School of Pharmacy

Characterisation of Genes Derived from Murine Malignant Mesothelioma by Suppression Subtractive Hybridization

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ABSTRACT

Malignant mesothelioma (MM) is an aggressive tumour, which is highly associated with previous asbestos exposure and is resistant to most conventional anticancer therapies.

Previous studies have used a mouse model of MM to develop effective approaches to induction of anti-tumour immunity using modification of tumour cells by the introduction of genetic constructs expressing genes such as that for B7-1 so that tumour growth can be inhibited *in vivo*. Transfectant clones, AC29 B7-7 and AC29 B7-6, which showed equal levels of expression of B7-1 but were markedly different in tumorigenicity were assessed using suppression subtractive hybridization (SSH) in order to isolate transcripts which may have been differentially expressed in the two clones. SSH allowed isolation of a number of cDNAs which were apparently differentially expressed in the cell lines. These required characterisation in order to determine their possible relevance to tumorigenicity.

Two cDNAs designated as 7-7-76 and 7-7-43 had been isolated previously and the aim of this project was to characterise these cDNAs by sequencing, searching for their homology relationships and investigating gene expression profiles. Preliminary searches revealed that clone 7-7-43 had homology to cyclin-dependent kinase regulatory subunit 1 which plays a role in the cell cycle. On the other hand, clone 7-7-76 showed only homology to an EST of hypertension related protein and therefore, further investigation was required to obtain the identity of clone 7-7-76.

The first part of this project was to investigate and evaluate gene expression on clone 7-7-43, using both relative RT-PCR and Northern blotting. In the second part of this project, a more intense study of clone 7-7-76 was conducted. Clone 7-7-76 was investigated for its homology relationships and its gene expression profile. Results obtained from relative RT-PCR suggested no difference in the expression of the either cDNA clone (7-7-43 and 7-7-76) between the MM clones AC29 B7-6 and AC29 B7-7, the cells used to derive these clones by SSH. Therefore, it was concluded that neither clone 7-7-43 nor 7-7-76 was differentially expressed in MM cells of differing immunogenicity.

RACE was employed in order to derive a longer sequence of clone 7-7-76 and the newly derived sequence of 7-7-76 was again used to search for homologies using a wider range of sequences for human and other species. These investigations on clone 7-7-76 showed it to correspond to the sequence of human mitofusin 2 which is involved in determining mitochondrial morphology.

The results determined in this project suggest that clones 7-7-43 and 7-7-76 are not differentially expressed in the range of MM cell lines tested. The data have however highlighted the potential of the SSH technique to easily derive cDNA clones worthy of investigation, but underline the possibility of false positive clones being isolated. The need for an efficient, accurate screening procedure such as real-time PCR is acknowledged.

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ABBREVIATIONS

AAP	Abridged Anchor Primer
ANGIS	Australian national genome information service
AUAP	Abridged Universal Amplification Primer
bp	Base pair
Cdk	Cyclin dependent kinases
CdkIs	Cyclin dependent kinase inhibitors
cDNA	Complementary deoxyribonuleic acid
Cks	Cdc kinase regulatory subunit
DD-PCR	Differential display polymerase chain reaction
DNA	Deoxyribonucleic acid
EST	Expressed sequence tags
HRP	Hypertension related protein
IFNs	Interferons
IL	Interleukin
Kb	Kilo base pair
m	milli
M	Molar
Mfn2	Human mitofusin 2
mg	milligram
MHC	Major histocompatibility complex
MM	Malignant mesothelioma
mRNA	Messenger RNA
MW	molecular weight

NR	Non-redundant
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
RACE	Rapid amplification of cDNA ends
RDA	Representational difference analysis
RNA	Ribonucleic acid
RT-PCR	Reversed transcription polymerase chain reaction
SAGE	Serial analysis of gene expression
SDS	sodium dodecyl sulfate
SSC	standard saline citrate
SSH	Suppression subtractive hybridization
TGF-β	Transforming growth factor-β
TIL	Tumor-infiltrating lymphocytes
TIM	Tumor-infiltrating macrophages
UV	Ultra violet

1.0 LITERATURE REVIEW

1.1 Malignant Mesothelioma

Malignant mesothelioma (MM) is a tumour known to be rare and invasive which grows rapidly and invades locally. It has been predicted that in the next 20 years, the number of people dying of mesothelioma will increase in Western Europe and Australia (Peto *et al,* 1999). The tumour normally affects the mesothelium of the pleural cavity but in some cases the peritoneal cavity is involved (Garlepp and Leong, 1995). MM is generally rapidly progressive but can remain stable for a long period of time (Robinson *et al,* 2001). However, it has been demonstrated to be fatal and the median time from diagnosis to death is about 9 months (Musk *et al,* 1992). Further studies have established that MM does not respond to any therapies such as radiotherapy, chemotherapy and surgery (Musk *et al,* 1992; Bielefeldt-Ohmann *et al,* 1996). However, based on animal studies and observations in some humans treated with immunotherapy, MM is believed to show some immunogenicity (Robinson *et al,* 2001). Therefore, it has been suggested that new therapies directed to immunotherapy may potentially aid in the eradication of MM (Caminschi *et al,* 1999).

1.1.1 Pathogenesis of MM

Much research has been done on MM. It has been suggested that the disease is caused by exposure to asbestos (Robinson *et al,* 1996), particularly in workplace environments and around the mining areas with high levels of asbestos fibres which have been associated with a high incidence of the disease (Anderson *et al,* 1991). Although much of the research has been done on the induction of MM as a

consequence of amphibole asbestos fibre exposure, little has been studied on the carcinogenicity of non-asbestos fibrous minerals (Kleymenova *et al,* 1999). There are patients who have not been exposed to excessive levels of asbestos, showing that other environmental agents might play a role in induction of the disease (Robinson *et al,* 1996).

A number of pathways can lead to the development of MM and other cancers (Weinberg, 1989). Mutation can result in activation or inactivation of genes. Genes which when activated lead to cancer development are known as oncogenes and inactivated genes are often tumour suppressor genes (Bertram, 2001). However, there is no consistent data showing that either oncogenes or tumour suppressor genes determine the development of MM (Robinson *et al,* 1996). Although a number of genetic aberrations have been demonstrated, there is no clearly defined set of genetic mutations that can be implicated in the pathogenesis of MM (Kleymenova *et al,* 1999). For example, there is some proof for activation of an unidentified non-ras oncogene in mesothelioma (Walker *et al,* 1992) and also reports showing p53 mutations or deletions in human MM and also in most of the murine asbestos-induced mesotheliomas (Cole *et al,* 1991; Kafiri *et al,* 1992; Mayall *et al,* 1992; Metcalf *et al,* 1992).

In vitro studies have shown that chromosomal damage occurred easily after exposure to asbestos (Lechner *et al,* 1985). The damage could be due to release of free radicals after absorption of asbestos fibres by mesothelial cells or by direct interaction of the fibres with the chromosomes (Wang *et al,* 1987; Mossman and Marsh, 1989). Mesothelial cells exposed to asbestos developed DNA single-strand

and double-strand breaks (Libbus *et al,* 1989) and also alterations of chromosomes (aneuploidy and other aberrations) (Rieder *et al,* 1991; Barrett, 1991; Yegles *et al,* 1993). Chromosomal instability in tumour cells, including deletions, triploidy and translocation of chromosomes can be seen in both human and murine asbestos-induced MM (Libbus and Craighead, 1988; Tianen *et al,* 1989; Funaki *et al,* 1991).

1.1.2 Identification Of Molecular Target In MM

An understanding of the biological process involved in enhancing the proliferation of tumour cells and the evasion of the immune system of the host, may benefit further studies on tumour-specific therapeutic regimens (Garlepp and Leong, 1995). Activation of characteristic oncogenes may be a primary event in tumour development or may be responsible for tumour progression and metastasis after tumour development. Therefore immunotherapy or antisense technology which target these oncogenes is a way to use as a treatment. However, there is still no strong evidence showing mutations in oncogenes in human MM or in the asbestos-induced animal model (Weinberg, 1989; Garlepp and Leong, 1995). Another possible therapeutic system is restoration of dysfunctional tumour suppressor gene activity. There is also no concrete evidence of tumour suppressor gene abnormalities in MM (Garlepp and Leong, 1995). The identification of genes which are preferentially activated or inactivated in MM cells would be useful as potential targets for therapy. Therefore, the application of advanced molecular technologies may provide clues to molecules which would be significant in developing immunological or antisense approaches to the treatment of MM in the future.

1.2 Animal Model

The establishment of a suitable animal model is a start to further study of the human malignancies in the area of development, evaluation and improvement of therapeutic approaches. There are a number of advantages in having an animal model to evaluate tumour biology and immunology. Evaluation can be done whether the tumour is at pleural, peritoneal or subcutaneous site and these tests can be done at any time in the animal's life or stage of tumour development. Besides that, an animal model is a useful model for evaluating new therapeutic modalities of diseases aimed at modifying tumour growth and bringing about tumour rejection by activating the host immune system. Tumour vaccines for diseases are also continuously being developed and an animal model of the particular disease can be used for examination of their efficacy.

The inadequate availability of representative cell lines and animal models encouraged the development of an asbestos-induced murine model of MM as *in vitro*-established cell lines and *in vivo*-passaged malignancy (Davis *et al,* 1992). Several studies have shown that the growth factor biology, major histocompatibility complex (MHC) antigen expression and tumour suppressor gene expression in the murine model of mesothelioma appears to be similar to its human counterpart (Manning *et al,* 1991; Davis *et al,* 1992; Garlepp *et al,* 1993a; Garlepp *et al,* 1993b; Robinson *et al,* 1996). Furthermore, these models were expected to be beneficial in cellular, molecular and genetic studies (Davis *et al,* 1992).

The establishment of different MM cell lines derived from each of 3 major strains of mice, Balb/C, CBA and C57B1/6, gives the opportunity to study the biological diversity and various aspects of immune responses to MM (Davis *et al,* 1992; Cora and Kane, 1993; Robinson *et al,* 1996). The MM cell lines were originally derived by injecting crocidolite asbestos into animals intraperitoneally. After 7-25 months of exposure, some of the mice developed mesothelioma and cell lines have been established from these primary tumours. The non-immunogenic mesothelioma cell line known as AC29 was derived from this process of inoculation of crocidolite asbestos into a CBA mouse, while the weakly immunogenic cell line (AB1) was generated from BALB/c mouse (Davis *et al,* 1992). Many experiments have been carried out using these cell lines with the aim of achieving long-term protective anti-tumour immunity.

1.3 Immunotherapy

An understanding of biological aspects of the immune system and the path to stimulate the system has opened the route for anticancer immunotherapy (Ribas *et al,* 2000).

1.3.1 Immune responses to tumours

T cells are a class of lymphocytes derived from the thymus that control cell-mediated immune reactions by cell-cell interactions and by secreting lymphokines (Cox, 2001). Cytotoxic T cells are believed to be major effector cells in anti-tumour immune responses. Cytotoxic CD8+ T cells have the potential to recognize peptide

antigens derived from cancer cells and presented by class 1 MHC molecules and subsequently kill the cancer cells (Riddell and Greenberg, 1995).

Many *in vitro* and *in vivo* experiments have shown that the immune system is able to recognize and respond to tumour cells (Townsend and Allison, 1993; Ramarathinam *et al*, 1994). In some tumour systems, immune responses depend on the expression of class I and class II MHC molecules by the tumour (Baskar *et al,* 1995). Therefore, a collaboration between CD4+ and CD8+ T cells may actually induce immune responses to tumour antigens and these interactions are particularly important when the levels of expression of tumour antigens are low. This may be of significance in nonimmunogenic cancer cells (Rosenberg and Singer, 1992; Mitchison, 1990).

1.3.2 Inefficient immune system in cancer

Despite the extensive evidence from many experiments that tumours can induce immune responses, cancers still develop and grow uncontrollably in many subjects suggesting that cytotoxic immune response towards tumour cell is inefficient in many cancers. This could be due to one or more of many factors. Inefficient activation of tumour specific T cell could be due to failure in the induction phase or active suppression of the immune response (Ioannides and Whiteside, 1993). Inappropriate signal transduction by tumour-infiltrating T cells, lack of MHC antigen expression, the lack of co-stimulatory molecules on tumour cells or the lack of potent target antigen may all lead to failure of anti-tumour responses (Zier *et al,* 1996; Jenkins and Johnson, 1993; Koeppen *et al,* 1993).

1.3.3 Cancer cell modification

Tumours are often classified as immunogenic, partially immunogenic or non-immunogenic. Rejection by the host requires minimal stimulation of the immune system with immunogenic cancer cells (Hellstrom *et al* 1997). Conversely, there is no induction of an immune response using non-immunogenic tumours (Embleton, 1987).

Genetic modification is often used to try to convert a non-immunogenic to an immunogenic tumour. This is often done by transferring genes which encode proteins involved in the induction of anti-tumour immunity, such as cytokines and co-stimulatory molecules or tumour antigens. Some tumours are successfully modified to become immunogenic, eg. AB1, while others remain non-immunogenic, eg. AC29. In this laboratory, genes such as B7-1 (Leong *et al,* 1996), B7-2 (Leong *et al,* 1997b) and interleukin-2 (IL-2) (Leong *et al,* 1997a) have been introduced into AC29 in order to increase immune responses to this cell line.

1.3.4 Expression of B7-1 Molecules by AC29

A range of different AC29 transfectant clones has been previously established through transfection of the expression vector PhbApr-neo-B7.1 (Leong *et al,* 1996). Results had demonstrated that all AC29-B7-1 transfectant clones expressed B7-1 *in vivo*. Some of the B7-1 transfectant clones were able to induce tumour-specific CTL responses and also were delayed in tumour outgrowth, eg. AC29 B7-6. However, one transfectant clone, AC29 B7-7 retained tumour growth analogous to the parental cell line, AC29 (Leong *et al,* 1996). Because these two clones demonstrated

extremes in tumorigenicity, they were examined closely to try to identify molecular differences between the two. Gene expression studies were conducted as part of the assessment. One of the gene expression techniques known as suppression subtractive hybridization was used to isolate differentially expressed genes in these murine clones.

1.4 Suppression Subtractive Hybridization

Interest in differentially expressed genes has encouraged the development of molecular techniques to identify and isolate over and under-expressed genes (Dey *et al,* 2001). Among the new techniques which have been developed are subtractive hybridization (Lee *et al,* 1991), differential display of mRNA (Liang and Pardee, 1992), suppression subtractive hybridization (Diatchenko *et al,* 1996), serial analysis of gene expression (SAGE) (Velculescu *et al,* 1995) and many more. The suppression subtractive hybridisation (SSH) method has been previously used to identify genes which were differentially expressed in murine MM (Fox, unpublished) and in this project two such genes are characterised.

1.4.1 SSH In The Study Of Differential Gene Expression

SSH has been used to identify genes which are differentially expressed in a number of tissues and cell lines of interest (Jin *et al,* 1997). SSH has proven to be a powerful method for generating cDNA libraries that are highly enriched for differentially expressed genes (Dey *et al,* 2001). Patterns of gene expression in cDNA libraries can be compared using microarrays where thousands of genes can be studied in one

hybridization (DeRisi *et al,* 1997; Schena *et* al, 1995; Schena et al, 1996). For example, in the experiment by Nguyen et al, (1995) a cDNA library of 800 000 clones from adult mouse thymus poly (A) RNA was used to study the differential gene expression among three cell types in the thymus using SSH technique.

1.4.2 Method

There are a few ways that subtractive hybridization can be used to identify and isolate cDNAs from differentially expressed genes (Duguid and Dinauer, 1990; Hara *et al,* 1991; Lee *et al,* 1991). SSH is a technique based on modifications of representational difference analysis (RDA) to normalize mRNA abundance (Diatchenko *et al,* 1996). The technique involves the selective amplification of differentially expressed cDNAs together with suppression of amplification of non-differentially expressed cDNAs (Diatchenko *et al,* 1996). This technique uses a combination of normalization and subtraction (Zhang *et al,* 2000). The subtraction step is said to enhance differentially expressed cDNAs of the target, while the normalization step normalizes the representation of genes with differing expression within one sample. Therefore, this technique creates a target sample-specific cDNA library (Dey *et al,* 2001).

This approach has been used to investigate the differences between the aggressive and the less aggressive B7-1 transfectant AC29 clones described above (Fox, unpublished). Several differentially expressed cDNAs have been isolated. Some encode known genes while others are yet to be identified. Two such unidentified cDNA clones are those designated as 7-7-76 and 7-7-43. A short preliminary

sequence of each clone was available at the commencement of this project and was used to search the GenBank database for homology identification.

1.5 Cyclin-dependent kinase gene

Based on the preliminary sequence and comparison from GenBank, it was suggested that clone 7-7-43 is homologous to *homo sapiens* Cks1 regulatory subunit gene, sequence entry X54941 submitted by Richardson, 1990.

Cyclin dependent kinases (Cdk) are a set of proteins which are important enzymes involved in the control of cell cycle processes (Toogood, 2001; Pines, 1994; Nigg, 1995; Harper, 2001). These kinases are composed of a catalytic Cdk subunit and a regulatory subunit (cyclin) which forms a heterodimer and is involved in the movement between phases of the cell cycle (Toogood, 2001; Morgan, 1997; Harper, 2001). Some Cdk form complexes with a small protein known as Cdc kinase regulatory subunit (Cks) which is vital for Cdk function and cell division in yeast (Pines, 1996). The absence of the function of Cks can lead to failure in mitosis progression (Tang and Reed, 1993; Patra and Dunphy, 1996). Series of structural studies have proved that Cks and cyclin proteins interact together with Cdk (Bourne *et al,* 1996). When Cks regulatory protein binds to Cdk, no inhibition or activation occurs but rather it alters the substrate target of phosphorylation or dephosphorylation (Lew and Kornbluth, 1996; Harper, 2001).

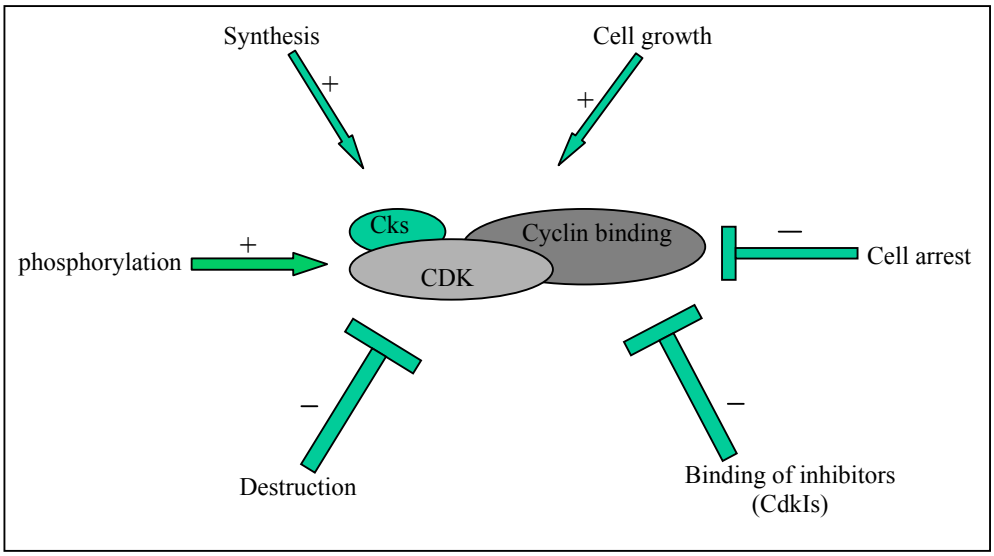

Figure 1.1 Principles of Cdk regulation. A simplified model of the major mechanisms used to regulate Cdk activity both negatively (-) and positively (+).

