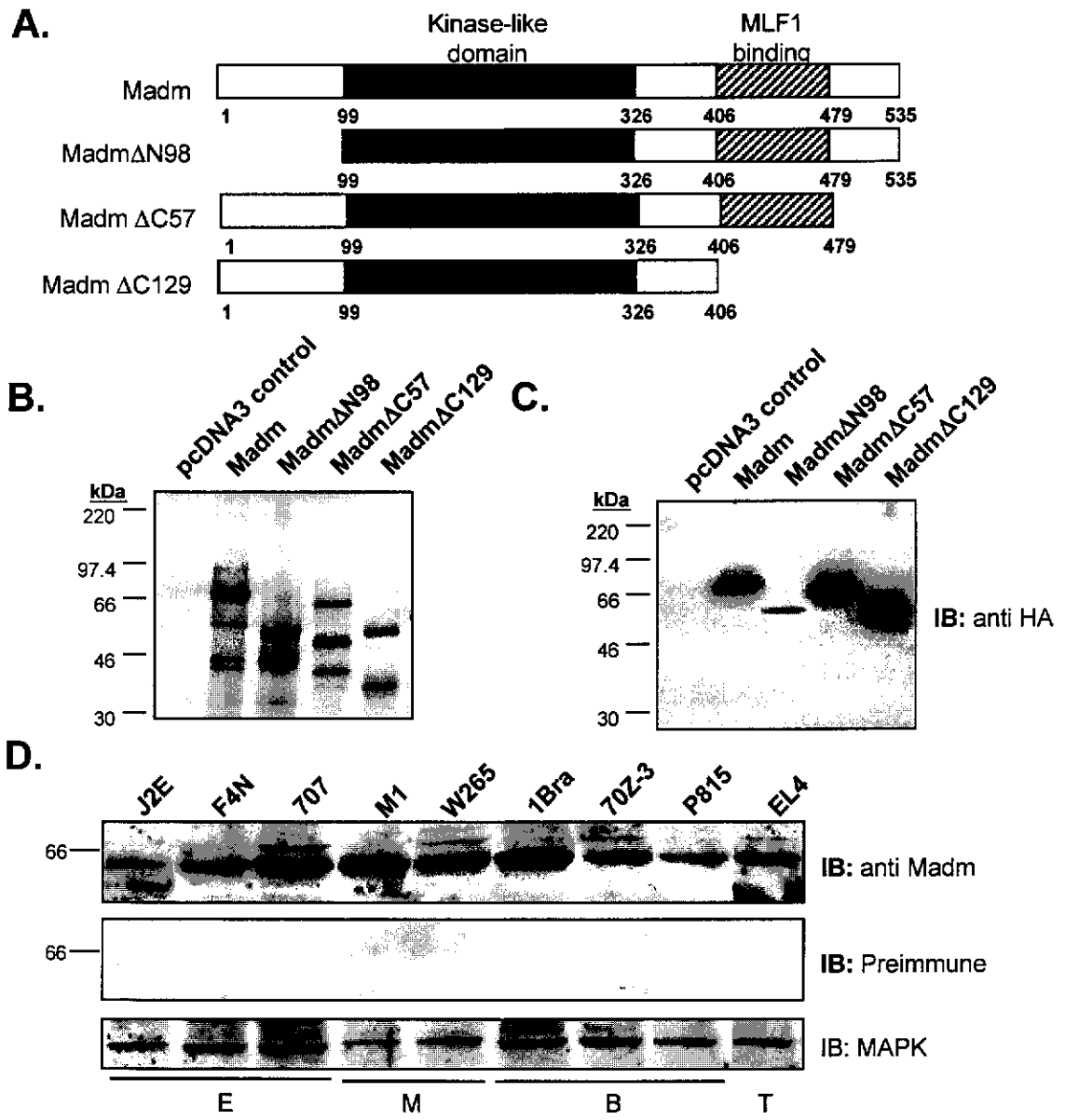


Figure 4.1. Expression of Madm protein. (A) Schematic diagram of Madm constructs. Shown are full length Madm (aa 1-535), MadmΔN98 (aa 99-535), MadmΔC57 (aa 1-479) and MadmΔC129 (aa 1-406). The kinase-like domain (black) and the Mlf1-binding region (hatched) are illustrated. (B) *In vitro* transcription/translation of Madm cDNA. The entire coding region of Madm and the deletion mutants shown in (A) were transcribed *in vitro* and translated using a reticulocyte lysate system. A vector only control (pcDNA3) is also shown. (C) Exogenous expression of Madm in COS7 cells. Madm constructs were synthesized with a N-terminal HA tag. Cells were transiently transfected and whole cell lysates immunoblotted with an anti-HA antibody. Molecular weight markers are shown at left. (D) Endogenous expression of Madm in hemopoietic cells. Whole cell lysates (100 μg/lane) were immunoblotted with Madm specific polyclonal antisera. Cell lines are labeled as erythroid (E), myeloid (M), B cell (B) or T cell (T). Preimmune serum and loading controls (MAP kinase) are shown.

predicted size of 49 kDa; this may indicate that by deleting the amino terminus, several sites for post-translational modification have been removed, thereby preventing the shift in electrophoretic mobility. As with the wild type protein, there were also faster migrating bands present in the deletion mutant samples. The smaller protein expressed with MadmΔN98 may have arisen from an initiation of translation at nucleotide 560, whereas the smaller proteins in samples MadmΔC56 and MadmΔC129 could have been generated by translation initiation from the AUG codon at nucleotide 359.

To determine if these translation products are expressed *in vivo*, wild type Madm and the deletion mutants were expressed using a mammalian cell system. Plasmid constructs bearing HA-tagged Madm, MadmΔN98, MadmΔC57 and MadmΔC129 were transfected into COS7 cells using Lipofectamine 2000. Twenty-four hours post-transfection, protein lysates were prepared and separated by SDS-PAGE. Immunoblotting using anti-HA antibodies revealed that only one polypeptide was expressed in mammalian cells, unlike the reticulocyte lysate (Figure 4.1B). This suggests that the shorter polypeptides shown in Figure 4.1A may be artifacts of the *in vitro* expression system and are not produced *in vivo*. Figure 4.1B demonstrates that the Madm, MadmΔN98, MadmΔC57 and MadmΔC129 polypeptides had molecular weights of approximately the same size of the full length proteins produced by the reticulocyte lysate viz. 65, 50, 60, 50 kDa, respectively.

To investigate the expression of endogenous Madm, polyclonal antibodies were raised in rabbits. The protein used for immunization was a GST fusion protein consisting of amino acids 1-55 of Madm. The GST-Madm recombinant protein was expressed in bacteria, but it was insoluble as the protein was packaged entirely into inclusion bodies. Therefore, the insoluble protein was resuspended in SDS and separated under reducing conditions. The band corresponding to GST-Madm was excised and used for immunization with Freund's adjuvant. Ten weeks after the primary inoculation, serum was collected and the presence of Madm-specific antibodies confirmed by immunoblotting total protein lysates from transiently transfected COS7 cells (data not shown). To examine Madm protein expression in hemopoietic cells, whole cell lysates were prepared and immunoblotted using the anti-Madm polyclonal antiserum. Figure 4.1C shows that a protein with a molecular weight of

approximately 65 kDa was detected in all cell lines examined; in contrast, this protein was not present in control blots probed with preimmune serum. Therefore, Madm was expressed in all cell lines examined and the level of expression was uniform across the different cell types, consistent with the mRNA expression profile (Figure 3.8).

4.2.2 Subcellular localization of Madm

As mentioned in Section 3.2.1, Madm contains a consensus bipartite NLS as well as a potential NES, suggesting that the protein may shuttle between the nucleus and cytoplasm. The potential NLS sequence in Madm was identified between amino acids 163 – 180 (Figure 4.2A) by examining the complete amino acid sequence using the PSORT computer program (http://psort.nibb.ac.jp). NLS sequences of this type are found in 56% of known nuclear proteins according to Dingwall and Laskey (1991); however, approximately 4.2% of non-nuclear proteins also contain this consensus sequence (Dingwall and Laskey, 1991). Comparison of the Madm NLS sequence among species (Figure 4.2B) showed that five out of five basic residues crucial for NLS function were conserved between mouse and human, while four out of five basic residues were present in the fly and worm proteins.

To examine if Madm is indeed nuclear, the pattern of subcellular localization was determined. Initially, investigation of both endogenous Madm protein in NIH3T3 cells, and exogenous protein from transiently-transfected COS7 cells, was performed by cell fractionation and immunoblotting. Transfected COS7 cells (Figure 4.3A) or untransfected NIH3T3 cells (Figure 4.3B) were separated into nuclear and cytoplasmic fractions. Exogenous Madm from COS7 cells was found predominantly in the cytoplasmic fraction; although, it was also detected in the nuclear fraction to a lesser extent. In contrast, endogenous Madm from NIH3T3 cells was found only in the cytoplasmic fraction, and not in the nuclear fraction. This difference may be due to the over-expression of Madm in COS7 cells, where excessive levels may have forced the protein into the nucleus. Alternatively, the levels of endogenous Madm protein in the nucleus of NIH3T3 cells may be below the limits of detection for the Madm-specific antiserum.

The intracellular distribution of Madm was then examined by confocal fluorescence microscopy. However, the polyclonal Madm antisera was unable to detect endogenous

A.

K K T K K N H K T M N E **K** A W **K R**	Madm
K K A V T K A Q K K D G **K** K R **K R**	Histone H2B
K K E L G H V N G L V D **K** S G **K R**	PHD
K K M K G P Q V L D F I **K** Q G **K R**	ZAP-70
K K - 10 aa - **K** **K R**	Consensus

B.

K K T K K N H K T M N E **K** A W **K R** W	$Human_{163-180}$
K K T K K N H K T M N E **K** A W **K R** W	$Mouse_{163-180}$
K R T K R N A K R L P L E S W **R R** W	$Fly_{205-222}$
Q **R** T R K A G S S L S I **K** A W **K K** W	$Worm_{140-157}$
K K - 10 aa - **K** **K R**	Consensus

Figure 4.2. NLS and NES sequences of Madm. (A) Alignment of the Madm NLS sequence with bipartite NLSs from other proteins. Shown are Histone H2B, the transcription factor PHD and the kinase Zap-70. (B) Comparison of the Madm NLS in human, mouse, fly and worm.

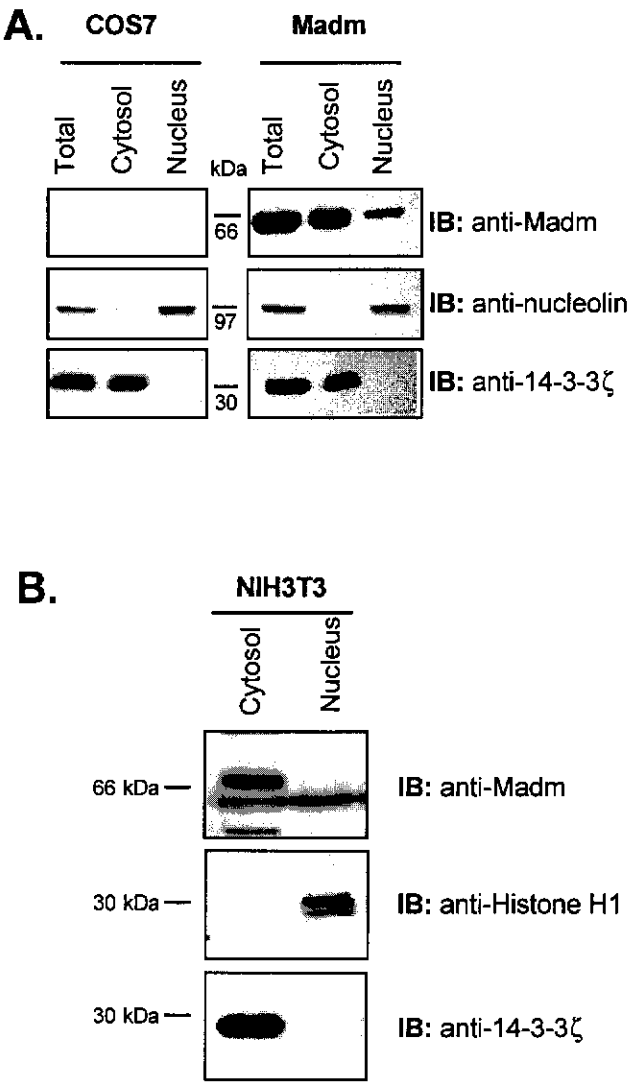


Figure 4.3. Cell fractionation analysis of Madm. Fractionation and immunoblot analysis of (A) COS7 cells transiently transfected with Madm or (B) NIH3T3 cells. The cell fractions were characterized by stripping and re-probing the blot for marker proteins, nucleolin or histone H1 as nuclear markers, and 14-3-3ζ as a cytoplasmic marker.

protein by indirect immunofluorescence (data not shown), and subcellular localization of Madm was determined in transiently-transfected COS7 cells. Several Madm constructs were synthesized to examine subcellular localization. These were (i) a N-terminal GFP-Madm fusion protein for direct immunofluorescence, (ii) a N-terminal HA-tagged Madm protein, and (iii) a C-terminal myc-tagged Madm (Figure 4.4A). Figure 4.4B shows that Madm protein was present in both nuclear and cytoplasmic compartments. Within the cytoplasm, Madm also localized in distinctive structures which may indicate binding to the cytoskeleton or microtubule complex. In addition, the diffuse nuclear localization of Madm did not include the nucleolus. The compartmentalization of Madm in transiently-transfected 293T, NIH3T3 and Hela cells was also found to be similar (data not shown). The presence of HA- and myc-tagged Madm in the nucleus demonstrated that the GFP tag did not influence nuclear localization. Thus, the Madm protein is probably actively transported to the nucleus. This observation implies that the protein either contains a functional NLS, or is transported by a protein that does. Furthermore, the exclusion of a large portion of Madm from the nucleus suggests that it may be exported from the nucleus, or is held in the cytoplasm by protein-protein interactions or post-translational modifications. It is worth reiterating that several potential phosphorylation sites exist in the vicinity the putative Madm NLS, suggesting that post-translational modification may be important in regulating Madm subcellular localization.

To determine if nuclear export was an important factor in regulating Madm localization, the amino acid sequence was analyzed and revealed several potential leucine-rich regions with features of an NES. These were located between amino acids 119-129, 457-467 and 488-499 (Figure 4.5A). The four key hydrophobic residues of the first NES are conserved between the mouse and human proteins, but only three of these are present in fruit fly and worm (Figure 4.5B); however, in the third NES, all four residues are present in the mouse, human and *D. melanogaster* proteins.

Previous studies investigating NES sequences have determined functionality by mutating two critical hydrophobic residues within the leucine-rich motif (Neufeld *et al.*, 2000). Therefore, to determine whether the first NES (amino acids 119 – 129) was able to direct nuclear export, the last two hydrophobic residues (Leu^{127} and Ile^{129}) were mutated to alanine and the affect on subcellular localization analyzed by confocal microscopy (Figure 4.5C).

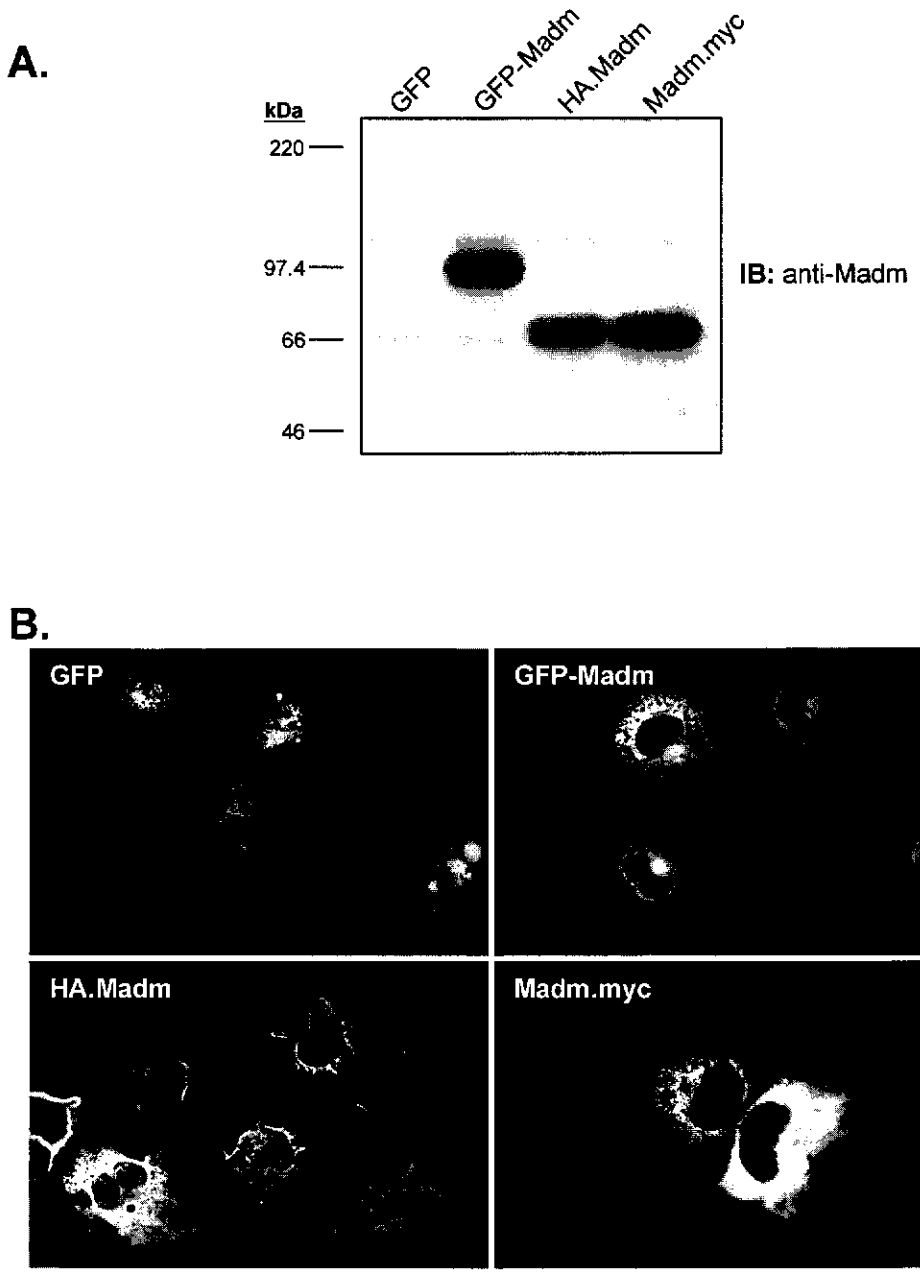


Figure 4.4. Subcellular localization of Madm. (A) Identification of GFP-Madm, HA- and myc-tagged Madm proteins expressed in COS7 cells by immunoblotting of whole cell lysates with Madm-specific antisera. (B) Fluorescence patterns of COS7 cells transiently transfected with GFP vector alone, GFP-Madm, HA-tagged Madm and myc-tagged Madm.

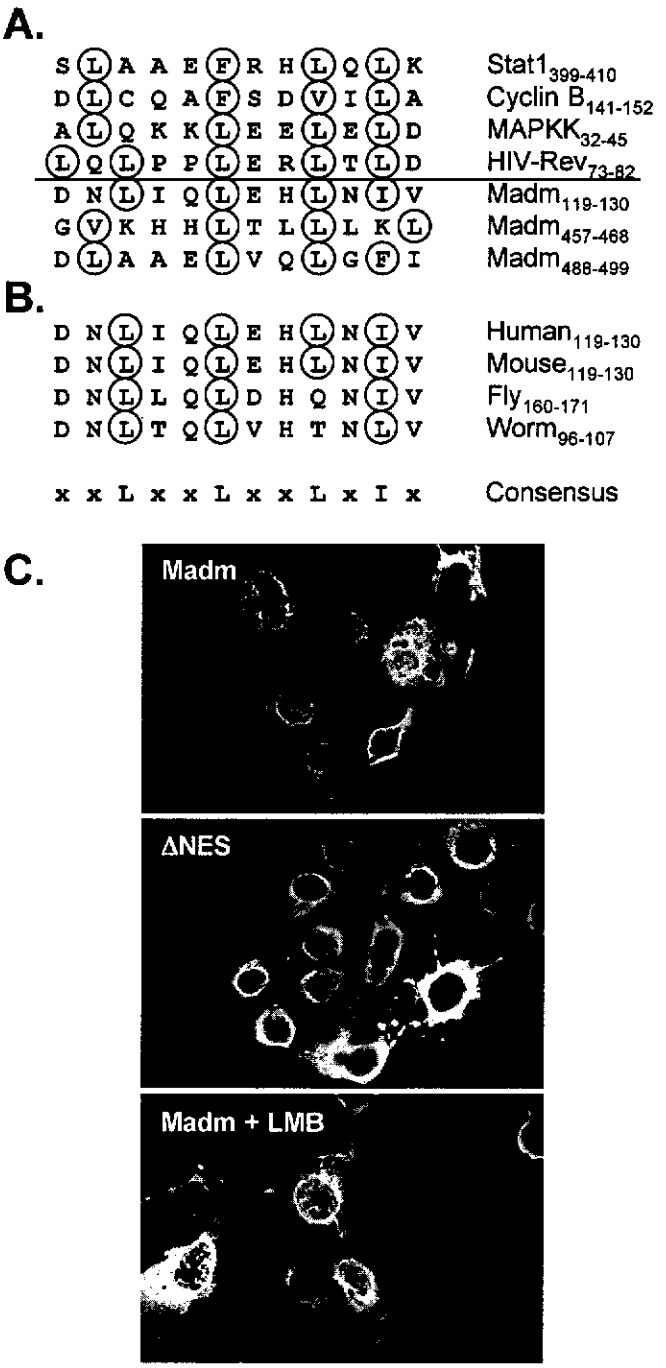


Figure 4.5. NES sequences in Madm protein. (A) Alignment of the three putative NES sequences of Madm with previously characterized leucine-rich NESs of Stat1, Cyclin B, Mitogen-activated protein kinase kinase (MAPKK) and HIV-Rev. Circled amino acids are important hydrophobic residues (leucine, isoleucine or phenylalanine). (B) Similarity of the first NES sequence of Madm protein from different species. (C) Immunofluorescence of COS7 cells expressing either HA-tagged Madm, a Madm protein with the NES mutated (ΔNES) and COS7 cells expressing HA-tagged Madm following treatment with Leptomycin B for 12 h to inhibit nuclear export.

