

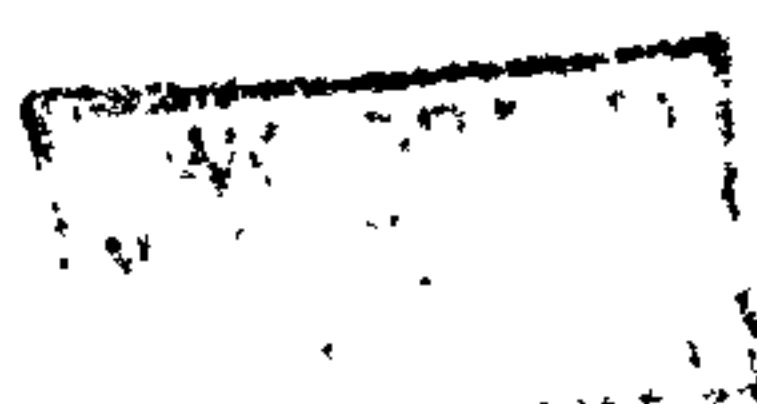
# **Identification of Proteins Interacting with the Human Mismatch Repair Protein MLH1**

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**A thesis submitted to the University of Glasgow in part fulfilment for the  
degree of Doctor of Philosophy.**

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# Declaration

I am the sole author of this thesis. All the references have been consulted by myself in the preparation of this manuscript. All the work presented in this thesis was performed by myself, except where otherwise stated.

**....any living cell carries with it the experience of a billion years of experimentation by its ancestors. You cannot expect to explain so wise an old bird in a few simple words.**

**Max Delbruck, Trans. Conn. Acad. Arts Sci. 38; 173. (1949)**

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## Abstract

Loss of expression of the human DNA mismatch repair (MMR) gene, hMLH1, is seen in a number of tumour cell lines resistant to a variety of cytotoxic drugs (Brown et al., 1997). Restoration of sensitivity to for example, cisplatin, has been demonstrated in MMR deficient cell lines, through the transfer of hMLH1 on chromosome 3 into these cells (Moreland et al., 1999). MMR has been implicated in playing a role in signalling apoptotic death pathways, via c-Abl, p53 and p73, in response to DNA damage (Duckett et al., 1999; Gong et al., 1999; Nehme et al., 1997). Exactly how MMR couples to these apoptotic pathways is unclear. The aim of this study was to identify other proteins that interact with hMLH1 to attempt to further elucidate its role in MMR and the engagement of downstream damage response pathways.

A yeast two-hybrid system, an *in vivo* system for detecting protein-protein interactions was utilised for this purpose. In this system, full length hMLH1 and a C-terminal fragment of hMLH1, amino acids 514-756, were shown to interact with two separate regions of hPMS2, amino acids 430-613 and amino acids 630-862. The hMLH1 hybrid protein was used to screen yeast two-hybrid cDNA libraries, from both normal mammary gland and ovarian tissue. Fifteen known and five unknown genes were identified as encoding proteins interacting with hMLH1. These included three known hMLH1 binding proteins, hMLH3, hPMS1 and MED1. Amongst the other genes identified was the proto-oncogene *c-MYC*, a gene previously implicated in genetic instability and apoptosis

Confirmation of the ability of MYC to interact with hMLH1 has been shown with GST pull-down experiments of human cell extracts using hMLH1 protein expressed in *E.coli*. Evidence for these proteins interacting in cells, is shown by c-MYC co-immunoprecipitating with hMLH1 from human and avian cell extracts. Using *in vitro* derived mutants of c-MYC, it has been shown that hMLH1 interacts with the leucine-zipper domain of c-MYC. This region of c-MYC is also required for the interaction with its major binding partner MAX. However the affinity of hMLH1 for c-MYC was shown not to be high enough to disrupt a c-MYC-MAX complex bound to its DNA recognition binding site, as shown by electrophoretic mobility shift assays (EMSA).

The effect of elevated c-MYC expression on functional MMR was examined. An inducible c-MYC expression system, Rat-1 fibroblasts expressing c-MYCER<sup>TM</sup>, a fusion of c-MYC to the hormone binding domain of the oestrogen receptor was utilised. Elevated



expression of c-MYC did not effect the mismatch specific binding complex activity in these cells as measured in EMSA experiments.

However c-MYC overexpression utilising the Rat-1 c-MYCER<sup>TM</sup> system was shown to result in a mutator phenotype in these cells. This was demonstrated through measuring the mutation rate at the selectable *HPRT* gene. Mutant clones were examined for mutations at a specific frameshift mutation hotspot, at a run of six guanines in exon 3 of the *HPRT* gene, previously associated with loss of MMR. No mutations were detected in the 22 mutant clones from cells grown in the absence of elevated c-MYC expression. Four of the 25 mutant clones from cells grown in the presence of elevated c-MYC expression, had a frameshift mutation at this hotspot, with five instead of six guanines.

The results suggest there may be a link between the mutator phenotype, induced through overexpression of c-MYC, and loss of MMR. It has been shown that c-MYC is capable of interacting with hMLH1 using *in vitro* protein interaction assays, and that c-MYC and hMLH1 can be co-immunoprecipitated from cells. Overexpression of c-MYC, which is associated with many cancers, may result in the sequestration of hMLH1 preventing functional MMR. The interaction between hMLH1 and c-MYC is proposed to act in a DNA damage response pathway which is disrupted upon aberrant c-MYC expression.

# Abbreviations

## Text

$\alpha\alpha$	Amino Acid
ADP	Adenosine Diphosphate
amp <sup>r</sup>	Ampicillin Resistance
ATM	<i>Ataxia telangiectasia</i> mutant
ATP	Adenosine Triphosphate
$\beta$ -Gal	$\beta$ -Galactosidase
bHLH-Zip	basic-Helix-Loop-Helix-Leucine Zipper
CAT	Chloramphenicol Acetyltransferase
CDDP	cis-diamminedichloroplatinum(II), cisplatin
CDK	Cyclin Dependent Kinase
cDNA	Complementary Deoxyribonucleic acid
CKII	Casein Kinase II
CPD	Cyclobutane Pyrimidine Dimers
CRC	Colorectal Cancer
CS	Cockayne's Syndrome
C-terminal	Carboxyl terminal
DHFR	Dihydrofolate Reductase
DNA	Deoxyribonucleic Acid
<i>E.coli</i>	<i>Escherichia coli</i>
EM	Electron Microscopy
EMSA	Electrophoretic Mobility Shift Assay
ENU	N-ethylnitrosourea
EXO1	Exonuclease 1
ES Cells	Embryonic Stem Cells
GFP	Green Fluorescent Protein
GST	Glutathione-S-Transferase
GTBP	G/T Binding Protein
HLH	Helix-Loop-Helix
HNPCC	Hereditary Nonpolyposis Colorectal Carcinoma
HPRT	Hypoxanthine Phosphoribosyl Transferase
IDL	Insertion/Deletion Loop

IR	Ionising Radiation
kan <sup>r</sup>	Kanamycin Resistance
MBI	MYC Box I
MBII	MYC Box II
MBD	Methyl-CpG-Binding Domain
MEF	Mouse Embryonic Fibroblast
MGMT	Methylguanine methyltransferase
MLH	MutL Homologue
MLX	<u>MAX</u> <u>L</u> ike Protein <u>X</u>
MMR	Mismatch Repair
MNNG	<i>N</i> -methyl- <i>N</i> '-nitro- <i>N</i> -nitrosoguanidine
MSH	MutS Homologue
MSI	Microsatellite Instability
MNU	<i>N</i> -methylnitrosourea
NER	Nucleotide Excision Repair
N-terminal	Amino-terminal
O <sup>6</sup> -meG	O <sup>6</sup> methyl Guanine
PAGE	Polyacrylamide Gel Electrophoresis
PCNA	Proliferating Cell Nuclear Antigen
PCR	Polymerase Chain Reaction
PMS	Post Meiotic Segregation
RER	Replication Error
RFC	Replication Factor C
<i>S. cerevisiae</i>	<i>Saccharomyces cerevisiae</i>
Ser	Serine
<i>S. pombe</i>	<i>Schizosaccharomyces pombe</i>
SSB	Single stranded DNA binding protein
<i>S. thypharium</i>	<i>Salmonella thypharium</i>
TAD	Transactivation domain
<i>T. aquaticus</i>	<i>Thermus aquaticus</i> (TAQ)
TCR	Transcription-Coupled Repair
TDG	Thymine DNA Glycosylase
6-TG	6-Thioguanine
TTD	Trichothiodystrophy
UAS	Upstream Activator Sequence

UV	Ultraviolet Radiation
XP	<i>Xeroderma pigmentosum</i>
Y2H	Yeast Two-Hybrid
<b>Reagents</b>	
Ade	Adenine
Amp	Ampicillin
APS	Ammonium Persulphate
3-AT	3-Amino-1,2,4-Triazole
BCA	Bicinchoninic Acid
CIP	Calf Intestinal Phosphate
CO <sub>2</sub>	Carbon Dioxide
His	Histidine
ddH <sub>2</sub> O	Double Distilled Water
DMSO	Dimethyl Sulphoxide
ECL	Enhanced Chemiluminescence
EDTA	Ethylenediaminetetra-Acetic Acid
HRP	Horse Radish Peroxidase
Kan	Kanamycin
LB	Luria-Bertani
Leu	Leucine
LiAc	Lithium Acetate
MES	2-( N-Morpholino) Ethane Sulfonic Acid
MOP	3-(N-Morpholino) Propane Sulfonic Acid
NIS	Non Specific Rabbit Serum
4-OHT	4-Hydroxy Tamoxifen
PBS	Phosphate Buffered Saline
PEG	Polyethylene Glycol
QDO	Quadruple Dropout Media
SC	Synthetic Complete Media
SD Medium	Synthetic Dropout Medium
SDS	Sodium Dodecyl Sulphate
TBE	Tris, Boric Acid, Ethylenediaminetetra-Acetic Acid
TE	Tris, Ethylenediaminetetra-Acetic Acid

TEMED	Tetramethylenediamine
Tris	2-amino-2-(hydroxymethyl) propane-1, 3-diol
Trp	Tryptophan
YPD	Yeast extract Peptone Dextrose YPD

**Units**

bp	base pair
cm	centimetre
Da	Dalton
g	gram
g	gravity
hr	hour
k	kilo
Kb	Kilobase
L	Litre
M	Molar
μ	micro
m	milli
m	metre
min	minute
n	nano
°C	degree Celsius
rpm	revolutions per minute
s	second
U	unit
v/v	volume for volume
w/v	weight for volume

# Chapter 1

## Introduction



## 1.1 DNA Mismatch Repair

Mismatch repair (MMR) of DNA plays a fundamental role in correcting replication errors i.e. mispaired bases that escape the proof-reading activity of DNA polymerases. MMR also corrects mispaired bases of recombination heteroduplexes that result from strand transfer between sequences that are related, but have diverged slightly at the nucleotide level. Mismatched base pairs can also arise through physical or chemical damage to DNA such as the deamination of 5-methylcytosine. A fundamental requirement of MMR is the ability to distinguish the correct base from the incorrect base. Functional MMR is essential to maintain genetic fidelity.

### 1.1.1 The Discovery of MMR in Prokaryotes

The role of MMR was first discovered over twenty years ago in bacteria. Loss of MMR was shown to produce increased spontaneous mutation rates (a mutator phenotype) (Modrich and Lahue, 1996; Nevers and Spatz, 1975). *Escherichia coli* (*E.coli*) strains were isolated with this mutator phenotype which led to the discovery of four mutator genes, *mutS*, *mutL*, *mutH* and *mutU/uvrD*. These MMR genes have been shown to play a role in the correction of replication errors by *in vitro* analysis of cell free extracts (Lu et al., 1983; Lu et al., 1984). Reconstitution of this methyl directed system was achieved *in vitro* (Lahue et al., 1989). By mixing the purified proteins, MutH, MutL, MutS, DNA helicase II, SSB (single stranded DNA binding protein), exonuclease I, DNA polymerase III holoenzyme, DNA ligase and co-factors ATP, deoxynucleoside-5'-triphosphates,  $Mg^{2+}$  and the ligase co-factor  $NAD^+$  together, repair mimicking that seen *in vivo* and in cell extracts was observed (Grilley et al., 1990; Lahue et al., 1989).

Initiation of *E.coli* MMR requires the MutS, MutL and MutH proteins (Modrich and Lahue, 1996). In wild type cells DNA is adenine-methylated at d(GATC) sequences by DAM methylase, however newly synthesised strands are not methylated until modified by this methylase. This allows the parent strand to be distinguished from the newly synthesised daughter strand and directs repair to the newly synthesised daughter strand.

MutS is an ATPase which functions as a homodimer. MutS recognises mismatched base pairs and insertion deletion loops in one DNA strand. MutH is an endonuclease which is both sequence and methylation specific. It acts by cleaving the DNA 5' to the unmethylated d(GATC) sequence in the hemimethylated duplex. The strand break is the

primary signal which directs correction to the unmethylated strand. MutL, as a homodimer, acts to mediate an interaction between the site of the mismatch bound by MutS and the d(GATC) sequence cleaved by MutH.

MutL has been demonstrated to physically interact with both MutH and UvrD (the protein product of *mutU*) (Hall et al., 1998; Hall and Matson, 1999). The d(GATC) specific endonuclease activity of MutH is stimulated by MutL, in the absence of MutS and a mismatch, but requires ATP (Hall and Matson, 1999). However further stimulation was observed with the addition of MutS in the presence of a mismatch and a hydrolysable form of ATP. The observed interaction between MutL and UvrD (DNA helicase II) led to the discovery that the helicase activity of this enzyme on incised duplex DNA is enhanced by MutS and MutL in a mismatch dependent manner (Yamaguchi et al., 1998).

DNA helicase II is one of several exonucleases required to excise the error carrying DNA strand, beginning at the nicked d(GATC) site. The bi-directional capability of the system means that the strand signal can be either side of the mismatch and explains why a number of exonucleases function in this repair process. The final step of the methyl directed repair reaction is the gap repair by DNA Polymerase III holoenzyme and ligation of the repair product.

### 1.1.2 The Discovery of MMR in Eukaryotes

The identification of MMR in yeast and fungi arose through observations of postmeiotic segregation (the result of un-repaired heteroduplex DNA generated during the process of meiotic recombination). Isolation of yeast mutants that displayed elevated post meiotic segregation (PMS) frequencies led to the discovery of MMR in *Saccharomyces cerevisiae* (Kramer et al., 1989; Williamson et al., 1985). Mutations in three loci the *PMS1*, *PMS2* and *PMS3* loci produced a mutator phenotype, with homozygotes displaying an elevated post meiotic segregation frequency and reduced spore viability.

Evidence for the recognition and repair of mismatches in yeast also emerged. By transformation of wild-type cells with covalently closed heteroduplex plasmid substrates it was shown that the yeast cells were able to repair single base substitution mismatches, a single base frameshift mismatch, and small insertion deletion heterologies (Bishop et al., 1989; Bishop and Kolodner, 1986; Grilley et al., 1990; Muster-Nassal and Kolodner, 1986). However mismatch correction was greatly reduced in the *pms1*, *pms2*, and *pms3*



mutant strains (Bishop et al., 1989; Bishop and Kolodner, 1986; Kramer et al., 1989; Muster-Nassal and Kolodner, 1986). Collectively these findings along with the observation that the *PMS1* gene product is homologous to the bacterial MutL protein provided strong evidence that these three genes encode components of a yeast MMR system and that this process in yeast also contributes to genetic stability (Kramer et al., 1989).

Mutations in the *PMS1* gene result in elevated rates of spontaneous mutation and increased levels of post meiotic segregation (Kramer et al., 1989). Additional yeast MMR genes were identified encoding proteins homologous to bacterial MutS, yMsh1p and yMsh2p and proteins homologous to bacterial MutL, yMlh1p (Prolla et al., 1994; Reenan and Kolodner, 1992). As in the case of *PMS1*, mutations in *MSH2* and *MLH1* affect nuclear mutation rates and PMS levels (Strand et al., 1993). yMsh1p was shown to play a role in mitochondrial DNA maintenance.

It was soon apparent that MMR is conserved from bacteria to humans. The crucial discovery in yeast of a mutator phenotype associated with increased instability of a poly(GT) tract, due to mutations in the MMR genes *PMS1*, *MLH1* and *MSH2* was paramount in the elucidation of phenotypes of human cells with deficient MMR (Strand et al., 1993).

### 1.1.3 DNA Mismatch Repair in Human Cells

Microsatellite instability (MSI) was observed in tumour DNA from a number of cancers at dinucleotide and trinucleotide repeat sequences (Thibodeau et al., 1993). The majority of tumours displaying this observed somatic instability, did so at numerous unrelated microsatellites. One study in particular demonstrated this MSI phenotype in tumour DNA from a familial colorectal cancer, hereditary nonpolyposis colorectal cancer (HNPCC) (Aaltonen et al., 1993). A significantly lower fraction of tumour DNA from sporadic CRC (colorectal cancer) examined showed this MSI phenotype. These studies suggested replication errors (RER) had occurred in these sequences during tumour development. Tumours with this associated replication error phenotype or microsatellite instability are designated RER<sup>+</sup> tumours. Genetic instability was therefore proposed to be a component of the familial cancer phenotype. These analogies to the yeast system led to the suggestion that microsatellite instability observed, for example in HNPCC tumour DNA, could be a

consequence of a mutation in a functional homologue of *PMS1*, *MLH1* or *MSH2* or another component of the MMR system.

HNPCC, a form of colorectal cancer, has been observed within familial groups. HNPCC kindred are defined as those in which at least three relatives in two generations have colorectal cancer, with one of these being diagnosed under the age of fifty (Lynch et al., 1985). Genetic linkage analysis was used to attempt to identify specific loci implicated in HNPCC families.

One gene localised to a HNPCC locus was a human MutS homologue *hMSH2* on chromosome 2 (Fishel et al., 1993; Leach et al., 1993). Mutations in this gene occurred in the germline of patients with RER<sup>+</sup> tumours, with or without classical HNPCC, and additional somatic alterations at this gene occurred in tumours. Results in these studies strongly suggested *hMSH2* mutations are responsible for HNPCC and the RER<sup>+</sup> phenotype apparent in these tumours.

Linkage analysis also provided evidence for strong linkage between a polymorphic marker on the short arm of chromosome 3 and the HNPCC disease locus (Lindblom et al., 1993; Peltomaki, 1994). Two studies identified three human *MutL* homologues, *hMLH1*, *hPMS1* and *hPMS2* (Bronner et al., 1994; Papadopoulos et al., 1994). The first gene was called *hMLH1* due to its high level of homology to the yeast *MLH1* gene. The second and third gene were more homologous to the yeast *PMS1* gene and were called *hPMS1* and *hPMS2*. *hMLH1* was mapped to chromosome 3, *hPMS1* to chromosome 2 and *hPMS2* to chromosome 7.