At every stage of the cell cycle, different types of cyclins have a function in regulation of kinase activity (Ninomiya-Tsuji *et al,* 1991). For instance, CDC28 (a major Cdk in the budding yeast or *Saccharomyces cerevisiae*), are activated by nine different cyclins during cell cycle (Levine *et al,* 1997). The high efficiency of functional activation of Cdc28 by the different cyclins raised an interesting question in molecular studies and subsequently, many studies have been directed towards the functions of CDC28 in the process of mitosis (Ninomiya-Tsuji *et al,* 1991; Levine *et al,* 1997). In addition uncontrolled cell proliferation/mitosis due to an error in regulatory pathways of cell cycles are believed to be the core of many diseases, particularly cancer (Toogood, 2001).

Therefore, interference during the cell cycle may be a good way to control abnormal cell proliferation (Toogood, 2001). A significant number of studies have demonstrated the G_1 events involved in regulation of phosphorylation as good targets

for cancer therapy (Fry and Garrett, 2000). Besides the enzymatic activities of Cdk being regulated by cyclin binding and Cdk phosphorylation or dephosphorylation, they are also regulated by association with inhibitory proteins, collectively known as the cyclin-dependent kinase inhibitors (CdkIs) (Morgan, 1995; Morgan, 1997; Sherr and Roberts, 1995). At the moment, there are two families of CdkIs known as the INK4 family and the KIP family (Zhu and Skoultchi, 2001; Izzard *et al,* 2002). For instance, $p16^{INK4a}$ which appears to be a member of the CdkIs-INK4 family, are produced after specific stimuli, causing tumour cell line divisional arrest in G_1 and inhibiting the process of the cell cycle (Sherr and Roberts, 1995).

Consequently, the expression of CdkIs or changes in regulatory subunit expression may be significant in cancer therapy. CdkIs have been considered to be of potential therapeutic use against proliferative diseases, particularly tumour cells (Toogood, 2001). CdkIs have also been proposed as therapeutic targets in mesothelioma as 55% of mesotheliomas are shown to have absence of CdkIs (Roussel, 1999).

Many genetic studies have been conducted with the aim of finding therapeutic targets for cancer therapy. One such genetic analysis has identified a protein, Cks1 that regulates Cdc28 kinase activity and associates with cyclin-Cdc28 kinase complexes *in vivo* (Hadwiger *et al,* 1989; Tang and Reed, 1993). AC29 cDNA clone 7-7-43 is highly homologous to the *homo sapiens* Cks1 regulatory subunit gene which is likely to have important function for regulating interactions with Cdk whose function might be significant in tumour progression. Cks proteins such as 7-7-43 generally bind to cyclin-Cdk complexes during cell cycle progression and hence, it is an essential component of the cell-cycle machinery. Therefore, based on preliminary evidence

using SSH that 7-7-43 (Cks1) is differently expressed in murine MM cell lines of different tumorigenicity, further investigation of 7-7-43 gene expression and its role in tumour progression was warranted.

Among the unknown clones isolated by SSH was 7-7-76. Preliminary database searches identified clone 7-7-76 is homologous to *Mus Musculus* cDNA EST clone (TC119995). Based upon preliminary evidence using SSH that 7-7-76 is differentially expressed in murine MM cell lines of different tumorigenicity, further investigation of 7-7-76 gene expression was required to identify or characterise the gene and confirm its differential expression.

1.6 Significance Of The Research

Analysis of expression patterns of genes associated with survival, growth and differentiation has become an important aspect of research studies in the area of biological processes and disease pathology at molecular level (Zamorano et al, 1996; Snider et al, 2001). The ultimate aim of the gene expression studies is to determine the roles of specific genes in biological regulation, to understand their function and to determine alterations in their expression which may promote tumour proliferation. Therefore, it is essential to study gene expression by various methods (Northern blots, relative RT-PCR) and processes (cell- and tissues- specific expression, differentiation). Tumour cells of a single tissue type that arise in the same host, or in different individuals, often differ morphologically and physiologically. For instance, some tumour progressions is dependent on particular hormones (eg. PDGF) or the immune system while others are not dependent on these factors. Therefore, it is

presumed that such tumours differ in the genes they express or the level of expression of these genes (Chiou et al, 2001).

This project will attempt to provide an investigation into the isolated differentially expressed cDNA clones in murine MM. Gene expression results obtained would greatly facilitate gene discovery and functional analysis which might involved in tumour progression.

1.6.1 Aims Of The Project

The overall objective of the work describe in this thesis was to characterise two cDNA clones isolated from murine MM cell line clones by SSH in order to determine their relevance to tumorigenicity. The specific aims of this project were :

1. To determine tissue and tumour specificity of the isolated cDNA.
2. To quantitated relative expression levels in tissue and tumours.
3. To determine of full coding sequences of the genes either by experimental or database searches.
4. To investigate the primary structure and homology relationships if unknown.
5. To asses the possible roles in tumour growth and immunogenicity in the cell lines under investigation.

This thesis is divided into two parts. The first part is to investigate and evaluate gene expression on clone 7-7-43, using both relative RT-PCR and Northern blotting. The second part of the thesis demonstrates a more intensive study on clone 7-7-76. Clone 7-7-76 was searched for its homology using GenBank database and gene expression was evaluated. RACE (Rapid Amplification of cDNA Ends) was completed in order

to achieve longer sequence of clone 7-7-76. The new sequence of 7-7-76 was again used to search for wider range of homology.

2.0 MATERIALS AND METHODS

2.1 Cell Lines

AC29 is a murine mesothelioma cell line developed from asbestos-induced malignant mesothelioma in CBA/CAH (H-2k) mice (Davis *et al,* 1992). The stable B7-1 transfectant clones AC29 B7-7 and AC29 B7-6 were previously generated after transfection of the expression vector, phbApr-neo-B7.1 (Leong *et al,* 1996). Both stably express the B7-1 molecule at similar levels but show different tumorigenicity in mice (Leong *et al,* 1996).

2.2 cDNA clones

The partial cDNA clones which were the focus of this project were originally from mRNA of cell lines AC29 B7-7 and AC29 B7-6. The two cDNAs designated as 7-7-76 and 7-7-43 were cloned in the pGEM-T vector and were previously isolated using SSH to identify differentially expressed genes.

2.3 Transformation

The E. coli JM109 High Efficiency competent cells (Promega, USA) were used for transformation according to the manufacturer's recommendations. The culture was then spread on LB agar plates containing 100 μg/ml ampicilin, 80 μg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) and 0.5mM isopropylthio-β-D-galactoside (IPTG), and

incubated at 37^0C overnight in an inverted position. Clones containing insert were identified using blue/white colour selection.

2.4 Amplification of Plasmid Inserts

Plasmid inserts were analysed by colony PCR amplification using universal M13 forward and reverse sequencing primers. Plasmid PCR was carried out in 10µl reactions containing 1µl of 10x reaction buffer (Biotech), 0.4µl of 5mM dNTP, 0.8µl of 25mM $MgCl_2$, 0.075µl of 5.5U/µl Taq polymerase (Fisher-Biotech) and 0.8µl of M13/pUC primer mix in a 0.5ml tube. The plasmid DNA was added by lightly touching the colony of interest with a 10µl pipette tip which was then ejected into the reaction mix. Once all the samples had been added in this way the tips were removed and amplification was performed using an PTC-100 (MJ Research) according to the following protocol: 94^0C for 5min, 25 cycles of 94^0C for 30sec, 60^0C for 30sec, 72^0C for 2min, then 3min at 72^0C and hold at 4^0C.

2.5 Plasmid Purification

Colonies of interest were isolated and were grown overnight at 37^0C in LB broth containing 100µg/ml ampicillin, in an incubator shaker rotating at 180 rpm. The overnight culture was subjected to plasmid purification. Plasmid purification was performed using the QIAprep^{TM} Spin Plasmid Kit (Qiagen). All buffers were supplied by the manufacturer and methods were done according to the manufacturer's

instructions. DNA concentrations were determined by spectrophotometry (Sambrook *et al,* 1989).

2.6 Sequencing

Cycle sequencing was performed in 10µl reactions containing: 400-800ng of template (depending on size of sequence), 1µl of 1.6pM of primer (generally the forward or reverse PCR primer for PCR products or relevant universal primer for plasmid) and 4µl of BigDye terminator mix (ABI). Reactions were carried out in PTC100 hot bonnet cyclers (MJ Research) under the following conditions: 94^0C for 3 min, followed by 25 cycles of amplification at 94^0C for 10 sec, 50^0C for 10 sec and 60^0C for 4 min. The sequencing products were precipitated with absolute ethanol and 3M sodium acetate, washed with 70% ethanol and dried. Gel electrophoresis was performed with the 373A DNA sequencer fitted with BigDye^{TM} filter wheel upgrade (PE Applied Biosystems) at the Australian Neuromuscular Research Institute. Table 2.1 lists all the sequencing primers used in the study. Sequences were then analysed using the programs Chromas V1.62 and DNA for Win V2.2.

2.7 Computer Analyses

The nucleotide sequences were used to search for homologous sequences in the non-redundant (NR) nucleic acid and protein databases using BLASTN and BLASTX which are maintained by the Australian National Genome Information Service (ANGIS) at the

website (http://www.angis.org.au). Expressed Sequence Tags (EST) were searched using BLASTX and EST databases maintained by ANGIS. The Mouse Gene Index Database (http://www.tigr.org/tab/tgi) of consensus EST sequences was also searched for sequence similarities. Primers were designed using website (http://www-genome.wi.mit.edu) to obtain new primers which were specific for the target sequences. The length of the primer designed was normally between 18-22 nucleotides and forward and reverse primers were indicated as "F" or "R" respectively. Primers were supplied by GeneWorks Pty. Ltd. and each primer was diluted into 2.5pmol using distilled water.

Primer	Sequence
M13/pUC-F	5’ CGC CAG GGT TTT CCC AGT CAC GAC 3'
M13/pUC-R	5' TTC ACA CAG GAA ACA GCT ATG ACC 3'
T7-F	5' TAG CGT GGT CGC GGC CGA GGT 3'
SP6-R	5' ACC TGC CCG GGC GGC CGC TCG A 3'
7-7-43F	5' CCG AAA ACC CAT CTG ATG TC 3'
7-7-43R	5' GAA AGA TGG CAG GGA GTG AG 3'
7-7-76 (207)F	5' TCA GGC AGC CAC TCC TAC TT 3'
7-7-76 (404)R	5' CCT GGC TAC CAA GCA CAA CT 3'
RACE-RT (274)R	5' CTC TAC TGT AGG TGT GCT 3'
RACE-1 (191)R	5' TGC CAT TCT GAG GAA AAC CT 3'
RACE-2 (140)R	5' AGC GTG ATG AGC AGA CAC AG 3'
RACE-A (44)R	5' TGC TCT CTG CTA AAT GTC TCT CA 3'
RACE-B (55)R	5' CTG TCC CAG CAA AAA GGG TA 3'
RACE-C (31)R	5' CTG AGG GTG GAG GCA GGA 3'
RACE-D (49)R	5' GCA AGA AGG GAG GCA AGT C 3'
Mfn-2NC-F	5' TCA CCC GGT TGA AAG TCA C 3'
Mfn-2NC-R	5' ACA AGA CAT GGG CAG GAG AG 3'
Mfn2B-R	5' AAC CCC TCA GTG CTA GGT CA 3'
Mfn-2R(rc)	5' CTC TCC TGC CCA TGT CTT GT 3'
Mfn2-splice F	5' CAC CAG GGA GAG CCT GTA TC 3'
Mfn2-splice R	5' TGC CAT TCT GAG GAA AAC CT 3'

Table 2.1 Primers used in this project.

2.8 RNA Extraction

Murine tissues (muscle, spleen, brain, intestine, heart, kidney, lung and liver) were available in our laboratory and were kept in the -80^0C freezer. RNA extractions were done using the Ultraspec^{TM} RNA Isolation System kit (Biotecx) according to the manufacturer's protocol. Murine tissues were homogenized with UltraspecTM RNA using a polytron homogenizer. The homogenate separates into two phases after extraction with chloroform. The total RNA which remained in the aqueous phase was precipitated by addition of isopropanol. Then the samples were washed with ethanol and solubilized in DEPC treated water and stored at -80^0C.

2.9 Reverse Transcription PCR

RT-PCR was performed using RNA from a range of mouse tissues (heart, brain, lung, intestine, liver, muscle, kidney) and cell lines (AC29, AB1, AC29, B7.1 2.6 (abbreviated as AC29 B7-6) and AC29 B7.1 2.7 (abbreviated as AC29 B7-7)).

2.9.1 DNAse Treatment of RNA

Prior to reverse transcription, contaminating DNA was removed by digestion in a 20µl reaction containing 10 µg RNA, 5µl of RQ1 RNase-free DNAse (Promega) and 2µl of 10x reaction buffer in an RNAse-free environment. Following incubation at 37^0C for 30 minutes, 2µl of RQ1 DNAse Stop Solution (Promega) was added and the mixture was

incubated at 65^0C for 10 minutes to inactivate the DNAse and placed on ice until required, or stored at -80^0C.

2.9.2 Reverse Transcription

Total RNA (1 μg) was added to a reverse transcription mix that contained 4μl of 25mM $MgCl_2$, 2μl of Reverse Transcription 10x buffer, 4μl of 5mM deoxyribonucleotide triphosphates (dNTPs), 0.5μl of Recombinant Rnasin® Ribonuclease Inhibitor, 1μl of avian myeloblastosis virus reverse transcriptase (AMVRT), 1μl of random primers and diethyl dicarbonate (DEDC; Sigma) treated water (all supplied by Promega) to a total of 20 μl. The mixture was incubated at room temperature for 10 minutes, followed by 42^0C for 30 minutes, 99^0C for 5 minutes and 4^0C for 5 minutes. The cDNA sample was stored at -80^0C until required.

2.10 PCR Amplification

The polymerase chain reaction (PCR) was performed for cDNA amplification for use in the detection of expression of the genes in tumours and cell lines. For each cDNA sample, 5μl was used for PCR amplification using primers (forward and reversed primers) designed based on sequencing results. PCR amplifications were carried out in 25μl reactions containing 2.5μl of 10x reaction buffer (Fisher Biotec), 1μl of 5mM dNTP, 2μl of 25mM $MgCl_2$, 1U Taq polymerase (Fisher Biotec) and 2.5pmol of each primer: forward and reverse. The samples were amplified using a PTC-100™

programmable Thermal Controller (MJ Research) according to the following protocol: 94^0C for 4min, 20-40 cycles of 94^0C for 30s, 55^0C for 30s, 72^0C for 1min then 5min at 72^0C and hold at 10^0C. Annealing temperature (55^0C – 64^0C) was optimized for each set of primers. Table 2.1 lists all the PCR primers used in this study.

2.11 Agarose Gel Electrophoresis

Agarose (Promega) gel electrophoresis was performed to analyse plasmid DNA and products of PCR amplification essentially as described by Sambrook *et al* (1989). A range of gel percentage (1% - 2%) was obtained by dissolving various amounts of agarose in Tris-acetate (TAE) buffer (Sambrook *et al,* 1989). DNA samples were diluted in Type II (Sambrook *et al,* 1989) loading buffer prior to loading. The electrophoresis process was carried out at 6-8 V/cm. After that, the gel was stained in Ethidium Bromide for 10 minutes. The bands were then visualised with the help of a UV transilluminator and photographed using a Kodak EDAS 120 system. Alternatively, gels were stained with 1/10000 dilution of SYBR gold (Molecular probes) in TAE buffer for 30 minutes.

2.12 Restriction Enzyme (RE) Digest

To prepare templates for RNA probe synthesis, a RE digest method was performed by adding 2μg of plasmid into the RE digest mixture which contained 5μl of 10x buffer (depending on the enzyme that was used), 5μl of 10x BSA (1mg/ml) and H_2O to 50μl.

10U of enzyme, SalI or NcoI were added depending on the sequence directions. The mixtures were left overnight and were purified by extraction with buffered phenol: chloroform, precipitated with isopropanol and resuspended in RNAse free water before running electrophoresis in 1% agarose gel.

2.13 Preparation of probes

Probes were made from the previously isolated SSH clones, 7-7-76 and 7-7-43.

2.13.1 RNA Probes

RNA probe labelling was done according to Strip-EZ protocol (Ambion). It was carried out in a 20µl reaction containing 2µl of 10x Transcription Buffer, 1µl of 10mM ATP, 1µl of 2mM modified CTP, 1µl of 10mM GTP, 0.67µl of 10mM UTP, 0.33µl of 25nmol DIG-11-UTP, nuclease-free water and RNA polymerase mix (either T7 or SP6). Following incubation at 37^0C for one hour, 1µl of DNase I was added and samples were left for incubation at 37^0C for 15 min. Samples were stored at -20^0C after the addition of 0.5M EDTA. Spot tests were done to estimate the yield of DIG labelled RNA probes (Roche Biochemicals). Serial probe dilutions were made and 1µl of each dilution was spotted onto a strip of nylon membrane. Methods of washing and incubating were done according to manufacturer's instructions. Spot intensities of the known control values were compared to the unknown probe. The control was a previously purchased DIG labelled RNA probe for β-actin supplied by the manufacturer (Roche Biochemicals).

2.13.2 DNA Probes

DIG-labelled DNA probes were produced by PCR according to our own protocol. It was carried out by preparing a 12µl reaction mix contained 1.2µl of 10x PCR buffer, 1µl of 25mM $MgCl_2$, 1.2µl of 10x PCR dNTP mix, primer mix, 0.7µl of DIG dUTP, 0.5U Taq DNA polymerase, plasmid template and sterile water. Mixtures were put into PCR amplification with PTC-100 (MJ Research) and a small aliquot was analysed on a 2% agarose gel. Then, samples were precipitated by adding 2µl of 4M LiCl and absolute ethanol. After that, it was incubated for -70^0C for an hour before centrifugation at 13000g for 15 min. The sample was later washed with 70% ethanol twice before being resuspended in 10µl of water. Spot tests were performed as for the RNA probes spot test except that the control was DIG labelled DNA supplied by Roche Biochemicals.

2.14 Northern Blotting

The Northern Max reagents and kit from Ambion were used for Northern blotting. This technique was performed using 7-7-76 and 7-7-43 clones and is briefly described below.

2.14.1 Electrophoresis

The total amount of RNA (5µg) was mixed with formaldehyde loading dye before incubating the mixture at 65^0C for 15 minutes. After that, the RNA samples were loaded into the wells of a 1% formaldehyde denaturing agarose gel and run at 5V/cm for 1 hour.

Then, the gel was stained in Ethidium Bromide before viewed on a UV transilluminator and photographed using a Kodak EDAS 120 system.

2.14.2 Northern Transfer

Positively charged nylon membrane (Roche Biochemicals) was placed on top of the blotting stack which contains paper towels and filter papers. Then, the gel was placed on top of the membrane followed by filter paper bridges. The RNA fragments were transferred to the membrane for about 3 hours by filter paper bridges in Northern Max transfer buffer (Ambion). The membrane was then rinsed in DEPC water before crosslinking the RNA using UV transilluminator.

2.14.3 Probes for Hybridization

The transcription reactions from Strip-EZ RNA Probe or PCR DIG-labelled DNA Probe (prepared as described in sections 2.13.1 and 2.13.2) were added directly to the hybridization mix. The quantity of probe to be added was determined according to the spot test results as described previously (Section 2.13.1).

2.15 Hybridization

Hybridization was performed using both RNA and DNA probes respectively at different hybridization temperatures before exposing to film for detection.