Cells transfected with Madm alone showed the characteristic cytoplasmic and nuclear localization patterns. Unexpectedly, mutation of the NES sequence did not change in Madm localization, suggesting that although a consensus NES sequence is present it may not be entirely responsible for directing nuclear export.

Since there was a possibility that Madm was being exported from the nucleus by some other mechanism, either via one of the other potential NES sequences or independent of a leucine-rich motif, further studies were performed utilizing the chemical leptomycin B (LMB), a potent inhibitor of the CRM1/exportin 1 pathway for nuclear export. To determine the affect of inhibiting CRM1/exportin 1 on Madm localization, transiently-transfected COS7 cells were treated with 10 μg/mL LMB for 12 h and cells were analyzed by indirect immunofluorescence. If the movement of Madm into and out of the nucleus is dependent on CRM1/exportin 1, then LMB should induce accumulation of Madm in the nucleus. However, upon treatment with LMB there was no change in the pattern of Madm localization (Figure 4.5C). This suggests that Madm nuclear transport is unlikely to be dependent on the CRM1/exportin 1 pathway.

To determine whether any particular region of Madm was involved in directing subcellular localization, cDNAs encoding Madm amino acids 1 – 99 and 326 – 535 (ΔKinase), 99 – 535 (ΔN98), 1 – 479 (ΔC57), 1 – 406 (ΔC129), 1 – 326 (ΔC209) and 99 – 326 (Kinase) were constructed with N-terminal HA tags and expressed in COS7 cells (Figure 4.6A and B). Examination of the Madm mutant proteins indicated that all protein shared similar localization, appearing in both nuclear and cytoplasmic compartments. Therefore, no discrete region of Madm appeared to specify localization.

Since all the immunofluorescence analyzes to date had been performed on cells that were growing asynchronously, Madm localization was examined during the course of the cell cycle. Transiently transfected cells were arrested at the G_1/S phase boundary of the cell cycle by a "thymidine" block. Cells were released into the cell cycle by removal of thymidine and the localization of Madm was monitored. Figure 4.7 shows that the localization of Madm did not change appreciably while the cells were synthesizing DNA (S phase), or preparing for cell division (G_2). The only time Madm compartmentalization altered significantly was 8 h

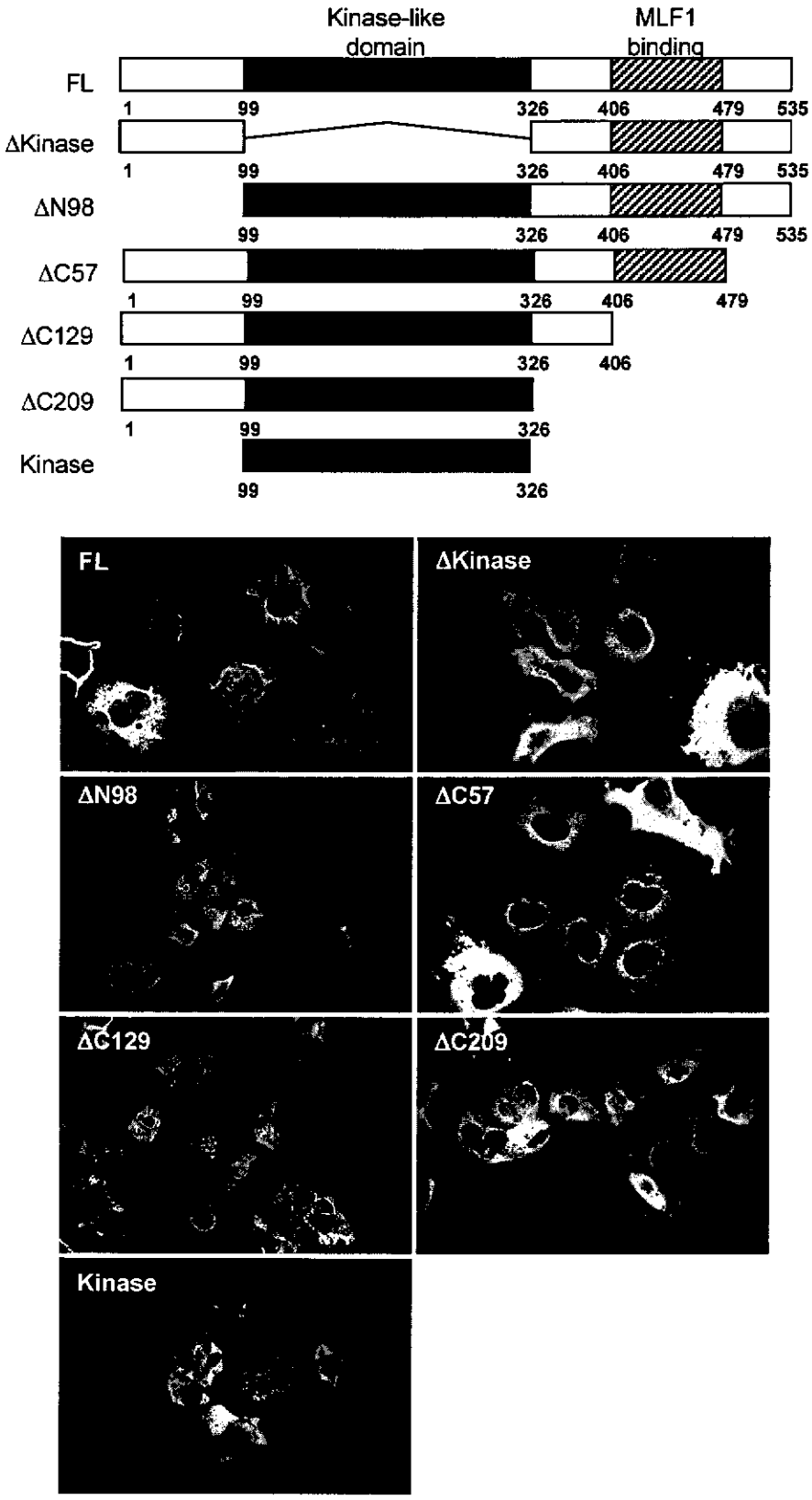


Figure 4.6. Localization of Madm deletion mutants. (A) Diagram of the Madm fragments generated. Each polypeptide contains an N-terminal HA tag. (B) Immunoluorescence of the Madm mutants after transient expression in COS7 cells.

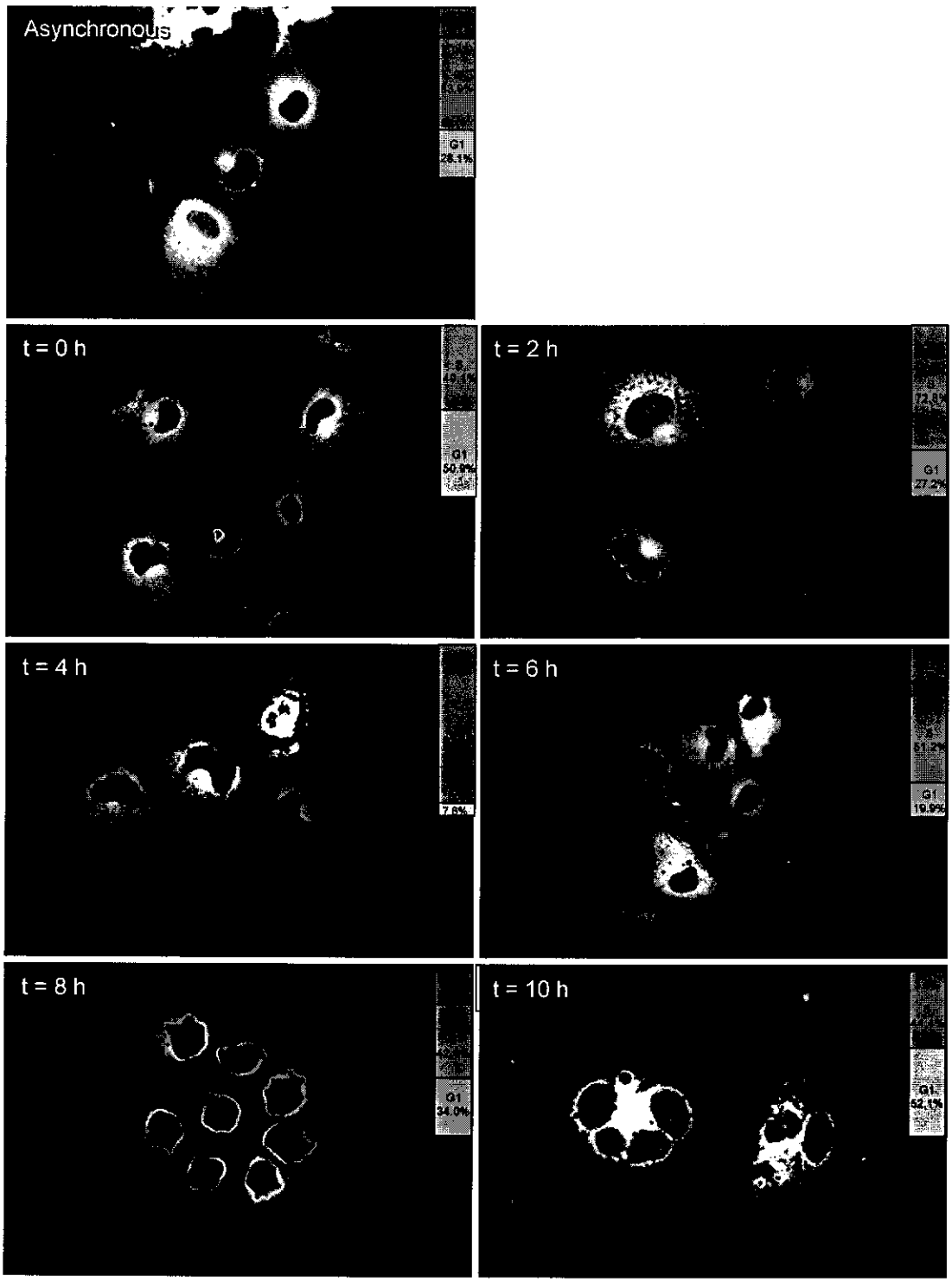


Figure 4.7. Madm localization throughout the cell cycle. COS7 cells transiently transfected with GFP-Madm were arrested at the G_1/S boundary of the cell cycle (t = 0 h) by overnight incubation with 2 mM thymidine. After washing the cells were allowed to progress through the cell cycle for the times indicated. Localization was monitored by confocal microscopy. The percentage of cells in each phase of the cell cycle was determined by flow cytometry of propidium iodide-stained cells (shown at the top right of each panel).

after the removal of thymidine; at this point the majority of cells were approaching the end of G_2 were preparing for cytokinesis. Intriguingly, Madm protein concentrated in the perinuclear region and was excluded from the nucleus.

4.2.3 Expression of recombinant Madm

To produce recombinant protein, Madm and a mutant form of Madm, deficient in the kinase-like domain (ΔKinase), were cloned into the bacterial expression vector, pET28a. Expression using this vector resulted in recombinant proteins containing an N-terminal 6 x His tag for purification. Recombinant Madm was expressed in large scale bacterial cultures, but constituted less than 1% of the total soluble protein (Figure 4.8A) as determined by Coomassie staining. The majority of Madm expressed was insoluble and packaged into inclusion bodies. The ΔKinase mutant was also expressed on a large scale and constituted ~10% of the total soluble protein (Figure 4.8A). When total bacterial lysates were probed with anti-Madm antiserum, full length Madm and ΔKinase were detected. In addition, bands with faster migration rates were also observed which may represent degradation products of the recombinant proteins (Figure 4.8A), or translation initiation from internal methionine codons as observed when Madm was expressed using reticulocyte lysate (Figure 4.1B). Proteins detected with the anti-Madm antibody were not synthesized in the vector control.

Recombinant Madm and ΔKinase proteins were purified from bacterial cultures, using metal chelate chromatography and elution with imidazole. The unbound fraction and two imidazole fractions were then analyzed by PAGE, Coomassie brilliant blue staining and immunoblotting with the anti-Madm antiserum. The majority of recombinant protein eluted in the first wash, with residual protein appearing in the second wash (Figure 4.8B). Examination of the protein that failed to bind the resin revealed that a proportion of Madm and ΔKinase was not purified (Figure 4.8B). It is possible that this fraction of protein was either insoluble or folded incorrectly, thus preventing the 6 x His tag from binding the metal ions. Some of the degradation products detected in the whole cell lysates (Figure 4.8A) were also purified on the resin (Figure 4.8B, E1 and E2). This indicates that these fast migrating proteins are most-probably C-terminal truncations that leave the N-terminal 6 x His tag intact. Alternatively, the bacterial translational machinery may have prematurely terminated

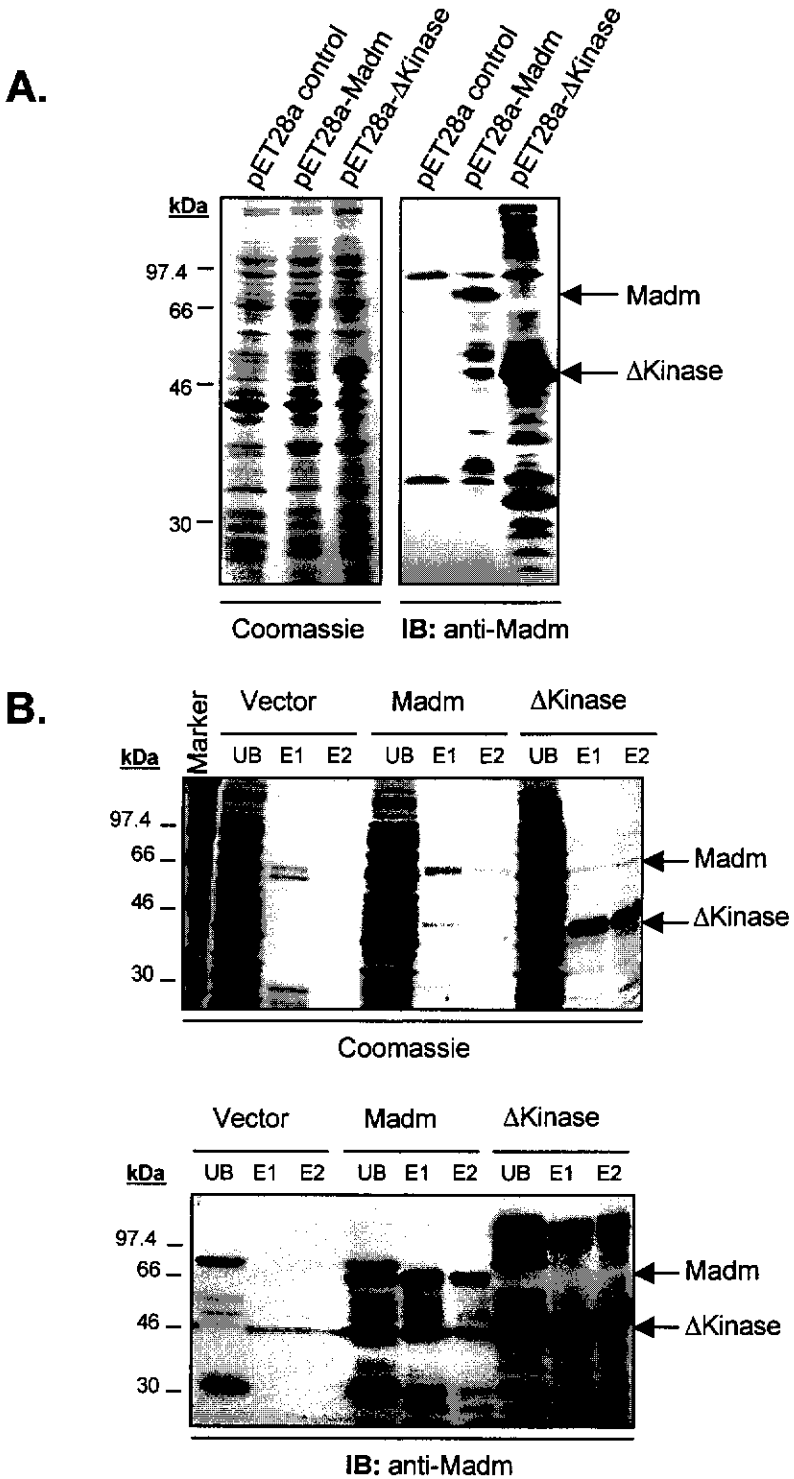


Figure 4.8. Production of recombinant Madm and MadmΔKinase mutant in bacteria. (A) Whole cell lysates of bacteria transformed with vector (pET28a), Madm (pET28a-Madm) and ΔKinase (pET28a-ΔKinase) constructs. The positions of the recombinant proteins are shown by arrows. (B) Metal chelate purification of lysates shown in (A). Shown are the unbound fraction (UB) and the two imidazole elution fractions (E1 and E2). Proteins were analyzed by Coomassie stain and immunoblotting. Madm and ΔKinase are identified by arrows. Molecular weight markers are at left

production of the full-length proteins. Coomassie staining of the eluted fractions detected the presence of several co-purifying proteins. These proteins are present in all three samples and are probably bacterial proteins that contain high percentages of histidine residues which have an affinity for the resin.

To eliminate contaminants from the purified proteins, ion exchange chromatography was performed. The two imidazole fractions from the metal chelate chromatography were pooled and dialyzed into 20 mM Tris.HCl pH 6.8. The isoelectric point of Madm was predicted to be 5.32, using the ExPASy "Compute pI/Mw Tool" web site (http://www.expasy.ch/tools/pi_tool.html). Since Madm was dialyzed in a buffer with slightly higher pH than the theoretical pI of Madm, the protein should have a negative charge; therefore, an anion exchange resin (MonoQ) was used for further purification. The majority of protein did not bind the column and was subsequently washed through. Bound proteins were eluted with a linear gradient of NaCl. Madm was the first major protein to elute from the column, followed by several other contaminants (Figure 4.9A). Fractions containing Madm were identified by immunoblotting using anti-Madm antiserum (Figure 4.9B). Madm eluted in the first major peak at a NaCl concentration of approximately 100 mM. A degradation product also detected by the anti-Madm antiserum eluted at a slightly lower NaCl concentration. The purity of the vector control, recombinant Madm and ΔKinase proteins was then determined on a Coomassie stained SDS-PAGE gel. Figure 4.9C shows that the Madm and ΔKinase proteins were ~50% and ~80% pure, respectively. However, several contaminating proteins in the vector control still remained even after two rounds of purification. A 67 kDa contaminating protein was present in both the Madm and ΔKinase samples differed slightly from the 68 kDa contaminant in the vector control. It is possible that this protein was co-purifying via interaction with the Madm/ΔKinase polypeptides, rather than with the metal ion or MonoQ resins. In addition, some degradation products of the recombinant proteins remained after purification.

4.2.4 Enzymatic activity of Madm

Although Madm has a consensus kinase domain, it lacks a typical ATP-binding motif (Figure 3.1); it was, therefore, important to determine whether Madm was a genuine kinase. Purified

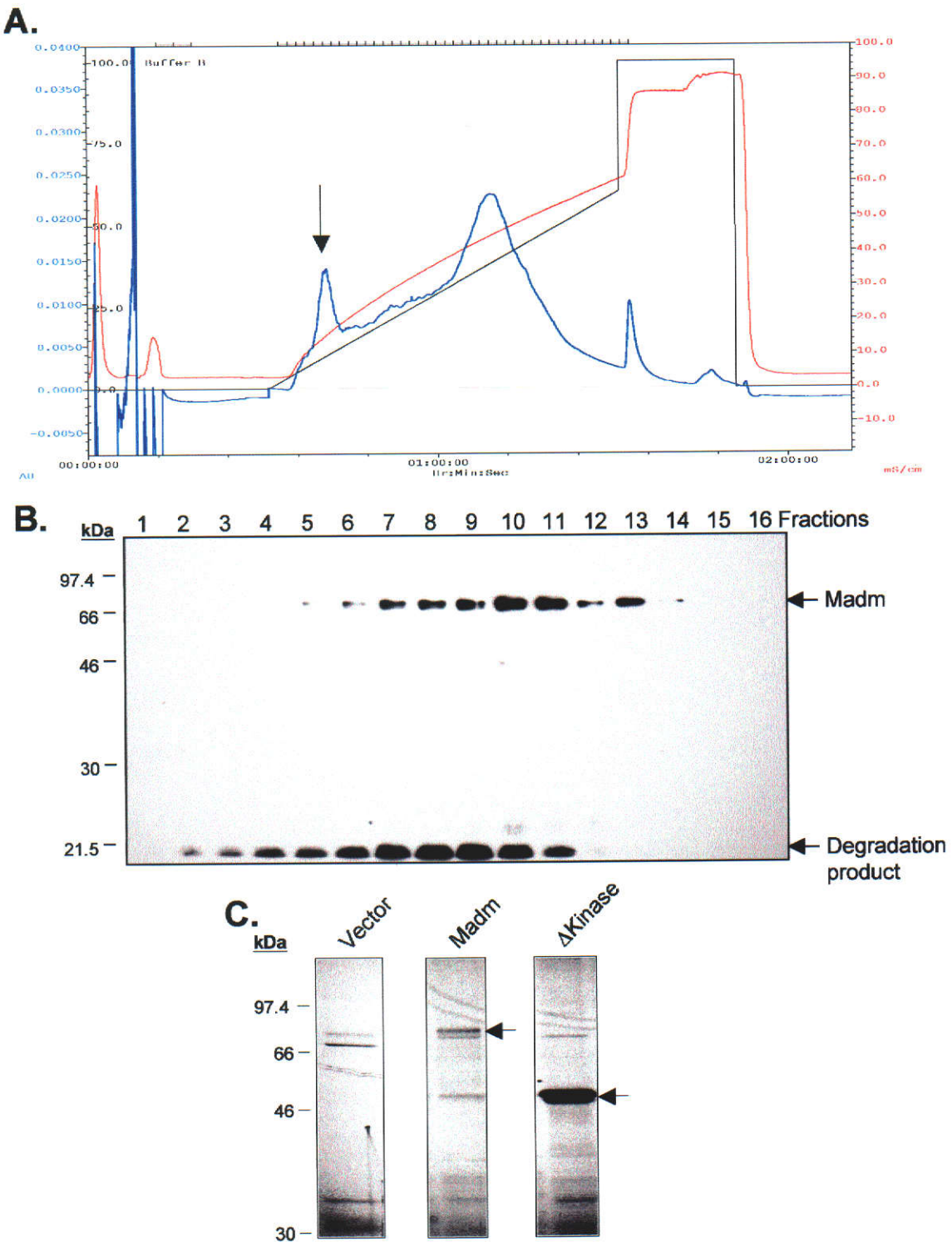


Figure 4.9. Ion exchange purification of recombinant Madm. (A) Elution profile of anion exchange purification. Absorbancy (OD_{254nm}) is indicated by the blue line, percentage of 1 M NaCl by the black line and conductivity (mS/cm) by the red line. The arrow indicates the Madm-containing peak. (B) Immunoblot of eluted fractions. Madm eluted in peaks 5 - 14 which is the peak indicated in (*A*) with an arrow. A degradation product of Madm also eluted at a slightly lower NaCl concentration in fractions 2 - 11. (C) Coomassie stained gel of protein purified from vector control, Madm and ΔKinase preparations. Full length recombinant Madm and ΔKinase proteins are indicated by arrows.

recombinant Madm was used to determine whether the protein possessed enzymatic activity in an autokinase assay. As controls, protein purified from vector only and ΔKinase samples were used. After exposure to radiolabled ATP a single protein was phosphorylated in the Madm sample that was not present in the vector control (Figure 4.10A). Although it was the same size as full length Madm, the phosphorylated protein was also present in the ΔKinase sample. It is likely to be the contaminant that was detected after Coomassie staining of the purified protein (Figure 4.9C). Immunoblotting with anti-Madm antisera confirmed that full length Madm was not present in the ΔKinase samples, and therefore was not contributing to the phosphorylation (Figure 4.10B). The band containing the contaminating protein was visualized using Sypro-Orange and excised from the gel for further analysis. Examination of trypsin-digested peptide fragments of this protein using mass spectrometry revealed that the contaminant was an *E. coli* protein named Colicin IB. This confirms that it was a different protein, not Madm, which is phosphorylated in the *in vitro* kinase assays.