Mutations in *hMLH1* were identified in HNPCC families. Following the discovery of mutations in both *hMSH2* and *hMLH1* in HNPCC affected individuals, a proportion of HNPCC cases remained unaccounted for (Service, 1994). Mutation screening in HNPCC cases is a demanding process, because the predisposing mutations vary between kindreds and are often scattered in different positions in the genes. Approximately 70% of HNPCC cases have mutations in *hMLH1* or *hMSH2* and with roughly equal proportions in the two genes (Peltomaki and de la Chapelle, 1997). The other *MutL* homologues, *hPMS1* and *hPMS2*, represented good candidates to account for the remainder of HNPCC cases. The discovery of an interaction between yeast Pms1p and yMlh1p was also suggestive of a role for a *PMS1* human homologue in HNPCC (Prolla et al., 1994).



Mutations were identified in both *hPMS1* and *hPMS2* genes in HNPCC cases (Nicolaides et al., 1994). Microsatellite instability was also observed in the tumour cell DNA from patients with these mutations. The somatic alteration of the remaining allele, in a tumour from a patient with a germline *hPMS2* mutation, supports the idea that inactivation of both alleles of an MMR gene is required for tumour formation (Nicolaides et al., 1994).

The inactivation of either yeast *MutL* gene (*yMLH1* and *yPMS1*) or the yeast *MutS* gene (*yMSH2*) results in a mutator phenotype (Strand et al., 1993). Inactivation of any of the three human *MutL*-related genes can result in HNPCC due to a deficiency in MMR and examination of tumour DNA demonstrates a related MSI (RER<sup>+</sup>) phenotype.

#### **1.1.4 MutS and MutL Homologues Involved in Eukaryotic DNA Mismatch Repair.**

The discovery of inherited mutations in a number of human MMR genes in HNPCC, has led to extensive studies of both the yeast and human MMR system. These studies have led to the identification of a range of MMR proteins and some understanding of how they function in this process (see Figure 1 and Table 1).

DNA polymerase slippage during replication results in characteristic insertion-deletion loop-type (IDL) mismatched nucleotides. In the absence of a functional MMR system these mismatches persist and following rounds of replication MSI results. In the bacterial system it has been demonstrated that nucleotide mismatches are recognised and bound by the bacterial MutS protein (Modrich, 1989; Su and Modrich, 1986). It was therefore of interest to identify whether MutS homologues in yeast or human cells could also recognise and bind mismatched DNA.

##### **1.1.4.1 MutS Homologues in Eukaryotes**

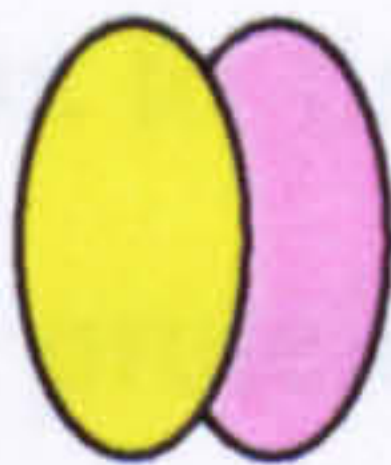
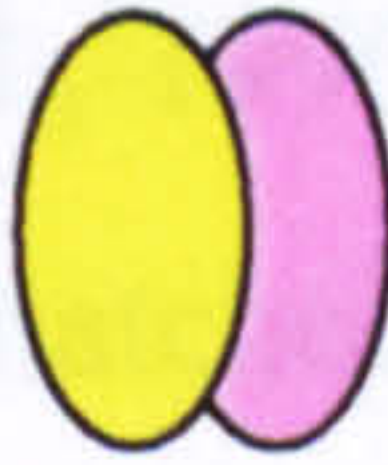
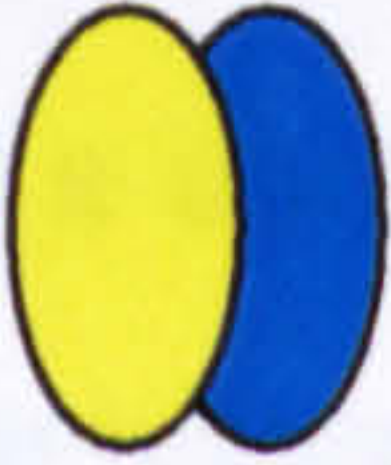
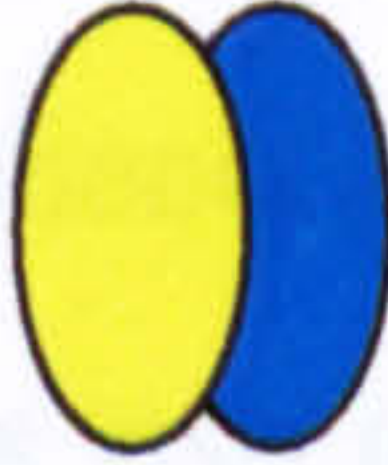
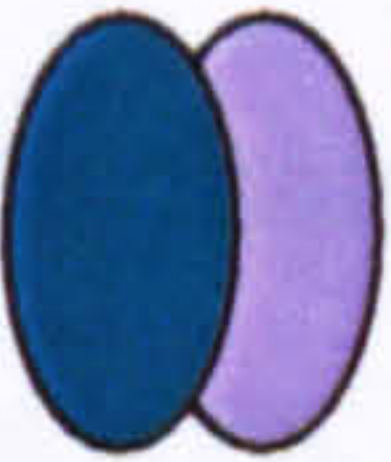
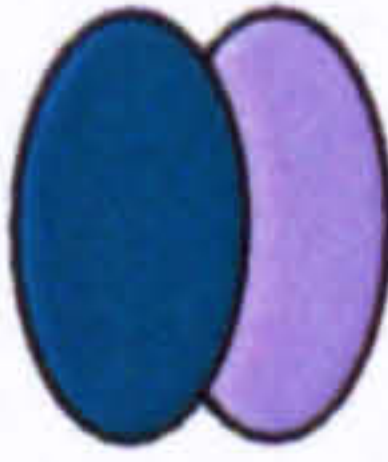
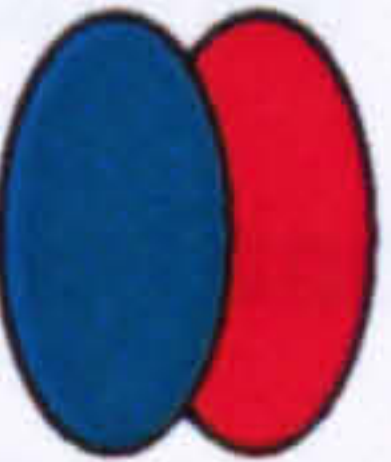
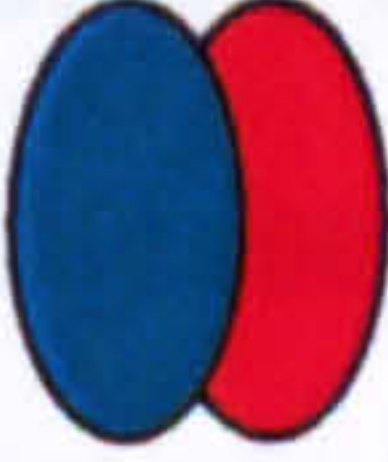
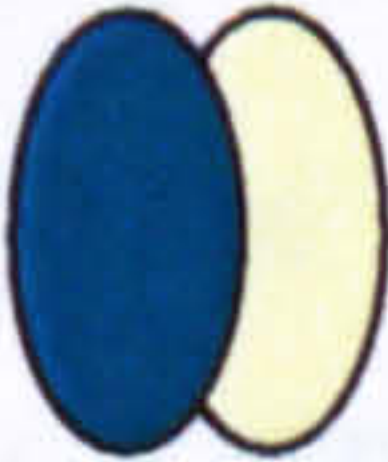
hMSH2 and its yeast homologue, yMsh2p, have also been shown to bind with high efficiency and specificity to single base pair mismatched nucleotides (Alani et al., 1995; Fishel et al., 1994; Fishel et al., 1994; Miret et al., 1993; Miret et al., 1996). Gel shift experiments with purified hMSH2 have also demonstrated binding to DNA containing IDL mismatches (Fishel et al., 1994). The specificity of this binding to the mismatch was visualised through electron microscopy. Further evidence for the role of hMSH2 in loop



<i>Escherichia coli</i>	<i>Saccharomyces cerevisiae</i>	<i>Homo sapiens</i>
MutS	yMsh2p	hMSH2
	yMsh6p	hMSH6
	yMsh3p	hMSH3
	yMsh1p	-
	yMsh4p	hMSH4
	yMsh5p	hMSH5
MutL	yMlh1p	hMLH1
	yPms1p	hPMS2
	yMlh3p	hPMS1
	yMlh2p	hMLH3
MutH	-	-
MutU	-	-

**Table 1: MMR Proteins Conserved from *Escherichia coli* to *Homo sapiens*.**

Yeast and human MutS and MutL homologues are listed. Human homologues are listed beside their yeast counterparts. hMLH3 is most closely related to yPMS1 and other non-mammalian MutL proteins.

 <div>yMutS<math>\alpha</math></div> <div>yMsh2p + yMsh6p</div>	 <div>hMutS<math>\alpha</math></div> <div>hMSH2 + hMSH6</div>
 <div>yMutS<math>\beta</math></div> <div>yMsh2p + yMsh3p</div>	 <div>hMutS<math>\beta</math></div> <div>hMSH2 + hMSH3</div>
 <div>yMutL<math>\alpha</math></div> <div>yMlh1p + yPms1p</div>	 <div>hMutL<math>\alpha</math></div> <div>hMLH1 + hPMS2p</div>
 <div>yMutL<math>\beta</math></div> <div>yMlh1p + yMlh3p</div>	 <div>hMutL<math>\beta</math></div> <div>hMLH1 + hPMS1</div>
	 <div>hMLH1 + hMLH3</div>

**Figure 1: List of the Yeast (y) and Human (h) MutS and MutL Complexes Formed by Yeast and Human MutS and MutL Homologous Proteins.**



repair and more specifically in mutation avoidance came from a study utilising an *in vitro* mismatch repair assay. Extracts from cells with normal *hMSH2* expression and from cells with deletions in both *hMSH2* alleles were tested in this assay and loss of repair for both loops and mismatches was demonstrated only in the MSH2 mutant lines (Umar et al., 1994).

*hMSH2* was shown to recognise and bind mismatched DNA, however the form of the protein active in MMR was not yet defined. The isolation of a hMutS protein complex from HeLa cells, that restores repair activity to the *hMSH2* defective LoVo cell line, gave some clues (Drummond et al., 1995). This complementing activity was named hMutS $\alpha$  and consists of two polypeptides of 105 kDa (*hMSH2*) and 160 kDa. Restoration of repair activity to another cell line with defective MMR, but no mutations in *hMSH2*, was also achieved, leading to the hypothesis that in this cell line the second polypeptide component of hMutS $\alpha$  must be mutated.

This polypeptide was identified as GTBP (for G/T binding protein) protein and is highly homologous to the MSH family of proteins (Acharya et al., 1996; Palombo et al., 1995). The GTBP protein complexes with *hMSH2* and binds mismatched DNA. The *GTBP* gene was also found to be mutated in a cell line which displayed no mismatch binding activity but in which neither *hMSH2* alleles were mutated. In addition *GTBP* was found to be mutated in a number of cell lines with an MSI (RER<sup>+</sup>) phenotype (Papadopoulos et al., 1995). Therefore suggesting that like *hMSH2*, GTBP is essential for maintaining the fidelity of the human genome. The GTBP protein was found to be homologous to yeast MSH6 and is therefore now referred to as *hMSH6*.

A third human MutS homologue was identified as a divergently transcribed gene next to the dihydrofolate reductase (*DHFR*) gene (Fujii and Shimada, 1989; Linton et al., 1989). Although the function of this gene was not immediately recognised, it was soon discovered to be a homologue of yeast *MSH3* and therefore named *hMSH3*. *hMSH2* also heterodimerises with *hMSH3* forming the hMutS $\beta$  complex (Palombo et al., 1996). This hMutS $\beta$  complex binds loops of one to four nucleotides. The discovery of this second hMutS complex gave some explanation of how cells lacking GTBP (*hMSH6*) are partially proficient in the repair of larger IDL mismatches. It also suggested that *hMSH3* and *hMSH6* are redundant in loop repair.



The role of MSH3 and MSH6 in MSH2 dependent mismatch repair was initially elucidated from genetic studies in the yeast *Saccharomyces cerevisiae*. Mutations in either *yMSH3* or *yMSH6* alone did not cause the extreme mutator phenotype seen in *ymsH2* mutants (Johnson et al., 1996; Strand et al., 1995). However when both genes together were mutated a comparable mutator phenotype to that seen in *ymsH2* mutants was observed. These observations support the suggestion that yMsh3p and yMsh6p have overlapping functions in the yMsh2p dependent repair pathway in yeast.

Another study examining the mutation rates in *ymsH2*, *ymsH3* and *ymsH6* mutants demonstrated the two pathways that exist in yeast for yMsh2p-dependent mismatch repair (Marsischky et al., 1996). One pathway consisting of yMsh2p and yMsh6p recognises single base mispairs and the second recognises insertion/deletion mispairs (IDLs) and requires either yMsh2p in combination with yMsh3p or with yMsh6p. This study also demonstrated the ability of yMsh2p to bind both yMsh3p and yMsh6p, suggesting that these two complexes may specifically bind mismatched DNA.

yMutS $\alpha$  was isolated in complex with mismatched DNA providing support for such a model (Iaccarino et al., 1996). The use of purified proteins, yMsh2p and yMsh6p, also clearly demonstrated the mismatch specific binding of this yMutS $\alpha$  complex, and suggest that the repair activity of the complex bound to the mismatch is dependent on its ability to use ATP hydrolysis to activate downstream steps of the repair pathway (Alani, 1996). Purification of the yMsh2p-yMsh3p heterodimer to near homogeneity allowed a demonstration of the binding activity of this complex as well (Habraken et al., 1996). This yMutS $\beta$  complex has low affinity for a G/T mismatch but binds insertion/deletion mismatches with high specificity.

hMSH2 also functionally interacts with hMSH3 and hMSH6 (Acharya et al., 1996). hMSH2 was able to associate with itself *in vitro* but at a lower efficiency than that seen with hMSH3 and hMSH6. hMSH2 is found in excess of these two proteins in proliferating cells and therefore it is possible that hMSH3 and hMSH6 are mostly if not wholly in complex with hMSH2. The excess hMSH2 may therefore form a homodimer complex active in other cellular functions. With gel shift experiments the hMSH2-hMSH3 complex was shown to recognise some +1 IDL and larger IDL substrates. The hMSH2-hMSH6 complex also recognised some +1 IDL substrates in addition to a G/T mismatch but failed to recognise the larger IDL substrates. Results from this study clearly suggest an overlapping mismatch recognition specificity of the two complexes.



Further evidence for the redundant roles of hMSH3 and hMSH6 in mismatch repair came from a study utilising the HHUA endometrial tumour cell line that is mutant in both *hMSH3* and *hMSH6* (Umar et al., 1998). These cells display elevated mutation rates at the *HPRT* locus and an MSI phenotype at a number of loci examined. However microcell-mediated transfer of chromosome 5 (*hMSH3*) or 2 (*hMSH6*) into these cells partially corrects the MSI phenotype seen in these cells and also the mutator phenotype at the *HPRT* locus. By using an *in vitro* mismatch repair system, the re-introduction of chromosome 2 (*hMSH6*) in the HHUA cell line was shown to restore repair activity to the cell line, specifically repair of single base mismatches or heteroduplexes containing up to four extra bases in one strand. An earlier study also showed that re-introduction of *hMSH3* on chromosome 5 can restore repair activity in these extracts for substrates with one to four extra nucleotide bases but not for base:base mismatches (Risinger et al., 1996).

Clearly hMSH2 acts with hMSH6 and to a lesser extent with hMSH3 to recognise and bind mismatched DNA at an early stage of the DNA mismatch repair pathway. hMutS $\alpha$  has previously been shown to be in excess of hMutS $\beta$  in proliferating cells (Drummond et al., 1997). 90% of nuclear hMSH2 has also been shown to be in complex with hMSH6 in mitotically active HeLa cells (Genschel et al., 1998). The binding specificities of the two complexes observed are in agreement with the information from genetic experiments that the hMutS $\alpha$  (hMSH2-hMSH6) complex preferentially recognises and binds base:base mismatches and short IDL substrates whereas the hMutS $\beta$  (hMSH2-hMSH3) complex recognises and binds specifically IDL substrates (Umar et al., 1998). There are clearly redundant roles for the two individual complexes in the recognition and binding of insertion/deletion loop substrates. In the absence of detectable hMSH2 polypeptide neither hMSH3 or hMSH6 polypeptides were detectable by western blot (Drummond et al., 1997; Genschel et al., 1998). These results suggest that perhaps hMSH2 regulates hMSH3 and hMSH6 levels, perhaps by stabilisation of the polypeptides through heterodimer formation.

The balance between the MutS $\alpha$  and MutS $\beta$  complexes is apparently crucial too. Overexpression of hMSH3 through amplification of the DHFR locus produced a significant change in the relative levels of these two complexes (Marra et al., 1998). *hMSH6* mRNA levels have been shown to be approximately four times as high as those of *hMSH3* (Chang et al., 2000). It is therefore not surprising that hMSH2 is found mainly in complex with hMSH6, and hMutS $\alpha$  is relatively abundant compared to low levels of hMutS $\beta$ . Overexpression of hMSH3 therefore results in an increased amount of hMutS $\beta$



being formed, which means that hMSH2 is sequestered mainly into this complex (Marra et al., 1998). This results in the degradation of hMSH6 which does not have a hMSH2 partner and hence depletion of hMutS $\alpha$  levels. Because of the different roles of these two complexes, a concomitant increase in the acquisition of transversions and transitions is seen. The effect of the change in balance between hMSH6 and hMSH3 levels therefore can clearly effect functional MMR.

Germline mutations in *hMSH6* have also been identified in a small number of HNPCC families (Akiyama et al., 1997; Miyaki et al., 1997). These tumours had reduced MSI compared to tumours with mutations in *hMSH2* or *hMLH1*. The recent discovery of mutations in *yMSH6* that can inactivate both yMutS $\alpha$  and yMutS $\beta$  dependent MMR, would suggest that similar germline mutations in *hMSH6* may be found in some HNPCC or sporadic cancers (Das Gupta and Kolodner, 2000). Such mutations would result in defective MMR, similar to that seen for *hmsH2* and *hmlh1* mutations, and could be responsible for MSI<sup>+</sup> HNPCC and sporadic cancers.