2.15.1 Hybridization Using RNA Probe

The membrane was pre-hybridized in ULTRAhyb hybridization solution (Ambion) in a rotating hybridization oven at 68^0C for 30 minutes. Different hybridization temperatures (64^0C, 60^0C) were performed in order to achieve the maximum sensitivity. For RNA probes, the probe was added directly without denaturing and hybridization was carried out at the respective temperatures overnight. The membrane was then washed with low stringency wash solution (2x SSC) twice for 5 minutes each wash. Then, two 15 minute washes at 68^0C were performed using high stringency wash solution (0.1x SSC). Detection steps were performed according to the DIG Luminescent Detection Kit (Roche Biochemicals). CSPD-Star was used as substrate solution (according to the manufacturer's instructions) before exposure to a Kodak BIOMAX^{TM} MS imaging film for 10 minutes. The probe was stripped according to the Strip-EZ kit protocol (Ambion). Briefly, the membrane was rinsed in DEPC water before incubating in stripping buffer for one hour at 68^0C. Stripping buffer was composed of 50% deionized formamide, 5% SDS and 50Mm Tris-Cl with pH 7.5 (Ambion). The membrane stripping step was repeated twice and the membrane was rinsed in 2x SSC before being stored at -20^0C for future use.

2.15.2 Hybridization Using DNA Probe

The membrane was pre-hybridized in ULTRAhyb hybridization solution (Ambion) in a rotating hybridization oven at 42^0C for 30 minutes. Different hybridization temperatures (45^0C, 50^0C) were performed in order to achieve the maximum sensitivity. For DNA probes, the probe was boiled for 5 minutes and chilled on ice for 10 minutes before

adding to the solution and hybridization was carried out at the respective temperatures overnight. The membrane was washed twice with low stringency wash solution (2x SSC) for 5 minutes each at room temperature. Then, two 15 minute washes at 42^0C were performed using high stringency wash solution (0.1x SSC). Detection steps were performed according to the DIG Luminescent Detection Kit (Roche Biochemicals). CSPD-Star was used as substrate solution (according to the manufacturer's instructions) before exposure to a Kodak BIOMAX^{TM} MS imaging film for 10 minutes. For stripping of the probe, the membrane was rinsed in DEPC water before incubating in 0.2M NaOH, 0.1% SDS for 20 minutes at 37^0C. The membrane stripping step was repeated twice and the membrane was rinsed in 2x SSC before being stored at -20^0C for future use.

2.16 Relative RT-PCR

Relative RT-PCR is a method for quantitative analysis of gene expression. It compares transcript abundance across multiple samples, using a co-amplified internal control for sample normalization. QuantumRNA^{TM} 18S Internal Standards were used in the following procedure and standards were composed of 18S primers and Competimers which were supplied separately. The 18S Competimers were modified to block DNA polymerase extension and mixing with 18S primers would maintain the relative quantitation. All buffer solutions and protocols were provided by the manufacturer (Ambion). The procedure followed is as below.

2.16.1 Linear Range Determination

The linear range of the reaction is defined as the period of the PCR in which the amplification efficiency is at its maximum and remains constant over a number of cycles. In other words, the amplification efficiency would fall and the rate of product accumulation would slow at the peak point. Firstly, the RNA sample which gave a median signal amongst the different tissues and cell lines in a initial screening reaction, was chosen and assembled into 10 identical PCR aliquots. The aliquots were subjected to PCR and were removed every two cycles between cycle 25 to cycle 35. The results were analyzed by densitometry.

2.16.2 Optimal Ratio Determination

The experiment was done to determine what ratio of Competimers to primers is needed to have both the target-of-interest, and the 18S control target amplify to give similar yields of product. Mixtures of primer : Competimer were prepared at ratios of 1:9, 2:8 and 3:7. After that, PCR was set up according to our own PCR protocol using total of 25µl instead of 50µl that was suggested in the manufacturer's instructions. Results were then assessed by gel electrophoresis.

2.16.3 Application to Cell Lines and Tissues

7-7-43

With 62^0C as the optimum annealing temperature, cycle 33 as the optimum cycle and 2:8 as the optimal 18S primer:Competimer ratio, relative, quantitative RT-PCR experiments were proceeded for all the cell lines and tissues.

7-7-76

With 62^0C as the optimum annealing temperature, cycle 31 as the optimum cycle and 3:7 as the optimal 18S primer:Competimer ratio, relative, quantitative RT-PCR experiments were proceeded for all the cell lines and tissues.

2.17 5' RACE

5'RACE technique was used to obtain flanking sequences of the cDNA clones. The following procedure was based on 5'RACE system (Life Technologies) and according to the manufacturer's instructions. Table 2.1 lists the primers used in this study. To start, first strand of cDNA synthesis was primed using a 5' RACE primer designed (based on sequencing results) to be highly specific for the target sequences. These formed stable duplexes with the target sequences free of secondary structure. After that, the first strand product was purified with GlassMax Spin Cartridge (Life Technologies) and followed by TdT (Terminal deoxynucleotidyl transferase) tailing of cDNA to add homopolymeric tails (poly C) to the 3' ends. Then, the tailed cDNA was amplified by PCR using Abridged Anchor Primer (AAP) (supplied by the manufacturer) and primer RACE-1 (191)R which was designed based on sequencing results. Lastly, the primary

PCR product was re-amplified using Abridged Universal Amplification Primer (AUAP) (supplied by the manufacturer) and primer RACE-2 (140)R (designed based on sequencing results). RACE product was subjected to purification using BRESAspin PCR Purification Kit (MO BIO) before ligation using pGEM-T Vector (Promega). Final RACE product sequences were aligned by a computer program known as Sequencher (Genecodes 3.0).

3.0 CHARACTERISATION OF CLONE AC29 B7-7-43

3.1 Introduction

The SSH technique has been used to investigate the differences between the aggressive and the less aggressive B7-1 transfectant AC29 clones. Several differentially expressed cDNAs have been isolated. Some encode known genes while others are yet to be identified. One cDNA clone designated as AC29 B7-7-43 was previously identified by partial sequencing as homologous to Human Cyclin-Dependent Kinase Regulatory Subunit 1 (Cks 1), which is involved in the cell cycle.

In the work described in this chapter, the AC29 B7-7-43 clone was subjected to a series of characterisations including relative RT-PCR, Northern blotting and computer analysis of its DNA sequence. Computer analysis was done by searching the GenBank databases for further homologous sequences. Relative RT-PCR and Northern blotting were conducted in order to investigate the level of expression and the tissue distribution of expression of the AC29 B7-7-43 transcripts.

3.2 Amplification of Existing Clone Sequence

Clone 7-7-43 was available as a cDNA insert in plasmid pGEM-T and was assessed by amplification and sequencing. Universal primers for pUC based vectors, M13/pUC (Table 2.1) were used as primers to amplify the region to be sequenced and analysed. The result of this amplification is shown in Figure 3.1. Lanes 1-4 demonstrated an expected fragment of approximately 700bp. The approximate size of the insert was 420bp as 265bp of the product were derived from the plasmid.

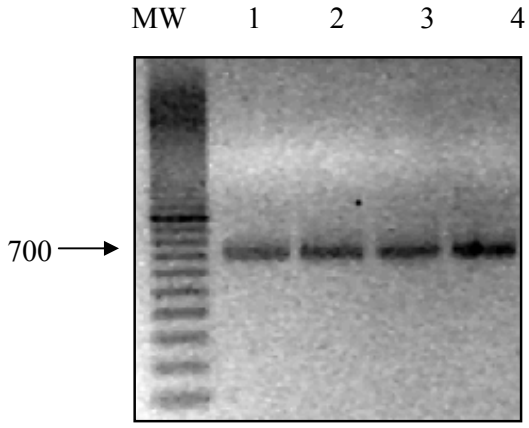

Figure 3.1 Amplification of clone 7-7-43. Primer M13/pUC was used to amplify plasmid clone 7-7-43. Lanes 1-4 - identical reactions. MW - 100bp molecular weight ladder.

3.3 Computer Analysis of Sequences

The 7-7-43 clone insert was sequenced in both directions using T7 and SP6 primers and contained 416bp insert. The complete sequence of clone 7-7-43 and its amino acids translation are shown in Figure 3.2. The nucleotide sequence of the clone was used to design 7-7-43F and 7-7-43R primers (Table 2.1) for further investigation of 7-7-43 expression.

```
gcctgcggcgcggggctgagcgccctcggacagagcaatcATGTCCCACAAACAAATCTA 60
                                        MetSerHisLysGlnIleTyr

CTATTCGGACAAATACGACGACGAGGAGTTCGAATACCGGCATGTCATGTTGCCCAAGGA 120
 TyrSerAspLysTyrAspAspGluGluPheGluTyrArgHisValMetLeuProLysAsp

CATAGCCAAGCTGGTCCCGAAAACCCATCTGATGTCTGAATCTGAATGGAGGAACCTCGG 180
 IleAlaLysLeuValProLysThrHisLeuMetSerGluSerGluTrpArgAsnLeuGly

CGTTCAGCAGAGTCAGGGATGGGTCCACTATATGATCCATGAACCAGAACCTCACATCTT 240
 ValGlnGlnSerGlnGlyTrpValHisTyrMetIleHisGluProGluProHisIleLeu

ACTGTTCCGACGGCCACTGCCCAAGAAGCCAAAGAAAtgaagccggcgaaccgccttccg 300
 LeuPheArgArgProLeuProLysLysProLysLys

gcctcgagcggtccacagctgtcctcactccctgccatctttctgatcgcgtgaccatgt 360
tgctttcttatttctcactttgatatgtaaaggatacactgtttgaatgtgctggt
```

Figure 3.2 The complete nucleotide sequence and amino acid translation (in bold) of the 416bp clone 7-7-43.

The full length nucleotide sequence of clone 7-7-43 was used to search for homologous sequences in the non-redundant (NR) nucleic acid and protein databases using BLASTN and BLASTX which are maintained by the Australian National Genome Information Service (ANGIS). Sequence analysis revealed that clone fragment 7-7-43 is homologous to the regulatory subunit 1 (Cks1) of human cyclin dependent kinase, accession number X54941 GenBank entry by Richardson. The human Cks1 was published by the same author, Richardson *et al*, 1990. Recently, a new mouse EST clone of Cks1 (accession number NM_016904) has been established and 7-7-43 is 100% homologous to the coding region of this clone. The mouse Cks1 is unpublished and only database was available in GenBank. Figure 3.3 shows alignments of sequences between clone 7-7-43, mouse Cks1 and human Cks1. Additional 303bp at the 3' end of mouse Cks1 is not shown and analogous nucleotides are indicated by dots.

Literature review (Section 1.5) was done on this kinase-like gene and suggested that this gene plays a key role in regulation of the eukaryotic cell cycle (Ninomiya-Tsuji *et al,* 1991). Therefore, further studies on clone 7-7-43 were undertaken to investigate its differential expression in tissues and MM cell lines.

```
                *        20         *        40         *        60
7-7-43 : ----------------------------------------GCCTGCGGCGCGGGGCTGAGCG
mCKS1  : AGAGGGTGGGAGCGCGTGCTGCTGGGAGTTGTCTGGAG......................
hCKS1  : ------------------------------------------------------------

                *        80         *       100         *       120
7-7-43 : CCCTCGGACAGAGCAATCATGTCCCACAAACAAATCTACTATTCGGACAAATACGACGAC
mCKS1  : ............................................................
hCKS1  : ---------.....G........G...........T.........................

                *       140         *       160         *       180
7-7-43 : GAGGAGTTCGAATACCGGCATGTCATGTTGCCCAAGGACATAGCCAAGCTGGTCCCGAAA
mCKS1  : ............................................................
hCKS1  : ........T..G..T..A.........C.............................T...

                *       200         *       220         *       240
7-7-43 : ACCCATCTGATGTCTGAATCTGAATGGAGGAACCTCGGCGTTCAGCAGAGTCAGGGATGG
mCKS1  : ............................................................
hCKS1  : ..................................T..T.......................

                *       260         *       280         *       300
7-7-43 : GTCCACTATATGATCCATGAACCAGAACCTCACATCTTACTGTTCCGACGGCCACTGCCC
mCKS1  : ............................................................
hCKS1  : .....T................................G........G..C.....A...

                *       320         *       340         *       360
7-7-43 : AAGAAGCCAAAGAAATGAAGCCGGCGAACCGCCTTCCGGCCTCGAGCGGTCCACAGCTGT
mCKS1  : ............................................................
hCKS1  : .....A..............T...A.G.TA.T..T.A.....A...TT.A.........

                *       380         *       400         *       420
7-7-43 : CCTCACTCCCTGCCATCTTTCTGATCGCGTGACCATGTTGCTTTCTTATTTCTCACTTTG
mCKS1  : ............................................................
hCKS1  : ...T...T...AA............AA.A.T.TT.......C.....G............

                *       440         *       460
7-7-43 : ATATGTAAAGG-------ATACACTGTTTGAATGTGCTGGT
mCKS1  : ...........-------.......................
hCKS1  : ....T....A.ATGTTCA.......................
```

Figure 3.3 The complete sequence of clone 7-7-43 aligned with mouse Cks1 (accession number NM_016904) and human Cks 1 (accession number X54941). mCKS1 – mouse Cks1, hCKS1 – human Cks1.

3.4 Northern Blotting

A DIG-labelled RNA probe was initially used in experiments aimed at detecting 7-7-43 mRNA in various tissues and cell lines. Those investigated initially were AB1, AC29, brain, kidney and liver. Both orientations of RNA probe were used. However, the 7-7-43 expression was undetectable in these tissues and cell lines although a range of hybridization temperatures and different exposure times were investigated (as described in Section 2.15.1). The labelling efficiency was at the expected level so that the sensitivity of detection was likely to be adequate. Additionally, a DIG labelled β-actin probe was obtained (Roche Biochemicals) and performed as expected (Figure 3.4).

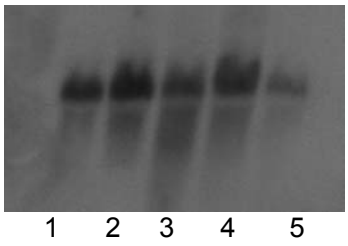

Figure 3.4 Northern blotting using β-actin probe. Lane: 1 - AB1; 2 - AC29; 3 - Brain; 4 - Kidney; 5 - Liver.

Subsequently, a DIG-labelled DNA probe was prepared and used for detection. A subsequent membrane with RNA from the cell lines, AB12, AC29, AB1, AC29 B7-7 and AC29 B7-6 was tested with a DNA probe. The 7-7-43 RNA was detected in most cell lines with faint band detected in AC29 B7-6 after exposing to film for 15 mins (Figure 3.5). High expression was shown in the AB12 and AC29 cell lines and lower levels of expression in AB1, AC29 B7-7 followed by AC29 B7-6. Size of

transcripts were consistent with Northern analysis described by Urbanowicz-Kachnowicz (1999), in their experiment on expression of Cks1 in normal and malignant cells.

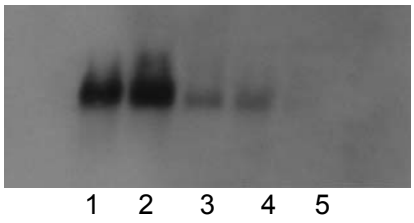

Figure 3.5 Northern blotting for 7-7-43 using DIG-labelled DNA probe. Lane: 1 - AB12; 2 - AC29; 3 - AB1; 4 - AC29 B7-7; 5 - AC29 B7-6.

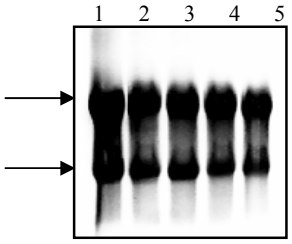

Figure 3.6 Ethidium bromide stained gel used for the Northern blot shown in Figure 3.5. Lane: 1 – AB12; 2 – AC29; 3 – AB1; 4 – AC29 B7-7; 5 – AC29 B7-6. The 28S RNA (4.7Kb) and 18S RNA (1.9Kb) are indicated by arrows.

An RNA probe was first used in hybridization experiments due to the fact that RNA probes are believed to be more sensitive than cDNA probes. However, the expression of 7-7-43 was undetectable using the RNA probe although the apparent labelling efficiency was at the expected level. The problem may have been due to the RNA probe degrading over the process of hybridization. Therefore, a DNA

probe which was easier to handle and more stable, was introduced and expression was detected.

Although Northern blotting was successfully conducted to study the differential expression of 7-7-43 in cell lines and tissue, it was a highly time consuming procedure which was not recommended for further investigation. An alternative technique known as relative RT-PCR was introduced and was successfully used to analyse the differential expression of the gene encoding 7-7-43 (Section 3.5).

3.5 Optimisation of PCR Assay

As part of the process to optimise amplification conditions, differing annealing temperatures were used to determine the optimum temperature for efficient amplification of the 7-7-43 product. Initial experiments were conducted using cDNA derived from mouse liver by reverse transcription. cDNA equivalent to 250ng of starting total RNA was used in each reaction.

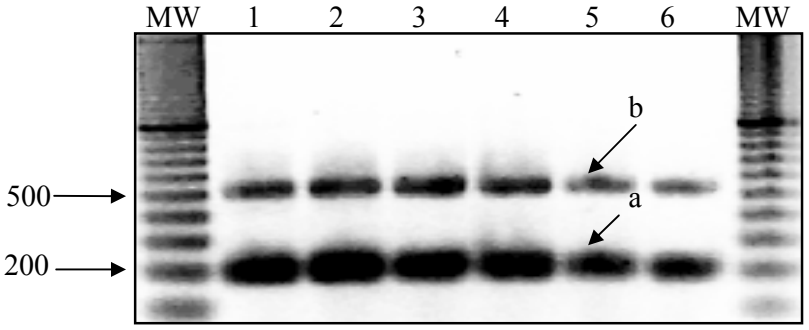

Figure 3.7 Effect of annealing temperature on 7-7-43 amplification. Liver cDNA was amplified using primer pairs 7-7-43F and 7-7-43R. Lanes 1-2 - 60^0C, lanes 3-4 - 62^0C, lanes 5-6 - 64^0C. MW - 100bp molecular weight ladder. (a) -expected 7-7-43 fragment size (200bp) (b) - additional band.

From the result in Figure 3.7, an annealing temperature of 62^0C allows efficient amplification of the expected fragment and was used for subsequent relative RT-PCR reactions.

From the same Figure 3.7, two bands appeared on the gel. The presence of the additional band that equates to approximately 500 bp was suspected to correspond to residual genomic DNA after RNA extraction. This was confirmed by the following relative RT-PCR experiments with no visible additional bands in figures (eg. Figure 3.8). All RNA was treated by DNAse prior to RT-PCR for all following PCR reactions.

3.6 Relative RT-PCR

3.6.1 Linear range determination

Aliquots of PCR samples using heart RNA were prepared before PCR to discover the linear range of the 7-7-43 amplification. The aliquots were removed from the thermal cycler starting from cycle 25 and ending at cycle 35. Figure 3.8 shows the effect of the different numbers of cycles on the PCR product in experiments conducted to determine the linear range of amplification. Signal strength detected from cycle 31 to 35 was determined by densitometry and cycle 33 was selected as the most suitable cycle in the linear range and was used in the following relative RT-PCR reactions.

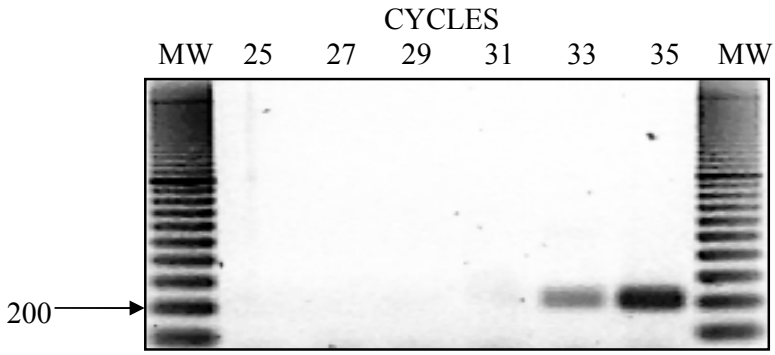

Figure 3.8 Relative PCR: Linear range determination. Reactions were removed at 2 cycle intervals. MW-100bp molecular weight ladder.