There are several factors that may contribute to a lack of kinase activity in bacterially-expressed Madm: (*i*) the protein may not be folded correctly by the bacterial translational machinery, (*ii*) Madm may require interaction with an activator protein, such as that seen for cyclin-mediated activation of cdc2 kinase (Draetta *et al.*, 1989) and (*iii*) Madm kinase activity may be regulated by phosphorylation, in a similar manner to mitogen-stimulated S6 kinase (Ballou *et al.*, 1988). To further examine the hypothesis that Madm does possess kinase activity, kinase assays were performed on protein expressed in mammalian cells. Several HA-tagged mutant Madm constructs were transiently transfected into COS7 cells to characterize domains of Madm possibly required for kinase activity. The various constructs included full length Madm (FL), a protein lacking the kinase domain (ΔKinase) and several truncations removing increasing amounts of the C-terminal (ΔC57, ΔC129 and ΔC209). Immunoprecipitation after exposure to radiolabeled ATP revealed that full length Madm was highly phosphorylated in this *in vitro* kinase assay, suggesting that Madm possessed autokinase activity. However, all the Madm mutants, including the ΔKinase protein, were also phosphorylated indicating that a separate kinase co-precipitated with, and phosphorylated, these proteins (Figure 4.11A). Indeed when myelin basic protein was added to Madm immunoprecipitates, its phosphorylation was increased seven fold over the control (Figure

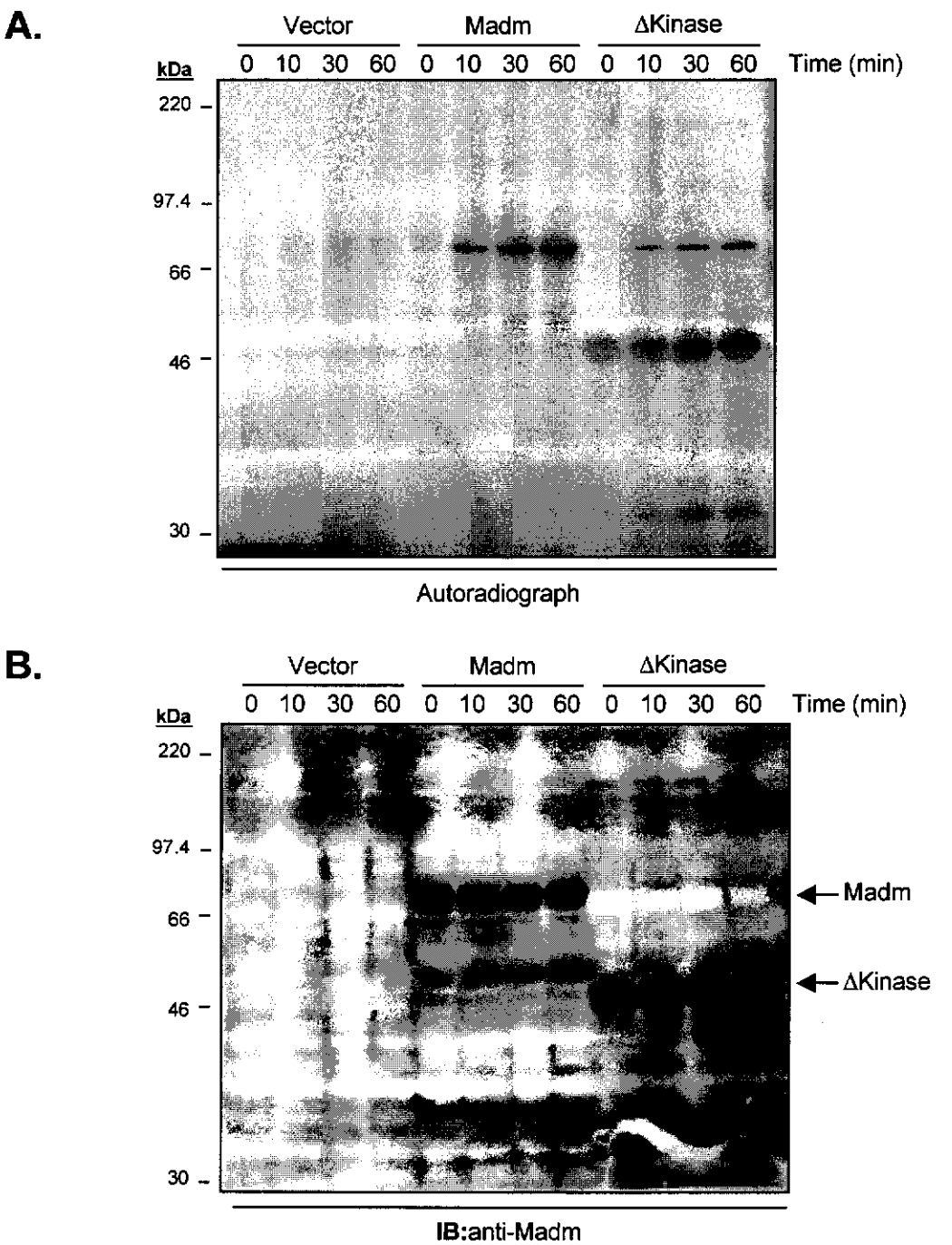


Figure 4.10. Enzymatic activity of recombinant Madm. (A) Bacterially-expressed Madm was incubated in the presence of [γ^{32}P]-labeled ATP for the times indicated. Protein purified from Vector only and ΔKinase samples were used as controls. (B) The presence of Madm and ΔKinase proteins was determined by immunoblotting with Madm-specific antisera. The size of molecular weight markers are shown at left.

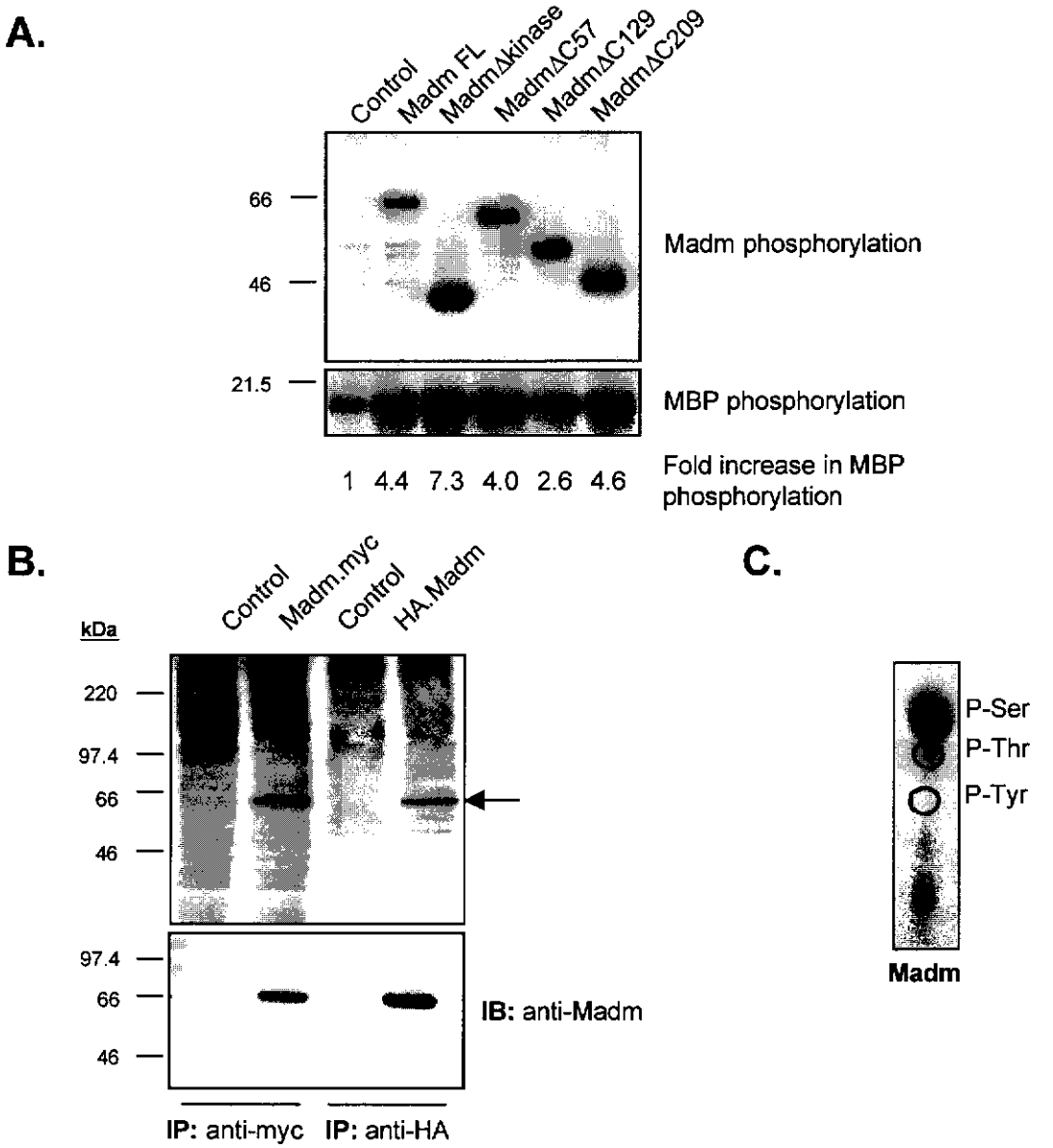


Figure 4.11. Madm is phosphorylated *in vitro* and *in vivo*. (A) Madm and deletion mutants were immunoprecipitated and subjected to *in vitro* kinase assays in the presence of 1 μg myelin basic protein (MBP). Radiolabeled protein was separated using SDS-PAGE and visualized by autoradiography. (B) COS7 cells transiently transfected with myc- or HA-tagged Madm were radiolabeled with [^{32}P]phosphoric acid for 7 hours before lysates were prepared. Madm was immunoprecipitated with the indicated antibodies and analyzed by SDS-PAGE and autoradiography (upper panel). The location of Madm protein on the autoradiograph (arrow) was confirmed by immunoblotting (lower panel). (C) Phosphoamino acid analysis of *in vivo* phosphorylated Madm. Phosphorylated Madm was treated with hydrochloric acid and hydrolyzed amino acids separated using one-dimensional thin layer chromatography. Radiolabeled amino acids were visualized by autoradiography. The position of ninhydrin-stained amino acid standards circled.

4.11A). The absence of Madm kinase activity was also supported by the inability of mammalian-expressed, and bacterially-produced, proteins to autophosphorylate under the reducing conditions of in-gel kinase assays (data not shown).

To determine if Madm was phosphorylated *in vivo*, COS7 cells transiently expressing either HA- or myc-tagged Madm were cultured in the presence of [^{32}P]-labeled phosphoric acid. Madm was then specifically immunoprecipitated using anti-HA or anti-myc antibodies. Figure 4.11B shows that the immunoprecipitates contained phosphorylated Madm protein, demonstrating that phosphorylation of Madm does occur *in vivo*. Phosphoamino acid analysis was performed to determine which Madm residues were phosphorylated. The results of this experiment shown in Figure 4.11C revealed that the only amino acid that was radiolabeled was serine, indicating that the Madm immunoprecipitates contain a serine kinase.

To characterize the activity of this kinase, cells transfected with full length Madm were stimulated with several known activators of serine/threonine kinases. Cells were treated with vanadate, okadaic acid, or were serum-starved before re-stimulation with serum, before lysis and immunoprecipitation. Figure 4.12A and B shows that these treatments did not alter Madm phosphorylation. Similarly the addition of several inhibitors of known serine/threonine kinases, such as PKA inhibitor, EGTA and staurosporine, did not result in any change of Madm phosphorylation (data not shown).

Madm phosphorylation was also monitored at different stages throughout the cell cycle. Cells were synchronized at the G_1/S phase of the cell cycle using a thymidine block. Following the removal of thymidine from the culture medium, cells progressed into S phase and samples were collected over a 9 h period. Madm was immunoprecipitated and its phosphorylation monitored using *in vitro* kinase assays. Figure 4.12C demonstrates that there was no apparent change in overall Madm phosphorylation throughout the cell cycle.

4.2.5 Madm forms homodimers

Preliminary experiments with the yeast two-hybrid system indicated that Madm was capable of homodimerization. To identify the region responsible for self-association, full length Madm

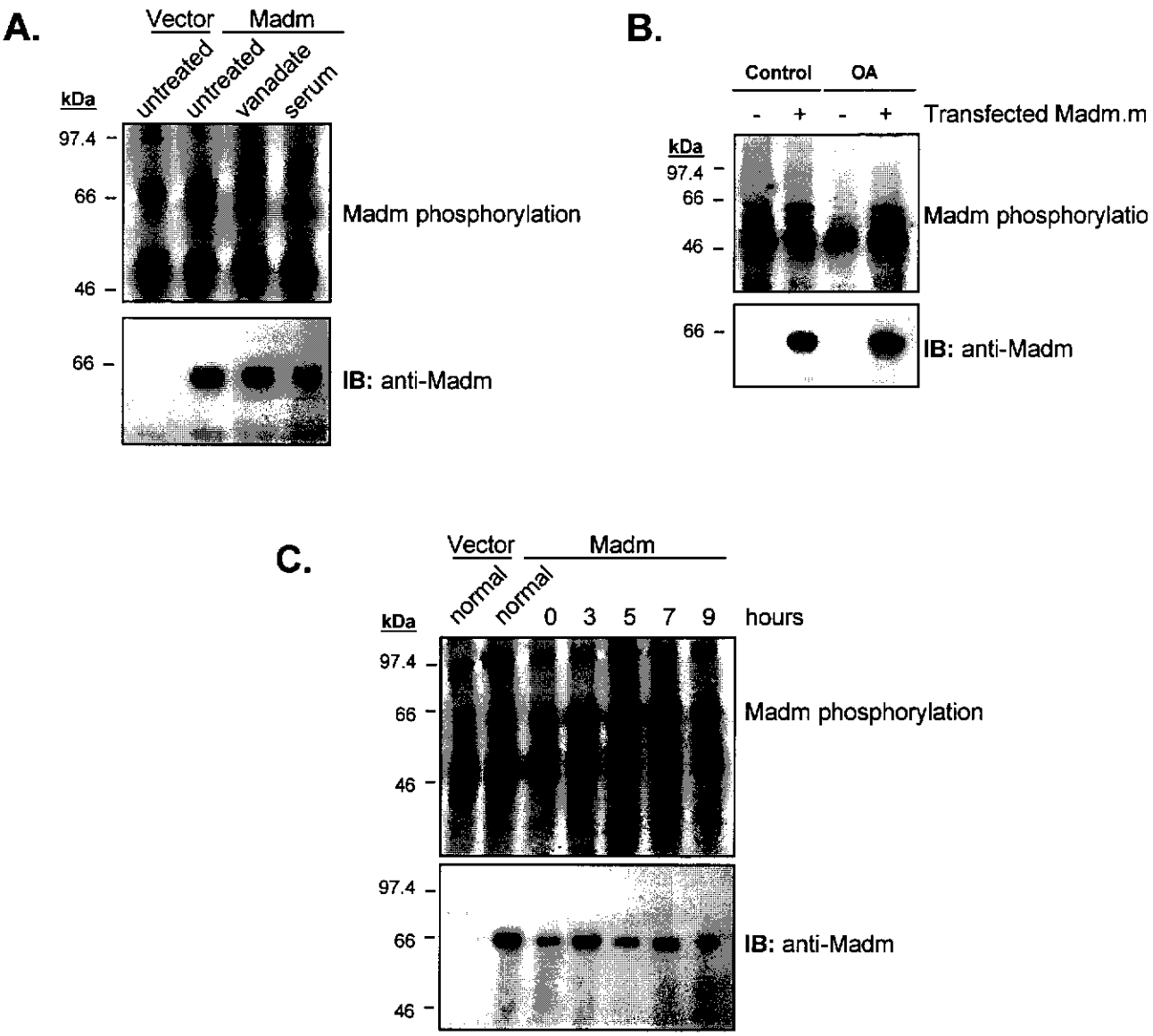


Figure 4.12. Madm phosphorylation in different culture conditions. COS7 cells were transiently transfected with vector alone or Madm constructs and stimulated using (A) vanadate (0.1 M), serum (10%) and (B) okadaic acid (1 μM). (C) Transiently transfected COS7 cells were arrested at the G_1/S phase boundary of the cell cycle by a 2 mM thymidine block and protein was analysed at the indicated time points following release into the cell cycle. Madm phosphorylation was monitored by *in vitro* kinase assay. Immunoblots using Madm-specific antisera show the amount of Madm protein loaded.

was screened for binding to several Madm deletion mutants using the yeast system. Binding was determined by β-galactosidase assay and these results are summarized in Figure 4.13A. Full length Madm was able to associate with the wild type construct and the ΔC57 mutant, but was unable to bind the ΔC209 mutant or a protein consisting of the kinase-like domain alone (Kinase). Therefore, the dimerization domain is within the carboxyl terminal region. Significantly, a mutant consisting of only the C-terminal Mlf1-binding region also associated with the full length protein. To confirm that Madm could dimerize in mammalian cells, Madm constructs tagged with either myc or HA were co-transfected into COS7 cells and immunoprecipitated with anti-myc or anti-HA antibodies. The data presented in Figure 4.13B confirmed that Madm is indeed able to self-associate in mammalian cells.

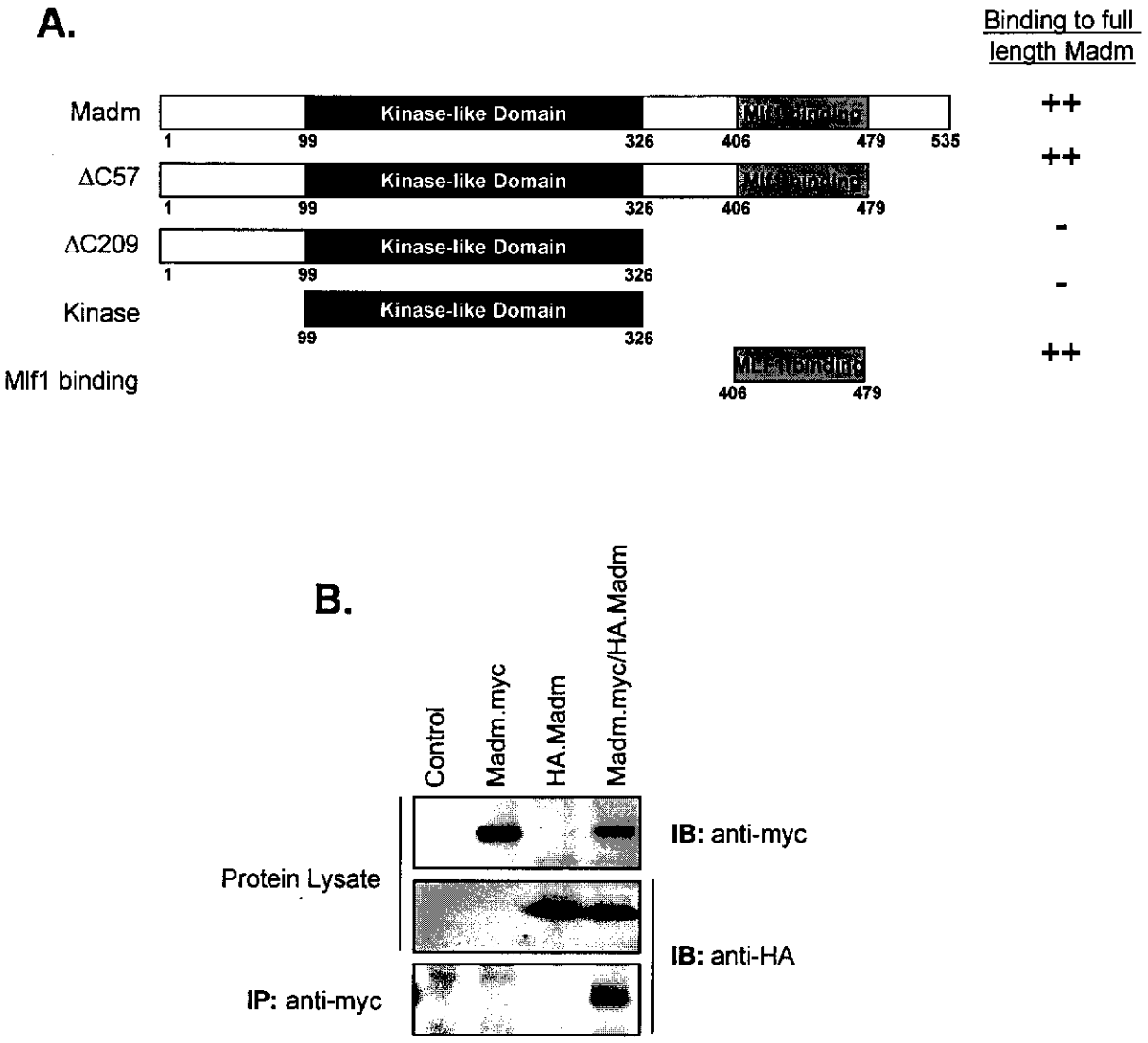


Figure 4.13. Madm is able to form dimers. (A) Madm was analyzed for self-association with full length and deletion mutant proteins using the yeast two-hybrid system. The symbols on the right indicate the intensity of color development in β-galactosidase assays. (B) COS7 cells were transiently transfected with vector, myc-tagged and/or HA-tagged Madm constructs. Cells were lysed and immunoblotted with anti-myc or anti-HA antibodies (upper panels). To demonstrate dimerization, Madm was immunoprecipitated using anti-myc antibodies and immunoblotted with anti-HA antibodies (lower panel).