#### 1.1.4.2 MutL Homologues in Eukaryotes

The yeast *PMS1* gene product was found to be homologous to the bacterial MutL protein (Kramer et al., 1989). From this initial discovery it soon became clear that there are multiple homologues of MutL expressed in eukaryotic cells (designated PMS and MLH). The existence of multiple MutL homologues therefore mirrors the complexity seen in eukaryotic mismatch repair with respect to the MSH proteins

Isolation of yMlh1p another MutL homologue in *Saccharomyces cerevisiae* demonstrated that unlike the bacterial system two MutL like proteins are required for MMR (Prolla et al., 1994). The *ymlh1* mutant displayed a mutator phenotype, as well as an overall inefficient repair of heteroduplex DNA, generated during genetic recombination (elevated PMS).

The inactivation of either yeast *MutL* gene (*yMLH1* and *yPMS1*) resulted in a mutator phenotype similar to that seen in *ymsH2* mutants (Prolla et al., 1994). *ymlh1*, *ypms1* and *ymlh1 ypms1* double mutants displayed a similar phenotype. This would suggest that these two proteins are acting in the same DNA mismatch repair pathway.

In *Saccharomyces cerevisiae* genetic studies show mismatch repair requires bacterial MutS homologues yMsh2p, yMsh3p and yMsh6p and two bacterial MutL homologues yMlh1p



and yPms1p. In the *E.coli* MMR pathway, MutL forms a homodimer in solution, that interacts with MutS in the presence of heteroduplex DNA (Grilley et al., 1989). Therefore the physical interactions between yMsh2p, yPms1p and yMlh1p were examined (Prolla et al., 1994).

Initially a yeast two-hybrid system was used to examine *in vivo* interactions between these proteins. yMlh1p and yPms1p were shown to interact using this system. *In vitro* binding assays also demonstrated an interaction between yPms1p and yMlh1p but not between either of these two proteins and yMsh2p (Prolla et al., 1994).

In addition a gel retard assay was used to test the DNA binding properties of these three proteins (Prolla et al., 1994). A 108bp homoduplex and a heteroduplex containing a G-T mismatch were constructed for use in this assay. This assay was used to assess whether yMlh1p and yPms1p proteins interact with yMsh2p bound to heteroduplex DNA. The affinity of yMsh2p binding for a G-T containing heteroduplex was 10-20 times greater than for G-C containing homoduplex. Alone or together, both yMlh1p and yPms1p showed low affinity binding for either homoduplexes or heteroduplexes. However when all three proteins were mixed together, the characteristic yMsh2p-heteroduplex band shift was replaced by a lower mobility species. This suggested the presence of a protein-DNA complex of a higher molecular weight, requiring all three proteins to be present. The occurrence of this band shift was more prevalent in the presence of heteroduplex DNA.

yMLH1 and yPMS1 genes were over-expressed in yeast, and the yMlh1p-yPms1p complex that resulted purified (Habraken et al., 1997). The purified yMlh1p-yPms1p complex was examined for DNA mismatch recognition activity but no stable nucleoprotein complex formed. A yMsh2p-yMsh3p complex was also purified, which is known to bind specifically to DNA fragments containing extra-helical loops. Subsequently it was examined whether addition of yMlh1p-yPms1p would affect the mismatch recognition properties of this second complex. yMlh1p-yPms1p was discovered to specifically enhance the mismatch binding ability of yMsh2p-yMsh3p.

A second study utilising purified yMlh1p-yPms1p and yMsh2p-yMsh6p complexes demonstrated that these two MMR complexes could form a ternary complex on mismatched DNA (Habraken et al., 1998). The yMsh2p-yMsh6p complex had been previously shown to bind a DNA substrate with a G/T mismatch and also to IDL mismatches, with decreasing affinity as the size of the extra-helical loop increases (Alani,



1996; Iaccarino et al., 1996). Addition of ATP or a non-hydrolysable form of ATP led to a significant decrease in binding of this yMutS $\alpha$  complex to the mismatched substrate. Formation of this ternary complex between yMutS $\alpha$  and the yMlh1p-yPms1p complex, on a mismatch containing DNA substrate, however required ATP, which can also be substituted by a non hydrolysable form of ATP. It is proposed that ATP binding by the yMutS $\alpha$  complex induces a conformation allowing an interaction with the yMlh1p-yPms1p complex, which results in the formation of the ternary complex.

Two additional yeast MutL homologues, yMlh2p and yMlh3p, were identified from the *Saccharomyces cerevisiae* genome sequence project. A C-terminal yMlh3p fragment was also identified as interacting with yMlh1p from a yeast two-hybrid library screen with yMlh1p as bait (Pang et al., 1997). This interaction would suggest a role for yMlh3p in a yMlh1p-dependent reaction of some sort. *ymlh3* mutants displayed a mutator phenotype. *ymlh3 ymsh6* double mutants had a synergistic effect on the mutator phenotype observed whereas the *ymlh3 ymsh3* double mutants had the same mutation rates as seen in the single mutants. These observations would suggest that a proportion of the repair of specific IDL mismatches, by the yMsh3p-dependent MMR pathway, utilises a heterodimeric yMlh1p-yMlh3p complex instead of yMlh1p-yPms1p (Flores-Rozas and Kolodner, 1998).

Three separate yMlh1p based heterocomplexes are now known to form. Using yMlh1p as a bait in a yeast two-hybrid screen, interactions were identified between yMlh1p and three other MutL homologues yPms1p, yMlh2p and yMlh3p (Wang et al., 1999). These interactions were confirmed using co-immunoprecipitation experiments.

An interaction between hMLH1 and hPMS2 was discovered in human cells (Li and Modrich, 1995). The hypermutable H6 colorectal tumour cell line is defective in strand specific mismatch repair, and both alleles of the *hMLH1* gene are mutated. A specific activity was purified from HeLa cells that complements H6 nuclear extracts to restore repair efficiency to a set of heteroduplex DNAs. This activity fractionated as a single species and co-purified with proteins of 85 and 110 kDa. Sequencing allowed the identification of these two proteins to be hMLH1 and hPMS2. The 1:1 stoichiometry of the two polypeptides indicate they form a heterodimer, hMutL $\alpha$ .

A single individual from a family with a clear cancer predisposition was shown to carry a mutation in the human *mutL* homologue *hPMS1* gene (Nicolaidis et al., 1994). However this mutation could not be shown to segregate with the disease as clinical samples were not



available from the other family members. Therefore it is not clear whether this mutation is causative, but this discovery does suggest hPMS1 may play a role in MMR. Support for this hypothesis has come from the discovery that in yeast, yMlh3p, the closest *Saccharomyces cerevisiae* homologue of hPMS1, interacts with yMlh1p. Further evidence for such a role has come from the identification of the human MutL $\beta$  (hMutL $\beta$ ) heterodimer which consists of hMLH1 and hPMS1 in complex (Leung et al., 2000; Raschle et al., 1999). However unlike the hMutL $\alpha$  complex, the hMutL $\beta$  complex was unable to complement extracts of MMR deficient cell lines lacking hMLH1 in an *in vitro* MMR assay. Therefore a direct role for the hMutL $\beta$  complex in MMR is yet to be demonstrated.

Interestingly, although not proving a role for this hMutL $\beta$  complex in MMR, two complexes between hMutS $\alpha$  and hMutL $\alpha$  and between hMutS $\alpha$  and hMutL $\beta$  have been identified from HeLa nuclear extracts (Matton et al., 2000). The interactions between these complexes occurred without the addition of ATP and the complexes were shown to specifically bind to heteroduplex DNA but not to a similar homoduplex oligonucleotide. Upon addition of ATP the interaction between hMLH1 and hPMS2 and/or hPMS1 or with the hMutS $\alpha$  complex is no longer seen.

A new MMR protein, human MLH3, has recently been identified (Lipkin et al., 2000). This hMLH3 protein has been shown to be most closely related to yMlh3p protein and other non-mammalian MutL homologues. Similar to other MutL homologues the N-terminal of the protein has a putative ATPase domain and the C-terminal has a predicted hMLH1 interaction domain. The hMLH3 mRNA was found to be ubiquitously expressed. An interaction between hMLH1 and hMLH3 was demonstrated in a mammalian two-hybrid system. Expression of a dominant negative form of hMLH3 in cultured cells resulted in microsatellite instability.

How this protein functions in MMR is yet to be discovered. By analogy to the yMlh3p protein, hMLH3 could act in complex with hMLH1, and with the hMutS $\beta$  complex, play a role in the repair of IDL mismatches. MSI in MMR knockout mice is lower in PMS2<sup>-/-</sup> mice than in MLH1<sup>-/-</sup> mice, even though both mice lack the hMLH1/hPMS2 complex (Yao et al., 1999). hMLH3 could be responsible for this residual IDL repair activity.

Many HNPCC cancers and sporadic colon cancers have no identifiable mutations in MMR genes that have been implicated in these diseases previously. A group of patients



displaying characteristics of hereditary colorectal cancer but without any mutations identified in MMR genes were tested for germline mutations in *hMLH3* (Loukola et al., 2000). None were found. A group of MSI-positive tumours were also examined for somatic mutations in eight exonic mononucleotide repeats of *hMLH3*, to test for somatic inactivation as is seen for *hMSH3* and *hMSH6*, but none were seen. Therefore from this study *hMLH3* mutations and hence inactivation, does not seem to be selected for in MSI-positive tumours.

Little is known of the functional domains of MMR proteins. The yMlh1p and hPms1p protein domains required for them to interact with each other have been described (Pang et al., 1997). This study identified the yMlh1p-interactive domain of hPms1p as a 260 amino acid domain located at the carboxyl terminus of the protein. The hPms1p-interactive domain of yMlh1p is within its final 212 amino acids. These domains are sufficient for yMlh1p-hPms1p interactions. No interaction is seen when deletions are located within these domains. Some *ymlh1* and *ypms1* mutant alleles caused a dominant negative mutator effect when over-expressed. This observation was particularly important as mutations in homologues of these genes in humans have been shown to abolish functional MMR and have been implicated in HNPCC. This may occur in a dominant negative manner leading to early tumour development. For example expression of a mutant form of hPMS2 which can still bind hMLH1, could result in complexes between these two proteins that are not functional. This complex formation, by sequestering the majority of hMLH1 in the cell, may also result in the loss of interactions between hMLH1 and other proteins such as hPMS1 and hMLH3 which may play a role in MMR.

The interaction domains of the hMutL $\alpha$  complex components has also been examined. In hMLH1 the interaction region has been shown to lie between amino acids 506 and 675 and in hPMS2 between amino acids 675 and 850 (Guerrette et al., 1999). The majority of mutations associated with HNPCC examined in hMLH1 lead to a decreased ability to bind hPMS2. This finding confirms that functional deficiencies in the interaction of these two proteins are associated with HNPCC. Interestingly there have been no reports of single-amino acid substitutions in hPMS2 located within this interaction domain (Guerrette et al., 1999). Perhaps as in the hMutS system hMLH1 can function with, for example hPMS1, in MMR, such that hPMS1 has a redundant function with hPMS2.



### 1.1.4.3 How Does MutS Function?

In the bacterial system it has been demonstrated that nucleotide mismatches are recognised and bound by the bacterial MutS protein (Modrich, 1989; Su and Modrich, 1986). However the exact function and the dependence on ATP of this reaction remains a contentious issue. It is accepted that MutS recognises and binds heteroduplex DNA leading to downstream events resulting in repair of the heteroduplex.

MutS homologues (MSH) have all been identified on the basis that they carry a highly conserved region incorporating a Walker-A adenine nucleotide binding motif. This conserved region encompasses approximately 150 amino acid residues which includes a helix-turn-helix domain associated with the Walker A type adenine nucleotide binding motif and a Walker B type magnesium binding motif (Walker et al., 1982). This Walker-A box represents over 80% of identifiable homology between MutS homologues (Fishel and Wilson, 1997).

Both purified bacterial and yeast MutS homologues have been shown to possess an intrinsic low level ATPase activity (Alani et al., 1997; Chi and Kolodner, 1994; Haber and Walker, 1991). This ATPase function is likely to be important for MutS homologue function, as mutation of these conserved amino acid residues in the adenine nucleotide binding domain result in a dominant mutator phenotype in both bacteria and yeast (Alani et al., 1997; Haber and Walker, 1991). The precise function and dependence on ATP of the MutS complex is unclear. A range of studies with bacterial MutS and also eukaryotic MutS homologues have given some clues. A number of models have been proposed to explain how this complex functions.

In both bacteria and eukaryotes MutS homologues fail to form a specific complex with a mismatch containing oligonucleotide in the presence of ATP (Alani, 1996; Iaccarino et al., 1996; Marsischky and Kolodner, 1999). For example in a study utilising purified yeast proteins, yMutS $\alpha$  was shown to bind a G/T mismatch, but this ability was lost upon addition of ATP (Habraken et al., 1998). However formation of a ternary complex between yeast MutL $\alpha$  and MutS $\alpha$  was also demonstrated and requires the addition of ATP analogous to the observations in bacteria (Allen et al., 1997; Habraken et al., 1998). However ATP hydrolysis is not required. No such complex was formed in the presence of ADP and it has been previously shown that yMutS $\beta$  forming a ternary complex with yMutL $\alpha$  does not require ATP to be present.



It has been proposed by Modrich *et al* that MutS recognises and binds the heteroduplex, initiating downstream events, which results in repair of the mismatch and ATP-dependent translocation of MutS away from the site. This complex is proposed to dissociate from the DNA until it again encounters and binds another mismatch. *E.coli* MutS incubated with heteroduplex DNA has been shown to form  $\alpha$ -loop shaped structures, with the mismatch in most cases localised within the loop structure (Allen et al., 1997). This structure is only formed upon addition of ATP to the reaction, as visualised by electron microscopy. The MutS protein was found to localise most probably as a dimer at the base of the loop. MutL co-localised to the base of the loop with MutS. Once the loop had formed no ATP was required. MutL binding was shown to enhance the rate of loop formation and the loop size increased with time. These observations led to the proposal that ATP activates the formation of secondary DNA binding sites, used for the movement of the complex along the helix by a translocation mechanism, which would require ATP hydrolysis.

A second study supporting this model came from observations of nucleotide promoted release of human MutS $\alpha$  from heteroduplex DNA (Blackwell et al., 1998). In this experiment a blocked end heteroduplex substrate was utilised. Addition of ATP to these blocked end substrates in complex with hMutS $\alpha$  did not result in dissociation of hMutS $\alpha$  from these substrates. When ATP hydrolysis is prevented either by the omission of magnesium ions or by using a non-hydrolysable form of ATP, addition of ATP or these analogues still induces the dissociation of the complex from the heteroduplex. This would imply that hydrolysis of ATP by at least one subunit of the complex, must be necessary to allow resistance of the complex to ATP induced dissociation from the heteroduplex. Therefore a model was proposed whereby each component of hMutS $\alpha$  has two sites that function in the movement of the complex along the DNA. Movement of the complex along the DNA is restricted by the ATP or ADP occupancy of the hMutS $\alpha$  subunit nucleotide binding site. This study argues that ADP is not actually required for mismatch recognition but changes the dynamics of formation of complex versus dissociation of the complex from DNA.

The hMutS $\alpha$  complex (hMSH2-hMHS6) has been shown to have an intrinsic ATPase activity (Gradia et al., 1997). This along with the associated adenine nucleotide binding domain, function to regulate mismatch binding, and are proposed to act as a molecular switch (Fishel, 1998; Gradia et al., 1997; Gradia et al., 2000). Using purified hMSH2 and hMSH6 protein which form a stable complex, the intrinsic DNA-dependent ATPase

activity of this heterodimer was demonstrated and shown to require magnesium as a co-factor. It was also observed that this ATPase function was most active in the presence of a G/T mismatch.

In this study hMutS $\alpha$  was released upon addition of ATP to the reaction but the specific binding affinity of the complex for the mismatch was restored gradually upon addition of ADP (Gradia et al., 1997). ATP hydrolysis by the hMutS $\alpha$  complex also resulted in the recovery of the binding activity of the complex. Binding of the hMutS $\alpha$  complex to a G/T mismatch was found to stimulate the exchange of ADP $\rightarrow$ ATP resulting in release from the mismatch and recycling of the protein complex.

The molecular switch model proposes that this switch is 'ON' when the complex is in an ADP bound form and is able to bind the mismatch and 'OFF' when in an ATP bound form which prevents mismatch binding (Gradia et al., 1997). How exactly this switch model signals downstream repair events is not known. Two possibilities were proposed. The first proposes that when hMutS $\alpha$  is in its active ADP bound form and binds the mismatched DNA, this triggers the assembly of the required repair machinery in close proximity to the mismatch, followed by ADP $\rightarrow$ ATP exchange leading to release of the hMutS $\alpha$  complex from the DNA. The latter proposes that mismatch recognition and binding triggers ADP $\rightarrow$ ATP exchange, which results in hydrolysis independent, DNA associated diffusion of the complex away from the mismatch, concomitant with assembly of the repair components, which upon binding by the hMutS $\alpha$  complex engages repair. In both cases the common end point is the release of the hMutS $\alpha$  complex which hydrolyses the ATP and therefore the complex is recycled.

As in the study by Modrich and colleagues (Blackwell et al., 1998) this study has demonstrated the requirement for magnesium ions for the formation of a stable complex of hMutS $\alpha$  on heteroduplex DNA in the presence of ATP (Gradia et al., 1999). Dissociation induced by the addition of a poorly hydrolysable form of ATP in the absence of magnesium would suggest that specifically magnesium and not ATP hydrolysis is required for formation of this stable complex. It was also shown that ADP $\rightarrow$ ATP exchange is normal where these blocked end substrates are utilised. However the hMutS $\alpha$  ATPase levels are reduced to levels seen in the absence of DNA. This strengthens the molecular switch model whereby MutS $\alpha$  binds the mismatch, which leads to ADP $\rightarrow$ ATP exchange, but because this complex is blocked in an ATP bound form due to the blocked ends of the



heteroduplex substrate, no dissociation and reduced ATP hydrolysis are observed, as would be seen if the ends were free.

A circular heteroduplex substrate was also utilised to examine these effects (Gradia et al., 1997). With this substrate the same binding patterns are seen. Upon addition of ATP however a number of more slowly migrating species were seen by gel shift assays suggesting a number of complexes bound to the substrate. Electron microscopy (EM) showed a number of MutS $\alpha$  protomers bound on the DNA upon addition of ATP. Upon addition of ATP this group proposes MutS $\alpha$  forms a clamp on the DNA. When the circular DNA substrate is nicked by addition of an endonuclease i.e. producing a free end, dissociation of the complex from the DNA is seen. It is proposed that this is by diffusion as translocation would require ATP hydrolysis which has been shown to be decreased. Also a number of forms of the complex bound to DNA were observed by EM. The majority of which were either pac-man ('C') shaped (open) or doughnut shaped (closed). By EM it is shown that addition of ATP to hMutS $\alpha$  produces a mixture of this closed and open forms but addition of a poorly hydrolysable form of ATP produces mostly closed forms. Addition of the circular heteroduplex DNA produces no binding of the heteroduplex in the presence of the non-hydrolysable form of ATP which this group argues gives further credence to the molecular switch model.

hMutS $\beta$  was also demonstrated by this group to dissociate from DNA upon addition of ATP, an effect which does not require ATP hydrolysis (Wilson et al., 1999). Limited proteolysis also demonstrated conformational changes induced by binding of the complex to ATP and ADP. This supports the idea that conformational transitions are associated with adenosine nucleotide binding.