3.6.2 Optimal ratio for 18S Primers:Competimers

To determine what ratio of Competimers to primers was needed to have both the target-of-interest and the 18S control target amplify to give similar signal intensity, PCR reactions with different ratios of primer:Competimer were prepared. As the EtBr staining is a poor method to quantify, SYBR gold was used to visualise the DNA on agarose gels. From Figure 3.9 it can be seen that the optimal ratio of 18S Primers:Competimers is 2:8, which is the ratio recommended for rare transcripts. Therefore, a ratio 2:8 of 18S Primers:Competimers was used in the following relative RT-PCR for 7-7-43 expression.

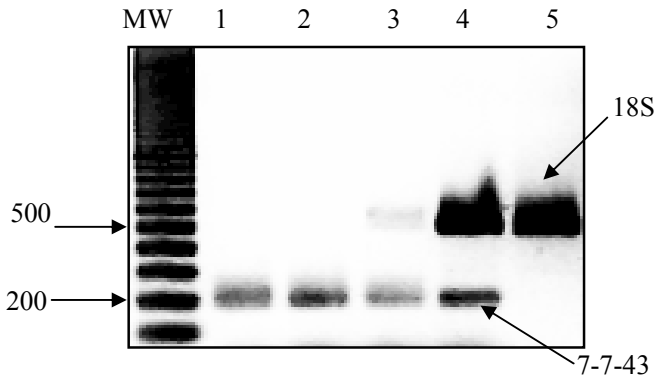

Figure 3.9 Relative PCR: Optimal ratio of 18S Primers:Competimers determination. In this experiment, different ratios of 18S Primers:Competimers were used with all the other conditions remaining identical to those used in Section 3.5 and Section 3.6.1. Each of the lanes contained 18S Primers:Competimers except lane 1. Lane : 1 – 7-7-43 primers; 2 – ratio 1:9; 3 – ratio 2:8; 4 – ratio 3:7; 5 – ratio 3:7 without 7-7-43 primers. MW -100bp molecular weight ladder.

3.6.3 Application to Cell Lines and Tissues

The method enabled investigation of potential differential expression of 7-7-43 in RNA from cell lines and mouse tissues. The 7-7-43 signal for each tissue and cell line was normalized against the 18S signal and the mean of two experimental results was determined. The results were expressed as a ratio relative to the signal for kidney, which was arbitrarily chosen as the calibrator because it had the lowest signal (Table 3.1).

Tissue	Ratio vs. kidney
spleen	1.91 ± 0.03
heart	1.35 ± 0.17
intestine	1.19 ± 0.00
brain	1.26 ± 0.21
muscle	1.30 ± 0.05
liver	1.47± 0.08
lung	1.07 ± 0.16
kidney	1.00 ± 0.09
AC29 B7-6	1.44 ± 0.37
AC29 B7-7	1.67 ± 0.07
AB1	1.84 ± 0.22
AC29	2.33 ± 0.01

Table 3.1 Ratio of net intensity in each sample versus kidney

From the results on Table 3.1, generally there was not a lot of variation between the normal mouse tissues. The greatest variation was observed between spleen and kidney, which was approximately 2-fold. Overall, there are no large differences between normal mouse tissues and the tumour cell lines. Among the tumour cell lines, AC29 was clearly revealed as having the highest expression followed by AB1, while AC29 B7-7 had slightly higher expression then AC29 B7-6. Variation was detected among tumour cell lines. AC29 and its B7-1 expressing clones (B7-6 and B7-7) showed approximately a 1.6 fold difference.

The results obtained from Northern blotting (Section 3.4) were consistent with the results achieved from relative RT-PCR (Section 3.6.3) in that AC29 was revealed as having the highest expression followed by AB1. Both relative RT-PCR and Northern blotting detected that AC29 B7-7 had a slightly higher expression then AC29 B7-6 but was not shown to be significant (further discussion in Chapter 5).

4.0 CHARACTERISATION OF CLONE AC29 B7-7-76

4.1 Introduction

As mentioned in Chapter 3 (Section 3.1), the SSH technique has been used to investigate B7-1 transfectant AC29 clones and several differentially expressed cDNAs have been isolated. One such unidentified cDNA clone was designated as 7-7-76. At the commencement of this project, the clone had been partially sequenced and some preliminary database searching had been performed.

In the work described in this chapter, the 7-7-76 clone was subjected to a series of characterisation and gene expression investigations including relative RT-PCR, Northern blotting, computer analysis of its DNA sequence and 5' RACE. Computer analysis was done by searching the GenBank database for homologous sequence. Relative RT-PCR and Northern Blotting were conducted in order to investigate 7-7-76 level of expression. 5' RACE was performed to elongate the 5' cDNA ends in order to obtain additional sequence to aid identification.

4.2 Amplification of Existing Clone Sequence

Clone 7-7-76 was available as a cDNA insert in plasmid pGEM-T and was assessed by amplification and sequencing. M13/pUC vector primers were used to amplify the region to be sequenced and analysed. The result of this amplification is shown in Figure 4.1 where product is around 900bp to 1Kb. The estimated size of the insert was 700bp as 265bp of the product were derived from the plasmid.

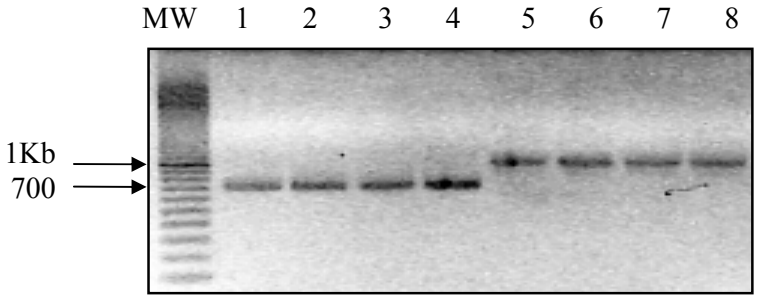

Figure 4.1 Amplification of clone 7-7-76. Primer M13/pUC was used to amplify plasmid clone 7-7-76 (lanes 5-8). Lanes 1-4 - clone 7-7-43 which was discussed earlier in Section 3.1. MW- 100bp molecular weight ladder.

4.3 Sequencing of Original 7-7-76 Clone

The PCR product was purified and sequenced using T7 and SP6 primers. Sequencing results revealed about 250bp forward sequence and 250bp reverse sequence. Therefore, another internal primer (7-7-76 (207)F) was designed in order to obtain the whole clone 7-7-76 sequence. The nucleotide sequence of clone 7-7-76 insert was assembled and comprised 701bp (Figure 4.2). The nucleotide sequence of the clone was used to design a reverse primer compatible with 7-7-76 (207)F for further investigation of 7-7-76 expression by PCR. This was 7-7-76 (404)R (Table 2.1).

```
                 *        20         *        40         *        60
7-7-76 : ACCAGTTGCTCCCTACCCCCTGCAGGAAGACCCCTGGCTCATACCCTAACGGAGACCAAC

                 *        80         *       100         *       120
7-7-76 : AAGGACTGGACAGCTCGGCTCCGAGGAGTTATGCATGTGTCTGTGTCTGCTCATCACGCT

                 *       140         *       160         *       180
7-7-76 : CACCCGGTTGAAAGTCACTGTGCATTTGATAAGGTTTTCCTCAGAATGGCACCCTGCAGA

                 *       200         *       220         *       240
7-7-76 : ATGACTGATGGGCGCCTTCACAGGCATCAGGCAGCCACTCCTACTTTCTCAGCCTGAGCA

                 *       260         *       280         *       300
7-7-76 : CACCTACAGAGAGACAGTGTTGGGGGTGACAGGATGCCCCAGAAAAATTCAGGATACTTC

                 *       320         *       340         *       360
7-7-76 : TGAAATTGCCATGTTTGTCTCAGAACTGGTATCTTGCGTTTGGCCCTCAGCTTTAGCACT

                 *       380         *       400         *       420
7-7-76 : TTTTCCCACTTGCAGAGTCTTTCAGTTGTGCTTGGTAGCCAGGCTCAACACACCGGGGCT

                 *       440         *       460         *       480
7-7-76 : TTAGACTCAAGCAGCTTATATGGGAAGCTGGAGACCCCTGCCAGCCAGAGCACAGTAAGG

                 *       500         *       520         *       540
7-7-76 : GCTCGGAGAAGGTATGTGAACTAGGGTATCTGATGTGGTAGTGCAGTTGGTGTCTGGCAT

                 *       560         *       580         *       600
7-7-76 : TGTGACGACAAGGGACTTGCCTCCCTTCTTGCCACTGAGGCCCTTGCTTTTCCTTCAGCT

                 *       620         *       640         *       660
7-7-76 : CTTGGCCTGTTGGTCCGGTTTATTAGTGAGAGCTAGTTTGCTCCAGGTGGTTAAAGGATG

                 *       680         *       700
7-7-76 : TAGCAGGAGGAATGGTGGAAGATGGGAGCTGCCCGGGTTAG
```

Figure 4.2 The entire nucleotide sequence of the 701bp insert of clone 7-7-76.

4.4 Computer Analysis of Sequences

The nucleotide sequence of clone 7-7-76 was used to search for homologous sequences in the nucleic acid and protein databases using BLASTN and BLASTX. Database searches identified that clone fragment 7-7-76 was homologous to a sequence in the 3' non-coding region of GenBank entry accession number U41803 submitted by Chen and Tang on the 20th of January 1996, a putative *rattus norvegicus* hypertension related protein (rat HRP). Figure 4.3 shows alignment between clone 7-7-76 and U41803. According to TIGR mouse gene index database searches, clone 7-7-76 has more than 90% homology to a Mus Musculus cDNA EST clone (TC119995). As clone 7-7-76 sequence was at the 3' non-coding region, 5' RACE technique was utilised to extend the sequence of this gene into the coding sequence (discussed later at Section 4.8).

```
                *        20         *        40         *        60
U41803 : ACCAGTTACTCCCTACCCCCTGCAGGAAGACCCCCAGCTCATACCCAGTGG-AGACCAAC
7-7-76 : ACCAGTTGCTCCCTACCCCCTGCAGGAAGACCCCTGGCTCATACCCTAACGGAGACCAAC

                *        80         *       100         *       120
U41803 : AAGGACTGGACAGCTCAGCCCCAAGGAGTCATGCATGTGTCTGTGTCTGCTCATCATGCT
7-7-76 : AAGGACTGGACAGCTCGGCTCCGAGGAGTTATGCATGTGTCTGTGTCTGCTCATCACGCT

                *       140         *       160         *       180
U41803 : CAGTCGGTTGGAAGTCACTGTGCATTTGATAAGGTCTTCCTCAGGATGCCATCCTGCAGA
7-7-76 : CACCCGGTTGAAAGTCACTGTGCATTTGATAAGGTTTTCCTCAGAATGGCACCCTGCAGA

                *       200         *       220         *       240
U41803 : ACGACTGATGGGCGCCTTCACAGGCATCAGGCAGGCACTCC-----TTTCAGCCCGAGTA
7-7-76 : ATGACTGATGGGCGCCTTCACAGGCATCAGGCAGCCACTCCTACTTTCTCAGCCTGAGCA

                *       260         *       280         *       300
U41803 : CACCTACAGAGAGAGAATGTTGGGGGTGACTGGACTCCCCAGAAAAGTTCAGGGTACTGA
7-7-76 : CACCTACAGAGAGACAGTGTTGGGGGTGACAGGATGCCCCAGAAAAATTCAGGATACTTC

                *       320         *       340         *       360
U41803 : ----------------------AGAACTTGTGTCTTGCATTTGGCCCTCAGCTTTAGCAC
7-7-76 : TGAAATTGCCATGTTTGTCTC-AGAACTGGTATCTTGCGTTTGGCCCTCAGCTTTAGCAC

                *       380         *       400         *       420
U41803 : TTTTTCCCACTTGCAGAGTCTTTCAGTTGTGCTTGGTGGCCAGGCTCACACACCCTGGGC
7-7-76 : TTTTTCCCACTTGCAGAGTCTTTCAGTTGTGCTTGGTAGCCAGGCTCAACACACCGGGGC

                *       440         *       460         *       480
U41803 : TTTAGACTCAACCAGCTTATAGGGAAAGCTGGAGACCCTTGCCAGCCAGAGCAAAGTAAG
7-7-76 : TTTAGACTCAAGCAGCTTATATGGGAAGCTGGAGACCCCTGCCAGCCAGAGCACAGTAAG

                *       500         *       520         *       540
U41803 : AGCTCAGGGGACGGTATGTGAACTAGGGTGTCCGATGTGGTAGTGAGGTTGGTGTCTGGC
7-7-76 : GGCTCGGAGAAGGT-ATGTGAACTAGGGTATCTGATGTGGTAGTGCAGTTGGTGTCTGGC

                *       560         *       580         *       600
U41803 : ATTGTGATGACAAGGGACTCGCCTCCCTTCTTGCA------------------------
7-7-76 : ATTGTGACGACAAGGGACTTGCCTCCCTTCTTGCCACTGAGGCCCTTGCTTTTCCTTCAG

                *       620         *       640         *       660
U41803 : ----------------------------------AGTTTGCTCCAGGTGGTTAAAGGA
7-7-76 : CTCTTGGCCTGTTGGTCCGGTTTATTAGTGAGAGCTAGTTTGCTCCAGGTGGTTAAAGGA

                *       680         *       700         *
U41803 : TGTTGCAGGAGGAATGGTGGAAGATGGGAGCTGCCCGGGTTAGTACAGAAGAGGT
7-7-76 : TGTAGCAGGAGGAATGGTGGAAGATGGGAGCTGCCCGGGTTAG------------
```

Figure 4.3 Sequence alignment between clone 7-7-76 and U41803. The entire sequence of U41803 (rat HRP) consists of 4160bp. Sequence number 1 of clone 7-7-76 is correspond to sequence number 2543 of rat HRP. Open reading frame or coding region of rat HRP is at sequence number 453 to 2438. Clone 7-7-76 clearly aligns in the 3' non coding region of rat HRP. Alignment generated using BLASTN.

4.5 Northern Blotting

A DIG-labelled RNA probe was initially used for detection of 7-7-76 RNA in extracts of various tissues and cell lines. Those investigated initially were AB1, AC29, brain, kidney and liver. Both orientations of RNA probe were used. However, the presence of 7-7-76 in these tissues and cell lines was not detected although different hybridisation temperatures and different exposure times were investigated. This was despite the labelling efficiency being within the expected level. As already described, a β-actin probe worked satisfactory (refer to Figure 3.4).

Following from that, a DIG-labelled DNA probe was prepared and was used for detection. A subsequent membrane with RNA from the cell lines, AB12, AC29, AB1, AC29 B7-7 and AC29 B7-6 was tested with a DNA probe. No signal was obtained although differing hybridisation temperatures and exposure times were exhaustively investigated. Therefore, it was concluded that the 7-7-76 transcript was probably below the detection sensitivity of the present Northern assay. This result contrasted with successful detection of 7-7-43 using a similar assay as described in Chapter 3. As differential 7-7-76 expression was being successfully determined by relative RT-PCR at the time (Section 4.6), no further development of the Northern assay was undertaken.

4.6 Optimisation of PCR Assay

As part of the process to optimise amplification conditions, differing annealing temperatures were used to determine the optimum temperature for efficient

amplification of the 7-7-76 product. Using the same RNA sample and cell lines, the annealing temperature was increased from 55^0C to 58^0C, 60^0C, 62^0C and 64^0C in separate reactions. From the above observations, an annealing temperature of 62^0C allows efficient amplification of the expected fragments and was used for the subsequent 7-7-76 RT-PCR reactions (Figure 4.4).

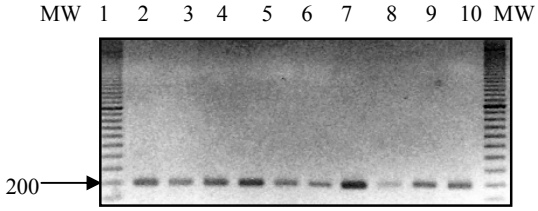

Figure 4.4 Detection of 7-7-76 by RT-PCR. 62^0C was used as the optimal annealing temperature for RT-PCR on various tissue and cell line with primers 7-7-76(207)F and 7-7-76(404)R. Lane: 1 – kidney; 2 – brain; 3 – heart; 4 – lung; 5 – liver; 6 – muscle; 7 – AB1; 8 – AC29; 9 – AC29 B7-6; 10 – AC29 B7-7. MW - 100bp molecular ladder.

4.7 Relative RT-PCR

4.7.1 Linear range determination

Using cDNA prepared from muscle RNA, aliquots of a PCR master mix were subjected to PCR to discover the linear range of the 7-7-76 amplification. The aliquots were removed from the thermal cycler every two cycles starting from cycle 25 and ending at cycle 35. Figure 4.5 shows the effect of the different number of cycles on the PCR product in experiments conducted to determine the linear range of

amplification. Signal strength detected from cycle 29 to 35 was determined by densitometry and cycle 31 was selected as the most suitable cycle in the linear range and was used in the following relative RT-PCR reactions.

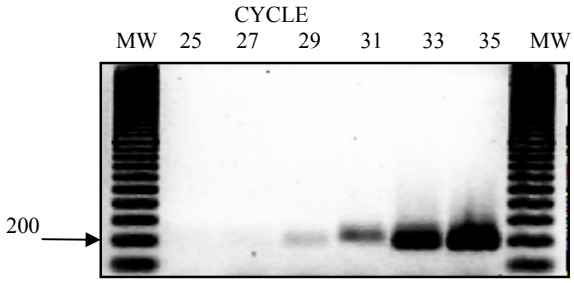

Figure 4.5 Relative PCR: Linear range determination. Reactions were removed at 2 cycle intervals. MW-100bp molecular weight ladder.

4.7.2 Optimal ratio for 18S Primers:Competimers

To determine what ratio of Competimers to primers was needed to have both the target-of-interest and the 18S control target amplify to give similar signal intensity, PCR reactions with different ratios of primer:Competimer were prepared. As the EtBr staining is a poor method to quantify, SYBR gold was used to visualise the DNA on agarose gels. As can be seen from Figure 4.6 the optimal ratio of 18S Primers:Competimers is 3:7, which is the ratio recommended for moderately expressed transcripts. An intermediate ratio (2.5:7.5) was also tested to attempt to derive more similar concentrations of 18S and target gene PCR products. However this did not produce improved results (data not shown). Therefore, a ratio 3:7 of 18S

Primers:Competimers was used in the following relative RT-PCR investigation of 7-7-76 expression.

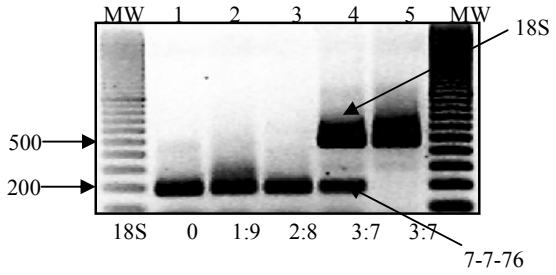

Figure 4.6 7-7-76 Relative PCR: Optimal ratio of 18S Primers:Competimers determination. In this experiment, different ratios of 18S Primers:Competimers were used with all the other conditions remaining identical to those used in section 4.6 and 4.7.1. Each of the lanes contained 18S Primers:Competimers except lane 1. Lane: 1 – 7-7-76 primers only; 2 – ratio 1:9; 3 – ratio 2:8; 4 – ratio 3:7; 5 – ratio 3:7 without 7-7-76 primers. MW – 100bp molecular weight ladder.