4.3 Discussion

In this chapter the features of Madm protein were investigated. *In vitro* transcription/translation of the protein revealed that the protein product migrated at 65 kDa, which is larger than the predicted molecular weight of 60 kDa. Since the Madm sequence does not contain glycosylation, myristylation or palmoylation consensus sequences, the increase in size may be due to phosphorylation. In support of this notion, dephosphorylation of protein lysates with calf intestinal alkaline phosphatase increased the electrophoretic mobility of Madm (data not shown). Immunoblotting with polyclonal antisera against Madm also demonstrated that the size of endogenous Madm in hemopoietic cells was approximately 65 kDa, comparable with the cell-free expression system.

Expression of Madm in the cell-free system and in mammalian cells identified some interesting properties of the protein. *In vitro* translated Madm deletion mutants were expressed at reduced amounts compared with full length Madm. This was also observed in mammalian cells, where the ΔN98 mutant was expressed at considerably lower amounts than the full length, ΔC57 or ΔC129 proteins. Additionally, expression of a mutant Madm protein consisting of only the kinase-like domain (aa 99 – 326) was expressed at decreased levels, suggesting that some element in the amino-terminus of Madm may be important for its translation efficiency or stability. Analysis of the Madm sequence with a different algorithm (available at http://emb1.bcc.univie.ac.at/embnet/tools/bio/PESTfind/) revealed that there were two PEST domains between aa 1 – 63 and 430 – 442. PEST sequences are rich in proline, glutamic acid, serine and threonine residues and are thought to regulate protein degradation. However, in contrast to Madm, it has been shown that deletion of PEST sequences can extend the half-life of proteins, e.g. IκBα (Shumway *et al.*, 1999). The PEST sequences were also identified in NRBP, the human homolog of Madm and it was suggested that the presence of these sequences may indicate that NRBP is rapidly degraded *in vivo* (Hooper *et al.*, 2000); however, no experimental data was provided to support this theory.

Recently, PEST domains have been attributed functions other than regulating protein degradation. For instance, the PEST domain of transcription factor PU.1 is required for normal myeloid development (Fisher *et al.*, 1998). It was shown that a specific stretch of

amino acids high in glutamine within the PEST domain was essential for myelopoiesis (Fisher *et al.*, 1998). Although the function of this region was unclear, the authors concluded it was probably involved in protein-protein interaction. Since the Madm PEST domain consists of mainly serine and glutamic acids residues that may bind SH2 domains (Malek and Desiderio, 1994, Malek *et al.*, 1996), it is conceivable that this part of the molecule is important for protein-protein interaction, rather than protein degradation.

The subcellular localization of Madm was investigated to define where it was acting in the cell. Madm contains consensus sequences for both nuclear localization and nuclear export. Interestingly, key residues in the Madm NLS are conserved between human and mouse, but only partially retained in the fruit fly or worm homologs. Approximately 56% of known nuclear proteins contain this type of NLS and it is only found in 4% of non-nuclear proteins (Dingwall and Laskey, 1991), indicating a likely nuclear function for Madm. When over-expressed in COS7 cells, subcellular fractionation showed that Madm was present in both cytoplasm and nucleus; this was confirmed by confocal microscopy. Madm localization was investigated using three different tags, N-terminal GFP, N-terminal HA and C-terminal myc, to ensure that localization was not affected by these modifications to the protein. In all cases Madm displayed cytoplasmic staining, as well as diffuse nuclear staining. In the nucleus, Madm was excluded from the nucleoli (regions identified by intense Hoescht staining), whereas in the cytoplasm Madm had a mottled appearance suggesting it may be tethered to fixed cytoplasmic structures.

While over-expressed Madm was cytoplasmic and nuclear, subcellular fractionation of NIH3T3 cells showed that endogenous Madm was only present in the cytoplasm. Two major factors that may regulate subcellular localization of Madm are interactions with nuclear transport machinery, and association with proteins that reside stably in either the cytoplasm or nucleus. It is possible that in NIH3T3 cells, Madm fails to enter the nucleus due to strong interactions with cytoplasmic anchor proteins that may mask the Madm NLS. However, when excess protein is produced (e.g. during over-expression in COS7 cells), these cytoplasmic regulatory mechanisms may be unable to retain Madm and it enters the nucleus. It is important to note that Madm must be actively transported into the nucleus, since proteins larger than about 50 kDa are unable to passively diffuse through the nuclear pore complex

and have to bind the importin α/β complex via an NLS to enter the nucleus in a GTP-dependent manner (Cyert, 2001).

While nuclear localization is an important factor in regulating a protein's compartmentalization, the importance of nuclear export has also become apparent. Early research in this field based on the HIV Rev protein and protein kinase A inhibitor led to the identification of the leucine-rich NES and the CRM1/exportin 1 pathway of nuclear exit (Fornerod *et al.*, 1997). CRM1/exportin 1 directly binds proteins that contain the specific leucine-rich motif and, with the help of both Ran-GTP and nucleporins (Floer and Blobel, 1999), efficiently exports such proteins into the cytoplasm. However, even though a protein may contain an NES it can still reside stably in the nucleus due to tight interactions with nuclear proteins.

Madm has several regions with high leucine content, especially in the carboxyl-terminus where the putative nuclear receptor binding motifs (consensus LXXLL) are located. There is also a region in the kinase-like domain that resembled the NES of HIV Rev. Although the final amino acid in the Madm motif is an isoleucine rather than leucine, NESs from other proteins have substituted phenylalanine, isoleucine and even methionine and still retained nuclear export activity (Rodriguez and Henderson, 2000, Seimiya *et al.*, 2000). To determine if this region (amino acids 119 – 129) was a functional NES, Leu^{127} and Ile^{129} were mutated to alanine. The localization of this mutant (ΔNES) was examined by confocal microscopy and was shown to be identical to the wild type protein. Therefore, either this motif is not the NES, or Madm does not contain a functional NES. To clarify this issue, cells transfected with Madm were treated with LMB, which specifically inhibits CRM1 (Kudo *et al.*, 1998) by interfering with the formation of RanGTP-CRM1-NES complexes, leading to nuclear accumulation of NES-containing proteins (Askjaer *et al.*, 1998). LMB has shown nuclear translocation of a variety of proteins, some which have predominantly extranuclear localization and function, e.g. actin (Wada *et al.*, 1998) and 14-3-3 (Brunet *et al.*, 2002). In this study, treatment with LMB did not change Madm localization, suggesting that Madm is not regulated by nuclear export, nor does it bind a protein that contains an NES.

To identify regions of Madm that regulate its subcellular localization, several deletion constructs were made; however, the localization of each mutant did not vary significantly from the full-length protein. In all cases the staining revealed nuclear localization, excluding the nucleolus, and an irregular cytoplasmic pattern. This would suggest that multiple elements of the protein regulate its localization. Interestingly deletion of the kinase domain, including the NLS and potential NES, had no effect on subcellular localization. It is possible, therefore, that the nuclear import of Madm may be regulated by interaction with another protein, which may contain an NLS. Althernatively as Madm can form homodimers the truncated mutants may dimerize with endogenous Madm protein and translocate to the nucleus.

The potential kinase activity of Madm was investigated using bacterially-produced recombinant protein. As a control, a mutant lacking the kinase domain was also generated. However, purification of these recombinant proteins using metal chelate chromatography was unable to remove all bacterial proteins. These contaminants most likely consist of proteins with high histidine content, or metal ion binding capability. While the majority of contaminating proteins were removed by ion exchange chromatography, some bacterial proteins still remained. When subjected to *in vitro* kinase assays it was found that the recombinant Madm and ΔKinase proteins did not possess kinase activity. The presence of a phosphorylated band at the approximate size for Madm was identified by peptide mass fingerprint to be the bacterial protein colicin IB. Since colicin IB was not present in the control samples it is plausible that the bacterial contaminant was binding to both the Madm and ΔKinase proteins. The affinity of the interaction must be quite strong, as it was not disrupted by the high salt or low pH washes.

Colicins are polypeptide toxins produced by *E. coli* and closely related bacteria (Cramer *et al.*, 1990). Channel forming colicins, including colicins A, B, E1, IA, IB, and N, are transmembrane proteins that depolarize the cytoplasmic membrane, leading to dissipation of cellular energy (Cramer *et al.*, 1990). These proteins have not been shown previously to possess kinase activity, or nucleotide binding ability. The genome of *E. coli* laboratory strain

K12 (4.6 Mbp) has revealed the presence of three kinase-type molecules (Shi *et al.*, 1998), which may be co-purifying with and phosphorylating colicin IB.

The lack of kinase activity in recombinant Madm could also be due to improper folding of the molecule or lack of activation. Many kinases contain a phosphorylation site which is activated in response to a cellular event (Hanks *et al.*, 1988, Jones *et al.*, 1991). However, when expressed in COS7 cells, full length Madm and each of the deletion constructs, including the ΔKinase mutant were phosphorylated. It was concluded that Madm did not possess kinase activity, and that the immunoprecipitates contained an associated serine kinase as Madm was phosphorylated on serine residues.

Prediction software (Blom *et al.*, 1999) indicated that Madm has 18 serine residues in the correct context for phosphorylation. This includes seven potential sites in the amino-terminus, within a potential SH2-binding region which has been shown in other proteins to be regulated by serine phosphorylation (Malek *et al.*, 1996, Malek and Desiderio, 1994). The kinase-like domain contains five phosphorylation sites, two adjacent to the NLS, which may be significant given that nuclear translocation of several proteins is regulated by phosphorylation near the NLS (Zhang *et al.*, 2000). The dimerization/Mlf1-binding domain also contains three potential phosphorylation sites, indicating that self-association and complex formation may also be regulated by phosphorylation, while the carboxyl-terminal contains three additional phosphorylation sites. Hence, it is not surprising that all the Madm-deletion mutants were phosphorylated *in vitro*. Identification of which serines are phosphorylated will be important in determining the significance of phosphorylation to Madm function.

Properties of the Madm-associated kinase appear quite unique when compared to most known serine/threonine kinases. Treatment with known activators of kinase pathways, such as vanadate and okadaic acid, had no affect on Madm phosphorylation. Strikingly, staurosporine, a broad specificity kinase inhibitor, had no affect on Madm phosphorylation. Addition of this inhibitor to kinase assays resulted in a marked decrease in the background phosphorylation; however, it did not decrease Madm phosphorylation or phosphorylation of MBP by Madm immune complexes. Staurosporine is a competitive inhibitor thought to bind

in the ATP pocket of kinases (Toledo *et al.*, 1999). It is therefore a possibility that the Madm-associated kinase does not contain a conventional ATP-binding pocket, such as the glycolytic kinases (Hanks and Hunter, 1995). Alternatively, the associated kinase may contain a unique structure in its ATP binding cleft that prevents intercalation of the inhibitor.

Chapter 5: CHARACTERIZATION OF THE INTERACTION BETWEEN MADM AND MLF1

5.1 Introduction

MLF1 is a recently described oncogene involved in acute myeloid leukemia and myelodysplastic syndrome (Yoneda-Kato *et al.*, 1996). It was initially identified in a translocation, between *MLF1* on chromosome 3 and *NPM* on chromosome 5, which generates the chimeric molecule *NPM-MLF1* (Yoneda-Kato *et al.*, 1996). Significantly, over-expression of wild-type MLF1 in acute myeloid leukemias, not involving the t(3;5), is associated with progression towards a malignant phenotype (Matsumoto *et al.*, 2000). Previous studies have also highlighted the importance of Mlf1 in the lineage determination of hemopoietic cells (Williams *et al.*, 1999).

Analysis of the Mlf1 sequence identified no recognizable motifs or domains, except for a characteristic RSXSXP binding site for 14-3-3 proteins (Williams *et al.*, 1999, Hitzler *et al.*, 1999). Predictably, a yeast two-hybrid screen identified 14-3-3ζ as a Mlf1 interacting molecule. The interaction between 14-3-3ζ and Mlf1 was confirmed by co-immunoprecipitation studies (Lim *et al.*, 2002). 14-3-3 proteins control the activity of their partner molecules, in part, by sequestering them from their site of action (Muslin and Xing, 2000).

Since 14-3-3 binding is regulated by phosphorylation of serine residues within the 14-3-3 binding motif, the role of Madm and the Madm-associated kinase in phosphorylation of Mlf1 was investigated. This chapter describes the findings that Mlf1 is phosphorylated on a serine residue within the 14-3-3 binding site, and that Madm complexes contain a kinase responsible for this phosphorylation.

5.2 Results

5.2.1 Madm binds Mlf1 via a C-terminal motif

Madm was identified as a 219 bp cDNA fragment via a yeast two-hybrid screen to identify Mlf1 interactors. Since this is only a small fragment of Madm, it was important to verify that the full length protein would also bind Mlf1. Data presented in Figure 5.1A confirmed that full length Madm and Mlf1 interacted in the yeast system. To further characterize the domains required for this interaction, several deletion constructs of Madm were made. These mutants consisted of amino acids 1 – 479, 1 – 326, 99 – 326 and 406 – 479. Other deletion mutants previously described in Chapter 4 non-specifically activated the reporter genes and were not included in the study. When these mutants were co-expressed with Mlf1, it was observed that only the full length, 1 – 479 and 406 – 479 could activate the reporter genes (Figure 5.1A). Therefore, amino acids 406 to 479 of Madm, encoded by the cDNA fragment originally identified in the yeast two-hybrid screen, are necessary and sufficient to bind Mlf1.

Similarly, deletion mutants of Mlf1 were examined for their Madm-binding ability. The Mlf1 constructs used contained amino acids 1 – 227, 1 – 163, 1 – 145, 1 – 128 and 122 – 267. Wild-type Mlf1 and C-terminal deletions up to residue 227, bound Madm (Figure 5.1B). The inability of the 1 – 163 mutant to bind Madm suggested that amino acids between 163 and 227 were important for this association. However, a mutant lacking the N-terminal 122 amino acids of Mlf1 also failed to bind Madm. Thus, at least two regions of Mlf1 appear to be involved in the Madm association.

Binding of the fusion molecule NPM-MLF1 to Madm was also ascertained. This was performed in collaboration with Dr. Steve Morris's group at St. Jude Children's Research Hospital, Memphis, USA. Both full-length NPM-MLF1 and a C-terminal truncated protein were able to interact with Madm (Figure 5.1C). In addition, deletion of amino acids 281 to 362 of NPM-MLF1 (corresponding to amino acids 121 to 202 of MLF1) retained Madm binding; however, removal of the N-terminal residues of MLF1 present in the fusion protein (corresponding to amino acids 17 to 120 in the normal MLF1 protein) prevented binding. From these data, it appears Mlf1 and NPM-MLF1 share an N-terminal region that interacts

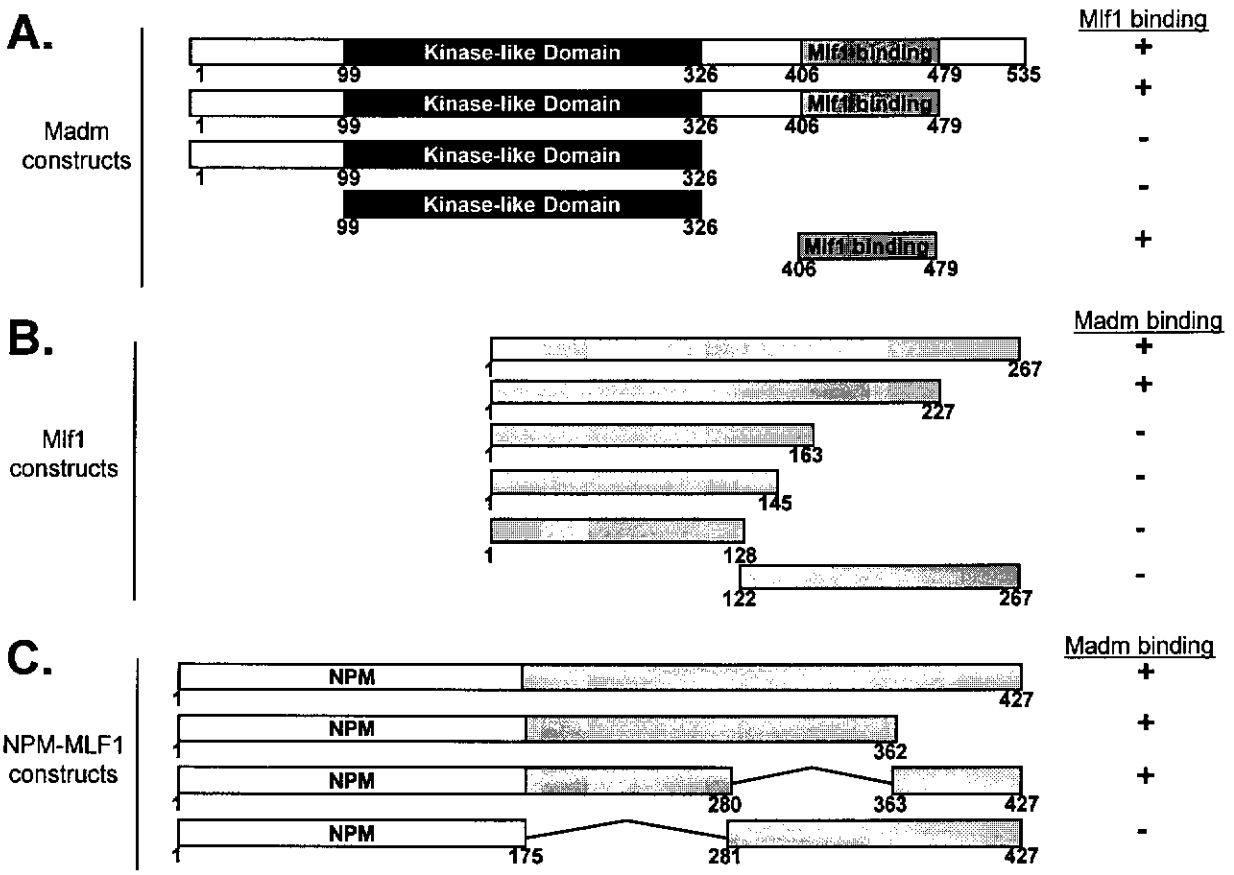


Figure 5.1. Domains of interaction between Madm, Mlf1 and NPM-MLF1. (A) Full length Mlf1 was analyzed for binding to Madm domains by HIS3 and LacZ reporter gene expression in yeast. (B) Full-length Madm was analyzed for binding to Mlf1 domains by reporter gene expression in yeast. (C) Full-length Madm was co-expressed in COS cells with MLF1 mutants of the chimeric NPM-MLF1 protein and binding was analyzed by co-immunoprecipitation.

with Madm, but that the second, C-terminal, region identified in Mlf1 may be dispensable for the interaction of NPM-MLF1 with Madm.

5.2.2 Madm binds Mlf1 in vivo

To investigate the interaction between Madm and Mlf1 further, COS7 cells were transfected with plasmids expressing full-length Mlf1 and myc-tagged Madm. Total protein lysates were prepared and Madm was immunoprecipitated using anti-myc antibodies. Transfection of both Madm and Mlf1 resulted in co-immunoprecipitation of the two proteins (Figure 5.2A). A reciprocal co-immunoprecipitation verified the interaction (Figure 5.2B). Binding of Madm to an Mlf1 mutant that had an altered 14-3-3 binding site (S34A) was also examined. Co-immunoprecipitation studies revealed that mutation of this amino acid did not affect the ability of Madm to bind Mlf1 (Figure 5.2A). These data confirm the association between Madm and Mlf1 identified by yeast two-hybrid analysis.

To extend analysis of binding between these proteins in mammalian cells, the interaction of Madm with various mutants of Mlf1 and S34A was also ascertained in transiently transfected COS7 cells. The yeast two-hybrid system had shown that Madm only interacted with full length Mlf1 and a mutant consisting of amino acids 1 – 227 (Figure 5.1A). Similar results were obtained for the S34A mutant (data not shown). Unexpectedly, it was found that Madm could co-precipitate several mutants, including some which did not interact directly in the yeast system (Figure 5.2C). One explanation for these observations is that although Madm is able to bind full length Mlf1 directly, other proteins present in the cell may bring Mlf1 and Madm together in a complex.