To attempt to define the role of the ATP binding domains conserved in MutS homologues, a number of studies were carried out utilising mutated purified proteins, with a range of mutations, which are still capable of forming stable MutS $\alpha$  complexes. Yeast Msh2p and yMsh6p proteins with mutations in the conserved C-terminal region, which includes a helix-turn-helix (HTH) domain and an ATP binding motif, were produced (Alani et al., 1997). From experiments with these purified mutant yMutS $\alpha$  complexes, it was proposed that both the ATP binding and hydrolysis modulation of mismatch recognition, and the interaction between the two sub-units of the yMutS $\alpha$  complex, is crucial for the ATPase role of the complex. Also ATP binding, ATP hydrolysis or both resulted in a



conformational change in the complex which was mediated through a domain that is clearly important for yMsh2p-yMsh6p interactions.

Yeast *msh2* and *yms6* mutants were made by mutating a conserved phosphate loop (p-loop) which is also conserved in purine nucleotide binding proteins and predicted from studies with prokaryote MutS protein to disrupt ATP binding or ATP hydrolysis or both (Studamire et al., 1998). Studies with these mutants in yMutS $\alpha$  complexes have demonstrated the requirement for both sub-units through their ATP binding domains in mediating ATP-dependent conformational changes in the complex. Upon addition of ATP to these complexes bound to heteroduplex DNA, no dissociation of the mutant yMutS $\alpha$  complexes is seen. These observations would support an equivalent role for the two sub-units of the yMutS $\alpha$  in ATP dependent release from the mismatch site.

yMutS $\alpha$  with these mutations in yMsh6p has a lower ATPase activity than with a mutated yMsh2p. Apparently only the yMsh6p ATPase is responsive to the presence of heteroduplex substrate. An analogous study with the purified human proteins utilised a lysine to arginine mutation in yMsh2p and yMsh6p (Iaccarino et al., 1998). Mutation of this conserved residue in the *Salmonella typhimurium* MutS homologue inactivated the ATP binding but not the ATPase activity of the protein. UV crosslinking experiments with the hMutS $\alpha$  complex with a heteroduplex substrate showed that hMSH2, hMSH6 and DNA were present in the complex but that hMSH6 alone was covalently bound to the mismatch. Mutation of hMSH6 led to a decreased ATPase activity of the hMutS $\alpha$  complex, whereas mutation of the hMSH2 protein had little effect on the ATPase activity of the complex. These results along with those in the yeast study would suggest a role for the MSH6 protein in steps prior to the proposed release of the complexes from the mismatch site

As shown in the study by Gradia *et al* (Gradia et al., 1999) ATP binding and not hydrolysis is shown to be required for the dissociation of hMutS $\alpha$  from DNA. The lysine to arginine mutation analogous to the yeast mutation described by Studamire *et al* (Studamire et al., 1998) in the hMutS $\alpha$  components also led to severe attenuation of the ATP-dependent dissociation of these complexes from heteroduplex oligonucleotides. This must be due to a reduced affinity of the mutant proteins for ATP. Contrary to expectations based upon the results with the *Salmonella* mutants this mutation apparently effects ATP binding of hMutS $\alpha$ . The addition of the double mutant hMutS $\alpha$  complex to cell extracts proficient for MMR led to a decreased ability of these extracts for MMR using an *in vitro*



MMR assay. Overexpression of the mutant yeast proteins in wild type yeast also produced a dominant negative phenotype (Studamire et al., 1998).

Overall the combination of the two studies with mutant yeast and human MutS $\alpha$  would suggest that the ATPase activity is not required for mismatch binding but for mismatch correction and that ATP binding is required for displacement of MutS $\alpha$  from mismatched DNA.

Another mutation utilised was seen in an MMR deficient cell line MT1 which has an aspartate (D) $\rightarrow$ valine (V) mutation in the Walker B motif of hMSH6 (Iaccarino et al., 2000). This Walker B motif has been seen in GTPases to play a role in co-ordinating divalent metal ions into the catalytic site of the GTPases with Walker A and B motifs. The D $\rightarrow$ V *msh6* mutant in complex with hMSH2 showed the same binding and specificity for heteroduplex DNA. ATP induced dissociation of this hMutS $\alpha$  complex from heteroduplex was also still seen and at even lower concentrations of ATP than seen for the wild type complex. Where the ends of the heteroduplex were blocked, and this mutant hMutS $\alpha$  complex bound to the heteroduplex, addition of ATP in the presence or absence of magnesium led to dissociation of the complex from the DNA. This has led to the proposal of two different methods of dissociation of the complex from DNA. The first method is dissociation by translocation where hMutS $\alpha$  requires the occupancy of both the Walker A and B sites by ATP and magnesium ions respectively. The second method is by direct dissociation as in the case where the ends of the oligo are blocked and this requires only nucleotide binding. Limited proteolysis experiments also demonstrated two distinct conformations of hMutS $\alpha$ , 'dissociation prone' and 'translocation prone'. The latter only apparent where both ATP and magnesium or manganese are present.

Another yeast mutant examined utilised a phenylalanine (F) to alanine (A) mutation in yMsh6p (Bowers et al., 1999). The mutation of this conserved residue F $\rightarrow$ A in MutS from *Thermus aquaticus* had been shown to terminate the crosslinking of MutS to DNA. The F $\rightarrow$ A mutation in yMsh6p and the over-expression of this mutated protein in a wild type yeast background gave a dominant negative phenotype in yeast. It also led to defective MMR by the wild type yMsh2p-yMsh3p and yMsh2p-yMsh6p complexes probably through sequestering yMsh2p. Both crosslinking and gel shift experiments demonstrated that this *msh6* mutant protein could not bind a DNA heteroduplex in a stable manner.

An analogous F→A N-terminus mutation to that described by Bowers *et al* was utilised in hMSH6 and hMSH2 (Dufner et al., 2000). Loss of mismatch binding was observed only for the *msh6* and double mutants with loss of the binding affinity for either homoduplex or heteroduplex. The *msh6* mutant complex has a normal ATPase activity but upon addition of homo or heteroduplex DNA the ATPase activity of the *msh6* mutant unlike that of the *msh2* or wild type complex does not increase. Neither of these two mutants can restore MMR activity to MMR deficient extracts. In yeast the double mutant can still be crosslinked to DNA, but unlike the human protein which cannot, it has a second conserved phenylalanine closeby.

The structure of co-crystals of *Thermus aquaticus* MutS proteins with DNA shows this phenylalanine residue is located on an alpha helix which contacts DNA in the vicinity of the structural distortion and the phenylalanine residue partially intercalates into the DNA at the site of the mismatch (Obmolova et al., 2000). This explains the key role of the residue in mismatch recognition. Again only one subunit contacts the substrate DNA via its N-terminal alpha-helix while the equivalent alpha helix of the second subunit is turned away from the DNA. Also in the absence of DNA, much of the protein is unstructured, but mismatch recognition induces formation of a dimeric clamp on the substrate.

The way in which MutS functions remains controversial, yet is key to understanding the MMR pathway. In summary, Modrich *et al* suggest MutS complexes act to recognise and bind heteroduplex DNA and subsequently signal downstream events, leading to repair of the mismatch and ATP-hydrolysis dependent translocation of the complex away from the mismatch (2000; Allen et al., 1997; Blackwell et al., 1998). Fishel *et al* argue against this model suggesting instead a ‘sliding clamp’ model, whereby a mismatch provokes ADP→ATP exchange within the MutS complex. Binding of ATP by this complex is proposed to induce a conformational change in the MutS complex, followed by translocation in an ATP-hydrolysis-independent manner away from this mismatch and signals to the repair machinery (Fishel, 1998; Gradia et al., 1997; Gradia et al., 2000; Gradia et al., 1999).

Recent crystal structure studies of prokaryote MutS complexes propose yet another alternative model for how MutS functions (Lamers et al., 2000; Obmolova et al., 2000). A model proposed from the results of the elucidation of the crystal structure of *Thermus aquaticus* (TAQ) argue against the ‘translocation model’. Structural observations suggest that the MutS dimer proposed to facilitate this ‘translocation model’ actually represents a



tetramer which is not believed to be the functionally relevant form of MutS and which does not occur often under physiological conditions. Biochemical and crystal structure studies both suggest that in fact it is a MutS dimer that recognises a mismatch. These crystal structure studies also argue against the 'sliding clamp' model. This model proposed a conformational change induced through ADP→ATP exchange. However the mobility of a number of domains of MutS increases when the complex is not bound to DNA which is proposed therefore to make interpretation of conformational changes difficult. Also the MutS complex is believed to be tightly bound to the heteroduplex, arguing against sliding.

The crystal structure studies suggest that MutS needs to remain bound to a mismatch site to invoke MMR. It is proposed that this complex is unable to retain the mismatch-bound conformation upon leaving the mismatch. Both studies propose that stabilisation of MutS binding to DNA by addition of MutL is facilitated by binding of MutL to a specific domain of the MutS protein (Lamers et al., 2000; Obmolova et al., 2000). They also propose an allosteric interaction between the distant ATP- and DNA-binding sites on MutS which acts as a 'transmitter' of information between the two sites.

Overall the precise function and ATP-dependence of the MutS complex remains unclear. However with further study a more unified model may emerge providing a fuller understanding of how MutS functions.

#### 1.1.4.4 How Does MutL Function?

The *E.coli* *MutL* gene product MutL was isolated, purified and shown to exist in solution as a homodimer (Grilley et al., 1989). This MutL protein was able to complement the defective MMR activity of MutL deficient cell extracts in an *in vitro* MMR assay. The MutS protein as described earlier, has been shown to bind DNA in the vicinity of mismatched DNA (Su et al., 1988; Su and Modrich, 1986). Using DNAase I protection experiments the level of protection of the mismatch containing DNA was shown to decrease significantly in the presence of ATP or ATP $\gamma$ S. In this study, only in the presence of ATP, the MutL complex has been demonstrated to interact with these MutS-heteroduplex complexes (Grilley et al., 1989).

Members of the MutL family have been found to have a conserved region of about 300bp at the N-terminus of the protein and more diverse C-termini. Most mutations producing a dominant mutator phenotype in *E.coli* MutL as well as greater than 50% of missense



mutations in the human homologue hMLH1 of HNPCC patients are found within this N-terminus. The *E.coli* MutL protein is cleaved by thrombin into an N-terminal 40 kDa (LN-40) and a C-terminal 30Kd fragment (LC30) (Ban and Yang, 1998). The LN40 fragment contains the conserved residues of the MutL family. Unlike MutL, LN40 is monomeric in solution which is consistent with reports that the C-terminal is required for dimerisation of MutL and its eukaryotic homologues. In an *in vitro* assay for *E.coli* MutH activity, LN40 is able to induce MutH activity in the presence of MutS and DNA heteroduplex with a GATC site, in place of MutL (Ban and Yang, 1998).

MutL has also been shown to be a member of an emergent ATPase, the GHL family (Bergerat et al., 1997; Dutta and Inouye, 2000). Members of the family are identified by their conserved ATP-binding domains, characterised by three conserved sequence motifs, and this family includes DNA gyrase, HSP90, type II topoisomerases and MutL. The crystal structure of the LN40 fragment was elucidated and found to be homologous to the structure of the ATPase of DNA gyrase (Ban and Yang, 1998).

Both MutL and LN40 have been shown to bind ATP. MutL is also demonstrated to have ATPase activity. However the LN40 fragment does not have this ATPase activity which would suggest that the C-terminal half of MutL plays a role in this function, possibly through dimerisation of the protein. Site directed mutagenesis of MutL, based on homology to other members of this superfamily, of regions required for their ATPase function have eliminated the ATPase function of MutL (Ban and Yang, 1998). From this study it is proposed that ATP binding but not hydrolysis is required for activation of MutH by MutL but that ATP hydrolysis is required for mismatch dependent activation of MutH by MutS and MutL. ATP binding by the N-terminus of MutL also induced conformational changes in the protein.

A further study with this N-terminal LN40 fragment of MutL has given further clues to how it functions (Ban et al., 1999). Crystal structures of this fragment in complex with ADP demonstrated that LN40 does have a weak ATP hydrolysis activity. Pure LN40 had been incubated with ATP but as ADP was discovered in the final refined crystal, hydrolysis must have occurred. Also crystals of ADPnP complexed with LN40 were made. Studies with these crystals demonstrated a change in MutL conformation upon binding ATP, which together with the weak ATPase activity (stimulated by DNA) of this protein, are hypothesised to act as a switch involved in co-ordinating DNA mismatch repair.



Functional requirements for this ATPase activity of MutL were identified (Spampinato and Modrich, 2000). A mutation in the N-terminal conserved nucleotide binding motif had previously been shown to produce a dominant negative phenotype in *E.coli*. Using *in vitro* assays this mutant was shown to be defective in the ATPase activities, and the MutS and MutL dependent activation of the MutH endonuclease, seen with the wild type MutL protein. The dominant negative effect of the mutation results from inhibition of the MutL mediated signal from MutS mismatch recognition to the MutH endonuclease activation through the formation of inactive ternary complexes with the mutant protein.

The requirement for this ATPase activity in eukaryotes was elucidated through the use of mutant yeast MutL $\alpha$  components. The importance of the N-termini of MutL homologues in yeast have been previously demonstrated (Pang et al., 1997). Two sets of mutations of conserved residues of the nucleotide binding motif in the yMlh1p and yPms1p were made which eliminated ATP binding and ATP hydrolysis in the MutL protein respectively (Tran and Liskay, 2000). The double hydrolysis and ATP binding mutants gave the same mutator phenotype observed for a double *ymlh1<sup>-</sup> ypms1<sup>-</sup>* mutant suggesting that these mutations eliminated functional MMR. The individual ATP binding mutants had a stronger effect on mutation avoidance than the individual ATP hydrolysis mutants.

Limited proteolysis experiments were utilised to demonstrate that the amino terminus of yMlh1p in yMutL $\alpha$  undergoes an ATP-binding-dependent conformational change (Tran and Liskay, 2000). Yeast two-hybrid analysis has previously shown that the N-termini of yMlh1p and yPms1p cannot interact (Pang et al., 1997). Mutations of the ATP binding motif have been identified in other GHL family members which eliminate the ATP hydrolysis function of the protein but not the ATP binding function. Based on the idea that hydrolysis mutations in the yeast MutL homologues may act to prolong the double ATP-bound state *in vivo*, and allow interaction of the N-termini of the two proteins, a yeast two-hybrid system was utilised to examine the interactions of mutant N-terminal fragments of yMlh1p and yPms1p (Tran and Liskay, 2000). These N-terminal mutant fragments clearly interacted in this system. However by repeating this assay with either of the two proteins expressing a mutant ATP binding domain in addition to the double ATPase mutations no interaction between the two proteins was observed. These observations suggest that the ATP binding function of both proteins but not hydrolysis is required for this interaction between the amino termini of the two proteins.



### 1.1.5 Other Proteins Involved in MMR.

In *E.coli*, MutL acts to mediate an interaction between the site of the mismatch bound by MutS and the d(GATC) sequence cleaved by the endonuclease MutH. MutL is known to activate this endonuclease activity of MutH and the helicase activity of UvrD (MutU) (Hall et al., 1998; Hall and Matson, 1999; Yamaguchi et al., 1998). MutL and its eukaryotic homologues are also known to enhance the binding of MutS proteins to DNA (Drotschmann et al., 1998; Habraken et al., 1997). Little else is understood of the function of MutL.

In the eukaryotic MMR system there is some evidence to suggest that a single-strand nick either 3' or 5' to the mispair determines strand discrimination in eukaryotic cells (Holmes et al., 1990; Thomas et al., 1991). Eukaryotic cells do not have the d(GATC) methylation signal seen in *E.coli* nor has there been a MutH homologue identified. As eukaryotic MMR proteins act in complexes, a number of groups searched for proteins interacting with known MMR proteins, with the aim of identifying potentially novel proteins involved in this repair pathway. For example no MutH homologue has been identified in eukaryotic cells. Therefore using MutL (known to interact with MutH) homologues for interaction studies could potentially lead to the discovery of a MutH homologue in eukaryotic cells.

*Saccharomyces cerevisiae* Exo1p has been shown to interact with yMsh2p protein in a yeast two-hybrid study (Tishkoff et al., 1997). This is a homologue of *Saccharomyces pombe* Exo1p, which is a double-stranded DNA specific 5'→3' exonuclease. *Saccharomyces cerevisiae* *exo1* mutant strain had a moderate mutator phenotype. Epistasis experiments with an *exo1* mutant and mutations in *yMSH2*, *yMLH1* and *yPMS1* showed that these genes encode products that act in the same pathway, as no difference was seen in the mutation rate of the double mutants compared to the single *exo1* mutants. These observations would suggest a possible role for hEXO1 in MMR. An interaction between hMSH2 and hEXO1 a human homologue has also been demonstrated (Schmutte et al., 1998).

In *E.coli*, a number of exonucleases (ExoI, ExoVII or RecJ) act with the helicase UvrD in the excision step of bacterial MMR. In yeast a number of candidate exonucleases have been suggested to act in this pathway, including yExo1p. A second exonuclease proposed to function in this pathway is the *Saccharomyces cerevisiae* Rad27p (FEN1 in humans) 5'→3' exonuclease and flap endonuclease, known to play a role in processing Okazaki



fragments. Once again mutations in *RAD27* produce a moderate mutator phenotype (Johnson et al., 1995; Tishkoff et al., 1997). Mutations in both genes, i.e. *rad27 exo1* double mutations are lethal (Tishkoff et al., 1997). These genetic results with *RAD27* and *EXO1* support the possibility that they act as redundant 5'→3' endo/exonucleases in the MMR pathway. No 3'→5' exonucleases have yet been implicated in eukaryotic MMR.