4.7.3 Application to Cell Lines and Tissues

The method enabled investigation of potential differential expression of 7-7-76 in cell lines and mouse tissues. The 7-7-76 signal for each tissue and cell line was normalized against the 18S signal and the mean of two experimental results was determined. Table 4.1 shows the ratios derived from each sample versus AC29 B7-6. The results were expressed as a ratio relative to the signal for AC29 B7-6, which was arbitrarily chosen as the calibrator because it had the lowest signal.

Tissue	Ratio vs AC29 B7-6
Spleen	1.84 ± 0.45
Heart	3.05 ± 0.87
Intestine	1.26 ± 0.50
Brain	2.58 ± 0.74
Muscle	1.95 ± 0.79
Liver	1.79 ± 0.16
Lung	1.21 ± 0.66
Kidney	1.32 ± 0.32
AC29 B7-6	1.00 ± 0.34
AC29 B7-7	1.00 ± 0.21
AB1	2.53 ± 0.13
AC29	2.68 ± 0.16

Table 4.1 Ratio of net intensity in each sample versus AC29 B7-6

From the results in Table 4.1, variation can be observed between the normal mouse tissues. The greatest variation was noticed between heart and lung, which was approximately 2-3 fold. The highest expression was seen in heart and brain compared to the lowest expression in lung and intestine. Among tumour cell lines, the highest expression was seen in AC29 and AB1. The expression by cell lines AB1 and AC29 was within the range of normal tissue expression although at the upper end. No variation was seen between AC29 B7-7 and AC29 B7-6. On the other hand, variation was observed between AC29 and the clones AC29 B7-7 and AC29 B7-6, the parental cell line expressing approximately 2-3 fold more of the 7-7-76 transcript.

4.8 5' RACE

5' Rapid Amplification of cDNA Ends (RACE) was used to amplify nucleic acid sequences between a known sequence site and unknown sequences towards the 5' end of the mRNA. The sequence of clone 7-7-76 that was obtained as described in

Section 4.4 was likely to be in the 3' non-coding region based on EST database data. Therefore, in order to obtain further sequence towards the coding region of the gene, the RACE technique was used.

Primers were designed for the following PCR procedures based upon the sequence derived clone for 7-7-76. Figure 4.7 shows the locations of the primers designed. Three antisense gene-specific primers (GSP) were designed for 5' RACE, which were identified as RACE-RT (274)R, RACE-1 (191)R and RACE-2 (140)R (Table 2.1). The first strand of cDNA was synthesised according to the manufacturer's protocol (Life Technologies) using primer RACE-RT (274)R. Then, RNA was degraded from cDNA before purification of cDNA using the GlassMax DNA Isolation Spin Cartridge procedure.

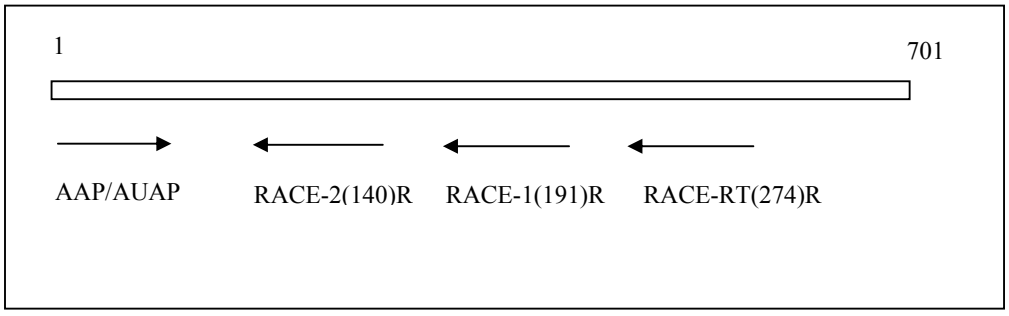

Figure 4.7 Diagrammatic of designed primers located in 7-7-76 clone fragment. The numbers in the figure shows the locations of each primer that are designed for RACE based on the clone fragment of 7-7-76 sequence. AUAP/AAP – primers supplied by the manufacturer. The arrows indicate the direction and extent of sequence.

Homopolymeric tailing of cDNA was conducted to create the abridged anchor primer binding site on the 3'-end of the cDNA. Efficient tailing was necessary and was performed according to the manufacturer's instruction (Life Technologies). Tailed cDNA obtained were then amplified by PCR according to manufacturer's protocol using AAP and primer RACE-1 (191)R. A typical cycling protocol for cDNA was initially performed with either 35 or 40 cycles. After analyzing both PCR products, the 40 cycle PCR product was used for subsequent nested PCR.

The nested PCR procedure was conducted according to the manufacturer's protocol and optimization was carried out in order to get the best result. Primary PCR product from above was diluted 1:100 and 1:1000 before conducting nested PCR using AUAP and primer RACE-2 (140)R with either 35 or 40 cycles. After evaluating both PCR products, the 40 cycle PCR product was used for subsequent RACE analysis. The final RACE product showed two bands on gel electrophoresis (Figure 4.8). The larger band of the RACE product and the smaller band deemed as residues of RACE product.

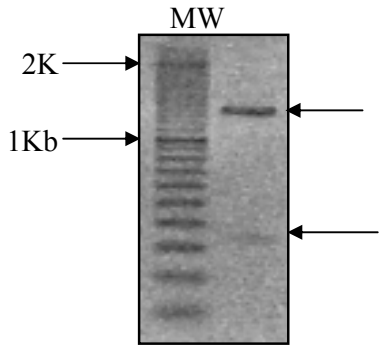

Figure 4.8 Gel electrophoresis of 7-7-76 5'RACE nested PCR products following purification with BRESAspin PCR Purification Kit. MW - 100bp molecular weight ladder.

The RACE product was ligated into pGEM-T vector followed by transformation before being subjected to colony PCR amplification and analysis using agarose gel electrophoresis (Figure 4.9).

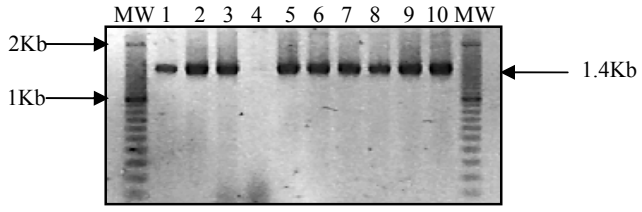

Figure 4.9 Colony PCR gel. Lane 1-10 –colony PCR product. MW - 100bp molecular weight ladder

Colonies of interest (lane 2, 3 and 5) were picked and grown overnight in LB broth before plasmid purification using QIAprep™ Spin Plasmid Kit. Universal primers T7 and SP6 were used to conduct sequencing in order to acquire the orientation of the RACE product. Primer RACE-2 (140)R was used to perform internal sequencing and subsequently primers were then designed to determine the unknown sequences. After 5 rounds of sequencing, the entire sequence of RACE clone 7-7-76 was determined. All the sequence results of 7-7-76 and RACE product were assembled and results can be seen in Figure 4.10.

```
                *        20         *        40         *        60
76RACE : CCATGTTTGTCTCAGAACTTGTATCTTGCGTTTGGCCCTCAGCTTTAGCACTTTTTCCCA

                *        80         *       100         *       120
76RACE : CTTGCAGAGTCTTTCAGTTGTGCTTGGTAGCCAGGCTCAACACACCGGGGCTTTAGACTC

                *       140         *       160         *       180
76RACE : AAGCAGCTTATATGGGAAGCTGGAGACCCCTGCCAGCCAGAGCACAGTAAGGGCTCGGAG

                *       200         *       220         *       240
76RACE : AAGGTATGTGAACTAGGGTATCTGATGTGGTAGAGCAGTTGGTGTCTGGCATTGTGAACG

                *       260         *       280         *       300
76RACE : GACAAGGGGACTTGCCTCCCTTCTTGCCACTGAGGCCCTTGCTTTTCCTTCAGCTCTTGG

                *       320         *       340         *       360
76RACE : CCTGTTGGTCCAGTTTATTAGTGAGAGCTAGTTTGCTCCAGGTGGTTAAAGGATGTAGCA

                *       380         *       400         *       420
76RACE : GGAGGAATGGTGGAAGATGGGAGCTGCCCGGGTTAGTACAGAAGAGGTCTGTCTATCTGA

                *       440         *       460         *       480
76RACE : CCTAGCACTGAGGGGTTCACAAACCTGTGAGGTGCACCTCACTAATTTTGGCTCATCCCT

                *       500         *       520         *       540
76RACE : GCCTTGCTACAAGCTCCCTGTGGCCACCATTCTCTGGACTGTACCGTGCTGATGACTGCA

                *       560         *       580         *       600
76RACE : GCTGGCTACAAAACAGCATGAGAACCTGGCCGCTGTTCTCCTACTGCCTCCACCCTCAGG

                *       620         *       640         *       660
76RACE : CAGGCCTCCTCCTCTTCTGCCCCTGAAACCTCGTGCTGCTTGCTCCTCCTGCCTCCACCC

                *       680         *       700         *       720
76RACE : TCAGGCAGGCCTCCTCCTCTTCTGCCCCTGAAGCCTCCTGNTGCTGCTCCTCCACCTCAG

                *       740         *       760         *       780
76RACE : TCAGTGCTGAACTTCTCACTGTTGAGTGTTGAAATTGTTAATGGTCAAAGCAGGGAGGAT

                *       800         *       820         *       840
76RACE : GCCAGTCACCAAGGTTGATTGTCTTGGGAAAGCAGTTTTAGAGTGATGAATCCTTTGTAG

                *       860         *       880         *       900
76RACE : GACAGGTAGAGAGTATCTTAGAGATAAAAAAATGGGCTATGTCCTCTCCTACCCATGTCT
```

```
                *       920         *       940         *       960
76RACE : TGTGCTCACTCGCCCCTCAGTCTACCCTTTTTGCTGGGACAGTGATGGTCTTGGCCAAGG

                *       980         *      1000         *      1020
76RACE : TCAGCATGTAATGGAAAGACAGTGTATTGCCAGGCTGGATAGATGGGGTCATCTCACAGT

                *      1040         *      1060         *      1080
76RACE : GGGTCACGTGAAAATCAGTGTCACGTAGTCCCAGTGAATGTTGTGTTCTTTCTGGGGGGA

                *      1100         *      1120         *      1140
76RACE : GGGGGGAATAGAATAAACTCAAATTCTTTTTCAGTATAGCCCCTGAGTAATGAATGAAAA

                *      1160         *      1180         *      1200
76RACE : TTTGAGCTTATGTAAGTAAAGATTCATTTATGCCACCAGGGAGAGCCTGTATCTGAGAGA

                *      1220         *      1240         *      1260
76RACE : CATTTAGCAGAGAGCACTTTAGTTTTTTGCTGCTAACTGTTGTGTGATCCTTTGCTGGGG

                *      1280         *      1300         *      1320
76RACE : TGCAAAAGGCACCGGACACGGCCACGCGTCGACTAGTACACCAGTTGCTCCCTACCCCCT

                *      1340         *      1360         *      1380
76RACE : GCAGGAAGACCCCTGGCTCATACCCTAACGGAGACCAACAAGGACTGGACAGCTCGGCTC

                *      1400         *      1420         *      1440
76RACE : CGAGGAGTTATGCATGTGTCTGTGTCTGCTCATCACGCTCACCCGGTTGAAAGTCACTGT

                *      1460         *      1480         *      1500
76RACE : GCATTTGATAAGGTTTTCCTCAGAATGGCACCCTGCAGAATGACTGATGGGCGCCTTCAC

                *      1520         *      1540         *      1560
76RACE : AGGCATCAGGCAGCCACTCCTACTTTCTCAGCCTGAGCACACCTACAGTAGAGACAGTGT

                *      1580         *      1600         *      1620
76RACE : TGGGGGTGACAGGATGCCCCAGAAAAATTCAGGATACTTCTGAAATTGCCATGTTTGTCT

                *      1640         *      1660         *      1680
76RACE : CAGAACTGGNATCTTGCGTTTGGCCCTCAGCTTTAGCACTTTTTCCCACTTGCAGAGTCT

                *      1700         *      1720         *      1740
76RACE : TTCAGTTGTGCTTGGTAGCCAGGCTCAACACACCGGGGCTTTAGACTCAAGCAGCTTATA

                *      1760         *      1780         *      1800
76RACE : TGGGAAGCTGGAGACCCCTGCCAGCCAGAGCACAGTAAGGGCTCGGAGAAGGTATGTGAA

                *      1820         *      1840         *      1860
76RACE : CTAGGGTATCTGATGTGGTAGTGCAGTTGGTGTCTGGCATTGTGACGACAAGGGACTTGC

                *      1880         *      1900         *      1920
76RACE : CTCCCTTCTTGCCACTGAGGCCCTTGCTTTTCCTTCAGCTCTTGGCCTGTTGGTCCGGTT

                *      1940         *      1960         *      1980
76RACE : TATTAGTGAGAGCTAGTTTGCTCCAGGTGGTTAAAGGATGTAGCAGGAGGAATGGTGGAA

                *      2000
76RACE : GATGGGAGCTGCCCGGGTTAG
```

Figure 4.10 Nucleotide results of 7-7-76RACE. 7-7-76RACE consists of 2001bp. 1300bp to 2001bp of nucleotides are the original clone fragment of 7-7-76. The nucleotides in bold are the newly obtained sequences from RACE.

The RACE technique was successfully conducted and new sequence had developed from the primary sequence. These sequences (both clone 7-7-76 and RACE product) were assembled using a computer software known as Sequencher (Genecodes 3.0). Sequencher Program revealed a new alignment of sequence which was different from the expected result.

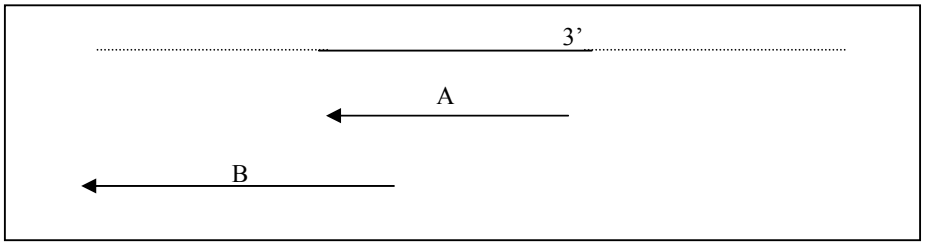

Figure 4.11 Expected results on RACE and 7-7-76 sequence alignment. Arrows indicate the direction and extent of sequence determination. Arrow A indicates the 7-7-76 product and arrow B indicates the expected 5' extension of 7-7-76 product using 5' RACE.

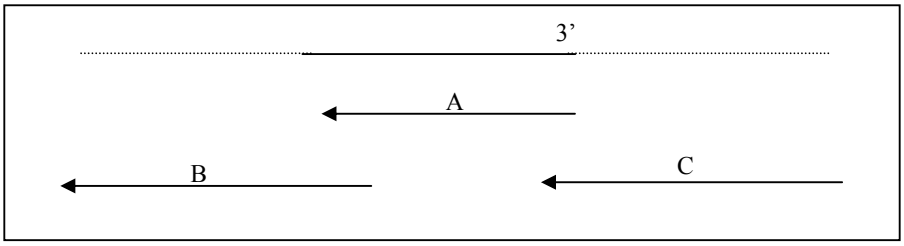

Figure 4.12 Observed results on RACE and 7-7-76 product sequence. Arrows indicate the direction and extent of sequence determination. Arrow A indicates the original 7-7-76 and arrow B indicates the 5' extension of 7-7-76 product using 5' RACE. Arrow C indicates the 5’ end of the RACE clone.

RACE was conducted for the purpose of extending the 7-7-76 sequence in a 5' direction. But, Sequencher results showed that although the 5' end of the original 7-7-76 sequence (A in Figure 4.11 and 4.12) was extended as expected, the very 5' end of the RACE sequence (C in Figure 4.12) actually aligned with the 3' end of the original sequence. This new assembled sequence was thoroughly examined and rechecked manually to ensure that individual sequencing results had been correctly assembled. It was confirmed and concluded that Figure 4.10 was established as 7-7-76RACE sequence. Since the 7-7-76RACE did not portray an expected alignment, new primers were designed to examine if the 7-7-76RACE sequence was due to some duplication or other possibility, and further Genbank investigation was also conducted.

4.9 7-7-76RACE vs. Hypertension Related Protein

The 7-7-76RACE product was used to search GenBank. BLASTN searches revealed that 7-7-76RACE was 99% homologous to a recent sequence entry of *mus musculus* hypertension related protein (AF384100, mouse HRP) submitted by Chen and Tang. The search showed that the 7-7-76RACE clone was homologous to the non coding region of AF384100. However, the 7-7-76RACE clone and AF384100 did not have the same sequence arrangement. Therefore, new primers were designed to examine both sequence arrangements in order to determine which sequence was biologically correct (Figure 4.13).

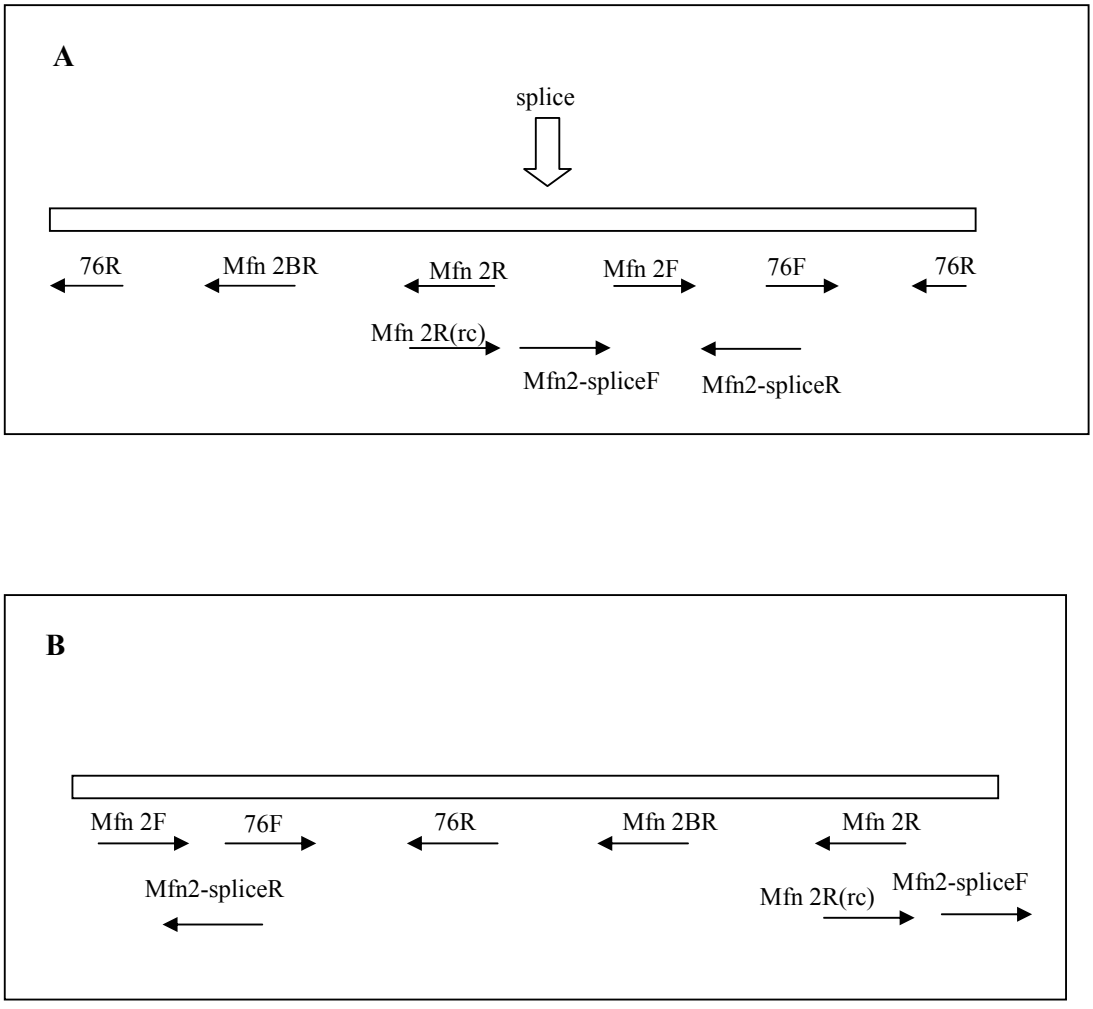

Figure 4.13 Diagrammatic location of primers on 7-7-76RACE and AF384100 (mouse HRP) sequences. In order to determine the actual arrangement of the mouse HRP 3' non coding region sequence and clarify the disagreement between 7-7-76RACE and mouse HRP, PCR was performed using the existing and newly designed primers shown. **A** – Location of primers on 7-7-76RACE product. **B** – Location of primers on mouse HRP. Arrows indicate the direction and extent of sequence. Splice arrow indicate the possible duplications of sequence. Results shown in Table 4.2.