5.2.3 Madm and Mlf1 co-localize in the cytoplasm and at the centrosome

Mlf1 has been shown previously to be localized primarily in the cytoplasm, but with some distribution in the nucleus (Yoneda-Kato *et al.*, 1996, Williams, 1999 #5). Confocal microscopy was employed to determine if the subcellular localization of Madm overlapped with that of Mlf1. Therefore, COS7 cells were transiently transfected with constructs expressing Mlf1 and myc-tagged Madm. Figure 5.3A shows that Mlf1 and Madm co-localized primarily in the cytoplasm, with concentration in a perinuclear area which may include

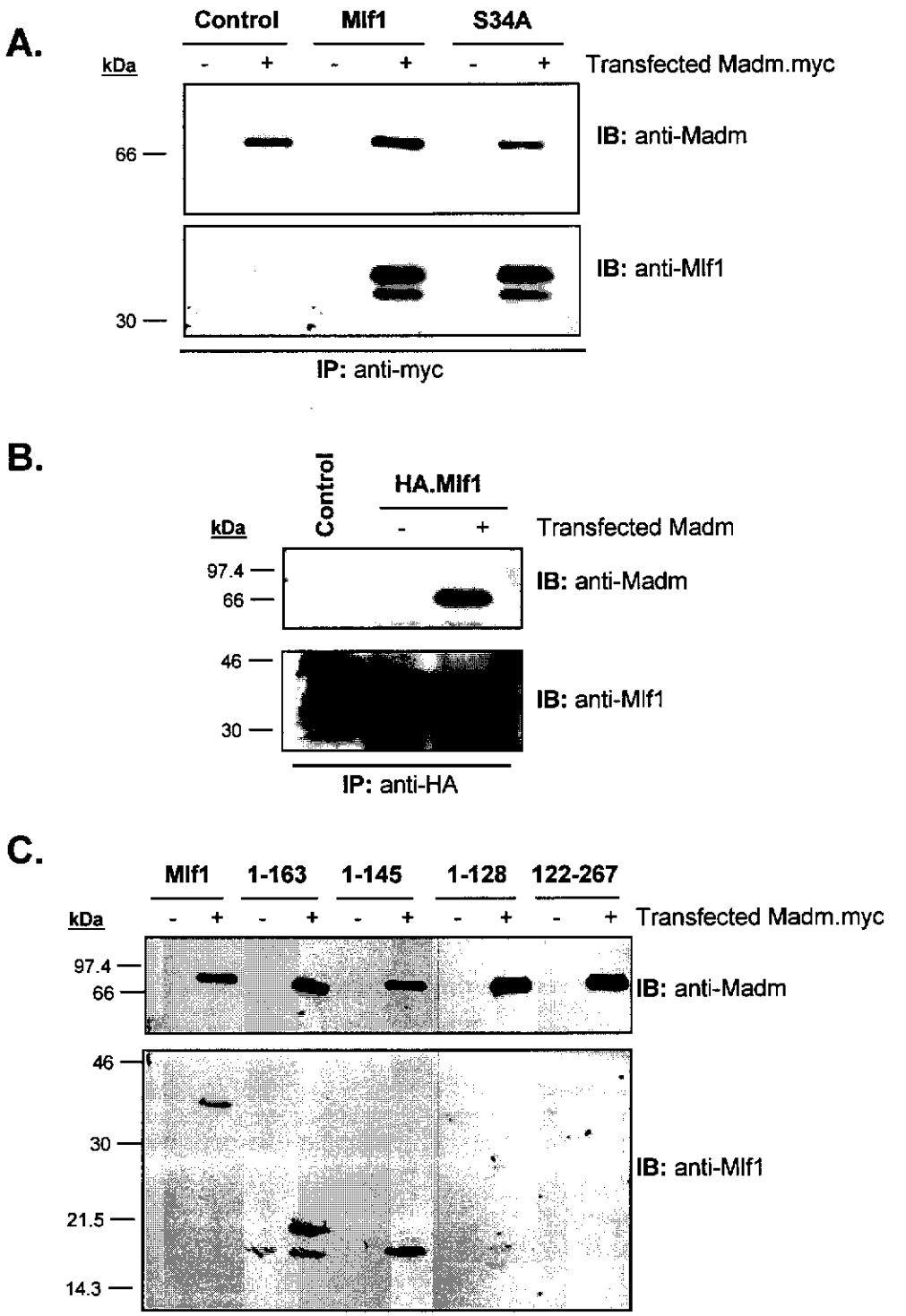


Figure 5.2. Madm and Mlf1 co-immunoprecipitate. (A) COS7 cells were transiently transfected with vector, Mlf1 or S34A, myc-tagged Madm or simultaneously with Mlf1/S34A and myc-tagged Madm. Protein lysates were immunoprecipitated with an anti-myc antibody for Madm and immunoblotted as shown. (B) COS7 cells were transiently transfected with vector, HA-tagged Mlf1, Madm or simultaneously with HA-tagged Mlf1 and Madm. Protein lysates were immunoprecipitated with anti-HA for Mlf1 and immunoblotted for Madm or Mlf1. (C) COS7 cells were transiently transfected with vector, Madm, Mlf1 and deletion mutants of Mlf1. The amino acids of each Mlf1 mutant are indicated. Protein was immunoprecipitated for Madm using an anti-myc antibody and immunoblotted as shown.

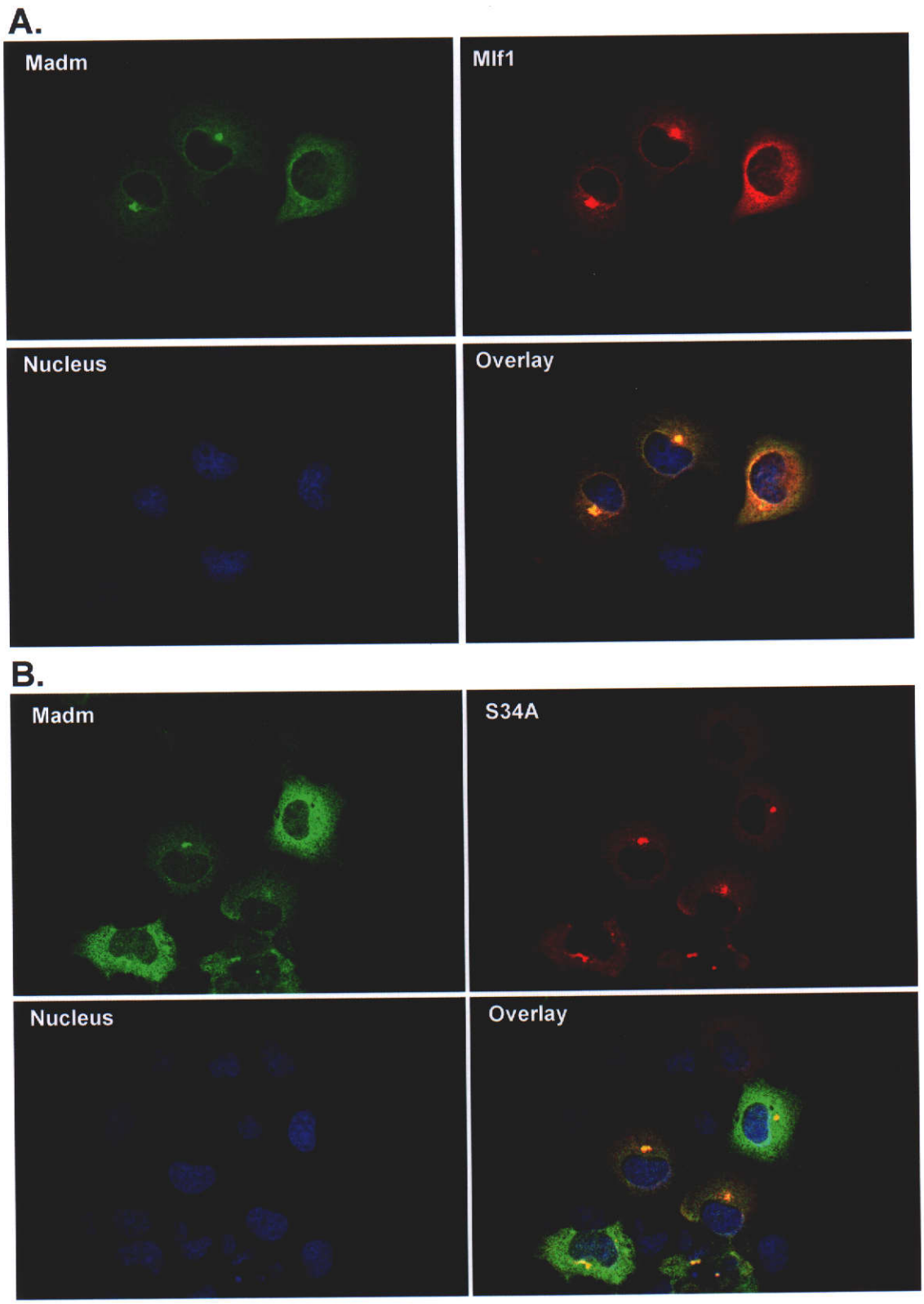


Figure 5.3. Madm co-localizes with Mlf1/S34A. COS7 cells were co-transfected with Madm and (A) Mlf1 or (B) S34A then fixed and analyzed using confocal microscopy. Madm was detected using anti-myc antibodies and a green fluorescent secondary antibody, while Mlf1/S34A was detected with anti-Mlf1 polyclonal antisera and a red fluorescent secondary antibody. Nuclei were stained with Hoechst 33258 (shown in blue).

centrosomes. A similar pattern was observed for Madm and the S34A mutant of Mlf1 (Figure 5.3B). Therefore, Madm and Mlf1 associate in the yeast two-hybrid system, co-immunoprecipitate and share subcellular locations.

5.2.4 Madm binds and co-localizes with the fusion protein, NPM-MLF1

To investigate whether Madm could bind the leukemogenic fusion protein NPM-MLF1, transient transfection into COS7 cells and immunoprecipitations were performed. It was observed that NPM-MLF1 was present in Madm immunoprecipitates demonstrating an *in vivo* interaction (Figure 5.4A).

As Madm co-precipitated with NPM-MLF1, co-localization was examined next. It has been reported that NPM-MLF1 has an exclusively nuclear localization (Yoneda-Kato *et al.*, 1996) and Madm was shown to have some localization in the nucleus by subcellular fractionation and confocal microscopy (Chapter 3). Figure 5.4B shows that NPM-MLF1 was indeed nuclear, with some protein present in the perinuclear region. When the fluorescence patterns of Madm and NPM-MLF1 were overlaid, limited co-localization was detected. Therefore, despite co-immunoprecipitation (Figure 5.4A) it appears that only a small fraction of Madm and NPM-MLF1 reside in the same subcellular compartment.

5.2.5 Madm/Kinase complexes phosphorylate Mlf1

It is possible that the kinase that co-precipitated with and phosphorylated Madm (Figure 4.11A) is also present in the Madm/Mlf1 complex. To test this hypothesis and the possibility that this kinase may also phosphorylate Mlf1, *in vitro* kinase assays were performed. Figure 5.5A illustrates the phosphorylation of Madm as previously observed, as well as Mlf1 and the S34A mutant of Mlf1. Phosphoamino acid analysis of Mlf1 and S34A following *in vitro* kinase reaction revealed that these proteins were also phosphorylated on serine residues (Figure 5.5B). It was concluded from these experiments that Madm recruits a serine kinase capable of phosphorylating both Madm and Mlf1.

To ensure that the phosphorylation observed in the *in vitro* kinase assays was genuine, the *in vivo* phosphorylation status of Mlf1 and NPM-MLF1 was examined next. Figure 5.6A demonstrates that a proportion of each protein normally exists in a phosphorylated state.

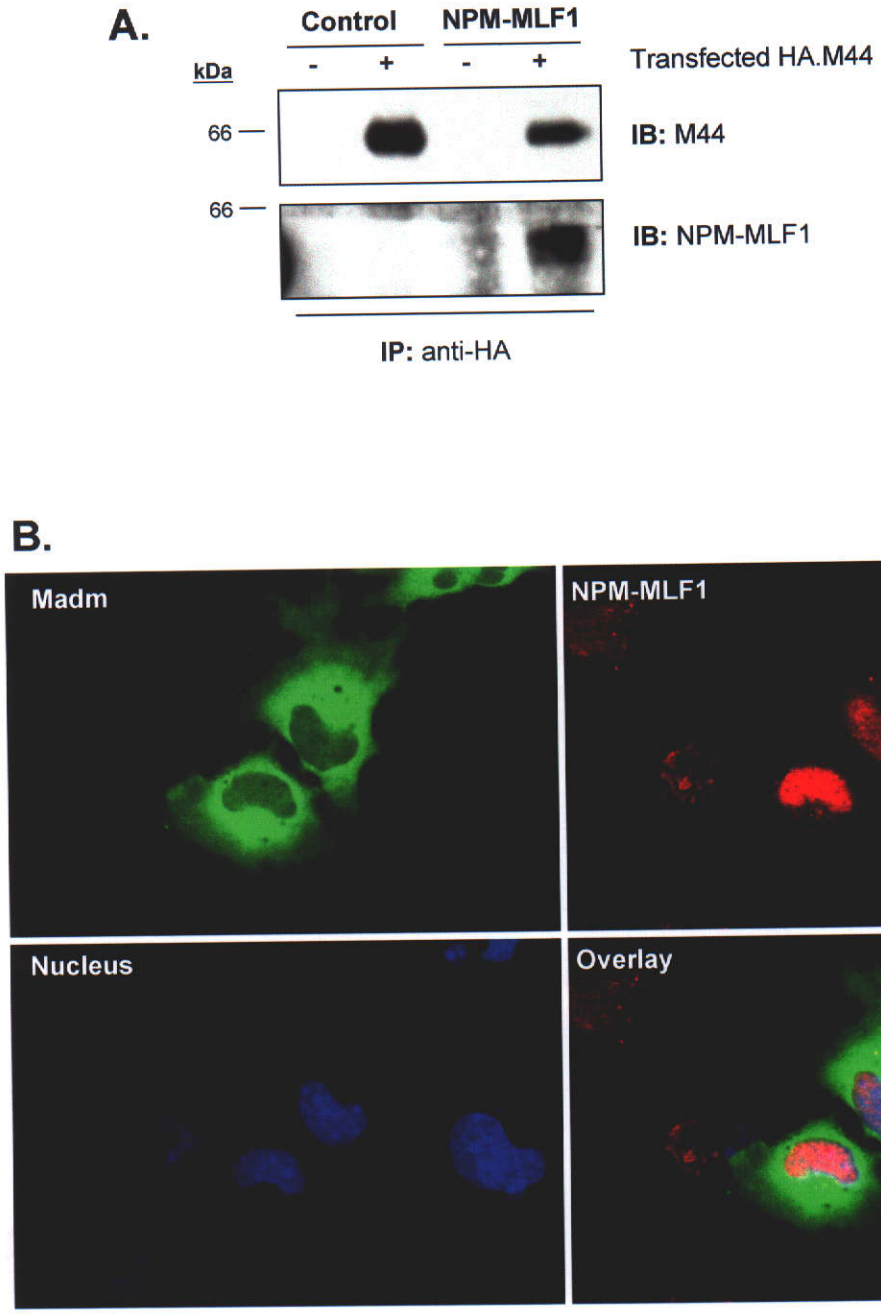


Figure 5.4. Madm binds the fusion protein NPM-MLF1. (A) COS7 cells were transfected with Madm and NPM-MLF1. Protein was immunoprecipitated for Madm using an anti-HA antibody and immunoblotted as shown. (B) COS7 cells were co-transfected with Madm and NPM-MLF1 then fixed and analyzed using confocal microscopy. Madm was detected using anti-myc antibodies and a green fluorescent secondary antibody, while NPM-MLF1 was detected with anti-MLF1 and a red fluorescent secondary antibody. Nuclei were stained with Hoechst 33258 (shown in blue).

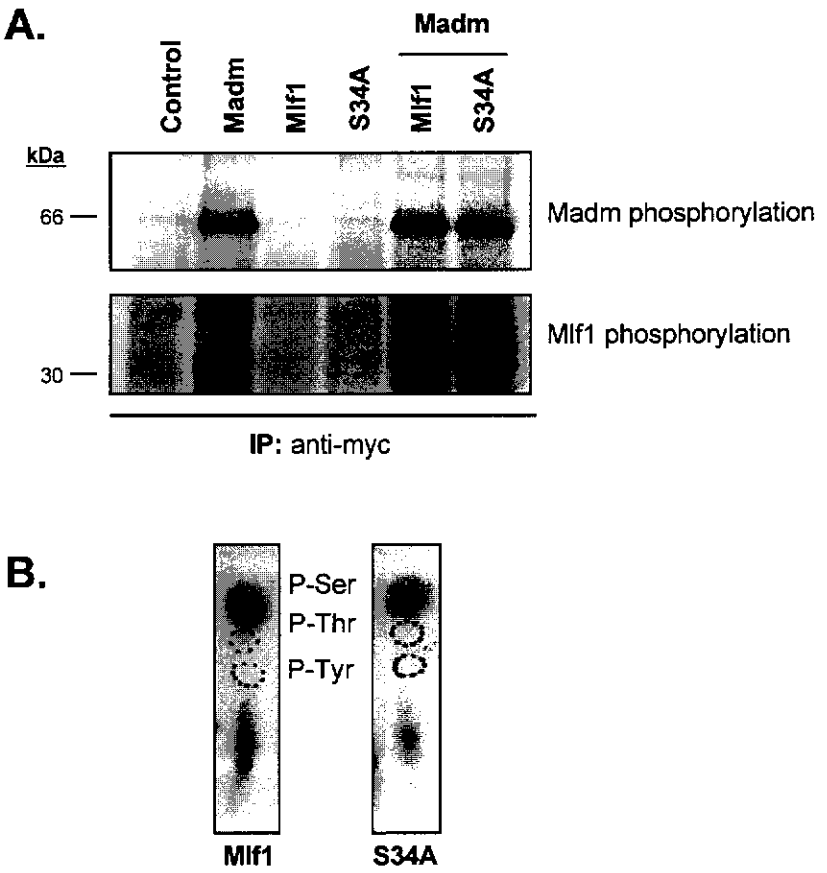
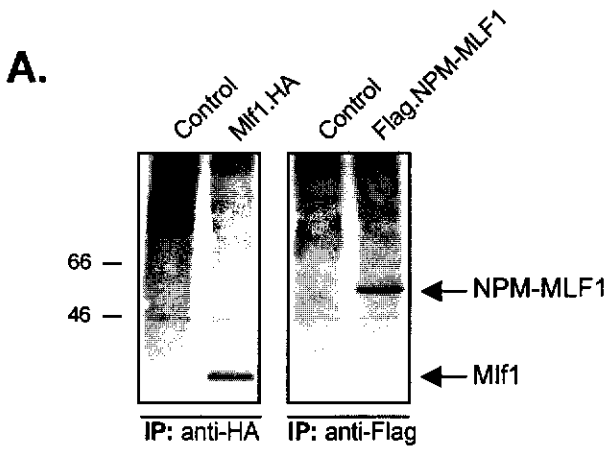


Figure 5.5. Madm immunoprecipitates phosphorylate Mlf1. (A) COS7 were transfected with vector, Mlf1/S34A and Madm. Madm immunoprecipitations were performed using an anti-myc antibody and protein was subjected to an *in vitro* kinase assay using [γ^{32}P]-labeled ATP. (B) Phosphoamino acid analysis of phosphorylated Mlf1 and S34A. Radioabeled protein was hydrolyzed and amino acids separated using one-dimensional thin layer chromatography followed by autoradiography. The positions of ninhydrin-stained amino acid standards are circled.



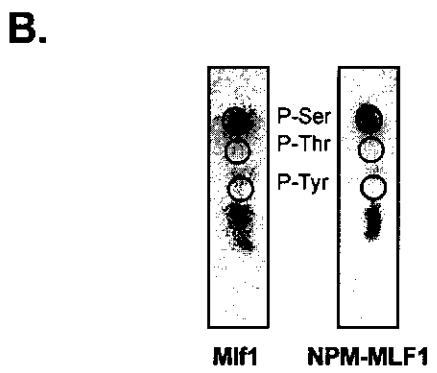


Figure 5.6. Mlf1 and NPM-MLF1 are serine phosphorylated *in vivo*. (A) COS cells transfected with Mlf1 or NPM-MLF1 were metabolically labeled with [^{32}P]-Phosphoric acid and protein immunoprecipitated as shown. (B) Phosphoamino acid analysis of *in vivo* phosphorylated Mlf1 and NPM-MLF1. The positions of ninhydrin stained amino acids standards are shown by circles.

Phosphoamino acid analysis following *in vivo* labeling confirmed that phosphorylation of Mlf1 occurred on serine residues, and that NPM-MLF1 was also phosphorylated on serine residues (Figure 5.6B). Thus, both proteins are affected by serine phosphorylation which may well involve Madm and its associated serine kinase.

5.2.6 Madm/Kinase complex phosphorylates the RSXSXP motif

To determine whether the Madm/kinase complex was capable of phosphorylating the serine residues within the 14-3-3-binding domain of Mlf1, peptides were used as substrates in an *in vitro* kinase assay. Figure 5.7 shows that, in addition to the phosphorylation of Madm, a concentration-dependent phosphorylation of the MLF1 peptide bearing the 14-3-3 binding site was observed. Although a small amount of peptide phosphorylation by endogenous kinase(s) was observed in non-transfected COS7 cells, phosphorylated peptide levels were 4-6 fold higher with exogenous Madm. These data demonstrate that Madm binds a kinase capable of phosphorylating the RSXSXP motif of MLF1, potentially influencing its association with 14-3-3ζ.

In an attempt to gain possible insights into the kinase present in Madm immunoprecipitates, other previously described 14-3-3 binding proteins were used as substrates. Binding of the Raf1 protein kinase to 14-3-3 occurs via a consensus RSXSXP motif and phosphorylation of S259 is required for this interaction (Li *et al.*, 1995). To determine if the Madm/kinase complex could phosphorylate the same motif from Raf1, peptides spanning S259 were expressed as fusion proteins with GST. When added to Madm immunoprecipitates and incubated with radiolabeled ATP, minimal phosphorylation of these peptides was observed (data not shown). Several proteins which bind 14-3-3 do not contain the classic RSXSXP motif, including cbl (Robertson *et al.*, 1997). When added to Madm immunoprecipitates no change in phosphorylation of recombinant cbl was observed (data not shown). These data suggest that the kinase recruited to Mlf1 by Madm displays substrate specificity with respect to 14-3-3 motifs.