Proliferating cell nuclear antigen (PCNA) plays a role in both replication and repair. PCNA, encoded by the *POL30* gene, is a homotrimeric sliding clamp which is loaded onto primer-template junctions in an ATP-dependent manner by the RFC (replication factor C) complex (Kelman, 1997; Lee and Hurwitz, 1990; Tsurimoto and Stillman, 1990). PCNA acts as a processivity factor for both DNA polymerase  $\delta$  and  $\epsilon$ . It acts to harness the catalytic unit of the polymerase to the DNA template allowing rapid and processive DNA synthesis (Kelman, 1997). PCNA has been shown to interact with a number of proteins involved in replication and repair including, the CDK inhibitor p21, a protein which can block entry into S-phase of the cell cycle and hence replication. This role of p21 is believed to be partially facilitated through its interaction with PCNA (Flores-Rozas et al., 1994; Kelman, 1997). Using an *in vitro* repair system the selective effect of p21 on PCNA was demonstrated (Li et al., 1994). p21 can arrest DNA replication while permitting active DNA repair. This supports the proposal that DNA damage leads to inactivation of chromosomal replication, but allows damage repair. PCNA has also been shown to interact with and activate FEN1 (Li et al., 1995). However p21 inhibits binding of FEN1 to PCNA (Chen et al., 1996; Gomes and Burgers, 2000). Whether p21 inhibits PCNA-dependent FEN1 activity is yet to be established.

A yeast two-hybrid library screen identified interactions between yPcnap and the two yeast MMR proteins yMlh1p and yMsh2p (Umar et al., 1996). A yeast strain with a *PCNA* mutation has an elevated mutation rate at a dinucleotide repeat. The mutation rate at this repeat did not increase further upon introduction of mutations into *yMLH1*, suggesting the two proteins act in the same pathway. These observations were supported by epistasis experiments with another *pcna* mutant and mutations in *yMSH2*, *yMLH1* and *yPMS1* (Johnson et al., 1996). The requirement for PCNA in MMR was demonstrated more directly utilising an *in vitro* MMR assay that does not require DNA repair synthesis. Upon addition of p21 protein, known to bind and inhibit PCNA activity, MMR activity as measured by this assay was inhibited. However on the introduction of excess PCNA, MMR activity was restored.



Human MSH2, hMLH1 and hPMS2 have been shown to form a ternary complex on heteroduplex DNA (Gu et al., 1998). Formation of this complex requires the addition of exogenous ATP. hPCNA was also found to specifically co-immunoprecipitate with this complex (Gu et al., 1998). In agreement with earlier studies this group found hPCNA to be required for repair initiation but also in the repair DNA re-synthesis step. Affinity binding studies using purified proteins demonstrated the ability of yPcnap to bind the yMsh3p-yMsh2p complex but not with yMsh2p alone (Johnson et al., 1996). As yMsh2p heterodimerises with yMsh3p or yMsh6p and acts in these complexes in MMR this result supports a role for yPcnap in MMR. hPCNA has also been shown to bind a number of proteins involved in replication and repair such as FEN1, UDG, XPG, p21 and DNA ligase 1 (Kelman, 1997). These proteins share a conserved amino acid motif. This consensus sequence has recently been identified in the N-terminal of both MSH3 and MSH6 proteins in yeast and humans but not in MSH2 (Clark et al., 2000). Human PCNA has been shown using GST pull-down assays to bind short peptide sequences with this consensus sequence from hMSH3, hMSH6, yMsh3p and yMsh6p. An interaction between yPcnap and the yMsh2p-yMsh6p complex was also observed. Introduction of mutations into this consensus binding sequence in yMsh3p and yMsh6p generated a mutator phenotype in these strains. These observations suggest that these interactions between PCNA and MSH3 and MSH6 play a role in maintaining genomic stability. Further support came from *in vitro* MMR assays, where N-terminal MSH3 and MSH6 peptides with this consensus binding sequence were added to the reaction, and inhibited the repair process at a point prior to DNA re-synthesis. These proteins are predicted to compete with the native MSH3 and MSH6 proteins for binding to PCNA, preventing functional repair.

Most recently a complex formed between yPcnap and the yMsh2p-yMsh6p complex has been identified and implicated in playing a role in mismatch recognition (Flores-Rozas et al., 2000). This interaction was found to be mediated through a specific binding site on yMsh6p. Mutation of this site results in defective binding to yPcnap and also produces a mutator phenotype. Specific binding of the yMutS $\alpha$  complex to mismatched DNA was demonstrated upon addition of yPcnap. It has been proposed from these studies that yPcnap plays a role in mispair recognition with yMutS $\alpha$ .

With the aim of identifying novel eukaryotic MMR genes, another yeast two-hybrid screen was utilised to identify proteins interacting with the hMLH1 protein (Bellacosa et al., 1999). From this screen, hMLH1 was shown to interact with the newly cloned MED1 (methyl-CpG binding endonuclease 1) protein. MED1 (or MBD4) structure was predicted



from the protein sequence to include an amino terminal methyl-CpG-binding domain (MBD), a carboxy-terminal catalytic domain with homology to bacterial DNA damage-specific glycosylase/lyases, and a central region with five putative nuclear localisation signals. In this study, the MED1 protein was shown to be capable of binding both hemimethylated and fully methylated DNA and also to have a weak endonuclease activity. Transfection of an MMR proficient cell line with a mutant form of *MED1* (with a deleted MBD domain,  $\Delta$ MBD) which acts in a dominant negative manner produced an MSI (microsatellite instability) phenotype in these cells. This would suggest MED1 plays a role in DNA repair.

MSI is detected in HNPCC tumours as well as in many sporadic tumours. The MSI phenotype (RER<sup>+</sup>) is associated with loss of functional MMR. The *MED1* coding sequences contains four potential hypermutable tracts. A number of studies have identified mutations in the polyadenine microsatellites in the central region of the coding sequence of *MED1* in human endometrial, colon and pancreatic carcinomas defective for the MMR genes *hMLH1* and *hMSH2* (Bader et al., 1999; Riccio et al., 1999). These mutations are predicted to result in the synthesis of truncated proteins lacking the C-terminal catalytic domain.

Further studies with the MED1 protein have disputed the weak endonuclease activity of this protein and instead shown it to be a mismatch specific T/U DNA glycosylase (Hendrich et al., 1999; Petronzelli et al., 2000). Cytosine methylation in a CpG context is a common cause of mutations. This mutability arises through hydrolytic deamination of cytosine. Cytosine deamination produces a guanine-uracil mismatch whereas deamination of methylated (m<sup>5</sup>) cytosine produces a guanine-thymine mismatch. The mismatched uracil can be removed by uracil glycosylase. The latter mismatches, m<sup>5</sup>CpG.TpG mismatches may be corrected by a second enzyme, thymine DNA glycosylase, TDG. MED1 has been shown *in vitro* to remove both thymine or uracil from a mismatched CpG site (Hendrich et al., 1999; Petronzelli et al., 2000). The MED1 protein like a number of other DNA glycosylases, such as TDG and the adenine glycosylase have been shown to exhibit single turnover. That is each active enzyme molecule is proposed to remove one mismatched thymine and/or adenine from mismatch containing substrates (Petronzelli et al., 2000). They also exhibit pre-steady state kinetics due to the fact that they tightly bind the apurinic/apyrimidinic (AP) site and slowly release the reaction product (Petronzelli et al., 2000). This tight binding is believed to allow protection of the AP site from non-specific processing until repair activities are recruited to the lesion.

Although the MBD domain is not required for removal of these thymine or uracil bases by MED1, it is proposed that it may act to direct the protein to m<sup>5</sup>CpG rich regions of the genome. These are sites where deamination of m<sup>5</sup>C may be more frequent. Hence providing MED1 with a more distinct function in the repair of G·T mismatches compared to TDG which does not have such an MBD domain. Some of the proteins involved in MMR are described in a schematic of the MMR pathway (see Figure 2).

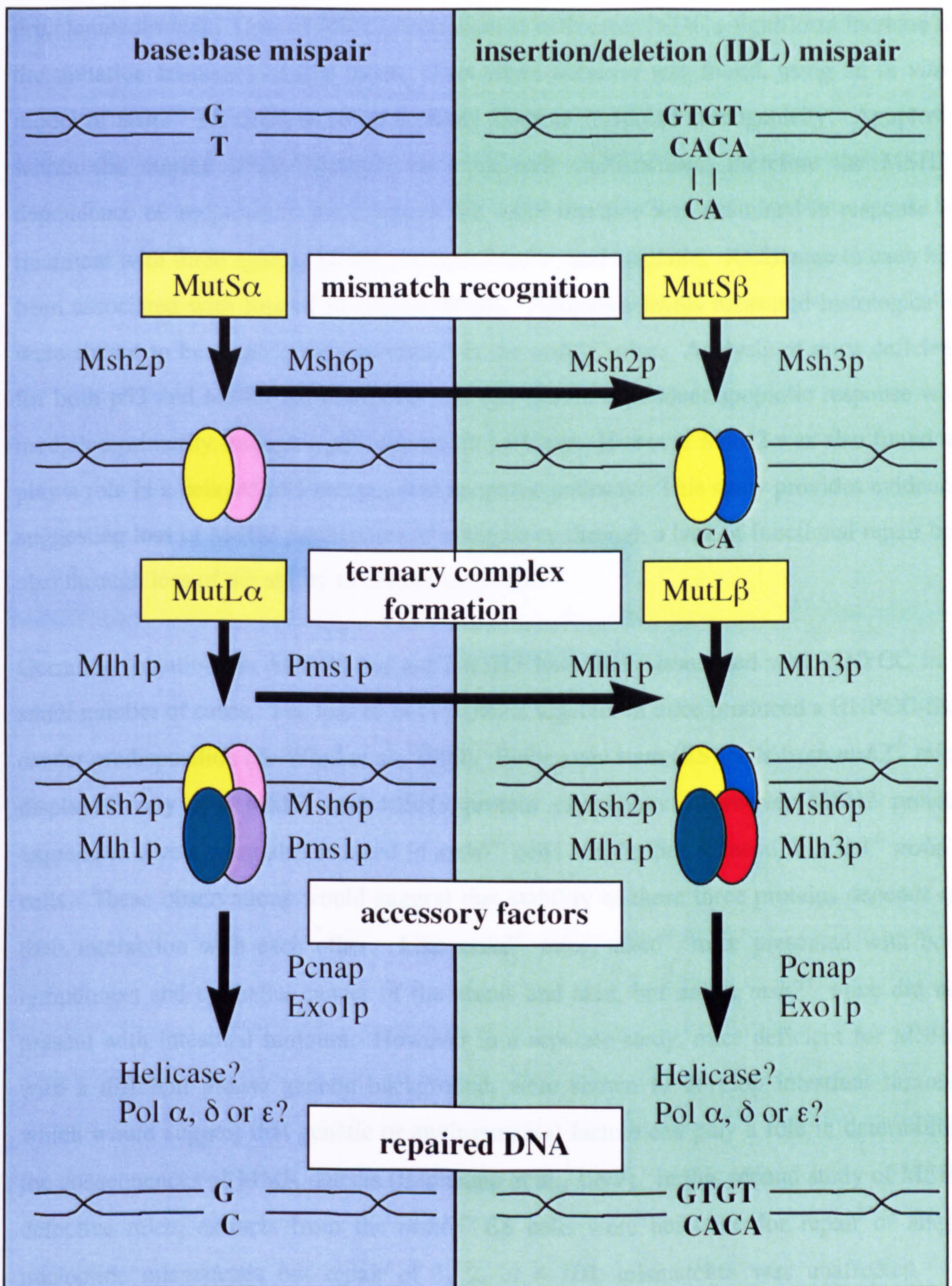
### 1.1.6 MMR Knockout Mice

To further understand the role of DNA mismatch repair in maintaining genome stability and tumorigenesis MMR deficient mice were generated and studied.

*msh2*<sup>-/-</sup> embryonic stem (ES) cells were generated (de Wind et al., 1995). Extracts of these MSH2 deficient cell lines were used in gel shift assays and the characteristic binding of proteins to mismatched DNA described earlier had been lost. This supported the idea that MSH2 is required for heteroduplex recognition and binding. These *msh2*<sup>-/-</sup> ES cells grew normally compared to wild type cells. Microsatellite instability at two loci was detected in genomic DNA extracts from these cells. The cells also displayed a mutator phenotype as measured by mutation frequencies at the *HPRT* locus in response to selection with 6-thioguanine compared to wild type cells. The cells were tolerant to treatment with MNNG (N-Methyl-N'-Nitro-Nitrosoguanidine). Treatment of cells with methylating agents such as MNNG produces DNA adducts such as O<sup>6</sup>-methyl guanine. MMR has been proposed to recognise the O<sup>6</sup>-methyl guanine·T mispair that occurs after the mis-incorporation of a thymidine nucleotide opposite this damaged base during replication. The *msh2*<sup>+/-</sup> mice generated transmitted the mutant allele through the germline so mice with a homozygous loss of *MSH2* could be examined. The heterozygotes were healthy at 8 months however 30% of the homozygous mice developed metastasising lymphomas at an average of 2 months. These results along with the studies of the MSH2 deficient cell lines established a role for MSH2 in mammalian MMR and also that MMR deficiency can lead to the accelerated malignant transformation of rapidly expanding cell populations.

MSH2 expression has also been shown to play a role in the control of apoptosis and genomic stability in the cells of the murine small intestine. Once again knockout *msh2*<sup>-/-</sup> mice have been utilised (Toft et al., 1999). The mutation frequency at the *DLB1* locus was measured in wild type mice versus *MSH2* knockout mice in response to endogenous levels of DNA damage and also in response to damage after treatment with the chemotherapeutic





**Figure 2: Schematic Describing the Proteins Involved in the Eukaryotic DNA Mismatch Repair Pathway.**

This figure describes the interactions between MutS and MutL-related proteins during MMR. The protein names used are for *Saccharomyces cerevisiae*. The closest human homologues of yPms1p and yMlh3p are hPMS2 and hPMS1 respectively. MutS $\alpha$  and MutL $\alpha$  both can play a role in the repair of IDL mispairs. Human MutL $\beta$  has not been shown to play a role in MMR as yet.



drug temozolomide. Loss of MSH2 expression in both cases led to a significant increase in the mutation frequency at this locus. This effect however was found, using an *in vitro* model of *msh2*<sup>-/-</sup> ES cells, to result partially from an increased clonogenicity. Apoptosis within the murine small intestine has been well characterised, therefore the MSH2-dependence of apoptosis in the crypts of the small intestine was examined in response to treatment with three agents, MNNG, temozolomide, and cisplatin. Resistance to each has been associated with loss of functional MMR. Apoptosis levels measured histologically were shown to be significantly decreased in the *msh2*<sup>-/-</sup> mice. Analysis of mice deficient for both p53 and MSH2 demonstrated that this MSH2 dependent apoptotic response was mediated primarily through a p53 dependent pathway. However MSH2 was also found to play a role in a delayed p53 independent apoptotic pathway. This study provides evidence suggesting loss of MSH2 predisposes to malignancy through a lack of functional repair but also through loss of the ability to engage apoptosis.

Germline mutations in *hMSH6* but not *hMSH3* have been associated with HNPCC in a small number of cases. The loss of both proteins together in mice produced a HNPCC-like cancer predisposition (de Wind et al., 1999). Embryonic stem (ES) cells from *msh2*<sup>-/-</sup> mice displayed very low MSH6 and MSH3 protein expression. However MSH2 protein expression levels were also reduced in *msh6*<sup>-/-</sup> cells and further reduced in *msh3*<sup>-/-</sup> *msh6*<sup>-/-</sup> cells. These observations would suggest that stability of these three proteins depends on their interaction with each other. Like *msh2*<sup>-/-</sup> mice, *msh6*<sup>-/-</sup> mice presented with both lymphomas and epithelial cancer of the uterus and skin, but unlike *msh2*<sup>-/-</sup> mice did not present with intestinal tumours. However in a separate study, mice deficient for MSH6, with a different mouse genetic background, were shown to develop intestinal tumours which would suggest that genetic or environmental factors can play a role in determining the consequences of MMR defects (Edelmann et al., 1997). In this second study of MSH6 defective mice, extracts from the *msh6*<sup>-/-</sup> ES cells were defective for repair of single nucleotide mismatches but repair of 1, 2, or 4 IDL mismatches was unaffected. In agreement with this observation there was no MSI phenotype observed in DNA from tumour cells from such mice.

MSH3 deficiency did not predispose the mice to cancer, but in combination with MSH6 deficiency i.e. in *msh3*<sup>-/-</sup> *msh6*<sup>-/-</sup> mice, intestinal tumours did develop (de Wind et al., 1999). In these knockout mice the role of MSH6 in MMR directed anti-recombination effects were demonstrated, however MSH3 does not seem to play such a role. MSH6 but not MSH3 deficiency was also shown to result in resistance to *N*-methyl-*N'*-nitro-*N*-



nitrosoguanidine. These differences in effect are most likely related to the functional redundancy between MutS $\alpha$  and MutS $\beta$ .

Mice defective in the *PMS2* gene were generated, which also supported a role for PMS2 in DNA mismatch repair (Baker et al., 1995). A fraction of *pms2*<sup>-/-</sup> mice developed either a lymphoma or a sarcoma. No tumours were seen in the wild type or the *pms2*<sup>+/-</sup> mice. Microsatellite instability was observed in tumour DNA, germline and tail DNA demonstrating a role for PMS2 in MMR in a range of tissue types (Baker et al., 1995; Narayanan et al., 1997). Male *pms2*<sup>-/-</sup> mice were infertile which is due to abnormal spermatogenesis resulting from defective chromosome synapsis during meiosis. Once again providing evidence for a link between MMR, recombination and chromosome synapsis in meiosis.

*mlh1*<sup>-/-</sup> mice initially generated, once again like the *msh2*<sup>-/-</sup> and *pms2*<sup>-/-</sup> mice are also defective for MMR as illustrated by a microsatellite instability phenotype (Edelmann et al., 1996). Extracts from *mlh1*<sup>-/-</sup> mouse embryonic fibroblasts (MEF) were demonstrated to have impaired MMR using an *in vitro* MMR assay. As in the *pms2*<sup>-/-</sup> mice the male mice are infertile although in this case no spermatozoa are made. However *mlh1*<sup>-/-</sup> female mice are also infertile.

The tumour susceptibility of *mlh1*<sup>-/-</sup> mice was examined in another study and compared to that of mice deficient in PMS2 and mice deficient in PMS1 (Prolla et al., 1998). Unlike the *pms2*<sup>-/-</sup> mice, *mlh1*<sup>-/-</sup> mice develop as well as lymphomas, intestinal adenomas and adenocarcinomas and to a lesser extent skin tumours and sarcomas. *pms1*<sup>-/-</sup> mice did not develop tumours. These results suggest distinct roles for the three proteins in tumour prevention. MEF cells deficient for MLH1 and PMS2 showed frequent microsatellite instability at di- and mono-nucleotide repeats unlike PMS1 deficient MEF cells which specifically showed increased MSI at only mono-nucleotide repeats. Similarly the MEF cells deficient for MLH1 and PMS2 showed a mutator phenotype (increased mutation rate) as measured by resistance to ouabain which was not observed in the PMS1 deficient MEF cells.