Primer Pair	Actual PCR Result	Expected 7-7-76RACE Result	Expected AF384100 Result
76F & 76R	+	+	+
Mfn 2F & 76R	+	+	+
Mfn 2F & 76F	-	-	-
Mfn 2F & Mfn 2R	-	-	+
76F & Mfn 2R	-	-	+
76R & Mfn 2R	-	-	-
Mfn 2F & Mfn 2BR	-	-	+
76F & Mfn 2BR	-	-	+
76R & Mfn 2BR	-	-	-
Mfn 2F & Mfn 2R(rc)	-	-	-
76F & Mfn 2R(rc)	-	-	-
76R & Mfn 2R(rc)	-	+	-
Mfn2-spliceF & Mfn2-spliceR	-	+	-
Mfn2-spliceF & 76R	-	+	-
Mfn 2R(rc) & Mfn2-spliceR	-	+	-

Table 4.2 PCR amplification using various primer pairs. RNA extracted from AC29 was amplified by RT-PCR using various primer combinations. Results are shown with either (+) or (-). (+) indicates bands obtained after gel electrophoresis and (-) indicates bands not detected from gel electrophoresis.

Analyses on both sequences (7-7-76RACE and AF384100) were achieved by PCR using the new primers that were designed and using gel electrophoresis to determine the bands. Table 4.2 showed the actual results obtained from bands and the expected results. Results were examined and it was concluded that either sequence could possibility be correct. Possible explanations for this disassembly in sequence were considered and one of the possibilities was duplication of sequence. The splice arrow has shown the possible starting of the sequence duplication (Figure 4.13).

Chapter 5 will further discuss the possibilities that could have caused the difference between sequences.

4.10 Identification of 7-7-76 as Mouse Mitofusin 2

The whole sequence of 7-7-76RACE was again used to search various databases using BLASTN and BLASTX searches. Sequence analysis revealed that clone fragment AC29 B7-7-76 was homologous to the original sequence of putative *rattus norvegicus* hypertension related protein with GenBank entry accession number U41803 submitted by Chen and Tang on the 20th of January 1996. A more recent entry of the sequence from Mus Musculus hypertension related protein with the sequence entry AF384100 was submitted on the 25th of June 2001 by the same investigators. Unfortunately, 7-7-76RACE sequence was still within the non-coding region at the 3' end of the gene after RACE technique (Section 4.9). With this non-coding region of clone 7-7-76RACE, BLASTN searches were limited to mouse gene index only. Therefore, coding region of the mouse hypertension related gene obtained from AF384100 was used to search GenBank again. Results revealed an additional homology with high score of 89% to an entry for human mitofusin 2 (accession number: BC017061), submitted by Strausberg, 18th December 2001. Figure alignments of sequences are shown in Appendix 3. This GenBank entry (mitofusin 2, BC017061) was not sufficiently homologous in the 3' non coding to 7-7-76RACE sequence for BLASTN searching to detect. It was concluded that in all likelihood clone 7-7-76 is identified as mouse mitofusin 2. Mitofusin 2 will be discussed further in Chapter 5.

5.0 GENERAL DISCUSSION

5.1 Introduction

During the course of this project, two cDNA clones designated as AC29 B7-7-76 and AC29 B7-7-43 obtained from murine MM were investigated. At the commencement of the project, these clones were believed to be differentially expressed in AC29 tumour clones which differed in their tumorigenicity. Furthermore at that time, one of the clones represented an as yet undescribed gene sequence. In order to characterise whether these genes were relevant to the tumorigenicity and/or immunogenicity of MM, it was necessary to characterise their expression profiles and gain further information on their sequence.

5.2 Limitations of SSH

The SSH technique has been used to identify genes which are differentially expressed in tissues and cell lines of interest (Jin *et al,* 1997). This approach has been utilised to investigate the differences between the aggressive and the less aggressive B7-1 transfectant AC29 clones described previously (Section 1.4). Several differentially expressed cDNAs have been isolated and two such cDNAs are designated as 7-7-43 and 7-7-76. The expression of these two isolated cDNAs was evaluated in cell lines and tissues. Gene expression studies are necessary to determine whether genes isolated by differential display are truly differentially expressed in the cell samples from which they are derived (Liang and Pardee, 1995).

Nishizuka *et al* (2001) suggested that SSH is a useful method for identifying differentially expressed genes which gives a high "true positive" detection rate. Besides that, experiments by Lo *et* al (2001) have successfully used SSH to identify 22 genes that are differentially expressed in one cell population. However, the gene expression studies discussed in this project (Section 3.6 and 4.7) have shown that both clone 7-7-43 and 7-7-76 were not differentially expressed in the murine MM clones and cell lines. These results proved that both 7-7-43 and 7-7-76 were false positive clones.

According to Ye and Connor (2000), although SSH is an effective method of subtraction analysis there is still a high possibility of obtaining false positive clones. These clones may prove to be either not differentially expressed or to be undetectable by Northern blotting analysis of mRNA from the derived cells (Luce and Burrows, 1998). False positive clones derived from differential display cannot be entirely avoided due to differential signal presented by false positive clones during primary screening procedure (Liang and Pardee, 1995; Rebrikov *et al,* 2000).

Desai *et al* (2000) suggested that differential screening using non-subtracted cDNA probes would minimise the isolation of false positive clones. It has also been reported that the combination of SSH and a recent technological advance known as cDNA microarray can be used successfully to identify differentially expressed genes (Yang *et al,* 1999). The potential advantage of this approach is allowing a rapid, high throughput analysis on a panel of cell lines with small DNA chips in a single hybridisation (Yang *et al,* 1999; Du *et al,* 2001). Therefore, improvement in the sensitivity and specificity of the screening process would alleviate the detection of false positive clones in the future.

5.3 Assessment of Techniques for Quantitation of mRNA Expression

The important element of all the gene expression technologies and techniques, is their ability to identify and confirm that transcripts are truly differentially expressed. However, it is this aspect of the method that is most time-consuming and labour intensive (Ali *et al,* 2001). In this thesis, Northern blotting and relative RT-PCR were used to assess both SSH clones.

5.3.1 Northern Blotting In Gene Expression Studies

Northern blotting was performed without using radioactive probes but using DIG-labelled RNA and DNA probes. Radioactive probes were considered to be more dangerous to handle than DIG-labelled probes. Besides, DIG-labelled probes provide detection sensitivity similar to radioactive probes. Furthermore, DIG-labelled probes are easier to remove from membranes which is beneficial if membranes are to be hybridized repeatedly.

7-7-76 expression was undetectable by Northern blotting. This could have been due to clone 7-7-76 being expressed at a level below the detection sensitivity of this assay. Melton *et al.* (1984) mentioned that the main limitation of the Northern blotting technique is its comparatively low sensitivity. Luce and Burrows (1998) also suggested that clones derived by differential display and undetectable by blotting techniques can represent exceptionally rare species of mRNA or immature RNA transcripts which would be undetectable by Northern blotting.

Clone 7-7-43 was however detected using a DNA probe and variation in expression of 7-7-43 between cell lines was observed. The results obtained were equivalent to the results from relative RT-PCR as highest detection was seen in AC29 while lower detection in AB1 and AC29 B7-7. Quantitation of level of expression and comparison between samples by Northern blotting is difficult and only allows detection of gross differences in expression. As with other techniques of assessment of expression, this requires comparison with expression levels of a ubiquitously expressed mRNA species (eg. β actin). For these reasons in this study it was prepared to assess relative expression of these gene using relative RT-PCR.

5.3.2 Relative RT-PCR In Gene Expression Studies

PCR and RT-PCR have been widely used in nucleic acid analysis studies and both techniques have been shown to perform sensitively on low abundance samples from as little as one cell (Rappolee *et al,* 1988; Wang and Brown, 1999; Bustin, 2000; Snider *et al,* 2001). Relative RT-PCR is a method that is widely used in quantitative analysis of gene expression. This technique was utilized here to determine whether clones 7-7-43 and 7-7-76 were differentially expressed in MM cell lines and tissues.

Expression of both clones was detectable in all cell lines and tissues. Using this technique, up to 3-fold variation in expression of 7-7-76 was demonstrated between cell lines and tissues and up to 2-fold variation was detected in expression of clone 7-7-43. These data do not suggest major quantitative differences in expression of the two genes

although the biological relevance of a two-three fold difference in expression of these genes is not known. Among the MM cell lines, AC29 had the highest expression of both clones. There was no difference in the expression of these cDNA clones between the MM clones AC29 B7-6 and AC29 B7-7, the cells used to derive these clones by SSH. Therefore, it was concluded that neither clone 7-7-43 nor 7-7-76 was differentially expressed in these transfectant clones of murine MM and neither gene was likely to play a major role in determining the differences in tumorigenicity and immunogenicity of these cell clones.

5.3.3 Northern Blotting vs. Relative RT-PCR

Both gene expression techniques, Northern blotting and relative RT-PCR, were used in this project and both methods were successfully accomplished. There were pros and cons for both methods that I have used and experienced. Technically, relative RT-PCR is preferable for gene expression studies in the future because this method was much easier to do compared to Northern blotting. It was less time consuming and labor intensive when relative RT-PCR was carried out while longer time was needed to perform Northern blotting. Screening for differentially expressed clones with Northern blotting normally involves a large amount of total RNA and is inefficient, tedious and expensive (Yang *et al,* 1999; Freeman *et al,* 1999; Du *et al,* 2001).

Relative RT-PCR was also recommended as a useful method because it has higher detection sensitivity compared to Northern blotting. Lockey *et al.* (1998) and Freeman *et al.* (1999) remarked that many target genes might not be detectable by Northern

blotting, as many mammalian mRNAs are expressed at relatively low levels. In addition to that, sharper bands were obtained from gel electrophoresis of relative RT-PCR products whereas Northern blotting usually gives more diffuse bands which complicate densitometry analysis.

Although relative RT-PCR was much preferred and beneficial than Northern blotting, this technique has its disadvantages. Suitable conditions such as number of PCR cycles and optimum annealing temperature were required to be established before relative RT-PCR can be reliably used. Besides, the ideal ratio of primers:competimers needed to be defined in great detail in order to achieve optimal bands for densitometry. In addition, staining gels with ethidium bromide at times causes residual (background) that can affect the quantitation of targets during densitometry. The development and advancement of real-time PCR, which is discussed later in this chapter, is likely to improve comparison of mRNA expression levels between samples.

5.4 Assessment Of RACE And 7-7-76RACE Product

The 5' RACE technique was used to amplify nucleic acid sequences between a known sequence site and unknown sequences towards the 5' end of the mRNA. Preliminary sequencing of clone 7-7-76 and comparison with database sequences suggested that 7-7-76 represented sequence in the 3' non-coding region and therefore RACE was utilized to obtain further sequence of clone 7-7-76. Technically, I personally think that RACE is a time-consuming technique and sometimes the expected results were unachievable. In

this research, I have successfully obtained a new sequence (7-7-76RACE) using RACE but the result was not as expected.

The developed sequence of 7-7-76RACE product was not in the same sequence order as hypertension related protein submitted by Chen and Tang to GenBank (rat HRP, U41803) (Section 4.9). There could be a few reasons for this to occur. One of the possibilities was mentioned in section 4.9 that duplication of sequence could have taken place (refer to Figure 4.13) and this could have been missed by the Chen and Tang when they were doing sequencing. I have designed some new primers to examine both sequence arrangements but final conclusion could not be made due to another possibility that will be discussed later.

Another possibility that could have caused the difference in sequence of 7-7-76RACE and hypertension related protein would be the rearrangement of plasmid during the 5'RACE experiments in this project. This could have caused the designed primers to attach onto different locations of the sequence instead of the target site during PCR. However, no matter which of these possibilities is correct, it was not possible to determine which sequence (7-7-76RACE and hypertension related protein) was right or wrong. In order to clarify this, I would have done a few more experiments on these sequences, such as to do a 5'RACE on Chen and Tang's hypertension related protein sequence. Apart from that, I would have designed some new primers for further sequencing or even done a 3' RACE on the whole hypertension related protein sequence if that was necessary. All these were not carried out because 7-7-76 clone was not

deemed to be significantly over expressed in murine MM (Section 4.7) and the two transfectant AC29 clone (B7-7, B7-6) did not differ in their expression of the gene.

5.5 Clone Identification And Expression

A complete sequence of clone 7-7-43 was obtained and gene expression of this clone in cell lines and tissues was analysed. Clone 7-7-76 was successfully identified after obtaining a longer sequence by RACE and 7-7-76 gene expression studies were also determined.

5.5.1 Clone 7-7-43 Analysis

Based on the preliminary sequence and comparison from GenBank, clone 7-7-43 is homologous to *homo sapiens* Cks1 regulatory subunit gene. Literature review (Section 1.5) has mentioned that the Cks1 is involved in the cell cycle and further investigation was performed to analyse the differential expression of 7-7-43 in tumour cell lines and mouse tissues.

A complete sequence of clone 7-7-43 was successfully obtained and gene expression was assessed using relative RT-PCR and northern blotting. Both relative RT-PCR and northern blotting assessment revealed that there were only minor variations in expression among the tumour cell lines and mouse tissues. Therefore, clone 7-7-43 was concluded as not differentially expressed gene in murine MM.

5.5.2 Clone 7-7-76 Analysis

In Section 5.4 the development of 7-7-76RACE product from clone 7-7-76 was described. Gene expression was also assessed using relative RT-PCR and Northern blotting. Both gene expression techniques described no significant variations in expression of clone 7-7-76. It seems likely that this clone was a false positive of SSH as mentioned in Section 5.2. However, identification of clone 7-7-76 as mitofusin 2 was successfully achieved through RACE and GenBank analysis (Section 4.10). Both hypertension related protein gene (rat HRP and mouse HRP) and mitofusin 2 gene are actually the same gene but there were submitted to the GenBank by different authors.

5.5.3 Mitofusin 2

Mitochondria are believed to have their own genetic system which encodes for proteins that are responsible for the production of energy to meet cellular needs in eukaryotic cells (Richter, 1995). Therefore, mitochondria are present in almost every cell in the body and depending on the cell type and its energy requirement, their numbers vary from only a few to hundreds in each cell (Hermann and Shaw, 1998). Mitochondrial changes in size and arrangement depend on physiological and pathological conditions (Arbustini *et al,* 1998; Inagaki *et al,* 1992). For instance, mitochondria change size and morphology in yeast and these changes are regulated by fusion and fission events (Hermann and Shaw, 1998; Nunnari *et al,* 1997; Yaffe, 1999).

A gene known as mitofusin 2 (Mfn2) is believed to encode a possible mediator that supports mitochondrial fusion. This gene is a homolog of *Drosophila* protein *fuzzy onion* (Fzo), which associates with mitochondria as well as altering mitochondrial morphology when expressed by transient transfection in cell culture. Fzo was the first mediator of mitochondrial fusion identified, which is required during spermatogenesis (Hales and Fuller, 1997). Tang *et al* (2000) suggested that mitochondrial fusion also takes place in mammals. In cells transfected by Mfn2, over 90% of mitochondria have alterations in morphology (Santel and Fuller, 2000). Since Mfn2 (7-7-76) is associated with mitochondria, I propose that further study on controlling of mitochondria in cancer, especially MM, by Mfn2 would be warranted. For example, inducing foreign protein which affect the fusion and fission of mitochondria in tumour cells would probably affect tumour cells growth or tumour progression.

5.6 Differential Gene Expression Using Today's Technologies

In today's modern technology, gene expression studies to analyse the complex biological processes have become essential (Snider *et al,* 2001). New methods that are able to identify and quantitate differential mRNA profiles, provide the understanding of mechanisms of development and differentiation (Jurecic and Belmont, 2000). The oldest and well-established techniques that are still popular are Northern blotting and RNase protection assay (Snider *et al,* 2001). However, some of these traditional methods have advantages and limitations. For example, the RNase protection assays require less RNA but are unable to analyse large numbers of samples. Furthermore,

both Northern blotting and RNase protection assay methods are time consuming and most of the time require the use of radioactive labels (Snider *et al,* 2001).

5.6.1 Differential Display PCR

Differential display PCR (DD-PCR) is a versatile technique for the comparison of gene expression in mRNA populations (Liang and Pardee, 1992; Welsh *et al,* 1992) that is based on RT-PCR and denaturing polyacrylamide gel electrophoresis (Ali *et al,* 2001). DD-PCR allows identification of differentially expressed genes in either *in vitro* or *in vivo* systems. Besides, this technique can also be used to obtain mRNA fingerprint (Ali *et al,* 2001). The advantages of DD-PCR is its ability to identify and isolate differentially expressed transcripts in biological systems and the ability to emphasize lower abundance transcripts that are saturated by higher abundant transcripts during PCR (Guimaraes *et al,* 1995).

Although it has the advantage of being applicable to small amount of total RNA (Ali *et al,* 2001) it is limited by the fact that it generates short expressed sequence tags (ESTs) that are mainly at the 3' non-coding region (Jurecic and Belmont, 2000). Therefore, additional techniques allowing sequencing of full length cDNA, eg. 3' RACE, are essential to identify coding sequence (Jurecic and Belmont, 2000). As with all the gene expression techniques, the major limitation with DD-PCR is having a percentage of isolating false positive clones (Debouck, 1995; Wan *et al,* 1996) and the need to confirm the results (Ali *et al,* 2001). A few strategies have been recommended to reduce these problems (Luce and Burrows, 1998). For instance, to reduce isolation of false positive

clones, the total RNA used in these studies needs to be of high quality and DNase treated (Liang *et al,* 1993). In addition, simply performing triplicate RT-PCRs using three independent RNA preparations followed by choosing reproducible bands for further study can eliminate this problem (Luce and Burrows, 1998). By following the above suggestions during DD-PCR, minimizing false positives in differential display will be improved (Luce and Burrows, 1998).

5.6.2 Microarray Analysis

A new tool known as cDNA microarrays allows examination of gene expression on a genome-wide scale. cDNA microarray techniques provide cell-, tissue- and process-specific gene expression profiles and also quantitative analysis of expression level for thousands of genes (Duggan *et al,* 1999). Therefore, microarrays will be increasingly important in analysing the transcriptional profile of specific cell types in relation to development, differentiation and function (Jurecic and Belmont, 2000).