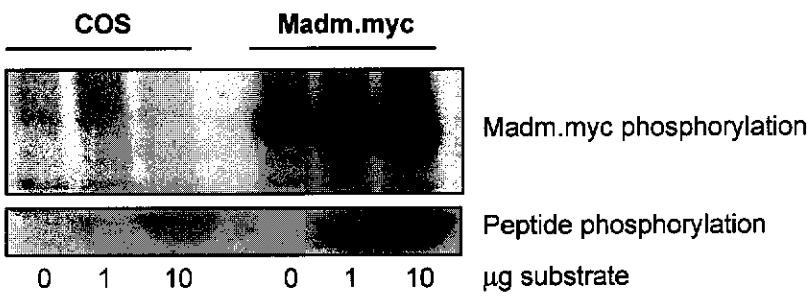


Figure 5.7. Madm immunoprecipitates phosphorylate the RSXSXP motif. COS7 were transfected with vector alone or Madm. Immunoprecipitations were performed using an anti-myc antibody for Madm and protein was subjected to an *in vitro* kinase assay in the presence of increasing amount of peptide corresponding to the 14-3-3-binding motif of Mlf1. The peptides spanned amino acids 26 - 41 of Mlf1 (M R Q M I **R S F S E P** F G R D L, where the RSXSXP motifs is in bold and serine 34 underlined) .

5.3 Discussion

The work described in this chapter characterized an interaction between the novel adaptor protein, Madm and Mlf1. The association of these proteins was first identified in a yeast two-hybrid screen using Mlf1 as bait, and the interaction was confirmed in mammalian cells by co-immunoprecipitation experiments. Furthermore, these two proteins shared subcellular locations.

Investigating the binding between Mlf1 and Madm revealed that the interaction is complex and most probably depends on secondary structure. While a discrete domain of Madm was able to bind Mlf1, a single region of Mlf1 that conferred Madm binding could not be identified. The results showed that neither the N-terminal, nor C-terminal, of Mlf1 was able to bind Madm. Instead, it appears that both regions are required for binding. Using the yeast two-hybrid system, a combination of residues between amino acids 39 – 81 and amino acids 164 – 227 was required for Madm association. In contrast, the interaction observed between Madm and NPM-MLF1 was less complex and could be localized to a region of the fusion protein corresponding to amino acids 17 – 121 of wild type Mlf1. Importantly, although the NPM-MLF1 fusion protein is phosphorylated *in vivo*, it no longer binds 14-3-3ζ (Lim *et al.*, 2002). Since the fusion junction is only 13 residues from the RSXSXP motif, it is conceivable that structural alterations may inhibit the binding of 14-3-3ζ, without affecting Madm interaction.

Some unexpected results were obtained when binding between Madm and Mlf1 was examined using co-immunoprecipitation experiments. These studies showed that while several Mlf1 deletion mutants were not able to bind Madm in the yeast two-hybrid system, they were still able to co-immunoprecipitate. Since co-immunoprecipitation can isolate complexes that may contain a number of different proteins, it is possible that Madm and Mlf1 may not only interact directly, but are also linked by other molecules in that complex which have the ability to associate with both Madm and Mlf1 simultaneously. This finding supports suggestions that Madm is a novel adaptor molecule, which recruits other proteins to Mlf1.

This adaptor function of Madm is emphasized by the observation that a Madm-associated kinase is also able to phosphorylate Mlf1 on serine residues. The importance of phosphorylation in Madm, Mlf1 and NPM-MLF1 function is highlighted by the finding that these proteins are constitutively phosphorylated on serine residues. Importantly, the Madm-associated kinase was able to phosphorylate the RSXSXP motif of Mlf1. This indicates that the Madm-kinase complex might regulate Mlf1 by enhancing binding to 14-3-3 proteins. The 14-3-3 family of proteins often control their partner proteins by binding them in the cytoplasm, and preventing their localization to the nucleus (Muslin and Xing, 2000). It is possible, therefore, that phosphorylation of the RSXSXP motif in Mlf1 by the Madm-kinase complex promotes 14-3-3 binding, thereby sequestering the molecule in the cytoplasm and restricting access to the nucleus. This model is consistent with primary localization of wild-type Mlf1 in the cytoplasm, with small amounts detected in punctate nuclear bodies (Yoneda-Kato *et al.*, 1996, Yoneda-Kato *et al.*, 1999, Williams *et al.*, 1999). While Madm was able to bind NPM-MLF1, this fusion protein has lost the ability to bind 14-3-3, and translocates to the nucleus where it is thought to have its oncogenic effect (Yoneda-Kato *et al.*, 1996).

The co-localization of Madm and Mlf1 suggests that these two proteins are intimately associated, especially in the perinuclear area. Furthermore, Mlf1 co-localizes with the centrosomal protein γ-tubulin (L. Winteringham, personal communication) and it appears that co-expression with Mlf1 is required to relocate Madm to the centrosomal regions. Interestingly, Mlf1 also interacts with dynein light chain (LC8/PIN) (L.Winteringham, personal communication), a microtubule-associated protein present in centrosomes. The centrosome plays an important role as the microtubule organizing center of the cell and forms the nucleation point for mitotic spindles. Importantly many human tumor types contain abnormal numbers of centrosomes (Roshani *et al.*, 2002, Sato *et al.*, 2001, Lingle *et al.*, 1998), which are thought to lead directly to aneuploidy and genomic instability through the formation of multipolar mitotic spindles. Curiously, NPM has been shown to have a role in centrosome duplication. NPM was identified as one of the substrates for CDK2/cyclin E in centrosome duplication (Okuda *et al.*, 2000), and that phosphorylation of NPM is essential for this process (Tokuyama *et al.*, 2001). It is still unknown whether the regulation of centrosomes by NPM plays a role in the oncogenic activity of the fusion protein, NPM-MLF1. If Mlf1 does

indeed have a role at the centrosome, this function may be enhanced or disrupted in t(3;5)-positive cells, resulting in their leukemic phenotype.

Chapter 6: EFFECT OF MADM EXPRESSION IN M1 MYELOID CELLS

6.1 Introduction

Molecular analysis of chromosomal translocations has enabled the identification of genes that are normally involved in the control of cell growth or differentiation. It has also been shown that translocations alter the function of these genes, which directly contributes to oncogenesis. To date, NPM has been identified as a translocation partner for three different genes in three types of cancer. These translocation partners consist of a tyrosine kinase, ALK (Morris *et al.*, 1994b), and a transcription factor, RARα (Redner *et al.*, 1996). In the third case, NPM was found as a fusion protein with the novel gene, MLF1 (Yoneda-Kato *et al.*, 1996). Initial studies showed that MLF1 was an important gene in hemopoietic differentiation (Williams *et al.*, 1999).

MLF1 expression mainly occurs in immature hemopoietic cell lines (Williams *et al.*, 1999) and in CD34+ human bone marrow cells (Matsumoto *et al.*, 2000). Furthermore, expression of Mlf1 decreases during the differentiation of M1 monoblastoid and MEL/707 erythroleukemia cell lines, consistent with its expression in less differentiated hemopoietic cells (Williams *et al.*, 1999). Enforced expression of Mlf1 in hemopoietic cells alters their morphology and differentiation capability, e.g. it inhibits differentiation of erythroid cell lines, and conversely enhances differentiation of myeloid cell lines *in vitro*. Similarly, in normal hemopoietic cells, Mlf1 decreased the number of erythroid progenitor cells which generated colonies, while simultaneously increasing the number of colony-forming myeloid cells (Williams *et al.*, 1999). Mlf1, therefore, plays an important role in normal hemopoiesis.

Given these observations and the identification of Madm as a potentially important regulator of Mlf1, the effect of Madm on hemopoietic differentiation, specifically myeloid cell maturation, was examined.

6.2 Results

6.2.1 *Generation of Madm retroviral constructs*

To over-express Madm in the M1 cell line, the coding region was cloned into the retroviral vector, MSCV 2.2 (MSCV-Madm). *Madm* cDNA (nucleotides 104 – 1711) was amplified by PCR and cloned into the *Bgl*II and *Eco*RI sites of the MSCV 2.2 vector, upstream of the PGK*neo* cassette (Figure 6.1A). In addition, an antisense *Madm* construct was generated (MSCV-αMadm). This construct consisted of a *Madm* cDNA fragment corresponding to nucleotides 22 – 269 and was cloned into the *Bgl*II site upstream of the PGK*neo* cassette such that the fragment was transcribed in the antisense orientation (Figure 6.1B). The aim was to generate a mRNA fragment, complementary to the endogenous *Madm* mRNA, that could hybridize over the initiation codon to reduce translation of Madm protein. Plasmids were linearized and transfected into the PA317 packaging cell line using calcium phosphate. Following selection in neomycin (G418), culture supernatants containing active retroviral particles were used to infect M1 cells.

6.2.2 *Analysis of transfected M1 clones*

M1 cells that had been retrovirally-infected were single cell cloned in semi-solid media. After several days growth, colonies were selected and expanded in liquid culture. Individual clones were analyzed by Southern blot to identify unique retroviral integration sites. Genomic DNA was prepared, digested with *Eco*RI and analyzed. Hybridization of digested genomic DNA was performed using a *Madm* probe (nt 104 – 1711) revealing the predicted two endogenous *Madm* genomic fragments. Of the clones infected with the MSCV-Madm construct, only two displayed unique integration sites (Figure 6.2). However, no retroviral integrations were observed with the antisense constructs.

For each of the M1 clones, infected with either MSCV alone, MSCV-Madm and MSCV-αMadm, the presence of viral mRNA was confirmed by Northern blotting of total mRNA. M1 clones containing MSCV alone expressed endogenous *Madm* transcript and no additional bands (Figure 6.3). M1 clones containing MSCV-Madm expressed endogenous *Madm* mRNA, as well as the larger MSCV-Madm transcript. The amount of viral transcript was

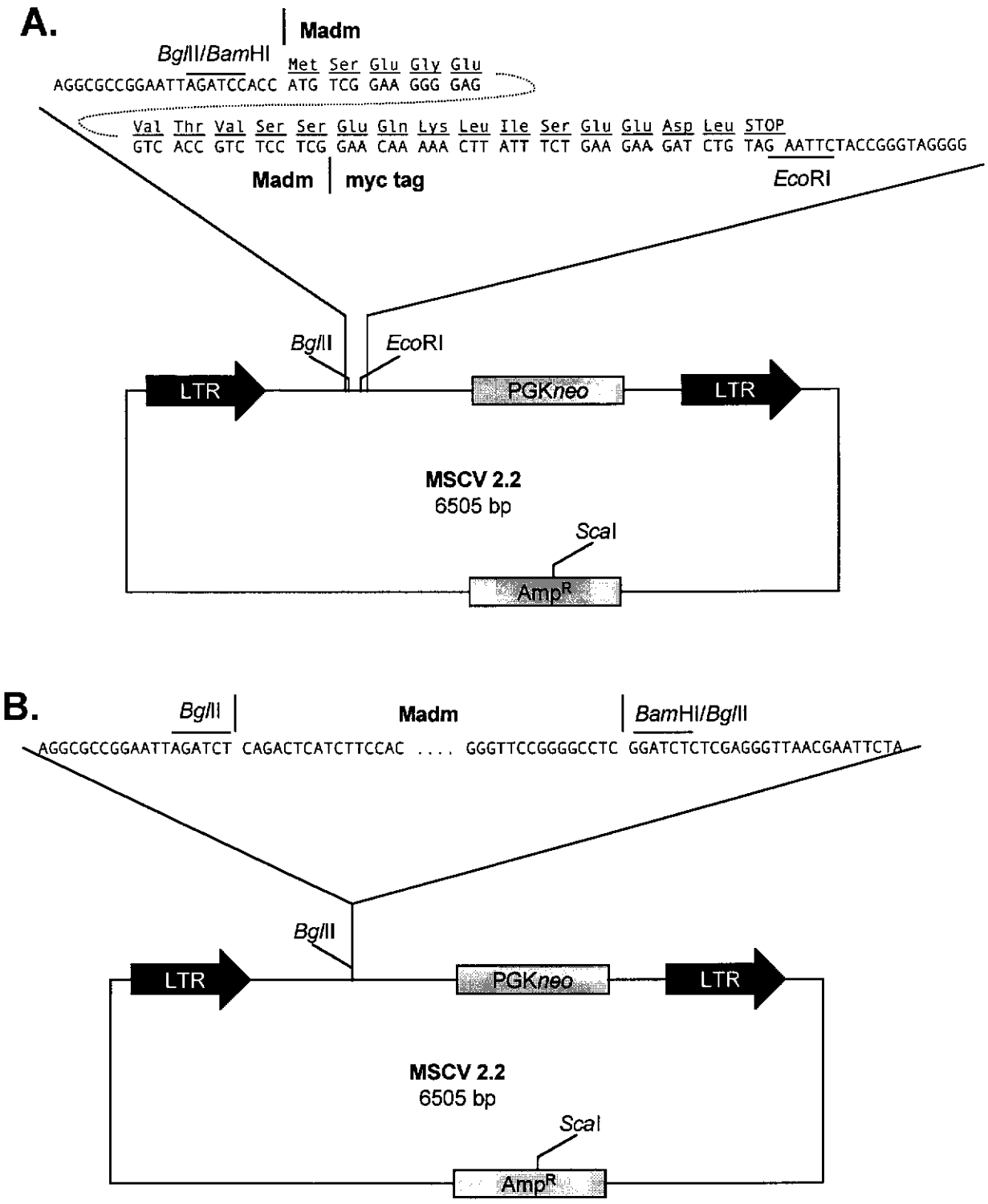


Figure 6.1. Detail of retroviral *Madm* constructs. (A) *Madm* was PCR-amplified using primers to incorporate a 5' *Bam*HI site and a 3' myc tag with *Eco*RI site. (B) *Madm* fragment (nt 22-269) was generated by *Bam*HI/*Bgl*II digestion and cloned in the antisense orientation with respect to the long tandem repeats (LTR). *Madm* fragments were cloned upstream of the PGKneo cassette and constructs were linearized using *Sca*I before transfection.

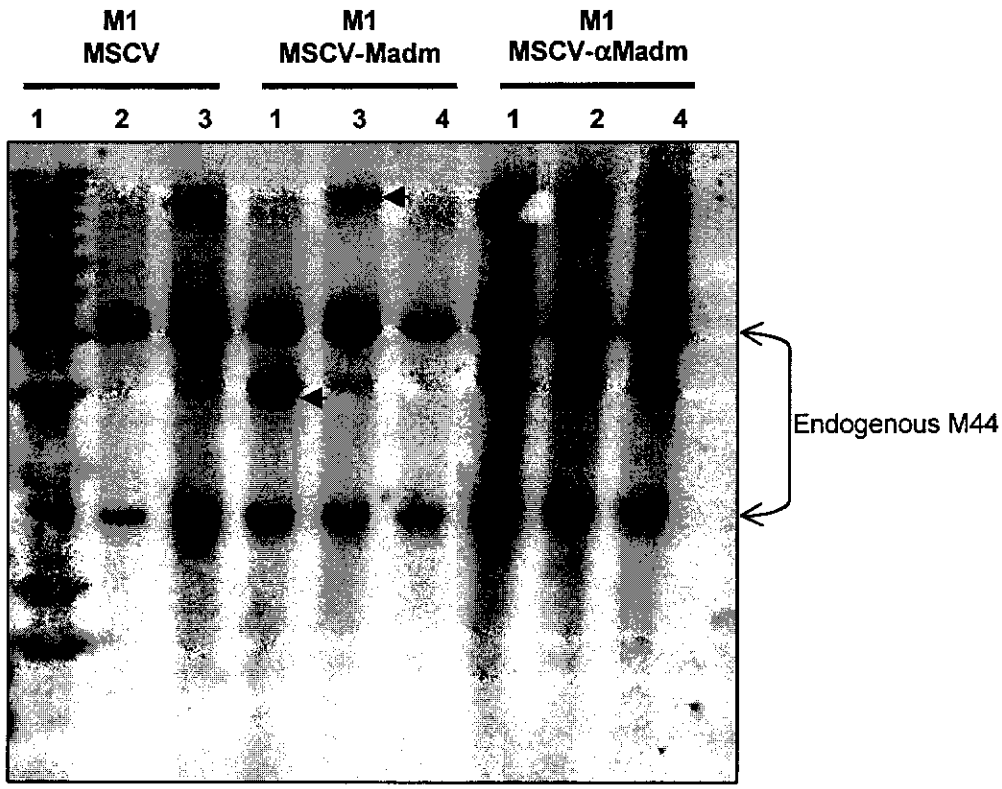


Figure 6.2. Analysis of retorvirally-infected M1 cell lines by Southern blot. M1 cells were infected with either MSCV alone, MSCV-Madm or MSCV-αMadm and individual cells cloned by methyl cellulose. Genomic DNA was prepared from unique clones (indicated by numbers), digested with *Eco*RI and hybridized with a radiolabeled Madm probe. Two bands corresponding to the endogenous Madm gene are evident. Unique integration sites of the viral DNA are arrowed.

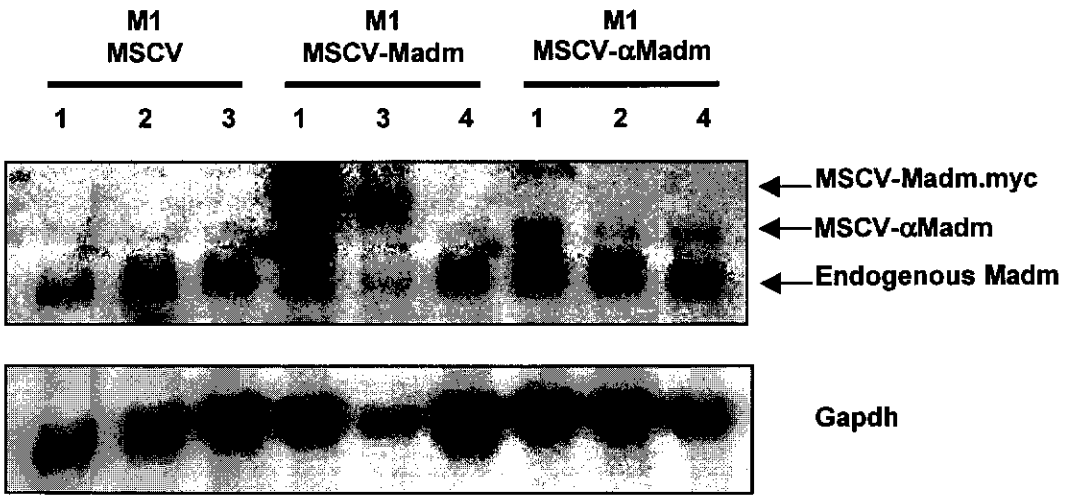


Figure 6.3. Expression of *Madm* mRNA in retrovirally-infected M1 cell lines. M1 cells were infected with either MSCV alone, MSCV-Madm or MSCV-αMadm and individual cells cloned in methyl cellulose. Total mRNA was prepared from each clone and hybridized with a radiolabeled *Madm* probe. Expression of the endogenous *Madm* and viral transcripts are indicated. Blots were hybridized with a *Gapdh* probe to indicate mRNA loading.

approximately double that of the endogenous *Madm* mRNA. While clones 1 and 3 expressed viral transcripts, clone 4 did not, confirming the absence of MSCV DNA indicated by the Southern blot analysis in Figure 6.2. Surprisingly, the M1-MSCV-αMadm clones also expressed a longer viral transcript. This was unexpected since the Southern blot failed to indicate the presence of integrated retroviral DNA; however, it is possible that the bands of retroviral DNA overlapped with the endogenous *Madm* bands. Of note was the lower expression of the MSCV-αMadm transcript, with levels approximately half that of the endogenous *Madm* mRNA.

Western blot analysis was carried out to determine the effect of the retroviral constructs on Madm protein levels. Total protein lysates were prepared from each of the M1 clones, electrophoresed, transferred to nitrocellulose and immunoblotted with various antibodies. The data presented in Figure 6.4 shows that M1-MSCV-Madm cells expressed between 50 and 80% more Madm protein than control cells. The presence of exogenous Madm was confirmed by the anti-myc antibody which recognizes the myc epitope tag. In contrast, no reduction in Madm protein content was detected in the cells bearing the antisense construct.

6.2.3 Effect of Madm expression on M1 proliferation

To examine if Madm over-expression affected M1 cell proliferation, serum-starved cells were stimulated in the presence of [^{3}H]-thymidine. The results shown in Figure 6.5 indicate that there was no significant difference in the DNA synthesis rate of Madm-expressing clones compared to parental M1 cells or M1 cells containing vector alone.

6.2.4 Effect of Madm expression on M1 differentiation

Over-expression of Mlf1 in M1 cells resulted in enhanced differentiation induced by IL-6 or LIF (Williams *et al.*, 1999). To determine the impact of Madm on myeloid maturation, the M1.MSCV and two M1.Madm clones were induced to differentiate using LIF. The extent of differentiation was measured by the presence of cell surface marker CD11b (Mac1) using flow cytometry (Williams *et al.*, 1999). In marked contrast to M1 cells over-expressing Mlf1 ((Williams *et al.*, 1999) and data not shown) both clones expressing exogenous Madm displayed lower Cd11b levels in the absence of LIF, or when exposed to sub-optimal

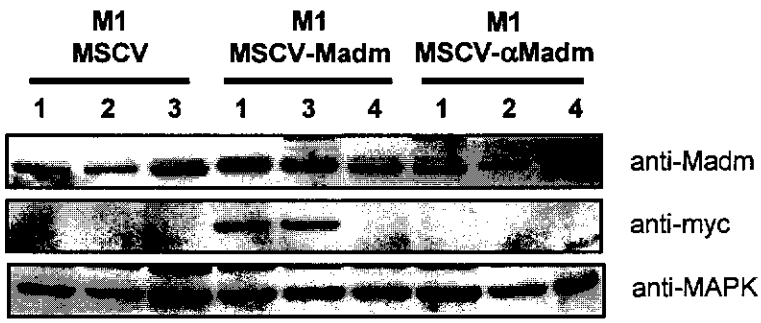


Figure 6.4. Expression of Madm protein in retrovirally-infected M1 cell lines. Clonal M1 cells infected with either MSCV alone, MSCV-Madm or MSCV-αMadm are shown. Total protein lysates from unique clones were analyzed for Madm protein expression by immunoblotting with anti-Madm antiserum, while the presence of exogenous Madm was detected using an anti-myc antibody. A control protein (MAPK) was used to indicate relative protein loading.