These results suggest MLH1 and PMS2 function in a similar way in the maintenance of genetic fidelity. This is consistent with the observation that homologues of these two proteins heterodimerise in human and yeast cells. The mononucleotide repeat mutation frequency in DNA from *mlh1*<sup>-/-</sup> mice was found to be 2-3 fold higher than that seen in



*pms2*<sup>-/-</sup> mice (Yao et al., 1999). Therefore making it possible that some residual repair capacity is existent in the *pms2*<sup>-/-</sup> mice. MLH1 protein expression was normal in *pms2*<sup>-/-</sup> mice, however in *mlh1*<sup>-/-</sup> mice no PMS2 protein expression was detected. The *mlh1*<sup>-/-</sup> mice had a similar mutator phenotype to the double knockout mice. The difference in tumour development in the *MLH1* and *PMS2* knockout mice may reflect this difference in mutator phenotype. Possibly the absence of one protein in comparison to the absence of both MLH1 and PMS2 in the *mlh1*<sup>-/-</sup> mice determines the tumour spectra observed.

The stable expression of human MLH1 in MLH1-deficient MEF cells was shown to complement a number of phenotypes, already described that result from loss of functional MMR (Buermeier et al., 1999). By transfecting hMLH1 cDNA into *mlh1*<sup>-/-</sup> MEF cells, mouse PMS2 protein expression was restored to normal levels, MSI was reduced, restoration of sensitivity to 6-TG was seen and restoration of 6-TG-induced cell cycle arrest also resulted. These observations along with those from the *MLH1* knockout mice support a role for MLH1 in maintaining genomic stability and in mediating sensitivity to 6-TG.



## 1.2      **Microsatellite Instability (MSI), Drug Resistance and Mismatch Repair.**

A larger number of mutations are seen in tumour cells, than can be accounted for by the spontaneous mutation rate in somatic cells (Loeb, 1991). Loeb proposed that if these multiple mutations observed in tumours actually play a role in tumorigenesis, rather than just accompanying cancer, then perhaps an early step in tumour progression is the induction of this observed mutator phenotype. A clear relationship between this mutator phenotype and tumour progression soon emerged.

The observation of the expansion or contraction of microsatellite (tandem repeat nucleotide sequences) in human tumour cells but not in non-malignant cells provided the first strong evidence to support a mutator phenotype in cancer (Peinado et al., 1992). This change in microsatellite length is believed to result from DNA polymerase slippage during replication. A subset of sporadic colorectal tumours as well as tumours from HNPCC cancer patients were both shown to display this MSI or replication error positive (RER<sup>+</sup>) phenotype (Aaltonen et al., 1993; Ionov et al., 1993; Thibodeau et al., 1993).

The biochemical basis of this MSI phenotype soon became apparent. The mutation rate at dinucleotide repeat sequences in RER<sup>+</sup> cells were shown to be significantly higher than those seen in RER<sup>-</sup> cells (Parsons et al., 1993). Importantly it was found that these RER<sup>+</sup> cells were deficient in functional MMR as demonstrated using an *in vitro* MMR assay. The loss of MMR leading to genomic instability was consistent with studies in bacteria and yeast. This RER<sup>+</sup> phenotype in particular was observed in yeast cells in which genes known to play a role in yeast MMR (*yMLH1*, *yPMS1* and *yMSH2*) had been mutated (Strand et al., 1993). The identification of the human MutS homologue, *hMSH2*, as a gene mutated in some HNPCC families, gave further credence to the proposal that loss of functional MMR was in fact the cause of this RER<sup>+</sup> phenotype (Leach et al., 1993). The *hMSH2* gene was shown to be heterozygous for the mutation observed in the somatic tissue of HNPCC patient but in tumour RER<sup>+</sup> cells an acquired mutation in the second *hMSH2* allele was observed (Leach et al., 1993).

Methylating agents such as MNU and MNNG are mutagenic and cytotoxic. O<sup>6</sup>-methylguanine (O<sup>6</sup>-meG) is the lesion produced in DNA by treatment with these agents (Karran and Bignami, 1994; Karran et al., 1990). Under normal circumstances O<sup>6</sup>-meG-DNA methyltransferase (MGMT), a repair enzyme, repairs this lesion directly. However in



many transformed cells this gene is not functionally expressed due to epigenetic silencing. In the absence of MGMT, cells are hypersensitive to killing by treatment with these methylating agents. This sensitivity is believed to be a direct consequence of the persistence of unrepaired O<sup>6</sup>-meG in DNA. However tolerance to treatment with methylating agents like MNU and MNNG has also been observed. This tolerance or resistance was found in both human and hamster cell lines to be associated once again with defective MMR (Branch et al., 1993). Loss of MMR, known to be associated with a mutator phenotype, was also shown in these methylation tolerant cell lines to result in increased mutation rates, as measured by increased rates of spontaneous mutations at selectable genes.

A recent study demonstrated an increase in hMSH2 and hMSH6 protein levels and G/T mismatch binding activity in the nucleus, in response to treatment with alkylating agents which produce an O<sup>6</sup>-alkyl guanine adduct, such as MNU, ENU and MNNG, due to the re-localisation of these proteins to the nucleus (Christmann and Kaina, 2000). This intriguing response was dose and time dependent. Treatment of cells lacking functional MGMT, with doses of alkylating agents which did not trigger cell death, resulted in increased translocation of pre-formed hMutS $\alpha$  complexes from the cytoplasm into the nucleus. The translocation mechanism is proposed to involve a putative nuclear localisation signal encoded by hMSH6. The results suggest that O<sup>6</sup>-meG adducts are the main trigger of this translocation of hMutS $\alpha$  into the nucleus. This discovery supports the idea that the loss of functional MMR can have a major impact on the response of cells to alkylating agents.

Another hypermutable cell line was identified that was also tolerant to treatment with such methylating agents (Kat et al., 1993). Once again the role for MMR in mediating methylating agent cytotoxicity was demonstrated, as these cells were shown using an *in vitro* MMR assay, to be defective in functional MMR. This MT1 lymphoblastoid B-cell line had a different mutation pattern to its alkylation sensitive parental line in response to treatment with MNNG and also in its spontaneous mutation spectrum. In particular a specific frameshift mutation hotspot in exon 3 of the *HPRT* gene was identified in the MT1 cell line in response to treatment with MNNG.

This hotspot at a run of six guanines in exon 3 was observed in a second study utilising a colorectal carcinoma cell line (Bhattacharyya et al., 1994). This particular cell line was again shown to be defective in MMR, had a RER<sup>+</sup> phenotype and an increased mutation rate at the selectable (on the basis of resistance to 6-thioguanine) *HPRT* gene. The



mechanism of 6-TG cytotoxicity begins by the metabolic processing of 6-TG by HPRT the product of the *HPRT* gene, to yield 6-thioguanosine monophosphate. This product is then phosphorylated to give 6-thioguanosine triphosphate which can be incorporated into genomic DNA. This incorporation step is the key to the cytotoxicity induced by this agent. After incorporation into DNA the thioguanine is methylated by S-adenosylmethionine to give S<sup>6</sup>-methylthioguanine (Swann et al., 1996). S<sup>6</sup>-methylthioguanine directs incorporation of thymine or cytosine into the newly made DNA strand during replication and the S<sup>6</sup>-methylthioguanine-thymine pairs that result have been shown to be recognised by the MMR system. From these observations it was proposed that this frameshift mutation hotspot in exon 3 of the *HPRT* gene specifically arises due to defective MMR (Bhattacharyya et al., 1994).

Overall it was clear from these studies that functional mismatch repair is required to maintain genomic stability. Loss of MMR leads to increased microsatellite instability, increased mutation rates at selectable gene (e.g. *HPRT* gene) but most intriguingly to resistance to treatment of cells with methylating agents such as MNNG. This resistance phenotype is also seen in mice which have defective MMR (de Wind et al., 1995; Prolla et al., 1998). How exactly functional MMR mediates sensitivity to these methylating agents is not understood.

One model proposed to explain how functional MMR mediates sensitivity to these agents suggests that methylated bases such as O<sup>6</sup>-meG do not actually block replication (Karran and Hampson, 1996). Rather, this model proposes that DNA polymerase inserts a base opposite the methylated guanine, which is the best match resulting in the least distortion to the DNA. In the case of O<sup>6</sup>-meG, O<sup>6</sup>-meG•T introduces the least amount of distortion into the DNA (Wood and Shivji, 1997). This base pair is proposed to be recognised by the MMR system as a mismatch. Support for this idea came from the discovery using *in vitro* gel shift assays that O<sup>6</sup>-meG base pairs are recognised by mismatch binding activities (hMutS $\alpha$ ) (Duckett et al., 1996; Karran et al., 1993). hMutS $\alpha$  recognises and binds O<sup>6</sup>-meG•T slightly less efficiently than a G•T mismatch. Repair synthesis is induced in the newly made strand i.e. opposite the O<sup>6</sup>-meG but as no suitable complementary base is available, it is proposed that these lesions lead to repeated attempts to repair this mismatch which somehow triggers cell death. Therefore in the absence of functional MMR no such attempts to repair these methylated bases are instigated, so the cells survive.



This resistant phenotype seen in tumour cells to treatment with MNU and MNNG and their clinical counterparts, such as temozolomide, is clearly associated with the loss of a functional MMR pathway. The possibility that this RER<sup>+</sup> phenotype associated with loss of MMR could be associated with resistance of tumour cells to a wider range of DNA damaging agents was next considered. From a number of studies it soon became apparent that functional MMR is essential to mediate sensitivity to a wide variety of clinically important chemotherapeutic agents.

The chemotherapeutic drug cis-diamminedichloroplatinum (II), or cisplatin (CDDP) is widely used and is effective against a variety of tumour types. CDDP exerts its cytotoxic effect by covalently binding DNA. This results in the formation of a range of adducts including intra-strand cross-links and inter-strand cross-links. The majority of adducts are believed to be intrastrand cross-links at the N-7 positions of adjacent guanine bases (1,2 d(GpG) adduct). Tumour cells selected for resistance to cisplatin were discovered to have an associated loss of hMLH1 protein expression (Anthoney et al., 1996). These cells had a concomitant RER<sup>+</sup> phenotype and an increased mutation rate at the *HPRT* locus. This cisplatin resistance phenotype was again demonstrated in isogenic pairs of cell lines proficient or defective for MMR as determined by *hMLH1* or *hMSH2* expression or lack thereof (Aebi et al., 1996; Anthoney et al., 1996). In the absence of either *hMLH1* or *hMSH2* gene expression, cells were shown to be resistant to treatment with cisplatin. Restoration of sensitivity in these cells was seen with chromosome transfer experiments, transferring the respective wild type MMR gene back into these cells.

Sensitivity of cells to treatment with a number of cisplatin analogues was tested in an MMR proficient versus deficient background again using isogenic tumour cell lines (Fink et al., 1996). Platinum drug resistance associated with loss of functional MMR was shown to be specific for cisplatin and carboplatin. No MMR associated resistance was seen with oxaliplatin, tetraplatin or transplatin. From this study it became apparent that the reason for this difference is due to the ability of MMR proteins to bind the adduct produced by treatment with each drug. Using gel shift assays, nuclear extracts incubated with cisplatin but not oxaliplatin formed protein DNA complexes which contained both hMLH1 and hMSH2.

Sensitivity of a wide range of chemotherapeutic agents was shown to be dependent on functional MMR. For example a similar study to those described above was carried out again using isogenic tumour cells and demonstrated the acquired resistance to doxorubicin



(a topoisomerase II inhibitor) in cells where functional MMR was lost (Drummond et al., 1996). A summary of the various agents, sensitivity to which is dependent on functional MMR, is listed in Table 2.

hMutS $\alpha$  binds lesions resulting from treatment with methylating agents such as O<sup>6</sup>-methylguanine or O<sup>4</sup>-methylthymine and binds platinated adducts such as the cisplatin induced 1,2 d(GpG) adduct (Duckett et al., 1996). The hMutS $\alpha$  complex has been shown to have different levels of affinity for different adducts depending on the base-pairing with the alkylated or platinated base (Yamada et al., 1997). hMutS $\alpha$  binds the 1,2 d(GpG) adduct with the highest affinity when it is opposite non-complementary bases i.e. when the complementary strand has a T opposite the 3', and a C opposite the 5' guanine. When the 1,2 d(GpG) adduct or the O<sup>6</sup>-methylguanine is base-paired with a C, recognition by hMutS $\alpha$  is poor, compared to when they are base-paired with a non-complementary base such as T. This would suggest that damaged DNA which has undergone replication is most likely to be recognised by the hMutS $\alpha$  complex. Recent studies of crystal structures of TAQ MutS bound to heteroduplex DNA suggest kinked DNA is a general feature of these complexes (Obmolova et al., 2000). Base stacking is less stable at a mismatch site and is therefore more susceptible to deformation. Therefore as DNA effected by cisplatin treatment is kinked, this in combination with the mismatch resulting from a round of DNA replication may aid binding of the adduct by hMutS $\alpha$ . Rapid induction of apoptosis in immature rat thymocytes in response to treatment with cisplatin was seen in proliferating cells (Evans et al., 1994). However in purified non-proliferating cells apoptosis levels in response to treatment with cisplatin were nominal. These two studies would suggest that the induction of apoptosis in sensitive cells in response to treatment with cisplatin requires the cells to be proliferating and therefore undergoing replication.

A model to explain how resistance arises due to loss of functional MMR must accommodate the different methods of action of the various DNA damaging agents described. The discovery of a range of agents, sensitivity to which is mediated through the MMR pathway, has led to a second model being proposed to explain how resistance occurs. This model proposes that enhanced replicative bypass, which is defined as the ability of the replicative complex of a cell to synthesise DNA past the DNA adduct, gives rise to the resistant phenotype (Mamenta et al., 1994). This process has also been referred to as postreplication repair because bypassing the adduct during S phase will allow survival



**Table 2: Mismatch Repair Defects Mediate Cellular Drug Response.**

Taken from Sedwick *et al* (Sedwick, 1999).

Drug or Agent	Response	Probable or Possible Mechanism
ALKYLATING AGENTS	Resistance	Prevents apoptotic signalling
<u>SN-1 Alkylators</u>		Critical proteins for apoptosis may not be expressed in some MMR defective cells.
MNNG		
MNU	Mutation	Prevents toxicity from futile DNA repair cycling in response to miscoding during DNA polymerase bypass synthesis.
Temozolomide		
<u>SN-2 Alkylators</u>		With MMS, apurinic sites may be recognised as miscoding lesions similar to base modifications
MMS		
Cisplatin	Resistance	Allows lesion by-pass to occur preventing toxicity at DNA segregation.
Carboplatin		Blocks recombination-dependent DNA repair.
Doxorubicin	Resistance	Unknown, may be similar to alkylator resistance mechanism.
6-Thioguanine	Resistance	Tolerance to mispairs from drug mis-incorporation mediated by same mechanisms as those seen for alkylating agents.
5-Fluoruracil (5-FU)	Resistance	Allows lesion by-pass, prevents apoptosis signalling.
<u>X-irradiation</u>		
Low dose rate	Sensitivity	Accumulation of toxic oxidative lesions and/or lesion leading to double strand break repair may kill cells.
		Interference with recombinational repair may predominate over other repair processes in response to low levels of toxic lesions.
High dose rate	Resistance or Sensitivity	Mixed results may reflect differing capacities of cells for repair of oxidative or recombinagenic lesions.



Acridine compounds	Sensitivity Mutation	Prevents lesion repair leading to toxic levels of frameshift events
Temozolomide + Benzimide	Sensitivity	3-aminobenzamide may alter capacity for lesion by-pass or affect other co-operating repair systems.
Cisplatin + Aphidicolon	Sensitivity	Aphidicolon prevents lesion by-pass at cisplatin lesions in MMR-defective cells.



of the cell into G<sub>2</sub> where cell cycle arrest can occur allowing repair of the damage before entering mitosis (Karran and Marinus, 1982).

Utilising parental and resistant human ovarian carcinoma cell lines the relationship between resistance and replicative bypass was analysed further (Mamenta et al., 1994). In the cisplatin-resistant cell lines replicative bypass of the platinated adducts was specifically observed. These observations suggest that this replicative bypass mechanism contributes in part to the resistant phenotype observed in these cells. This proposal is further supported by another study which utilised an *in vitro* system to look at steady-state replication which measures the rate of DNA synthesis across DNA lesions (Vaisman et al., 1998). Specifically the rate of bypass of cisplatin adducts in cells with defects in either hMLH1 or hMSH6 was significantly increased compared to MMR proficient cells.

The sensitivity of MMR defective cells to cisplatin and MNU has been shown to increase upon treatment of the cells with the polymerase inhibitor aphidicolin (inhibits polymerase  $\alpha$ ,  $\epsilon$  and  $\delta$ ) to a greater extent than seen in MMR proficient cells (Moreland et al., 1999). Polymerase  $\epsilon$  and  $\delta$  have been shown to bypass cisplatin induced 1,2 crosslinks in structures that resemble replication forks. Treatment with aphidicolin also partially restored the cisplatin induced G<sub>2</sub> arrest absent in MMR deficient cells compared to proficient cells. These observations are consistent with the model proposed above whereby aphidicolin is inhibiting replicative bypass preferentially in cells with defective MMR.

### **1.2.1 MMR Mediated Sensitivity to DNA Damaging Agents: Possible Pathways**

Overall a number of models have been proposed to explain how loss of functional MMR leads to a resistant phenotype. One model suggests that the lesion produced in response to treatment with e.g. methylating agents persists in the template leading to futile rounds of repair. This abortive repair produces excision intermediates which are proposed to somehow activate the damage response pathway. Therefore in the absence of functional MMR no such excision intermediates will arise. A second model proposes that due to the fact that MMR proteins recognise and bind these lesions this leads to the assembly of a signalling complex which directly triggers the damage response. Again in the absence of MMR the signalling to these downstream pathways will be lost. Finally another model suggests the mechanism of resistance involves enhanced replicative bypass of the lesion in the absence of functional MMR which allows the damage to persist. The models proposed



to explain how these resistant phenotypes arise suggest that a functional MMR pathway is required to trigger downstream cellular responses which result in an apoptotic response to treatment with these agents. How exactly MMR transduces signals leading to apoptosis is not clear.