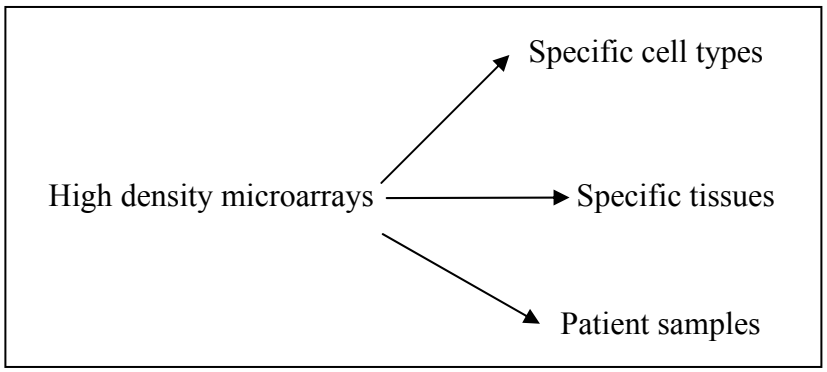

Figure 5.1 Advantageous of microarray techniques. Microarray techniques facilitate the study of cell-, tissue-, process-specific gene expression profiles and help identify disease-related target genes.

After isolation of sequences, confirmation of their differential expression often involves the handling of large number of cDNAs and therefore, the use of a rapid and high-throughput method such as microarrays will aid in screening cDNA libraries (Gardmo *et al,* 2002). The advantage of using cDNA microarrays is that it allows direct comparison of quantitative values between two RNA populations (Yang *et al,* 1999). However, the limitation of microarrays is the lack of ability to identify some genes that were known to be differentially expressed by northern blotting (Yang *et al,* 1999).

Technological improvements in cDNA microarrays such as probe labelling, hybridization and data analysis have facilitated the use of cDNA microarrays to incorporate with other methods to analyse gene expression (Jurecic and Belmont, 2000). For example, Yang *et al,* (1999), has successfully combined the technologies of SSH and cDNA microarrays to develop a powerful high throughput screening procedure to verify the SSH results and identify genes differentially expressed in breast cancer cell lines (Gardmo *et al,* 2002). Combination of SSH and microarrays has proved to be handy for analyzing expression in uncommon cell populations and patient samples where only low abundance RNA is available (Jurecic and Belmont, 2000). For example, combination of cDNA and tissue microarrays was used to identify genes related to progression of prostate cancer (Bubendorf *et al,* 1999). Such studies have helped in better understanding of development and pathogenesis that subsequently provides better diagnosis and treatment of cancer, which would be beneficial to the research community in the future (Jurecic and Belmont, 2000; Cox, 2001).

5.6.3 Nylon Membrane Array

Applications of nylon arrays in scientific research are now accepted as a tool that provides high throughput information on gene expression (Cox, 2001). This method is straightforward and relatively economical compared to other gene expression technologies (Cox, 2001). Array technology is also a powerful tool to investigate global cell gene expression *in vitro* or *in vivo* (Bertucci *et al,* 1999; Shim *et al,* 1998). Besides, it has the ability to detect changes in expression profile in a biological system affected by stimulants such as cytokines (Cox, 2001). Nylon arrays were used for research in diseases such as cancer (Gress *et al,* 1996) and evaluation of drug effects upon biological tissues (Cox, 2001). The use of arrays could identify drugs that regulate specific genes and also keep in the search for possible side effects of drugs *in vitro* (Cox, 2001). This technology will help to accelerate the understanding of disease and the development of drugs to fight against disease world wide (Cox, 2001). As many diseases are manifestations of the altered expression of specific genes, the development of various gene expression technologies will assist our understanding of the pathological processes and improve our knowledge in diseases in the future (Snider *et al,* 2001).

5.6.4 Real-time PCR

Although relative RT-PCR rather than Northern blotting was preferred to study gene expression (Section 5.3.3), a recent technological invention known as real-time PCR might be more effective for further study of gene expression. This powerful tool can be used to monitor gene expression by detection and quantitation of a fluorescent reporter (double –strand DNA-specific dye SYBR® Green), the signal of which increases in

direct proportion to the amount of PCR product in a reaction (Higuchi *et al,* 1993; Wittwer *et al,* 1997). Using this method, a single molecule of DNA can be detected from within a DNA mixture that contained a high background of genomic DNA (Beitner-Johnson *et al,* 2001).

This method is especially beneficial for providing quantitative numerical values for gene expression (Ali *et al,* 2001). It gives highly sensitive and accurate quantitation levels for specific mRNAs present in small samples (Lockey *et al,* 1998; Freeman *et al,* 1999). In addition, real-time PCR incorporates the ability to directly measure and quantify the reaction while amplification is taking place. Therefore, it is a multidimensional analysis assay which enables multiple studies simultaneously. In our laboratory research, numerous genes are now being studied using real-time PCR and these have ratified that real-time PCR has all the benefits as mentioned. As more laboratories and core facilities acquire the instrumentation required for real-time analysis, this technique may become the dominant RT-PCR-based quantitation technique in the future.

5.7 Conclusion

In summary, the goal to characterize the putatively differentially expressed genes isolated from murine MM has been successfully achieved. Gene expression studies on both clones 7-7-43 and 7-7-76 have proved that these genes were not differentially expressed, which could be due to false positive clones isolated during SSH. However, identification of these clones was achieved and the results accomplished may help in future studies. It also remains to be determined whether Cks1 (7-7-43) and Mfn2 (7-7-

76) are associated with processes relevant to the pathogenesis of MM or its future therapy.

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APPENDICES

Appendix 1

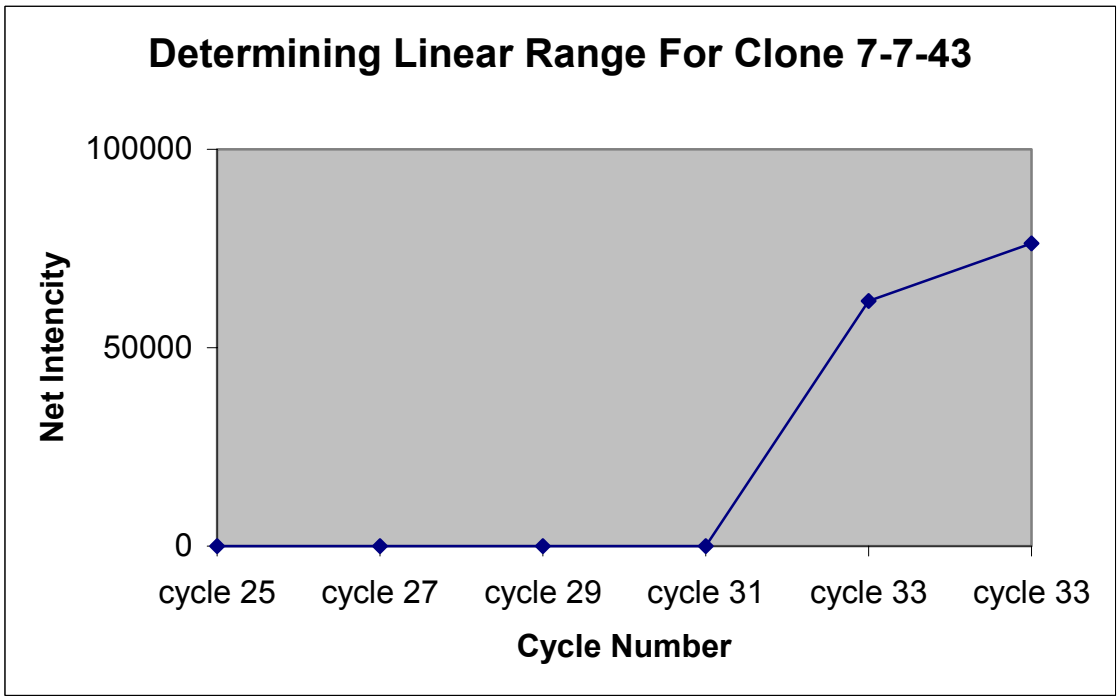

Figure A1.1 Determining linear range for clone 7-7-43. To determine suitable cycle number for relative RT-PCR.

Appendix 2

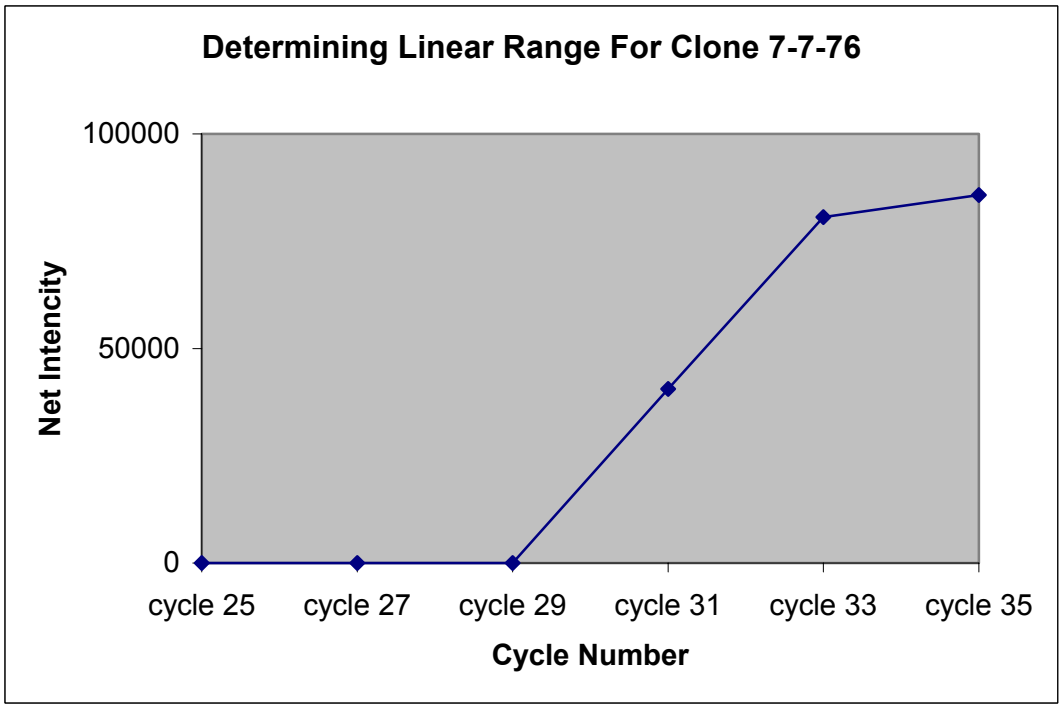

Figure A1.2 Determining linear range for clone 7-7-76. To determine suitable cycle number for relative RT-PCR.