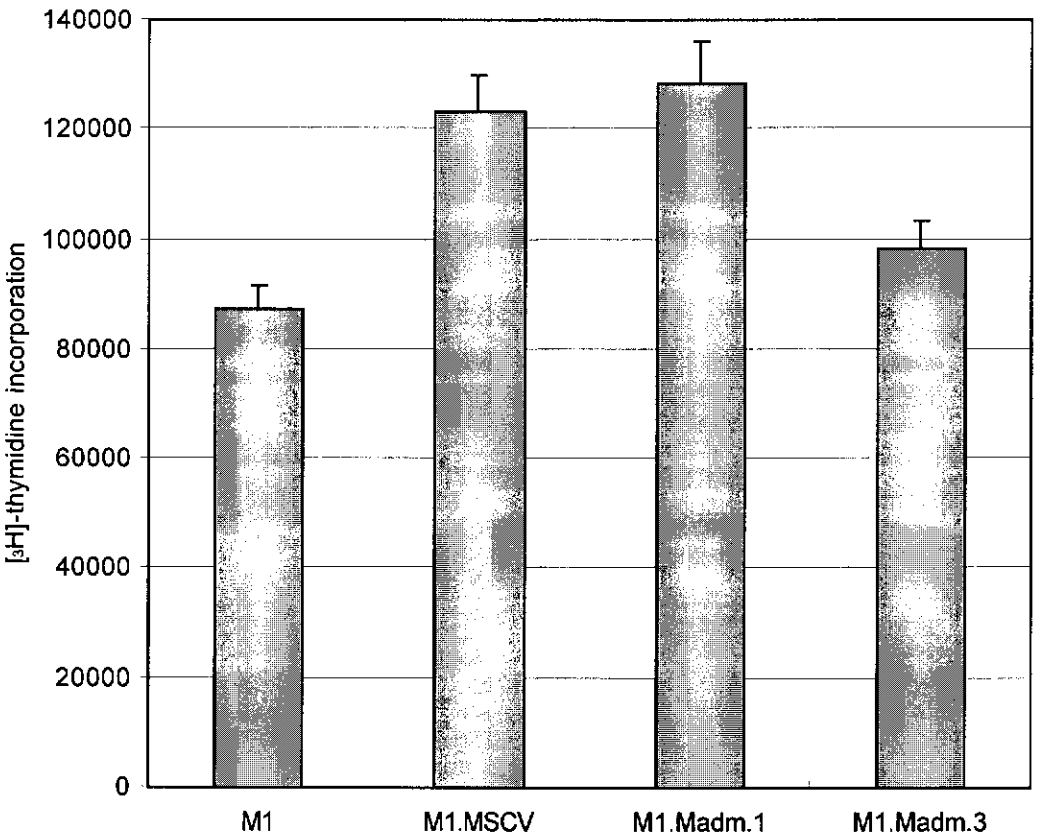


Figure 6.5. Over-expression of Madm in M1 cells does not affect proliferation. M1 cells were infected with either MSCV alone or MSCV-Madm. M1 clones were subjected to [^{3}H]-thymidine incorporation to measure DNA synthesis which is shown as disintegrations per minute (DPM) ± standard deviation. These data represent the cumulated results from five independent experiments.

concentrations of the cytokine (Figure 6.6). Clone M1.Madm.3 persisted with lower Cd11b expression even at higher concentrations of LIF. These results demonstrate that modest alterations to the concentration of Madm can influence the maturation of M1 cells, and that increasing the intracellular content of Madm had the opposite effect to Mlf1 on differentiation.

To determine if Madm over-expression could be affect morphological maturation, cytocentrifuge preparations of M1 cells and Madm-expressing M1 cells were examined after exposure to LIF. Figure 6.7 demonstrates the characteristic appearance of monoblastoid cells, with darkly-stained scanty cytoplasm, present in all uninduced cell lines. As M1 and M1.MSCV control cells differentiated, they enlarged and took on the appearance of macrophages, with round nuclei, visible endocytic vacuoles and ruffled plasma membranes. However, the morphology of the Madm-expressing cells differed from the parental cells two days post-induction; they appeared less morphologically mature by being smaller and containing more basophilic cytoplasm. This appearance is consistent with the cells being less differentiated by flow cytometry (Figure 6.6). Furthermore, at day four there were not as many endocytic vacuoles in the Madm-expressing clones and these cells had smaller cytoplasms. These observations support the flow cytometry data that over-expression of Madm impedes the maturation of M1 cells.

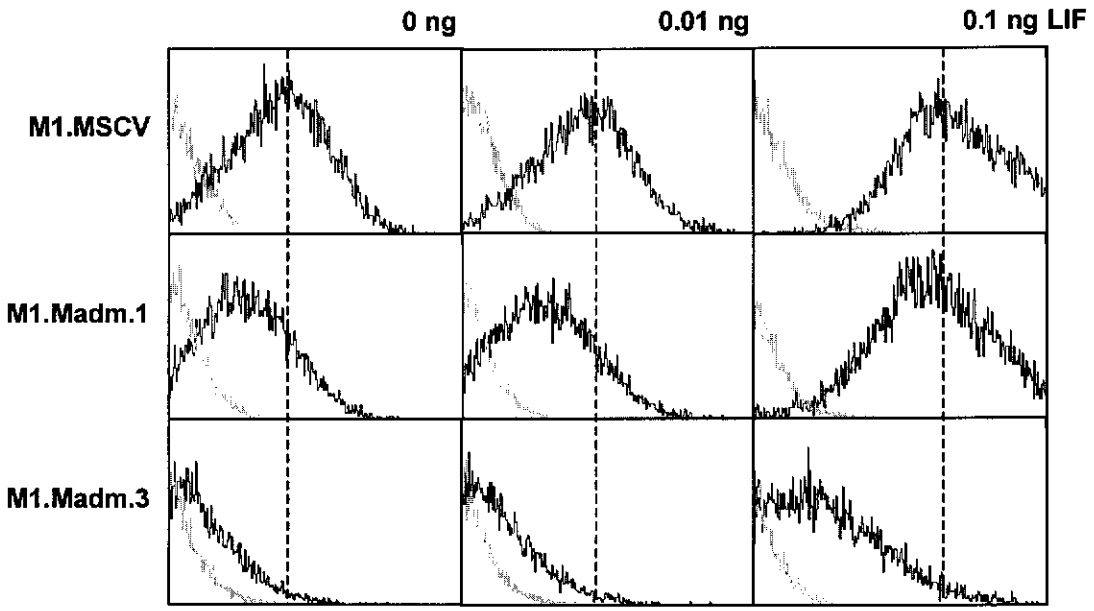


Figure 6.6. Madm inhibits M1 cell differentiation. Two independent M1 clones expressing exogenous Madm were analyzed after being stimulated to differentiate with different concentrations of LIF (0 - 0.1 ng/mL). Differentiation was assessed by measuring cell surface expression of Cd11b using flow cytometry (control, grey; with antibody, black).

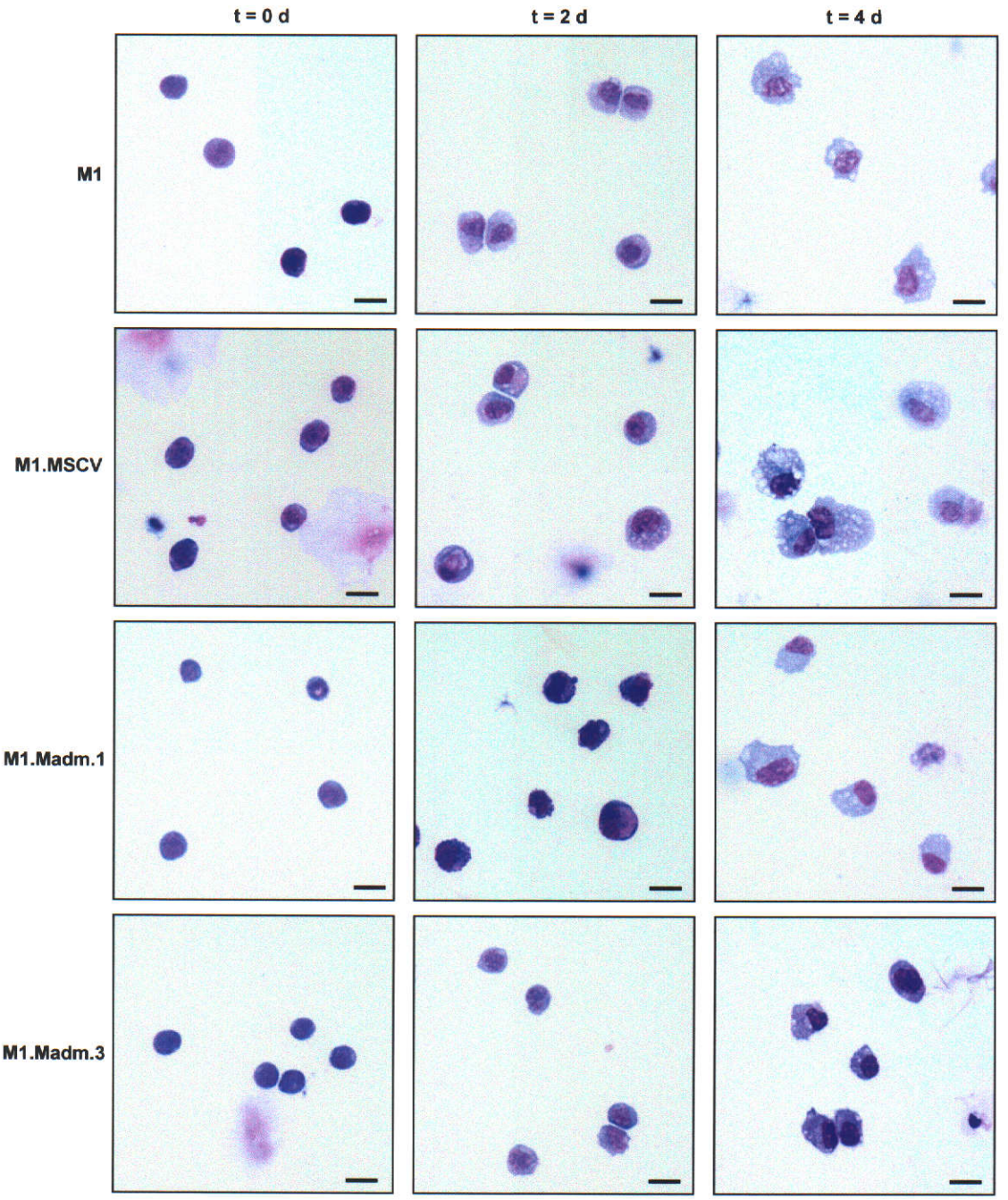


Figure 6.7. Expression of Madm in M1 cells affects morphological maturation. M1 cells were infected with either MSCV alone or MSCV-Madm. Cells were stimulated with 1 ng/mL LIF as indicated. Cytocentrifuge preparations were stained with hemotoxylin and eosin. The bar indicates 10 μm.

6.3 Discussion

Ectopic expression of Mlf1 potentiates myeloid maturation – this is manifest by increased myeloid colony formation by hemopoietic progenitors and elevated Cd11b on M1 cells (Williams *et al.*, 1999). Furthermore, when MLF1 was over-expressed in an erythroleukemic cell line, it reprogrammed the cells and induced a morphological change. The Mlf1-expressing cells displayed a monoblastoid appearance and took on myeloid characteristics, such as the ability to migrate in culture (Williams *et al.*, 1999). This illustrates that Mlf1 has a profound effect on the maturation of cells towards the myeloid lineage.

To investigate if the Mlf1-interacting protein, Madm, could also influence myeloid maturation, the cDNA was ectopically expressed in M1 cells. In the two Madm clones selected for analysis, the amount of exogenous Madm mRNA was double that of endogenous transcript. However, the amount of protein produced was only increased 50 – 80%, indicating that the amount of Madm in M1 cells is tightly regulated. Nevertheless, this modest increase in the concentration of Madm had a marked impact on the capacity of myeloid cells to mature, and was opposite to the effects of Mlf1, i.e. while expression of exogenous Mlf1 potentiated M1 maturation (Williams *et al.*, 1999), differentiation was inhibited in Madm-expressing cells. Although Madm affected the differentiation of M1 cells, no change in proliferation was detected suggesting that Madm specifically affects components of the differentiation pathway. Moreover, Mlf1 protein levels were unchanged in Madm-expressing cells (data not shown) indicating the decrease in differentiation was not due to down-regulation of Mlf1.

Results presented in Chapter 5 indicated that Madm was involved in serine phosphorylation of Mlf1 by the recruitment of a serine kinase. It was also shown that phosphorylation probably occurred at several sites of the protein; however, it specifically occurred on the consensus 14-3-3 binding site (Figure 5.7). It was, therefore, suggested that Mlf1 binding to 14-3-3 was regulated by phosphorylation at this site by the Madm-kinase complex. The opposing effects of Madm and Mlf1 on M1 cell differentiation are significant. One possibility is that when Madm is over-expressed in M1 cells it increases Mlf1 phosphorylation, which may have an inhibitory affect on Mlf1 function. This inhibition may be due to increased binding of 14-3-3ζ, thereby altering Mlf1 localization within the cell. In addition, it was noted

that the dimerization domain of Madm overlapped with the Mlf1-binding region; altering the Madm/Mlf1 ratio may well affect complex formation between these proteins. The composition of these complexes may, in turn, dictate the progress of terminal differentiation.

Chapter 7: GENERAL DISCUSSION

The molecular characterization of non-random chromosomal rearrangements unique to human cancers has identified several genes that control cellular growth or differentiation. MLF1 is a novel protein identified in the NPM-MLF1 chimeric protein produced by a (3;5)(q25.1;q34) chromosomal translocation, which is associated with MDS, and occurs in all AML subtypes, except for M3 (Yoneda-Kato *et al.*, 1996). The murine homolog of *MLF1* (*Mlf1*) was subsequently isolated independently as one of the genes involved in lineage switching from erythroleukemic cells to hemopoietic cells with a monocyte/macrophage phenotype (Williams *et al.*, 1999). The function of MLF1 is unclear because its amino acid sequence has no significant similarity to known proteins, and does not contain any identifiable functional domains apart from a 14-3-3-binding motif. However, the importance of this protein in regulating hemopoietic maturation is revealed in the (3;5) translocation, which exposes the oncogenic potential of the molecule (Yoneda-Kato *et al.*, 1996). Furthermore, over-expression of wild-type Mlf1 in patients with AML correlates with poor prognosis and reduced survival (Matsumoto *et al.*, 2000). Mlf1 has also been shown to influence hemopoietic lineage commitment in progenitor cells, alter the differentiation potential when over-expressed in myeloid cell lines and can reprogram erythroleukemic cells to develop a monoblastoid phenotype (Williams *et al.*, 1999),

A yeast two-hybrid screen identified two interesting proteins that interacted with Mlf1, viz. 14-3-3ζ and Madm. The identification of 14-3-3ζ as an Mlf1 partner protein was not surprising, as the only recognizable domain in Mlf1 is the 14-3-3 binding motif, RSXSXP (Williams *et al.*, 1999, Hitzler *et al.*, 1999). 14-3-3 proteins have been implicated in many diverse functions, and their role in promoting cytoplasmic localization of some partner proteins is well documented e.g. phosphorylation of cdc25c by Chk1 results in 14-3-3 binding, which masks an NLS and retains cdc25c in the cytoplasm (Peng *et al.*, 1998, Lopez-Girona *et al.*, 1999). It is possible, that phosphorylation of the RSXSXP motif in Mlf1 potentiates 14-3-3 binding, thereby sequestering the molecule in the cytoplasm and restricting its access to the nucleus. This model is consistent with primary localization of wild-type Mlf1 in the cytoplasm, with small amounts detected in punctate nuclear bodies (Yoneda-Kato *et al.*, 1996, Yoneda-Kato

et al., 1999, Williams *et al.*, 1999). Current studies showed that the phosphorylated RSXSXP motif associated with 14-3-3ζ, but not the unphosphorylated form (Lim *et al.*, 2002).

Data presented in this thesis demonstrate that Mlf1 is phosphorylated on several serine residues *in vivo* and that peptides spanning the 14-3-3 binding site are phosphorylated by a kinase recruited to Mlf1 by the novel adaptor protein, Madm. The properties of the co-purifying serine kinase are that its activity is independent of (i) cAMP and cGMP, (ii) phospholipids and (iii) calcium ions and calmodulin. This excludes it from several other highly expressed kinases, viz. cAMP-dependant protein kinase, cGMP-dependent protein kinase, protein kinase C and calcium/calmodulin-dependent protein kinase (Walsh *et al.*, 1968, Gill *et al.*, 1976, Ogawa *et al.*, 1981, Cohen *et al.*, 1978). Furthermore, the finding that the activity of this kinase is not inhibited by staurosporine excludes many other known serine/threonine kinases (Tamaoki *et al.*, 1986). Interestingly, Akt has also been shown to be serine phosphorylated by a staurosporine-insensitive kinase (Hill *et al.*, 2001). As both Madm and Akt have been implicated in regulating 14-3-3-binding proteins, it would be interesting to determine if they are phosphorylated by the same kinase. Identification of this kinase by yeast two-hybrid screening and *in situ* pull down experiments have been unsuccessful to date, but may be achieved using other techniques such as mass spectrometry.

The co-localization of Madm and Mlf1 suggests that these two proteins are intimately associated, especially in the perinuclear area. Although Madm has a clearly defined kinase domain, it does not contain a characteristic ATP-binding region (Hooper *et al.*, 2000). While a number of kinases that lack this domain have been identified, including Chk1 and cdc7 (Walworth *et al.*, 1993, Patterson *et al.*, 1986), our data indicate that Madm does not possess the catalytic activity of a kinase. Indeed, Hooper *et al.* (2000) considered that the human homolog of Madm was unlikely to possess functional kinase activity because it lacked 7 of the 15 highly conserved kinase domain residues (Hanks and Hunter, 1995).

The extremely high degree of Madm conservation between mouse and human (98% identity) is noteworthy. In addition, Mlf1 shares 79% identity with its human homolog, and 14-3-3 proteins are highly conserved among all species (Aitken *et al.*, 1992). This may indicate that Madm-Mlf1-14-3-3ζ complexes serve evolutionarily conserved functions.

Madm contains a potential bipartite NLS, as well as an NES. Confocal microscopy and biochemical analyzes revealed that Madm was present in both the cytoplasm and nucleus, indicating it may shuttle between the compartments. It is interesting to note that Madm has a potential phosphorylation site within the NLS, which could possibly regulate transport to the nucleus, as seen with IFI-16 and APC (Briggs *et al.*, 2001, Zhang *et al.*, 2000). However, deletion of the central region of Madm, including the NLS and NES, had no significant affect on Madm localization. These data suggest that the putative NLS and NES were non-functional. However, one possible explanation for these observations resides with ability of Madm to dimerize i.e. mutants of Madm that retain the dimerization domain could still bind endogenous Madm, and be transported to the nucleus.

While many adaptor proteins are associated with receptor-mediated signal transduction, others have also been shown to have a role in the nucleus. An example of this is Grb4, an adaptor protein consisting of three SH3 domains and a single SH2 domain that was identified as a interacting partner of Bcr-Abl and v-Abl via its SH2 domain (Coutinho *et al.*, 2000). Grb4 functions in the nucleus to inhibit v-Abl-induced transcription and this is dependent on an SH3 domain within the protein (Jahn *et al.*, 2001). The localization of Madm to the nucleus suggests that it has a functional role in this compartment; furthermore, the nuclear pool of Madm was shown to interact with the NPM-MLF1 fusion protein. The presence of two nuclear receptor-binding motifs introduces the tantalizing prospect of specific functions for Madm within the nucleus. However, no functional analyzes have been performed to determine if the protein does bind nuclear receptors (Hooper *et al.*, 2000). It is possible that Madm may not only regulate Mlf1 localization by the recruitment of a kinase, but may also regulate Mlf1 in the nucleus by binding to proteins via its LXXLL motifs. Further analysis of proteins that interact with these motifs may identify a nuclear role for Madm, as well as identify nuclear complexes that may associate with Mlf1.

There was no major change in localization of Madm, or its phosphorylation, throughout the cell cycle. In contrast, the localization of Madm's partner, Mlf1, oscillated during the cell cycle (L. Winteringham, personal communication) and has been shown to influence the cell cycle inhibitor $p27^{kip1}$ (Winteringham *et al*, submitted and (Yoneda-Kato and Kato, 1999)). It is

hypothesized that Mlf1 suppresses erythroid differentiation, in part, by preventing $p27^{kip1}$ degradation (Winteringham *et al*, submitted).

The opposing effects of Madm and Mlf1 on M1 cell differentiation are significant. Whereas expression of exogenous Mlf1 potentiated M1 maturation (Williams *et al.*, 1999), differentiation was inhibited by over-expression of Madm, even though the levels of Madm were raised only 50 – 80%. This modest increase in the concentration of Madm had a marked impact on the capacity of myeloid cells to mature, and was contrary to the effects of Mlf1. It is conceivable that an increase in Madm results in enhanced phosphorylation of endogenous Mlf1, promoting 14-3-3ζ binding and inhibiting the function of Mlf1. Alternatively, since the dimerization domain of Madm overlaps with the Mlf1-binding region, altering the Madm/Mlf1 ratio may affect complex formation between these proteins. The composition of these complexes may, in turn, dictate the progress of terminal differentiation.

The role of Madm will be further characterized with the generation of knockout mice. Functional redundancy is often a problem with knockout mice; however, there are no other genes in the mouse genome that share sequence similarity with Madm. Furthermore, the high degree of sequence conservation in Madm from fruit fly to humans suggests that the protein has an evolutionarily conserved function, and deletion or changes to the protein may not be tolerated. Therefore, it is likely that the knockout of Madm will produce a phenotypic change. Given the ubiquitous expression of Madm it is difficult to predict the phenotype of the knockout mice. However, since Madm had an effect on myeloid cell differentiation, this cell type may be a useful target for initial analyses. Since Mlf1 knockout mice are also being generated (S. Morris, personal communication), a comparison of the phenotype with Madm -/- mice may identify a common function for these molecules.