Alterations in the p53 gene are frequently observed in human neoplasia. p53 levels are elevated in the cisplatin resistant, MMR deficient A2780/CP70 ovarian carcinoma cell line compared to the parental cisplatin sensitive, MMR proficient A2780 cell line (Brown et al., 1993). Elevated expression of p53 is often associated with mutations in conserved domains of the protein. However p53 in the A2780/Cp70 cell line was shown to be wild type, i.e. no mutations were found upon sequencing. These cells also had a decreased apoptotic response to treatment with cisplatin compared to the parental cell line (Anthoney et al., 1996). In addition the p53 dependent G<sub>1</sub> arrest in response to ionising radiation (IR) was also significantly reduced in these cells compared to the parental cell line. This demonstration of loss of p53 functions was corroborated by the reduced amount of p21 mRNA expressed in these cells, a gene known to be partially regulated by p53 and required for the p53 dependent G<sub>1</sub> arrest in response to IR. Introduction of a dominant negative mutant form of p53 into the A2780 parental cell line or inactivation of p53 function in these cells using genetic suppressor elements results in acquired resistance to cisplatin (Anthoney et al., 1996; Gallagher et al., 1997). Restoration of functional MMR to these MMR deficient cells restores sensitivity to drugs such as cisplatin, but does not restore p53 function. From these studies a direct role for MMR in signalling to p53 has not been established in this model. Instead it is possible that both functional MMR and p53 are required for sensitivity to cisplatin but are not mutually exclusive as is suggested by other studies (Branch et al., 2000).

Phosphorylation of Ser-15 and Ser-392 of p53 has been implicated in playing a role in cellular responses to different types of DNA damage. Therefore p53 was used in another study as an *in vivo* substrate, to test whether damage-signalling kinases are activated in response to treatment with MNU or MNNG, in an MMR dependent manner (Duckett et al., 1999). Loss of hMutS $\alpha$  or hMutL $\alpha$  in cells results in loss of G<sub>2</sub> arrest and apoptosis in response to treatment with methylating agents or the anti-metabolite 6-thioguanine (Hawn et al., 1995; Kat et al., 1993). This G<sub>2</sub> arrest occurs in the second cycle after drug exposure in MMR proficient cells. This suggests that a round of replication is required, allowing incorporation of an incorrect base opposite e.g. the O<sup>6</sup>-methylguanine or the S6-methylthioguanine adducts that result from treatment with such agents, forming a mispair



recognised by the MMR system. The MMR system is proposed to recognise these adducts and signal downstream cellular responses. This observation further supports the requirement for translesion synthesis for sensitivity to these agents, i.e. to trigger cell death. Functional MMR, in particular both hMutS $\alpha$  and hMutL $\alpha$ , were found to also be required for phosphorylation of p53 in response to treatment with these methylating agents (MNU or MNNG) (Duckett et al., 1999). These results clearly implicate MMR as playing a role in signalling cellular responses to methylator damage through a p53 dependent pathway.

Analysis of mice deficient for both p53 and MSH2 demonstrated that MSH2 dependent apoptotic response to treatment with temozolomide (a methylating agent) is also mediated primarily through a p53 dependent pathway (Toft et al., 1999). However MSH2 was also found to play a role in a delayed p53 independent apoptotic pathway.

The c-ABL protein tyrosine kinase and the c-JUN NH<sub>2</sub>-terminal kinase (JNK) are activated in cells in response to treatment with the DNA-damaging agent cisplatin (Nehme et al., 1997). In MMR deficient cells the activation of the c-ABL kinase in response to treatment with cisplatin is completely lost. Activation of JNK has also been shown to be weaker in MMR deficient cells compared to proficient cells in response to treatment with cisplatin. However JNK activation by cisplatin was not seen in cells deficient for hMSH2 suggesting that JNK is not essential for activation of apoptosis in response to cisplatin. c-ABL activation is seen in response to a number of different forms of DNA damage. For example c-ABL contributes to the G<sub>1</sub> arrest of cells in response to IR in a p53-dependent manner (Yuan et al., 1996). c-ABL interacts with p53 in irradiated cells but does not phosphorylate the p53 protein. c-ABL can regulate DNA-damage induced apoptosis through a p53 - independent mechanism (Yuan et al., 1997). These observations have led to the proposal that c-ABL may act to couple damage detection, such as recognition of adducts by the MMR system, to an apoptotic response.

Further evidence for this proposed role for c-ABL has come from studies of the p53-related gene p73. The p73 gene like p53 encodes a protein with a transactivation, DNA-binding and oligomerisation domain and is significantly homologous to p53 (Clurman and Groudine, 1997). Different isoforms of p73 are expressed due to alternative splicing, some of which can transactivate p53 target genes, induce cell cycle arrest and apoptosis (White and Prives, 1999). A number of studies have demonstrated that p73 is a target of the non-receptor protein tyrosine kinase c-Abl.



A cell line deficient for hMLH1 expression was utilised in one study (Gong et al., 1999). Restoration of MMR activity in these cells and hence sensitivity to cisplatin was seen upon transfer of chromosome 3 (carries *hMLH1*) into these cells. Chromosome transfer of chromosome 2 into these cells had no such effect, these cells like the parent line remained resistant to treatment with cisplatin. However p53 induction on treatment with cisplatin was the same in each of these three cell types. Interestingly p73 was induced only in the chromosome 3 transfer line i.e. where there was proficient MMR upon treatment with cisplatin. In this cell line sensitivity correlates with the induction of both p53 and p73. p73 mRNA expression levels did not increase, instead the half-life of the protein was seen to increase.

This induction of p73 in response to treatment with cisplatin and the stabilisation of this protein in these cell lines was found to be dependent on the kinase activity of c-ABL. Using mouse embryonic fibroblasts deficient for c-ABL, hMLH1 and p53 it was found that although p73 is expressed in the absence of these proteins induction upon treatment with cisplatin requires both hMLH1 and c-ABL. p53 induction does not require expression of either hMLH1 or c-ABL. In the p53 deficient cells some killing by cisplatin is apparently mediated via p73.

Induction of apoptosis by p73 specifically requires c-ABL, in particular the kinase activity of this protein (Agami et al., 1999). p73 and c-ABL were shown to interact, via the SH3 domain of c-ABL and a PXXP domain within the linker region between the DNA-binding and oligomerisation domains of p73. Under physiological conditions these two proteins were shown to co-immunoprecipitate in untreated cells and in cells after  $\gamma$ -irradiation (Agami et al., 1999; Yuan et al., 1999). p73 was also shown to be a substrate for c-ABL mediated phosphorylation. c-ABL induced the transactivation function of p73 by phosphorylation of tyrosine at position 99 of this protein.

However this phosphorylation of p73 decreased in AT cells (ATM deficient) (Yuan et al., 1999). ATM is known to interact with and phosphorylate both c-ABL and p53 (Banin et al., 1998; Shafman et al., 1997). The activation of both proteins by ATM probably represents two separate pathways. Overall the c-ABL mediated phosphorylation of p73 results in increased apoptosis in response to DNA damage caused by IR, treatment with cisplatin, but not UV (Gong et al., 1999). c-ABL induction in response to cisplatin relies upon functional MMR. These studies together, provide some evidence for a role for mismatch repair proteins in the signalling of downstream responses to DNA damage.



### 1.2.2 Significance of MSI and Resistance.

Functional MMR is clearly required to mediate sensitivity to a range of clinically important anti-cancer agents such as cisplatin. However in the studies described the resistance to cisplatin is relatively low, about 2 fold, in the absence of functional MMR. The question of whether this level of resistance is clinically relevant must be asked.

Experiments with mixed populations of cells, with a minority of MMR deficient cells which are constitutively expressing GFP (green fluorescent protein), exposed a number of times to either cisplatin or oxaliplatin were carried out (Fink et al., 1998; Fink et al., 1997). Treatment with cisplatin but not oxaliplatin resulted in the gradual enrichment for MMR deficient cells. When grown as xenografts, cisplatin treatment of the tumours again resulted in significant enrichment for MMR deficient cells (Fink et al., 1998). These results would suggest that treatment with the DNA-damaging agent cisplatin results in selection of MMR deficient cells. The lack of enrichment by oxaliplatin for MMR deficient cells is in agreement with observations that MMR does not seem to play a role in mediating sensitivity to this agent (Fink et al., 1996).

Xenografts were also utilised to test whether loss of MMR causes resistance to cisplatin *in vivo*. *MSH2<sup>+/+</sup>* and *msh2<sup>-/-</sup>* ES cells were established as xenografts in nude mice and treated with either cisplatin or oxaliplatin after tumour implantation (Fink et al., 1997). The *msh2<sup>-/-</sup>* mice were considerably less sensitive to treatment with cisplatin compared to the *MSH2<sup>+/+</sup>* mice, whereas there was no difference in sensitivity to oxaliplatin. This provides evidence for *in vivo* resistance to cisplatin in MMR deficient cells.

Collectively these observations suggest that although the level of resistance to these drugs is quite modest in the absence of MMR, exposure to e.g. cisplatin can produce a significant enrichment for MMR deficient cells both *in vitro* and *in vivo*. It is proposed that this enrichment could foster additional mutations that underlie tumour progression.

This proposal is supported by the fact that loss of functional MMR is also associated with the emergence of microsatellite instability, an RER<sup>+</sup> phenotype. For example cancers associated with HNPCC, a hereditary cancer linked to loss of functional MMR, are characterised by instabilities of these simple repeat sequences (Aaltonen et al., 1993). This microsatellite instability (MSI) is not only observed in colorectal cancer but in a wide range of cancers including sporadic breast and ovarian cancers.



An RER<sup>+</sup> phenotype has been seen in a proportion of sporadic colorectal cancer tumours to be associated with good prognosis (Bubb et al., 1996). However this association of an RER<sup>+</sup> phenotype with good prognosis is not seen in all cancers with this phenotype. For example this RER<sup>+</sup> phenotype has also been shown to correlate with poor disease free and overall survival in sporadic breast cancers (Paulson et al., 1996). This difference in the effect of an RER<sup>+</sup> phenotype on prognosis between colon and breast cancer may reflect the different impact of this phenotype on these tumours. For example the RER<sup>+</sup> phenotype seen in these sporadic colon cancers may select for mutations in genes that normally facilitate tumour progression. In the case of these breast cancers the effect of the RER<sup>+</sup> phenotype may have more to do with the chemotherapeutic treatment administered, as loss of MMR and an RER<sup>+</sup> phenotype are associated with resistance to drugs such as cisplatin, which are commonly used in the treatment of sporadic breast cancer.

If cisplatin or other similar anti-cancer agents enrich tumour populations for cells deficient for MMR this may have important implications other than just the emergence of drug resistance. MMR deficient cells have a mutator phenotype which is indicative of increased mutation rates at a variety of alleles including e.g. transforming growth factor  $\beta$  receptor II, which can influence the tumour phenotype (Markowitz et al., 1995). This mutator phenotype, identified in a range of HNPCC and sporadic cancers has been attributed in many cases to mutations in MMR genes. In MSI-positive tumours, from HNPCC and sporadic cancers, somatic frameshift mutations have been detected in the repeat tracts of a range of genes including the MMR genes, *hMSH3* and *hMSH6*, the pro-apoptotic gene *BAX* and more recently the repair gene *MED1* (Riccio et al., 1999; Yamamoto et al., 1997; Yamamoto et al., 1998). These observations support the model of mutators mutations (Malkhosyan et al., 1996; Perucho, 1996). This model proposes that the mutator phenotype emerges through a number of steps. The primary step is a homozygous mutation in for example an MMR gene such as *hMLH1* or *hMSH2*, which results in mutations in secondary mutator genes, such as other MMR genes. As there is a higher occurrence of these somatic frameshift mutations in the repeat tracts of such genes, compared to other genes with these repeat sequences, a positive selective pressure for mutations in these specific genes appears to exist in tumorigenesis. This enrichment effect could therefore result in the accumulation of additional mutations in genes that underlie tumour progression and/or drug resistance.

Paired tumour samples from 38 ovarian cancer patients who had undergone at least three rounds of cisplatin or carboplatin based chemotherapy were examined histologically for



hMLH1 expression (Fink et al., 1998). In a large proportion of cases following treatment with either drug, the level of hMLH1 expressed in the tumour cells had decreased significantly. In a number of small retrospective studies looking at hMLH1 expression in breast and ovarian tumour samples, pre and post cisplatin-based chemotherapy, hMLH1 was also shown to be significantly reduced and this was associated with poor disease free survival (Mackay et al., 2000). Evidence for loss of hMLH1 expression other than through mutation has also emerged. Loss of hMLH1 in colon and ovarian tumour cells and also colon, ovarian and endometrial tumour cell lines arose through cytosine methylation of the hMLH1 promoter (Kane et al., 1997; Strathdee et al., 1999).



### 1.3 MMR and the Cell Cycle

A role for MMR in cell cycle regulation has been suggested from a number of studies. Re-introduction of chromosome 3 (carries *hMLH1*) into a *hMLH1* deficient cell line produced increased cell cycle arrest at G<sub>2</sub> in response to treatment with the antimetabolite 6-thioguanine compared to the parent cell line (Hawn et al., 1995). *hMLH1* has also been implicated in regulating a G<sub>2</sub>-M arrest in response to treatment with IR (Davis et al., 1998). Once again, using chromosome 3 transfer into a *hMLH1* deficient parental cell line, enhanced G<sub>2</sub>-M arrest was demonstrated in response to IR compared to the MMR deficient parental cell line. However as seen in studies utilising established mouse embryonic fibroblasts deficient for *PMS2*, *MLH1* or *MSH2* expression, these cells deficient for functional MMR did not show much difference in survival after IR compared to the MMR proficient cells (Fritzell et al., 1997).

Cell cycle expression of MMR genes was examined. In yeast mRNA expression of *yMSH2*, *yMSH6* and *yPMS1* were each found to increase at the G<sub>1</sub>-S boundary compared to other points in the cycle (Kramer et al., 1996). Expression levels of *yMLH1* and *yMSH3* were shown to be constitutive throughout the cell cycle.

Expression of human MMR genes has also been studied. One study of *hMSH2* expression in resting versus proliferating blood lymphocytes and thymocytes demonstrated that *hMSH2* protein levels increased 12 fold upon induction of proliferation (Marra et al., 1996). However throughout the cell cycle only minor variations in *hMSH2* protein expression were observed, in particular a slight increase in expression was seen at the G<sub>2</sub>/M replicative and post-replicative phases. A second study also reported little variation in *hMSH2* mRNA or protein levels throughout the cell cycle (Meyers et al., 1997). Expression levels of *hMLH1* showed a similar pattern, although as for *hPMS2*, protein levels did increase slightly at the G<sub>1</sub>/S transition phase.

Steady state levels of the human MMR proteins were also examined (Chang et al., 2000). Utilising competitive quantitative reverse transcription-polymerase chain reaction and western blotting. Results with both measurements gave similar ratios of expression levels between the different gene products. The *hMutS* homologues were found to be the most abundantly expressed proteins. In particular *hMSH2* was expressed at approximately 3-5 times the level of *hMLH1*. *hMSH6* was more highly expressed than *hMSH3* but the combined abundance of the two proteins was about equal to that of *hMSH2*. *hMLH1* was



about twice as abundant as hPMS2. In agreement with the earlier study with hMSH2 alone, expression levels of all the MMR genes examined increased in highly proliferative cells.



## 1.4 Transcription Coupled Repair and MMR

DNA repair and transcription are not two completely separate processes as demonstrated by the discovery that in *E.coli*, *Saccharomyces cerevisiae* and human cells, DNA damage is repaired more rapidly in the transcribed strand than from the non-transcribed strand in expressed genes (Hanawalt, 1994). This phenomenon has been called transcription-coupled repair (TCR). It is proposed that the presence of a bulky lesion in response to treatment with e.g. ultraviolet light (UV) leads to the stalling of RNA polymerase at this lesion, somehow signalling repair to this strand (Leadon, 1999; Mellon et al., 1987).

Nucleotide excision repair (NER) acts to repair bulky helix distorting lesions, such as cyclobutane pyrimidine dimers that result from UV exposure, or thymine glycols that result from oxidative DNA damage (Wood, 1997). Such bulky lesions can obstruct transcription and replication processes. A key part of the NER pathway is the incision of the damaged strand on both sides of the lesion and consequent removal of the damage site. It is believed that a proportion of NER is coupled to transcription (Leadon, 1999). A number of gene products involved in this TCR process have been found to be mutated in three human hereditary disorders, xeroderma pigmentosum (XP), Cockayne's syndrome (CS), and trichothiodystrophy (TTD). Two helicases, encoded by XPB and XPD which play a role in NER are also known to be components of TFIIH, a transcription initiation factor complex

NER repairs bulky adducts as described above. MMR functions to repair single base mismatches and small heteroduplexes. MMR proteins have been discovered to also play a role in TCR, which has led to the proposal that MMR may act to couple NER to transcription. Initial observations in *E.coli* demonstrated that mutations in MutS or MutL resulted in the abolition of TCR of cyclobutane pyrimidine dimers (CPD) in the induced lactose operon of cells treated with UV (Mellon and Champe, 1996). However the addition of MutL or MutS to an *in vitro* NER system did not have any effect on the efficiency of lesion removal (Leadon, 1999; Selby and Sancar, 1995).

Mutations in the yeast, *Saccharomyces cerevisiae*, *yMSH2*, *yMLH1*, *yPMS1* and *yMSH3* genes had no effect on TCR of UV induced DNA damage (Sweder et al., 1996). However the TCR of thymine glycols in response to oxidative damage was found to require yMsh2p, yMlh1p and yPms1p (Leadon and Avrutskaya, 1998). Interestingly in the single *mlh1* and *pms1* mutants no effect on TCR was observed. However loss of TCR of thymine glycols

was observed in the double *mlh1 pms1* mutants which would suggest that unlike for their role in MMR these two proteins act independently but have overlapping roles in TCR.

In contrast with these observations in yeast, hMLH1, hMSH2 and hPMS2 were each shown to play a role in TCR of cyclobutane pyrimidine dimers which arise in response to UV treatment (Mellon et al., 1996). However in the case of TCR of oxidative damage in human cells, hMSH2 was required, but hMLH1 was not required for the preferential removal of oxidative DNA damage from the transcribed strand of the active gene (Leadon and Avrutskaya, 1997). The human MutS $\alpha$  complex has also been shown to specifically bind such adducts (Wang et al., 1999). This differential involvement of the two proteins was the first example of a protein absolutely required for TCR of UV-induced damage of DNA but not for TCR of oxidative DNA damage. Recent observations of interaction between yeast Msh2p and a number of NER proteins suggests these proteins could act in complex (Bertrand et al., 1998).



## 1.5 Aims of the Thesis

The initial aim of the work in this thesis was to set up a functional yeast two-hybrid system. In particular it was hoped to construct a yeast two hybrid vector expressing a hMLH1 hybrid protein. Utilising this hMLH1 yeast two-hybrid construct the aim was to identify novel proteins involved in DNA mismatch repair. hMLH1 has been implicated in playing a role in signalling downstream cellular pathways in response to DNA damage. Screening a yeast two-hybrid cDNA library could also therefore potentially identify novel protein-protein interactions between hMLH1 and other proteins involved in these pathways.