Appendix 3

```
                    *        20         *        40         *        60
human_mito : GTGTGATGGCCGCCGCGAGGCCGGGAAGGTGAAGTCAGGACTGGTGGAGTCAACACAGTC
mouseHRP   : ------------------------------------------------------------
7-7-76     : ------------------------------------------------------------

                    *        80         *       100         *       120
human_mito : AATCAATAGCCAACCTCAACCTGAGACAGGACAGAAGAGAACTCAGAATCTTTTTGTCTT
mouseHRP   : ------------------------------------------------------------
7-7-76     : ------------------------------------------------------------

                    *       140         *       160         *       180
human_mito : TTGGACTTCAGCCATGTCCATGATGCCTACCCTGTGAAGATCTCTCACCATCCAAAAAAC
mouseHRP   : ------------------------------------------------------------
7-7-76     : ------------------------------------------------------------

                    *       200         *       220         *       240
human_mito : GCAATGTCCCTGCTCTTCTCTCGATGCAACTCTATCGTCACAGTCAAGAAAAATAAGAGA
mouseHRP   : ---ATGTCCCTGCTCTTTTCTCGATGCAACTCCATCGTCACCGTCAAGAAGGATAAGCGA
7-7-76     : ------------------------------------------------------------

                    *       260         *       280         *       300
human_mito : CACATGGCTGAGGTGAATGCATCCCCACTTAAGCACTTTGTCACTGCCAAGAAGAAGATC
mouseHRP   : CACATGGCTGAAGTGAATGCTTCCCCTCTCAAGCACTTTGTCACTGCCAAGAAAAAGATC
7-7-76     : ------------------------------------------------------------

                    *       320         *       340         *       360
human_mito : AATGGCATTTTTGAGCAGCTGGGGGGCCTACATCCAGGAGAGCGCCACCTTCCTTGAAGAC
mouseHRP   : AATGGAATCTTTGAGCAGCTGGGGGGCCTACATCCAAGAGAGCGCCAGCTTCCTTGAAGAC
7-7-76     : ------------------------------------------------------------

                    *       380         *       400         *       420
human_mito : ACGTACAGGAATGCAGAACTGGACCCCGTTACCACAGAAGAACAGGTTCTGGACGTCAAA
mouseHRP   : ACCCACAGGAACACAGAACTGGACCCGGTTACCACGGAAGAGCAGGTCCTGGACGTCAAA
7-7-76     : ------------------------------------------------------------

                    *       440         *       460         *       480
human_mito : GGTTACCTATCCAAAGTGAGAGGCATCAGTGAGGTGCTGGCTCGGAGGCACATGAAAGTG
mouseHRP   : GGGTACCTGTCCAAGGTCAGGGGTATCAGCGAAGTGCTGGCCAGGCGGCACATGAAGGTG
7-7-76     : ------------------------------------------------------------

                    *       500         *       520         *       540
human_mito : GCTTTTTTTGGCCGGACGAGCAATGGGAAGAGCACCGTGATCAATGCCATGCTCTGGGAC
mouseHRP   : GCTTTTTTTGGCCGGACGAGCAATGGGAAGAGCACCGTGATCAATGCCATGCTCTGGGAC
7-7-76     : ------------------------------------------------------------

                    *       560         *       580         *       600
human_mito : AAAGTTCTGCCCTCTGGGATTGGCCACACCACCAATTGCTTCCTGCGGGTAGAGGGCACA
mouseHRP   : AAAGTTCTGCCATCTGGGATTGGTCATACCACCAATTGCTTCCTGCGGGTTGGGGGCACA
7-7-76     : ------------------------------------------------------------

                    *       620         *       640         *       660
human_mito : GATGGCCATGAGGCCTTTCTCCTTACCGAGGGCTCAGAGGAAAAGAGGAGTGCCAAGACT
mouseHRP   : GATGGCCATGAGGCCTTCCTCCTCACAGAGGGCTCAGAAGAGAAGAAGAGTGTCAAGACT
7-7-76     : ------------------------------------------------------------

                    *       680         *       700         *       720
human_mito : GTGAACCAGCTGGCCCATGCCCTCCACCAGGACAAGCAGCTCCATGCCGGCAGCCTAGTG
mouseHRP   : GTGAACCAACTGGCCCATGCCCTCCATCAGGACGAGCAGTTGCATGCAGGCAGCATGGTG
7-7-76     : ------------------------------------------------------------
```

```
                    *       740         *       760         *       780
human_mito : AGTGTGATGTGGCCCAACTCTAAGTGCCCACTTCTGAAGGATGACCTCGTTTTGATGGAC
mouseHRP   : AGTGTGATGTGGCCCAACTCCAAGTGTCCGCTCCTGAAGGATGACCTCGTGCTGATGGAC
7-7-76     : ------------------------------------------------------------

                    *       800         *       820         *       840
human_mito : AGCCCTGGTATTGATGTCACCACAGAGCTGGACAGCTGGATTGACAAGTTTTGTCTGGAT
mouseHRP   : AGCCCTGGGATCGATGTTACCACGGAGCTGGACAGCTGGATTGATAAGTTTTGCCTGGAT
7-7-76     : ------------------------------------------------------------

                    *       860         *       880         *       900
human_mito : GCTGATGTGTTTGTGCTGGTGGCCAACTCAGAGTCCACCCTGATGCAGACGGAAAAGCAC
mouseHRP   : GCTGATGTGTTTGTGCTGGTGGCCAACTCAGAGTCCACGCTGATGCAGACGGAGAAGCAG
7-7-76     : ------------------------------------------------------------

                    *       920         *       940         *       960
human_mito : TTCTTCCACAAGGTGAGTGAGCGTCTCTCCCGGCCAAACATCTTCATCCTGAACAACCGC
mouseHRP   : TTCTTCCACAAAGTGAGTGAACGTCTCTCCCGGCCCAACATCTTCATCCTGAACAACCGC
7-7-76     : ------------------------------------------------------------

                    *       980         *      1000         *      1020
human_mito : TGGGATGCATCTGCCTCAGAGCCCGAGTACATGGAGGAGGTGCGGCGGCAGCACATGGAG
mouseHRP   : TGGGATGCGTCTGCCTCGGAGCCTGAGTACATGGAGGAGGTGCGGCGGCAGCACATGGAG
7-7-76     : ------------------------------------------------------------

                    *      1040         *      1060         *      1080
human_mito : CGTTGTACCAGCTTCCTGGTGGATGAGCTGGGCGTGGTGGATCGATCCCAGGCCGGGGAC
mouseHRP   : CGCTGCACCAGCTTTCTCGTGGATGAGCTGGGCGTGGTGGATCGAGCTCAGGCTGGGGAC
7-7-76     : ------------------------------------------------------------

                    *      1100         *      1120         *      1140
human_mito : CGCATCTTCTTTGTGTCTGCTAAGGAGGTGCTCAACGCCAGGATTCAGAAAGCCCAGGGC
mouseHRP   : CGGATCTTCTTCGTGTCTGCCAAGGAGGTTCTCAGCGCCAGGGTCCAGAAAGCCCAGGGC
7-7-76     : ------------------------------------------------------------

                    *      1160         *      1180         *      1200
human_mito : ATGCCTGAAGGAGGGGGCGCTCTCGCAGAAGGCTTTCAAGTGAGGATGTTTGAGTTTCAG
mouseHRP   : ATGCCAGAAGGAGGCGGCGCTCTCGCAGAAGGTTTTCAAGTGAGGATGTTTGAGTTTCAG
7-7-76     : ------------------------------------------------------------

                    *      1220         *      1240         *      1260
human_mito : AATTTTGAGAGGAGATTTGAGGAGTGCATCTCCCAGTCTGCAGTGAAGACCAAGTTTGAG
mouseHRP   : AATTTCGAGAGGCGGTTTGAGGAGTGCATTTCCCAGTCTGCAGTAAAGACCAAATTTGAG
7-7-76     : ------------------------------------------------------------

                    *      1280         *      1300         *      1320
human_mito : CAGCACACGGTCCGGGCCAAGCAGATTGCAGAGGCGGTTCGACTCATCATGGACTCCCTG
mouseHRP   : CAGCACACAGTCCGGGCCAAGCAGATTGCAGAGGCCGTTCGTCTCATCATGGATTCCCTG
7-7-76     : ------------------------------------------------------------

                    *      1340         *      1360         *      1380
human_mito : CACATGGCGGCTCGGGAGCAGCAGGTTTACTGCGAGGAAATGCGTGAAGAGCGGCAAGAC
mouseHRP   : CACATCGCAGCTCAGGAGCAGCGGGTTTATTGTCTAGAAATGCGGGAAGAGCGGCAAGAC
7-7-76     : ------------------------------------------------------------

                    *      1400         *      1420         *      1440
human_mito : CGACTGAAATTTATTGACAAACAGCTGGAGCTCTTGGCTCAAGACTATAAGCTGCGAATT
mouseHRP   : CGGCTGAGGTTTATTGACAAGCAGCTGGAGCTCCTGGCTCAAGACTACAAGCTGCGAATT
7-7-76     : ------------------------------------------------------------
```

```
                    *      1460         *      1480         *      1500
human_mito : AAGCAGATTACGGAGGAAGTGGAGAGGCAGGTGTCGACTGCAATGGCCGAGGAGATCAGG
mouseHRP   : AAGCAGATTACGGAGGAAGTGGAAAGGCAGGTGTCCACAGCCATGGCTGAAGAGATCAGG
7-7-76     : ------------------------------------------------------------

                    *      1520         *      1540         *      1560
human_mito : CGCCTCTCTGTACTGGTGGACGATTACCAGATGGACTTCCACCCTTCTCCAGTAGTCCTC
mouseHRP   : CGCCTCTCTGTGCTAGTTGACGAGTACCAGATGGACTTCCACCCATCCCCAGTTGTCCTC
7-7-76     : ------------------------------------------------------------

                    *      1580         *      1600         *      1620
human_mito : AAGGTTTATAAGAATGAGCTGCACCGCCACATAGAGGAAGGACTGGGTCGAAACATGTCT
mouseHRP   : AAGGTTTATAAGAACGAGCTGCACCGCCATATAGAGGAAGGTCTGGGCCGGAACCTGTCT
7-7-76     : ------------------------------------------------------------

                    *      1640         *      1660         *      1680
human_mito : GACCGCTGCTCCACGGCCATCACCAACTCCCTGCAGACCATGCAGCAGGACATGATAGAT
mouseHRP   : GACCGCTGCTCCACTGCCATTGCCAGTTCACTGCAGACTATGCAGCAGGACATGATAGAC
7-7-76     : ------------------------------------------------------------

                    *      1700         *      1720         *      1740
human_mito : GGCTTGAAACCCCTCCTTCCTGTGTCTGTGCGGAGTCAGATAGACATGCTGGTCCCACGC
mouseHRP   : GGCTTGAAGCCCCTTCTTCCTGTATCTATGCGGAATCAGATAGACATGCTGGTCCCTCGA
7-7-76     : ------------------------------------------------------------

                    *      1760         *      1780         *      1800
human_mito : CAGTGCTTCTCCCTCAACTATGACCTAAACTGTGACAAGCTGTGTGCTGACTTCCAGGAA
mouseHRP   : CAGTGTTTCTCCCTCAGCTATGACCTGAATTGTGACAAGCTGTGTGCTGACTTTCAGGAG
7-7-76     : ------------------------------------------------------------

                    *      1820         *      1840         *      1860
human_mito : GACATTGAGTTCCATTTCTCTCTCGGATGGACCATGCTGGTGAATAGGTTCCTGGGCCCC
mouseHRP   : GACATCGAGTTCCACTTCTCCCTTGGATGGACTATGCTAGTGAACAGGTTCCTGGGCCCC
7-7-76     : ------------------------------------------------------------

                    *      1880         *      1900         *      1920
human_mito : AAGAACAGCCGTCGGGCCTTGATGGGCTACAATGACCAGGTCCAGCGTCCCATCCCTCTG
mouseHRP   : AAGAATAGCCGCCGGGCCTTGCTAGGCTACAGTGATCAGGTTCAGCGTCCTCTCCCTCTG
7-7-76     : ------------------------------------------------------------

                    *      1940         *      1960         *      1980
human_mito : ACGCCAGCCAACCCCAGCATGCCCCCACTGCCACAGGGCTCGCTCACCCAGGAGGAGTTC
mouseHRP   : ACACCTGCCAACCCCAGCATGCCCCCCTTGCCACAGAGCTCCCTCACCCAGGAGGAGCTC
7-7-76     : ------------------------------------------------------------

                    *      2000         *      2020         *      2040
human_mito : ATGGTTTCCATGGTTACCGGCCTGGCCTCCTTGACATCCAGGACCTCCATGGGCATTCTT
mouseHRP   : ATGGTCTCCATGGTTACTGGCCTGGCCTCTCTGACGTCCAGGACCTCCATGGGCATTCTT
7-7-76     : ------------------------------------------------------------

                    *      2060         *      2080         *      2100
human_mito : GTTGTTGGAGGAGTGGTGTGGAAGGCAGTGGGCTGGCGGCTCATTGCCCTCTCCTTTGGG
mouseHRP   : GTGGTCGGAGGAGTGGTGTGGAAGGCAGTGGGCTGGAGACTCATCGCCCTCTCCTTTGGA
7-7-76     : ------------------------------------------------------------

                    *      2120         *      2140         *      2160
human_mito : CTCTATGGCCTCCTCTACGTCTATGAGCGTCTGACCTGGACCACCAAGGCCAAGGAGAGG
mouseHRP   : CTGTATGGCCTCCTGTACGTCTATGAGCGACTGACCTGGACCACCAAAGCCAAAGAGAGG
7-7-76     : ------------------------------------------------------------
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                    *      2180         *      2200         *      2220
human_mito : GCCTTCAAGCGCCAGTTTGTGGAGCATGCCAGCGAGAAGCTGCAGCTTGTCATCAGCTAC
mouseHRP   : GCCTTCAAGCGCCAGTTTGCGGAATACGCCAGTGAGAAGCTACAGCTCATCATCAGTTAC
7-7-76     : ------------------------------------------------------------

                    *      2240         *      2260         *      2280
human_mito : ACTGGCTCCAACTGCAGCCACCAAGTCCAGCAGGAACTGTCTGGGACCTTTGCTCATCTG
mouseHRP   : ACCGGCTCTAACTGCAGCCACCAAGTCCAGCAGGAATTGTCTGGGACATTTGCTCATCTG
7-7-76     : ------------------------------------------------------------

                    *      2300         *      2320         *      2340
human_mito : TGTCAGCAAGTTGACGTCACCCGGGAGAACCTGGAGCAGGAAATTGCCGCCATGAACAAG
mouseHRP   : TGCCAGCAAGTTGACATCACCCGAGATAATCTGGAGCAGGAAATTGCTGCCATGAACAAG
7-7-76     : ------------------------------------------------------------

                    *      2360         *      2380         *      2400
human_mito : AAAATTGAGGTTCTTGACTCACTTCAGAGCAAAGCAAAGCTGCTCAGGAATAAAGCCGGT
mouseHRP   : AAAGTCGAGGCTCTGGATTCACTTCAGAGCAGAGCCAAACTGCTCAGGAATAAAGCTGGC
7-7-76     : ------------------------------------------------------------

                    *      2420         *      2440         *      2460
human_mito : TGGTTGGACAGTGAGCTCAACATGTTCACACACCAGTACCTGCAGCCCAGCAGATAGTGG
mouseHRP   : TGGTTGGACAGCGAACTCAACATGTTCACACACCAGTACCTGCAGCCCAGCAGATAGTGG
7-7-76     : ------------------------------------------------------------

                    *      2480         *      2500         *      2520
human_mito : GCACCTGAGGCGGAGTCTGCGTGGAGAGGGGCGGTGCTGCCAGCCCTAAGTGCCGTGTGG
mouseHRP   : GCAGCCAGGGCGGACCTGCACGAAGAAGAGGCAGGGCCGACCTCCCATCAGCTCTAGTCC
7-7-76     : ------------------------------------------------------------

                    *      2540         *      2560         *      2580
human_mito : GCTCCCCCAGGGGCACGTGTGGCTCCTGCCCCCTGGCCACTGCCAAGAGAATGAAGCACC
mouseHRP   : TTGGCCGCTGCAGAGAGAAGGAAAGCACCCAGTCTTGTACCAGTTACTCCCTACCCCCTG
7-7-76     : ----------------------------------------ACCAGTTGCTCCCTACCCCCTG

                    *      2600         *      2620         *      2640
human_mito : CAGTCTCGTACCATTTTGAGCCCTCCAGCACTACTTATTTTCCCCCACCTTTGCCTGCTG
mouseHRP   : CAGGAAGACCCCTGGCTCATACCCTAATGGAGACCAACAAGGACTGGACAGCTCGGCTCC
7-7-76     : CAGGAAGACCCCTGGCTCATACCCTAACGGAGACCAACAAGGACTGGACAGCTCGGCTCC

                    *      2660         *      2680         *      2700
human_mito : TTGCTGGAAGAGCTGGCTCATACCCCCAAAGGACACTTTCAGCGACAGCTATGGACAGCA
mouseHRP   : AAGGAGTTATGCATGTGTCTGTGTCTGCTCCTCACGCTCACCCGGTTGAAAGTCACTGTG
7-7-76     : GAGGAGTTATGCATGTGTCTGTGTCTGCTCATCACGCTCACCCGGTTGAAAGTCACTGTG

                    *      2720         *      2740         *      2760
human_mito : TGGTACCAAGGAGTTAAGTTGAGGCTTTTTCCAGCTTTCTCTGGTTCATTTGATTGCTTG
mouseHRP   : CATTTGATAAAGTTTTCCTCAGAATGGCACCCTGCAGAATGACTGATGGGCGCCTTCACA
7-7-76     : CATTTGATAAGGTTTTCCTCAGAATGGCACCCTGCAGAATGACTGATGGGCGCCTTCACA

                    *      2780         *      2800         *      2820
human_mito : ATAAGGCCTCAGGATCTCAGCATTGCACAATGCCTCATGGAAGCCTTTGAGGGTATCACA
mouseHRP   : GGCATCAGGCAGCCACTCCTACTTTCTCCAGCCTGAGCACACCTACAGAGAGACAGTGTT
7-7-76     : GGCATCAGGCAGCCACTCCTACTTTCTCAGC-CTGAGCACACCTACAGAGAGACAGTGTT

                    *      2840         *      2860         *      2880
human_mito : CAGACACCCCCACCTTCCTCCAGCCTGTGCGCACCTGCCCTCCTTGCAGCCCAGCACACC
mouseHRP   : GGGGGTGACAGGATGCCCCAGAAAAATTCAGGATACTTCTGAAATTGCCATGTTTGTCTC
7-7-76     : GGGGGTGACAGGATGCCCCAGAAAAATTCAGGATACTTCTGAAATTGCCATGTTTGTCTC
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                    *      2900         *      2920         *      2940
human_mito : TGCAGGTGTAAGGGACGATTGGAGTTTCTTCCCAGAGAGTCTGTCCCAGAAGGACTGTGG
mouseHRP   : AGAACTTGTATCTTGCGTTTGGCCCTCAGCTTTAGCACTTTTTCCCACTTGCAGAGTCTT
7-7-76     : AGAACTGGTATCTTGCGTTTGGCCCTCAGCTTTAGCACTTTTTCCCACTTGCAGAGTCTT

                    *      2960         *      2980         *      3000
human_mito : CTTGTGTGTGTCCATCTCGCCTGTTGGCTCAGTGCTTCATCCCATTTGCAGAGCCTCAGA
mouseHRP   : TCAGTTGTGCTTGGTAGCCAGGCTCAACACACCGGGGCTTTAGACTCAAGCAGCTTATAT
7-7-76     : TCAGTTGTGCTTGGTAGCCAGGCTCAACACACCGGGGCTTTAGACTCAAGCAGCTTATAT

                    *      3020         *      3040         *      3060
human_mito : CACGTCTTGGTGGTGAGGCTCAGTTACCCCTGGGCTTAGGCTGAGGCGGGCCCTGTGCTG
mouseHRP   : GGGAAGCTGGAGACCCCTGCCAGCCAGAGCACAGTAAGGGCTCGGAGAAGGTATGTGAAC
7-7-76     : GGGAAGCTGGAGACCCCTGCCAGCCAGAGCACAGTAAGGGCTCGGAGAAGGTATGTGAAC

                    *      3080         *      3100         *      3120
human_mito : GGGGTGGTAGAAAGGATGCTGCTGAGGCAGCTGGAGGAGTGGGAGTAGCTCAGAGGGGAG
mouseHRP   : TAGGGTATCTGATGTGGTAGTGCAGTTGGTGTCTGGCACTGTGACGACAAGGGACTTGCC
7-7-76     : TAGGGTATCTGATGTGGTAGTGCAGTTGGTGTCTGGCATTGTGACGACAAGGGACTTGCC

                    *      3140         *      3160         *      3180
human_mito : GGCTGTTGGATGTATGGGGAGCTGGCAGAGCAGGTGGCAGTCACTGGGACAAGGAGGGAC
mouseHRP   : TCCCTTCTTGCCACTGAGGCCCTTGCTTTTCCTTCAGCTCTTGGCCTGTTGGTCCGGTTT
7-7-76     : TCCCTTCTTGCCACTGAGGCCCTTGCTTTTCCTTCAGCTCTTGGCCTGTTGGTCCGGTTT

                    *      3200         *      3220         *      3240
human_mito : TTGCCTCTCTTCTCATTATTGTGTCCTTTGCTTTAGTGTCAGTCCTGGACTTGTGCAGGC
mouseHRP   : ATTAGTGAGAGCTAGTTTGCTCCAGGTGGTTAAAGGATGTAGCAGGAGGAATGGTGGAAG
7-7-76     : ATTAGTGAGAGCTAGTTTGCTCCAGGTGGTTAAAGGATGTAGCAGGAGGAATGGTGGAAG

                    *      3260         *      3280         *      3300
human_mito : CTGTTTTGTGTAGATCTGTTTTGGAAGATGGCATGGTCTAGGTGGTTGAAGGATGTAGTA
mouseHRP   : ATGGGAGCTGTCCGGGTTAGTACAGAAGAGGTCTGTCTATCTGACCTAGCACTGAGGGGT
7-7-76     : ATGGGAGCTGCCCGGGTTAG------------------------------------------

                    *      3320         *      3340         *      3360
human_mito : GAAGGATGGATGGTGGAAGGTGGGGACGTTGGTGGCTGGCTGAGGTGCATGGCCCCCACA
mouseHRP   : TCACAAACCTGTGAGGTGCACCTCACTAATTTTGGCTCATCCCTGCCTTGCTACAAGCTC
7-7-76     : ------------------------------------------------------------

                    *      3380         *      3400         *      3420
human_mito : CAGGACAGCTGGAGAATGGGCCGTCCACTTGGCCTCGTTCTGCGAGGGGCTCATGGGTCT
mouseHRP   : CCTGTGGCCACCATTCTCTGGACTGTACCGTGCTGATGACTGCAGCTGGCTACAAAACAG
7-7-76     : ------------------------------------------------------------

                    *      3440         *      3460         *      3480
human_mito : GAGAGCCCCCACCCACTAGGCTTGATTGCATCCCTGTTGTGCCCTTTAAGAGACATGTTT
mouseHRP   : CATGAGAACCTGGCCGCTGTTCTCCTACTGCCTCCACCCTCAGGCAGGCCTCCTCCTCTT
7-7-76     : ------------------------------------------------------------

                    *      3500         *      3520         *      3540
human_mito : CCACCCCACCCCCAACCTTGTCCCAAGTGCCCTGGACTAAATTTCCTGTGCCAGTGACTG
mouseHRP   : CTGCCCCTGAAGCCTCCTGCTGCTTGCTCCTCCTGCCTCCACCCTCAGGCAGGCCTCCTC
7-7-76     : ------------------------------------------------------------

                    *      3560         *      3580         *      3600
human_mito : CAGTTGGCCAAGGGACAATGTGGAAAACCCAGTGTCCATCTTTCCACCCTCCCTGATCTC
mouseHRP   : CTCTTCTGCCCCTGAAGCCTCCTGCTGCTGCTCCTCCACCTCAGTCAGTGCTGAACTTCT
7-7-76     : ------------------------------------------------------------
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                    *      3620         *      3640         *      3660
human_mito : CAGAACCTTCGACTGACCCCCTTGTCTTTATGCTGATGTTGAGTTTTGGGATTGTTACTG
mouseHRP   : CACTGTTGAGTGTTGAAATTGTTAATGGTCAAAGCAGGGAGGATGCCAGTCACCAAGGTT
7-7-76     : ------------------------------------------------------------

                    *      3680         *      3700         *      3720
human_mito : GTTGAAGTGGGGGCAGATGCCTGTCACCAAGGTGTTGACTGTGTGAGAAAAGCAGTTTGG
mouseHRP   : GATTGTCTTGGGAAAGCAGTTTTAGAGTGATGAATCCTTTGTAGGACAGGTAGAGAGTAT
7-7-76     : ------------------------------------------------------------

                    *      3740         *      3760         *      3780
human_mito : GTGACAAATCCTGTGTGGCACAAGTTGGATCGCTTCCTAGAAATAAGCAACACCTCTCCC
mouseHRP   : CTTAGAGATAAAAAAATGGGCTATGTCCTCTCCTGCCCATGTCTTGTGCTCACTCGCCCC
7-7-76     : ------------------------------------------------------------

                    *      3800         *      3820         *      3840
human_mito : AAAAAGCAGCCCACAAGGCAGGGGCCCAGCAGCCCAGCCATCACTCATCTTTGAGGAAAT
mouseHRP   : TCAGTCTACCCTTTTTGCTGGGACAGTGATGGTCTTGGCCAAGGTCAGCATGTAATGGAA
7-7-76     : ------------------------------------------------------------

                    *      3860         *      3880         *      3900
human_mito : GAGTTGGTAGCCTCTGTGCACTGTTTGGTGGCCACATCACAGGTGATGTCCTGTTCACAT
mouseHRP   : AGACAGTGTATTGCCAGGCTGGATAGATGGGGTCATCTCACAGTGGGTCACGTGAAAATC
7-7-76     : ------------------------------------------------------------

                    *      3920         *      3940         *      3960
human_mito : ACCTGCTTGTATTTAAAGCCCTCAGTCTGTCCTGTTGTGTGGGGCGAAGTGATGGACTCT
mouseHRP   : AGTGTCACGTAGTCCCAGTGAATGTTGTGTTCTTTCTGGGGGGAGGGGGGAATAGAATAA
7-7-76     : ------------------------------------------------------------

                    *      3980         *      4000         *      4020
human_mito : GCCAGGTGGACATGCTGTGGGTGGATGTTCCCGGCGTGTGCCGGGCCTGAATGGACAGGG
mouseHRP   : ACTCAAATTCTTTTTCAGTATAGCCCCTGAGTAATGAATGAAAATTCGAGCTTATGTAAG
7-7-76     : ------------------------------------------------------------

                    *      4040         *      4060         *      4080
human_mito : GCCACTTCACAGCATGTCAGGGAAAATCACTGTCACACAATTCCAATGGATTTTGTGCTC
mouseHRP   : TAAAGATTCATTTATGCCACCAGGGAGAGCCTGTATCTGAGAGACATTTAGCAGAGAGCA
7-7-76     : ------------------------------------------------------------

                    *      4100         *      4120         *      4140
human_mito : TTTTTGAAAAAAAAAAATTCTTTAGCGTAAACATGAATTTTTTTTCAATGTAGCCCCTGG
mouseHRP   : CTTTAGTTTTTTGCTGCTAACTGTTGTGTGATCCTTTGCTGGGGTGCAAAAGGCACCGGA
7-7-76     : ------------------------------------------------------------

                    *      4160         *      4180         *      4200
human_mito : GGAATGAATGAAATTTTGAGCTTCTTCAATACGTAAAATTAAATTTATACCACTGAGGGA
mouseHRP   : CACAGTGTGTTTGCTTCTGGTATATTTAGGTGATGCATCCGCCTGGGTTACCTGTATCTC
7-7-76     : ------------------------------------------------------------

                    *      4220         *      4240         *      4260
human_mito : GAGACCCTTTCTGAAAGAAGTATGGCCAAAAGCACTTTAATGCTGCTGACATTGTTGTTT
mouseHRP   : TGCTGATCATATTGGAAACTAAAATAAAGCCCCATTTGTGACCCTGAAAAAAAAAAAAAA
7-7-76     : ------------------------------------------------------------

                    *      4280         *      4300         *      4320
human_mito : TTATGTTCATTTGCTGGAGCGCAAGACGTGCTGACACAGTGAGTTTTCTCTGATGTATTT
mouseHRP   : AAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
7-7-76     : ------------------------------------------------------------
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                     *      4340         *      4360         *      4380
human_mito : AAGGTGATGTATTTGCTTGAGTTACTCCTGTATCATTGCTCATAATATTGGAAACTAAAA
mouseHRP   : AAAAAAAAA-----------------------------------------------------
7-7-76     : ------------------------------------------------------------

                     *      4400         *      4420
human_mito : TAAAACCTAGTTGGAAAAAAAAAAAAAAA-----------------
mouseHRP   : ----------------------------------------------
7-7-76     : ----------------------------------------------
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Figure A1.3 Complete alignment of various sequence. Sequence alignment between human mitofusin 2, mouse HRP (AF384100) and original clone 7-7-76.