Adaptor molecules possess no enzymatic or transcriptional activity but instead mediate protein-protein interactions through different protein-binding domains (Pawson and Scott, 1997). Adaptor proteins can also contain multiple residues that are phosphorylated allowing them to be recognized by binding domains on other proteins (Norian and Koretzky, 2000). A yeast two-hybrid screen with Madm has identified a number of possible interacting proteins (Appendix 2). Interestingly, one of the proteins isolated from the screen was

calcium/calmodulin-dependent protein kinase II alpha (CaM kinase IIα), which is a serine/threonine kinase. CaM kinase II is composed predominantly of two subunits and is active as a holoenzyme (Kolb *et al.*, 1998). However, the Madm-associated kinase activity is unlikely to be due to CaM kinase IIα, since this enzyme is sensitive to staurosporine (Yanagihara *et al.*, 1991).

Other proteins identified in the screen have wide-ranging functions in signaling and provides additional evidence that Madm is indeed a novel adaptor protein. Interaction with proteins from diverse pathways is typical of adaptor molecules (Pawson and Scott, 1997) Interestingly, Madm bound to several proteins involved in endocytic pathways; these proteins are either membrane associated or cytoskeletal (e.g. Dynamin 2, Clathrin light chain II and glycosyl-phosphatidyl-inositol (GPI)-anchored membrane protein 1). It is noteworthy that the cytoplasmic localization of Madm was not diffuse and it appeared that Madm was tethered to intracellular structures. Further analysis of Madm localization with different markers for cytoskeletal structures, such as microtubules or actin fibers, may clarify intracellular elements that associate with Madm. It is possible that activation of Madm, either by phosphorylation or dephosphorylation, may alter localization of the protein to these structures. Furthermore, co-expression of these interactors with Madm may change its subcellular distribution.

Madm has also been shown to interact directly with the GTP-binding protein, Gem (unpublished observations), and a novel protein that contained a Dbl domain for guanine nucleotide exchange factors. During the preparation of this thesis, Madm was independently isolated from a yeast two-hybrid screen using Rac3 as bait (De Langhe *et al.*, 2002). Madm was also shown to interact with wild-type Rac and dominant negative and dominant active mutants (S. De Langhe, personal communication). Rac and Rac3 are highly related GTPases belonging to the Rho subfamily of Ras proteins (Didsbury *et al.*, 1989). The regulation of the actin cytoskeleton and membrane trafficking by these proteins has been described in detail (Nobes and Hall, 1995) and are known to influence transcriptional control, cell adhesion and cell cycle (Olson *et al.*, 1995, Chou and Blenis, 1996). Furthermore, in

macrophages, Rac is essential for activation of the NADPH oxidase, a multiprotein complex that produces superoxide in phagocytic cells (Abo *et al.*, 1991).

Several well characterized adaptor molecules interact with GTP-binding proteins e.g. Grb2, a ubiquitously expressed adaptor protein identified by its association with the phosphorylated epidermal growth factor receptor (EGF-R) via an SH2 domain (Lowenstein *et al.*, 1992). Grb2 also contains two SH3 domains that can interact with the guanine nucleotide exchange factor, Sos (Li *et al.*, 1993). Following EGF-R autophosphorylation, Grb2 binds phosphorylated tyrosine residues, bringing Sos to the membrane where it catalyses activation of the small GTPase, Ras (Rozakis-Adcock *et al.*, 1993). Cbl is another adaptor protein that associates with GTP-binding molecules and acts as a negative regulator of protein tyrosine kinase-mediated signaling (Rao *et al.*, 2002). The mechanism of Cbl action involves the E3 ubiquitin ligase activity of its RING finger domain (Sawasdikosol *et al.*, 2000). Cbl specifically associates with activated protein tyrosine kinases of the Src and Syk/ZAP-70 families, and ubiquitination of these kinases leads to their degradation, attenuating receptor signaling (Rao *et al.*, 2002). Cbl also interacts with guanine nucleotide exchange factors, such as Vav, which provides additional ways that it can down-regulate receptor signaling (Marengere *et al.*, 1997). Further characterization of Madm's interactions with GTP-binding proteins and guanine nucleotide exchange factors may illuminate its precise biochemical function.

Several Madm-partner proteins identified in the yeast two-hybrid screen have specific expression patterns, e.g. Mlf1 is expressed in hemopoietic cells, skeletal muscle, testis and heart (Hitzler *et al.*, 1999) and Gem is expressed in thymus, spleen, kidney, testis and lung (Maguire *et al.*, 1994); conversely, other Madm-interacting proteins have a ubiquitous expression, e.g. Rac3 (De Langhe *et al.*, 2002). It is possible that Madm may have a common function with ubiquitously expressed partner proteins, as well as have tissue-specific functions, determined by the restricted expression of other binding partners. Analysis of these Madm-interacting proteins may identify functions for Madm in tissues that do not express Mlf1.

There are several possible reasons for the leukemogenic actions of NPM-MLF1. One likely explanation is the inappropriate cellular compartment occupied by the fusion protein (Yoneda-Kato *et al.*, 1996, Yoneda-Kato *et al.*, 1999). Unlike wild-type Mlf1, NPM-MLF1 is found almost exclusively in the nucleus. Significantly, NPM-MLF1 no longer binds 14-3-3 despite the fusion junction being 13 residues N-terminal of the RSXSXP motif (Lim *et al.*, 2002). However, the fusion protein retains the ability to bind Madm, and is still phosphorylated *in vivo*. Therefore, it is conceivable that structural alterations in NPM-MLF1 may inhibit the binding of 14-3-3ζ. The inability to bind 14-3-3 may remove cytoplasmic retention and enable translocation of NPM-MLF1 to the nucleus. In addition, the NLS within the NPM portion of the fusion protein may facilitate nuclear translocation.

The identification of new adaptor proteins with the ability to interact with multiple signaling molecules is likely to be important in the understanding of how signaling pathways are modulated. While the precise biochemical function for Madm still awaits elucidation, the work presented in this thesis describes a novel adaptor protein for the oncogene Mlf1 which may play a pivotal role in regulating its subcellular localization, and potentially its function. Madm recruits a serine kinase that phosphorylates the RSXSXP motif of Mlf1, thereby promoting 14-3-3ζ binding. Complexes involving Mlf1, Madm and 14-3-3ζ are likely to influence the distribution of these proteins in the cell and affect their ability to influence hemopoietic lineage commitment.

Chapter 8: BIBLIOGRAPHY

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Appendix 1 – Sequence of the murine *Madm* gene

The full genomic sequence of *Madm* was determined by screening a 129/SvJ genomic library and sequencing by primer walking. This sequence has been deposited into GenBank under accession number AF302139.

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   1 GAATTCACAG AGATCTCTCT GGCATGCTTG TTGAAATTTC AGTTTTTAAA GCAAGCATTT
  61 TAAAGTGAAG AGATGAGAAA AAAATGAGTC TCTATAGGAT TTCTGTGTGA ACCCGTGAAC
 121 TAGGATTAAC TAGTCCCTTT AAGGAGAGTA AAATTGGTGA TTGTGACATT CTCAGATTCA
 181 GATGATTCAT CCTTCTTCCT TGATAATACA TGTATAATGG TATTATACAT CTCTTAAATT
 241 CTGCCTTTAA CTTTCTAAGA TCAATGGCTT ACCCAGAATC TATTGTAATT CTTTACACTC
 301 ACAGACAATG TAAACTCTTT CCAAATAATA CCTTTTCAAA GACAGTTTTG ATGGATCTTC
 361 TTACTTCGGA TTCTATTTTG GACTCATGTC AACTTAAATT TGAGTAACTT TTTTTTTTTG
 421 TTTTGTTTTG TTTCTTTTTC GAATTTGCCA ACTATCATTG GATAACTTCA ATGAGTTCTC
 481 CCTTATGTGG GTCGAATCAG GTTATGTCTG GGTAAATAAT GTTATGCAAA TCAGCAACTA
 541 TTAGTTTTCA TGTTAAGCGA TTAAAAAAAA GCTTTCTGAA AATTTGATGA CCAGTGCACT
 601 GTCTCTTTCC CAAATGGGGA ATGTGGGTGG GTTCGTTCCT CAGATTGAAA GGCTGCGTCT
 661 TCCGTTCCTT TTGTCTTCTT AGAGGATTAG GACTGTCAGG CGCTTTCAAA AAGAGAGAGA
 721 AGGCCTTCCT TTCGTCCGGC AGAATTACTT GGGGGCACCT CGACTGGGAG GAGGATGGAC
 781 AGAGAGACAA GAAGACAACG CTGGCAGGCA GTCTGCCGGA ACCCCGCAAG GGGAGGAGAC
 841 CCGGCAGTAG ACTGCCTTGA GAGCCTCGGT CTGTGGGAAC CGCCCCCCGC TGCCCTGTCC
 901 AATGGAAACC TGGCGGCGGT GCGGCACCGC CCACTGTTGG TCCGGACGCC GCGAGGGCGG
 961 GGCCCGCAGT TCGGTTGCGC TGCGGAGCGC AGCTGTGAGG GAGTCGCTGG GATCCGAGGC
1021 CCCGGAACCC GAGCTGGAGC TGAAGCGCAG GCTGCGGGCG CGGAGTCGGG AGGTGAGCTC
1081 CGGAGAAAGA GCCCTGGAGG GAGGGCTCG CGGAAGGGCG GCAGGATGAC CCCGAGCCGC
1141 GGGCCGGCAG GACGCGGCCG GCGGGTGCTG TCGGGCGGCC CTGCTTTCTC GGGAGTCAGG
1201 CCCGACCTAG CCCGGCCCGT CGCAGCTGAG GGGCCACCTT GGCCCCAGGG CGCCGCCCGC
1261 CCTCCTGGTG TCTCCGGCGT GGCCGCGGCG GCCCGGAATT CTGCGGGCGC GGGTCGGGGG
1321 GCCCCGGCGT TCTCTTGAAA CTCTGGCCGC GAGAGGCGGG GCAGTCGGCG CGGACGGGGG
1381 CGGGGCGGGG GCCTGGGCCC GAGGGCCTGC GGAAGGAAGC GCGGCGTCCG GGCCGGGCCT
1441 GCTCGCGGGG CGGCGCGCCG AGCGCGGGGG CGTGGTCTGC GGCCTGACGG CGGGGCAGGG
1501 CTGGCCTTTG ATGAGGTGGC TGTGGTGCTC GGTCTCCTGG CCGGCCACTG TCTGTCCGCC
1561 GCCGCTTCCT TCCAGATACT GGCCGGGCGA CCTGTTGGAG TGTTGACAAG CTGAGGGCCT
1621 CCTCCCTCCG AGCTTGCATT CTCTCCGAGT GAGCAGTTCT GTTGACCTCA GAGGCTTTCT
1681 GGGTTTCCTC AGAGCGGCCT GTGGCTGCGA GCTATGCGGG GTGATGTGCA GAGGGCTTTC
1741 AACTAATAAT GGAAGCCGGG AGAACAACTT TGCGAGATGA CAGACAATGC CCTGTAGTGA
1801 ATGTACTATG CGTCCTCAGG GTAGCTAGAG GCTTCGAGGA GATACCCTCA CCGATGCAAA
1861 TGAGCTTTTC CACAGTGATC CCTGTTAAAA CTGCCAGCAG CTCTGAGGCA GAATCCCCAG
1921 TGTTGCACAC GTTTCTGCAT CTCTTGCGGG AGTGCTTGGA GATCTAGTGC CTCAGAGATT
1981 GGAAAAGTCA GAGCCTTTGA GCAATGTAAA TGAAAGGAAA GCTGGAAACA AGGTCTTTGA
2041 TTTTTTTTTT TTTTAATTTG TGCTTAGGTA AGTGTAGGCA TTTCATTTAT TTTAATTCAC
2101 TGATCTATCT CAAGCACCCT CCCAGTGTGT GACGTACAAT AACCTACAAA TGTTACTAAA
2161 CATTTCAGTG AGTAAATGAA AAGAAATGTT GTTAAACAAT ACCCACTTGC CACACTTACA
2221 GATGGAAGTC ACGCCTGTCG GTCACTTGGC CCAAAGGCAG CTTCTACCTA TGTATAGGAA
2281 AGATGAATAA TGTCACTATT GGTCTAGTAG GATAAGTCAC CCTTGGGAGT TTCGGCTAGT
2341 TGGTTAAGGA TGGTATAGAC CAAGCTGGGC GTGGTGGTGC ACGCCTTTAA TCCCAGTACT
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2461 CAGGACAGCC AGGGCTATAC AGAGAAACCC TGTCTCAAAA ATAAATAAAT AAAAAAATAA
2521 AAAGGGATGG TATAGACCCA TAGGGTTGTC TGTCTCAGTG GACATTTTAG AGTGTAGAGT
2581 CATTTGCCTG TAATGCCTGT ACCCCTATTC TTGAGCACAG TCAGAATTGA GGTCCATTGA
2641 GAGCCAATGG GAAGTATATT AATGTAACCC TATTGGCTGA GAGGCTATGG GGCACCAGAT
2701 TTTACTGTAT ATTTGAGTTT GACTAAAGTG GACTATATTG TCATTATTCT CGGGTATGGC
2761 ACTTCTGTGT GGTCTTAAGC CAAAGTAACT CATCTTCTAG AAGCCAGAGA GTCATTCACA
2821 TCAGTATTTA GGTAAGAGTA CATTTGATTT TAGATTTAGA GGAAACAAGA TATATTTTTA
2881 AGGATGAAGA TGAAGAAGTT AAGACTATTA AAGTTTTATA CGTCTGTTTT TGACCTTTGT
2941 CTTCTGACTT TGCCCAACAA ATGCAGGCTG AGTGTTCATT CCAGCATGTC GGAAGGGGAG
3001 TCCCAAACAG TAGTCAGCAG TGGCTCAGAC CCAAAGGTAG AATCCTCATC TTTAGCTCCT
3061 GGCCTGACTT CTGTGTCACC TCCTGTGACC TCTACAACTT CAGCTGCTTC TCCCGAGGAA
3121 GAAGAAGAAA GTGAAGATGA GTCTGAGATC TTGGAAGAGT CACCCTGTGG GCGCTGGCAG
3181 AAGAGGCGAG AAGAGGTGAG GTGGTGGTGA TGGCGTTCCT GGGAGAGTCA ATTGTAAGCG
3241 GTGAGACCTA GAGTAGCTGT CCAGAAGAGC AGAACAGACA CGAGTGGGGC AGGGCTGGAA
3301 GATGAGCATA TGGAATACTA CAGTGGCAGC TTCTTGAAGT GCCTTAATTC CACTTGAATT
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3361 GCTTCTCAGG TGAATCAGCG TAATGTACCA GGCATTGACA GTGCATACCT GGCCATGGAT
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3481 AAACTGCAGG AGGTAGGTGA TGAGGCCTGG GGGCCTATTA TTTAAGCAGC TGCTGCTGTT
3541 GTTGGGAATG TGACTGTGAA GAAAGCTTCA TGATTAGATC ATCCATGGGG ATGGGTAGGG
3601 AATGGCTTTT CACATTATTA CAGTGATTTG TGAACTCTGT TGTCCCTCTG CCTCCCTGCT
3661 CCAGGAAAAG GTCCGTGCAG TGTTTGATAA TCTGATTCAG CTGGAACATC TTAACATTGT
3721 TAAGTTTCAC AAATATTGGG CTGATGTTAA AGAGAACAAG GCTAGGGTAA GATTTTTTTG
3781 CTTAGTATTT CTTGAATTTT CCAGGTGCAT ATATCGTCCA TTTTGCATTC AGAGGTTGAA
3841 ACTAGGTAAT TTTGAACTGA AATCTGCTGT GGCAGGGAAG AGTAGGGTTT CATCTGATAT
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4381 CAGGACACCT AAGGCACACT GTGTGTGTGT GTGTGTGTGT GTGTGTGTGT GTGTGTGTGT
4441 GTGTGTTTGT GTATGGTGAA ACCATAGAGA AGGAGGGTGG GTAGAGAGGG GAGAAGGAAG
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5641 ATGCAGTCGT GGGAGCAAGG CCTGTTCCCT GCTTCCTGTG CCTAATTATG CCTGCTTAGC
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5821 CTAAATCATA CTGTGCTCCT AGATTCTCTG TGATTTGTAG TCATTGTTAC GGTCAGAGCG
5881 AGTCAGCTTG GGCCTGGTCC CTCATTCAGC CTTGCGGTAG GTGGTGTTGA GTAAGTAAGT
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6001 GTACTGGTTC ACTTTAGAGC TTTTTGGGTT TTTTGTTTTA AGTTTCATTC ATCACAGATG
6061 AGTAAGGAAG AGGCTAGCTC CTTGGATCTA TGTTAATACA GCTTTGCCTT TCTTATCCTG
6121 GTTCATGAGG TCTCCAGTTG TCTCATAGTA GGAAAGGGTT GCCTGTATTA TGTTAATTGC
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6361 CCTTAGATGA TAATGGAGGC TGCAGAAAGG GAATCTAGGT GGTGTTTATA TGTCGTCTAT
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7261 CTTTCAAGTT TATTGTACTT TCCAAGAGGT CCCAAATGGT TTAAGAGCAA AAGAAGAGAA
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 7441 ACATAAAGGT GAGCTGATTG GGTGGTTACC TCAGGCAGCT GCTATACACT CTTATACAAT
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 7621 AACAAGAACG CTTGACTCAG GTCAGGCATG GAGGCTACAT CTATACGCCT AGCACTTAGG
 7681 AGGTAGAGAT AGAAGGATCG GAGTTTAAGG TAATCCTCCC CTACATAGAG GGTTCAAGAC
 7741 TAGCCTGGGC TATATTAGAC CCTGTCTCAA AAACAGTTAA TGTTAATCAG AGACAGTAGT
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 7861 ACTAGGGTGG GATTATTAGG TGAAAGATAA GGTGTGTAAC TTTCTTGTCC TTTAAAACTA
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 8521 CAAGCCAGCT TAAGCCATGT AATGAGACCC TGTCTTAGAG GAACAAAATG TTTAGAAAGA
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 8641 GAAATACATC AATGTTTCCC CAATAAAGTT GAATTCCAAG CATGTAGGGA ACATTTTGCT
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 9001 GGCATTGGGG TCTTTAGTTT TCTGTTACCA AAGGGATTCT TTTACCCCTG TCCTCTGCCT
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 9361 TGTCAGGTGA GATAGGGTGG GCTTCTGGGA GGAAGTAGGG ACAGGTGTCT TGAAGCTTTC
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 9481 TTTGGGCTAC CTCGGCCTCA GCAGCCACAG CAGGAGGAGG TGACATCACC TGTTGTGCCC
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 9601 CTGGTATGAT TGTATTTTTG GCCATGGGGA TAGAGACATC TTAACTAAGG CACTGTCCTG
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10081 AGACTGTATT CAAGCACCCC TCCTGTCTCT CTGTCAGATG AGAGCATCCC GGACTTGGCA
10141 GCTGAGCTGG TGCAGCTGGG CTTCATTAGT GAGGTGAGGG TCCTGCCTGT GTTCTGGGTG
10201 CCTCCCTGCC CCCCCTCTCT CAGCCCTGCC TTACTGAGCT GCTTCCCCAG GCTGATCAGA
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10321 CACTCAACAC AGCCACTGTC ACCGTCTCCT CGTAGAGCTC ACTTGAGCCA GGCCCCTAGC
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10561 GCCTGGGCCT TCTCAGCAGC CACCTTCTAG CTGGGGGCTA GTAGCTGACC TGCTGCCTCC
10621 TGCCCTACTT GTGTGGACAG GAGGCCCACG GGCACTGGGG AAGCTGAGTT CTACAATCCC
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10801 TGTGCTTGCG GCTCAGTGGG TGGCACCGCC GCTTCGGTTG GGGCGGGGCG GGCCCAGGTA
10861 GGGTGGCCCC TTGGAGGCCG GGCCTTAGGT CAGGGGCGGG GCGGGGCCAG GTGGAGCCGC
10921 CTGCGGCGGC GCGGAAGCGG CGCTTGAGGT CGGCGTCGGG ACAAAGCCAG GTCCAGGTAG
10981 AGCCTATGGG CGGAGGGAGT AGAGCGGCCA CGATGAGGTG CTGCGGCGTC TGCGCCTTCG
11041 GTAAGTTGTA CGGCTTTCTA CCGCACCTGT GGTCCCACAG GCCGACGCTG GGCCTGGGGC
11101 CCCGCCCTGC ACGGCTACTG TCCGCCCCTC CCCCTACCTT GCAGATGCCG CCCGAGGGCC
11161 CAGGCGGCTG ATGCCGCGTG GGACTCGCGC TGATCCTGGT GGGCCACGTG AACCTCCTGG
11221 TGGGAACCTG TGTTGCATGG TACTG
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Appendix 2 – Madm-interacting proteins

A yeast two-hybrid screen using Madm as bait was performed by Jim Tiao. Clones positive for β-galactosidase activity were sequenced and listed below with the binding region shown in amino acids (aa).

Protein	Accession number	Binding region (aa)	Number of clones
Calcium/calmodulin-dependent protein kinase II α	NP_033922	137-200	1
Chromodomain helicase DNA binding protein (CHD4)	Q14839	417-510	1
Clathrin light chain 2	U91848	1-160	1
Cleavage and polyadenylation specificity factor 1	AAG40326	755-915	1
Coatomer protein subunit a (COPA) (human)	NP_004362	870-926	1
Dynamin 2 (Dnm2)	NP_031897.1	519-563	3
Gem-GTP binding protein	NP_004362	1-128	1
GPI-anchored membrane protein 1	NP_058019	405-484	1
NS1 binding protein (human)	NP_006260	244-332	1
NY-REN-2 antigen (human)	AAD42861	350-447	1
Peptide N-glycanase PNGase	NP_067479	496-651	1
Protein 4.1	AAA37122	580-700	1
Protein disulfide isomerase/cellular thyroid hormone binding protein (p55)	X06453	1-299	1
SAP49 - splicosome assoc. protein	Q15427	3-207	3
Supressor of varl (S. cerevisiae) 3-like 1 (human)	XP_005981	45-211+	1
TGF-beta receptor interacting protein 1 (TRIP-1)	AAF01455	175-301	1
TRRAP (human)	NP_003487	450-609	1
TSC-22-like protein (human)	AJ133115	62-266	3
Novel - human KIAA1321	AB037742		2
Novel - RIKEN clone 2610511M14	AK012128		1
Novel protein – Dbl domain for GEFs	NP_060672		1
Novel (no homologies)			1