From these studies an intriguing interaction between hMLH1 and c-MYC was observed. This particular protein-protein interaction has been further characterised, and attempts to elucidate the functional significance of this interaction have been made. Therefore a brief introduction to c-MYC follows.

## 1.6 The c-Myc Oncogene

The proto-oncogene *c-MYC* was first discovered through its homology with the transforming gene, *v-MYC*, of avian myelocytomatosis virus, MC29 (Ryan and Birnie, 1996; Vennstrom et al., 1982). Members of the *MYC* oncogene family include, *c-MYC*, *N-MYC* and *L-MYC*. The latter two genes were discovered again through their homology with *v-MYC*, as amplified genes in neuroblastoma cells (*N-MYC*) and a small cell lung tumour cells (*L-MYC*) (Nesbit et al., 1999). The oncogenic activation of the *c-MYC*, *N-MYC* and *L-MYC* genes has been implicated in the establishment of a range of human neoplasias (Hayward et al., 1981; Nesbit et al., 1999). The most ubiquitous and best studied of the group, the *c-MYC* gene is associated with a range of cancers.

The *c-MYC* gene is found on chromosome 8 and consists of three exons (Battey et al., 1983; Dang, 1999). Exon I is a long untranslated exon, and Exon II and III encompass the protein coding sequences. Transcription initiates from two major promoters P1 and P2, found in Exon I with a gap of 174 nucleotides between them. Most transcript in tissues and cell lines examined originate from P2. Both P1 and P2 have a consensus TATA box element, and P2 also has a strong consensus initiator element (Inr). The major c-MYC protein (c-MYC 2) is translated from the AUG start codon in exon 2, and produces a 64 kDa protein. A second 67 kDa (c-MYC 1) protein is produced by translation from an alternative CUG codon in exon 1, 15 codons upstream of the AUG (Dang, 1999; Hann et al., 1992). The expression from the upstream start codon is inefficient compared to that from the major AUG start codon. However upon increased cell density in culture, initiation from this CUG upstream start codon increases, which is specifically due to amino acid deprivation in particular through lower availability of methionine (Hann et al., 1992). A more recently identified 45 kDa polypeptide, c-MYC short (c-MYC S) results from translation from an internal AUG initiation site (Spotts et al., 1997).

Expression of the c-myc gene is closely related to cellular proliferation (Obaya et al., 1999; Ryan and Birnie, 1996). In quiescent cells c-MYC expression is absent. Upon mitogen stimulation i.e. the addition of serum containing growth factors, *c-MYC* expression is rapidly induced (Cole, 1986; Eilers et al., 1989). *c-MYC* is an immediate early gene, as shown by the fact that c-MYC levels peak between 2 and 4 hours after serum stimulation of expression (Persson et al., 1985). Levels of c-MYC subsequently decrease to a lower constitutive level, which is maintained throughout the rest of the cell cycle (Rabbitts et al., 1985). Removal of serum containing growth factors, from cells at any point during



progress through the cell cycle leads to the down-regulation of *c-MYC* expression (Dean et al., 1986).

Altered *c-MYC* expression is associated with many cancers. *c-MYC* is capable of transforming cells *in vitro* and mouse xenografts with cells expressing activated *c-MYC* (i.e. increased *c-MYC* expression) from tumours (Keath et al., 1984). How *c-MYC* induces neoplastic transformation however is not fully understood. The observation that *c-MYC* could affect gene expression led to the idea that this protein may play a role in transcription control (Kaddurah-Daouk et al., 1987).

## 1.7 c-Myc Protein Structure

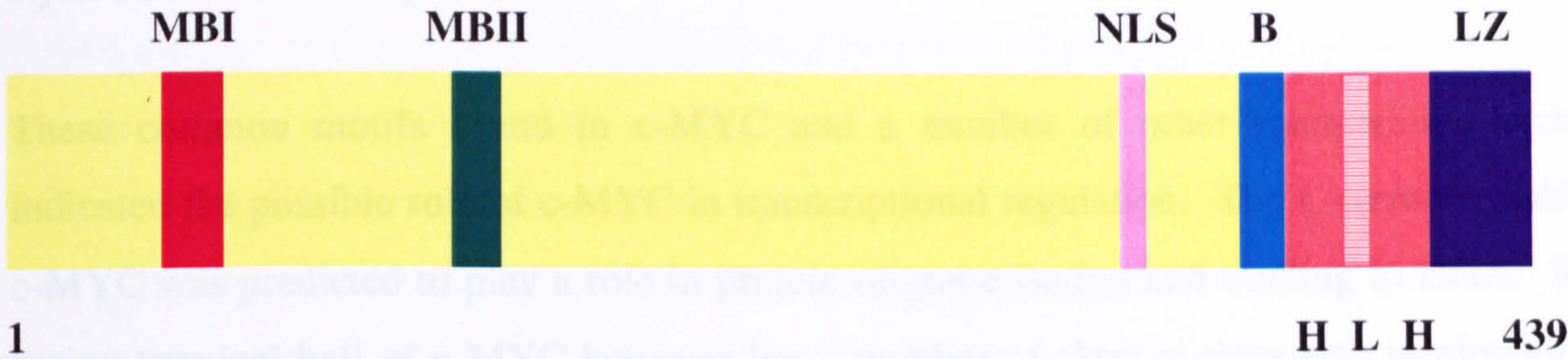
The c-MYC protein in avian and human cells was found to localise to the nucleus by immunofluorescence analysis (Dang and Lee, 1988; Eisenman et al., 1985). Further examination of the c-MYC protein revealed a number of motifs that were previously identified in a number of known transcription factors (see Figure 3). Immunofluorescence experiments with mutants of c-MYC, examining the subcellular localisation of these proteins, demonstrated the role of this conserved region in the nuclear localisation of c-MYC (Stone et al., 1987). c-MYC was also found to be a phosphoprotein, phosphorylated by kinases such as casein kinase II (CKII) (Luscher et al., 1989).

Amino acid sequence similarity was recognised between c-MYC and two other transforming proteins, JUN and FOS (Landschulz et al., 1988; Vogt et al., 1987). Periodic repeats of leucine residues were observed in the C-terminal sequences of each of these proteins (Landschulz et al., 1988). It was proposed that these repeated leucine residues extend from an extra long  $\alpha$ -helix. These leucine residues were proposed to establish amphipathy which aids the stabilisation of this long  $\alpha$ -helix. c-MYC has, beginning at a position 32 amino acids from the C-terminus, leucine residues at every seventh position over 8 proposed turns of the  $\alpha$ -helix. This motif is conserved in the N-MYC protein and also in three of the four positions in L-MYC. This proposed conserved motif structure is seen in other proteins including FOS, JUN and C/EBF. Only the leucine amino acids are conserved between these proteins. The leucine side chains (which are long, symmetrical and bulky) of one helix were proposed to 'interdigitate' with those of a matching helix from a second polypeptide, to allow stable non-covalent linking of the proteins.

Another structural motif found in the DNA binding domain of bacterial repressors and activators was identified in mammalian proteins. This DNA binding motif elucidated through X-ray crystallography is called a 'helix-turn-helix' motif. The characteristics of this motif are two consecutive amphipathic  $\alpha$ -helices juxtaposed at approximately  $90^\circ$  by a turn or 'loop' of four amino acids (Landschulz et al., 1988; Steitz et al., 1982). This helix-loop-helix (HLH) motif was soon identified in a range of mammalian proteins including c-MYC (Luscher and Eisenman, 1990). Proteins involved in transcription regulation have also been shown to carry this motif e.g. the transcription factors USF and MYOD1. This motif facilitates protein oligomerisation.



Examination of the MYC protein also led to the discovery of, in addition to the HLH domain, a group of basic residues adjacent to the HLH motif (Davis et al., 1990). This basic region was found to be required for sequence-specific binding of DNA. The c-MYC HLH motif located directly upstream of the Octamer binding site appears to interact on its amino-terminal side by a "basic region" (see Figure 3) (Lukatis and Eisenman, 1994; Ryan and Birnba, 1996).



- MBI:** MYC Box I ( $\alpha\alpha$  45-63)
- MBII:** MYC Box II ( $\alpha\alpha$  104-122)
- NLS:** Nuclear Localisation Signal ( $\alpha\alpha$  320-328)
- B:** Basic Region ( $\alpha\alpha$  355-368)
- HLH:** Helix-Loop-Helix Domain ( $\alpha\alpha$  369-410)
- LZ:** Leucine Zipper Domain ( $\alpha\alpha$  411-439)

**Figure 3: c-MYC Structure**

Structural domains of the c-MYC protein, amino acids ( $\alpha\alpha$ ) 1-439, are illustrated.



Examination of the MYOD1 protein also led to the discovery of, in addition to this HLH domain, a group of basic residues adjacent to the HLH motif (Davis et al., 1990). This basic region was found to be required for sequence specific binding of DNA. The c-MYC HLH motif located directly upstream of the C-terminus leucine zipper domain was flanked on its amino-terminal side by a 'basic region' (see Figure 3) (Luscher and Eisenman, 1990; Ryan and Birnie, 1996).

These common motifs found in c-MYC and a number of other transcription factors indicated the possible role of c-MYC in transcriptional regulation. The C-terminal half of c-MYC was predicted to play a role in protein oligomerisation and binding to DNA. The amino terminal half of c-MYC however has a number of short regions rich in glutamine, proline, and acidic residues, which are associated with a number of transactivation domains (Kato et al., 1990). GAL4-c-MYC fusion proteins were expressed in cells to test the ability of this protein to activate transcription. Fusion proteins consisting of the N-terminal region of c-MYC and the GAL4 binding domain of the yeast GAL4 transcription factor were demonstrated to have transactivation ability. By transient co-transfection of a vector expressing this fusion protein and a reporter construct consisting of the GAL4 DNA binding site upstream of a chloramphenicol acetyltransferase (CAT) reporter gene, transactivation was easily measured. This experiment clearly demonstrated the specific transactivation potential of the c-MYC protein. Three independent regions were identified, that lie between amino acids 1-143 of c-Myc, and are capable of activating transcription from the CAT reporter gene.

Multiple oncogenes are required to mediate the transformation of normal cells into tumorigenic cells (Land et al., 1983). Expression of either *c-MYC* or activated *RAS* alone does not lead to transformation of primary cells, but together can do so (Land et al., 1986; Lee et al., 1985). The transformation of rat embryo fibroblasts through both aberrant *c-MYC* expression and expression of a mutant *ras* gene serves as a classical example of cooperation between oncogenes (Lee et al., 1985). By utilising in frame insertion or deletion mutants of *c-myc* expressed from a retroviral promoter-enhancer, the regions of *c-MYC* required for co-transformation of rat embryo fibroblasts with a mutant *ras* gene were elucidated (Stone et al., 1987). This oncogenic activity of c-MYC was shown to depend on two specific regions of the protein, amino acids 105-143 and 321-439. The region between amino acids 1-104 could tolerate small insertions or deletions and the region between amino acids 144-320 was dispensable for this oncogenic role of c-myc. A number of conserved blocks of amino acids are found within the N-terminus of the c-MYC protein.



The most critical for the oncogenic transformation role of c-MYC is MYC Box II (MbII) which centres around amino acid 135 (see Figure 3) (Cole and McMahon, 1999; Stone et al., 1987).

The *N-MYC* and *L-MYC* genes also have the three exon structure of *c-MYC* with coding sequences in exons II and III. Both proteins encoded by these genes, N-MYC and L-MYC have a number of domains with at least 80% amino acid sequence homology to the c-MYC protein. These highly conserved regions include the N-terminal MYC Box I (MbI) and MYC Box II (MbII) domains and the basic helix-loop-helix and leucine zipper domains. The conservation of these domains between these three proteins and with homologous proteins from other species suggest they play an important functional role.

## 1.8 The MYC/MAX/MAD Family of Proteins

Evidence was accumulating in the early 1990s to suggest that c-MYC is a transcription factor. However it was discovered that c-MYC could only oligomerise and bind DNA when expressed at high concentrations, suggesting these observations are not physiologically relevant (Dang et al., 1989; Ryan and Birnie, 1996). The conserved bHLH-Zip (basic-helix-loop-helix-leucine zipper) domain of c-MYC was used to screen a cDNA expression library derived from a baboon lymphoblastoid cell line (Blackwood and Eisenman, 1991). This resulted in the identification of MAX, a protein that can specifically bind to c-MYC, L-MYC and N-MYC, in *in vitro* binding assays. The HLH-Zip domain of c-MYC was required for this interaction which supports the models proposed for how c-MYC exerts its function through oligomerisation and DNA binding.

Examination of MAX sequence suggested it also contains a putative C-terminal bHLH-Zip domain as well as a basic region. The basic region of c-MYC was found not to be required for this interaction. However the c-MYC-MAX heterocomplex was found to be able to bind to an oligonucleotide containing the sequence CACGTG, and this binding required the basic region of c-MYC. This confirms the hypothesis that this domain is required for specific binding of this protein to DNA.

MAX encodes two major polypeptides p21 (21 kDa) and p22 (22 kDa) that differ by the addition of 9 amino acids amino terminal to the basic region of the protein sequence (Blackwood et al., 1992). MAX is a highly stable nuclear protein and like c-MYC is phosphorylated by CKII (casein kinase II). Co-immunoprecipitation studies demonstrated an *in vivo* interaction between c-MYC and MAX (Blackwood et al., 1992). Under these conditions of immunoprecipitation most of c-MYC was found to be in complex with MAX. Formation of the complex did not effect the short half-life of the c-MYC protein. These immunoprecipitated complexes were also found to be capable of binding specifically to an oligonucleotide with the CACGTG sequence. Unlike c-MYC, MAX is expressed at constant levels when cells are quiescent, after mitogenic stimulation and in cycling cells (Berberich et al., 1992; Blackwood et al., 1992).

The MAX interaction with c-MYC was found to depend on functional HLH-Zip domains on both proteins (Kato et al., 1992). This supports the role of these domains in oligomerisation. Like c-MYC, MAX has a nuclear localisation signal but does not have any domains capable of transcriptional activation. The transcriptional activation abilities



of MAX were tested for in the same way as c-MYC previously described, utilising GAL4 fusion proteins. The C-terminal half of the c-MYC protein expressed as a recombinant protein was shown to be capable of binding the consensus E-box recognition binding sequence (CACG/ATG) in DNA. MAX dimers or c-MYC-MAX heterodimers also bind this E-box sequence, and bind with it specifically when mixed with a pool of random oligonucleotides (Berberich et al., 1992; Kato et al., 1992; Littlewood et al., 1992).

The interaction between c-MYC and MAX was a very interesting observation. The C-terminal bHLH-Zip domain of c-MYC has been shown to be essential for the oncogenic activity of c-MYC (Stone et al., 1987). Some suggestions of a link between this transforming ability of c-MYC and its potential role in transcriptional activation had also been proposed through the discovery of a requirement for the N-terminal conserved domains of c-MYC for both activities. The discovery of an interaction between MAX and c-MYC which facilitates binding of the c-MYC protein to the E-box derivative consensus sequence as part of this complex, led to the proposal that MAX could be mediating these functions of c-MYC.

An *in vivo* expression system in yeast was utilised to test the transcriptional activation ability of c-MYC and MAX (Amati et al., 1992). This system involved the expression of c-MYC, MAX or both proteins in yeast which had a *LACZ* gene with an E-box sequence upstream of the promoter sequence. MAX homodimers and c-MYC-MAX heterodimers could both bind this recognition sequence. However by measuring  $\beta$ -galactosidase activity from the reporter gene, it was shown that only the c-MYC-MAX heterodimers could activate transcription of this gene. Through using mutants of c-MYC in this system, it was shown that the basic region of this protein is required for binding to DNA and hence transactivation of the *LACZ* gene. The dimerisation ability is also required for transactivation as mutations in the HLH-Zip domain also resulted in the loss of transactivation function. Mutants in the N-terminal transactivation domain (TAD) of c-MYC also led to loss of transactivation function. Through these studies, the HLH-Zip domain of MAX was found to have higher affinity for the HLH-Zip domain of c-MYC than for itself and preferably formed heterodimers with c-MYC than forming homodimers with itself.

A similar *in vivo* reporter system in yeast was used to characterise the regions of c-MYC required for binding to MAX and subsequent transactivation functions (Crouch et al., 1993). By utilising c-MYC mutants in this system both the HLH and the leucine zipper

domains were shown to be required for MAX-dependent DNA binding and transactivation by c-MYC. In particular helix 2 of the HLH and the leucine zipper are required for the c-MYC-MAX interaction, whereas helix 1 of the HLH is required for the sequence specific DNA binding of the heterodimer. These mutants had been previously tested for their ability to transform higher eukaryotic cells. Comparison of the effect of c-MYC mutations on the transforming ability and on the transactivation role of the protein revealed a close correlation between the regions of c-MYC required for these functions (Crouch et al., 1993; Crouch et al., 1990).

Another reporter system, a CAT reporter gene expressed in fibroblast or epithelial cell lines, was used with a fourfold repeat of an E-box sequence upstream of the promoter (Kretzner et al., 1992). This system demonstrated that overexpression of the MAX protein leads to repression of transcription from the reporter gene, in contrast to overexpression of c-MYC which led to activation of expression. This overexpression of c-MYC can specifically release the repressive effect of overexpressed MAX.

In summary c-MYC in complex with MAX can activate reporter gene expression through binding of this complex to a specific DNA recognition sequence CACG/ATG, the E-box sequence. MAX homodimers can also bind this sequence and have a repressive effect on transcription of the reporter gene. As stated above the MAX protein was not found to encode a TAD domain. However MAX forms heterodimers with c-MYC preferably to homodimers. These heterodimers are more stable than the homodimers and have a higher affinity for the E-box sequence than the MAX-MAX dimers. This would therefore suggest that putative genes regulated by c-MYC are also dependent on the levels of MAX being expressed. The oligomerisation of the two proteins via their respective HLH-Zip domains produces a protein structure capable of binding DNA through the basic domains of the proteins. This binding of c-MYC-MAX to the E-box derivative sequence, CACG/ATG, facilitates the transactivation abilities of the c-MYC protein.

Further studies with mutants of c-MYC and MAX suggest that only the HLH-Zip domain of MAX is required for the transactivation function of c-MYC (Amati et al., 1993). This supports the proposal that the TAD of c-MYC is sufficient to drive the transactivational activity of the DNA bound c-MYC-MAX complex. MAX and its mouse homologue MYN were both found to be able to inhibit the transforming effect of MYC and RAS together when expressed at high levels (Amati et al., 1993; Prendergast et al., 1991). The possibility that MAX could heterodimerise with other proteins that could bind the same



recognition binding sequence and compete with MYC-MAX as do MAX homodimers was proposed from these observations. MAX therefore plays a role in mediating both the transactivation and oncogenic roles of c-MYC. However interestingly MAX can act as both suppressor and activator of these functions.

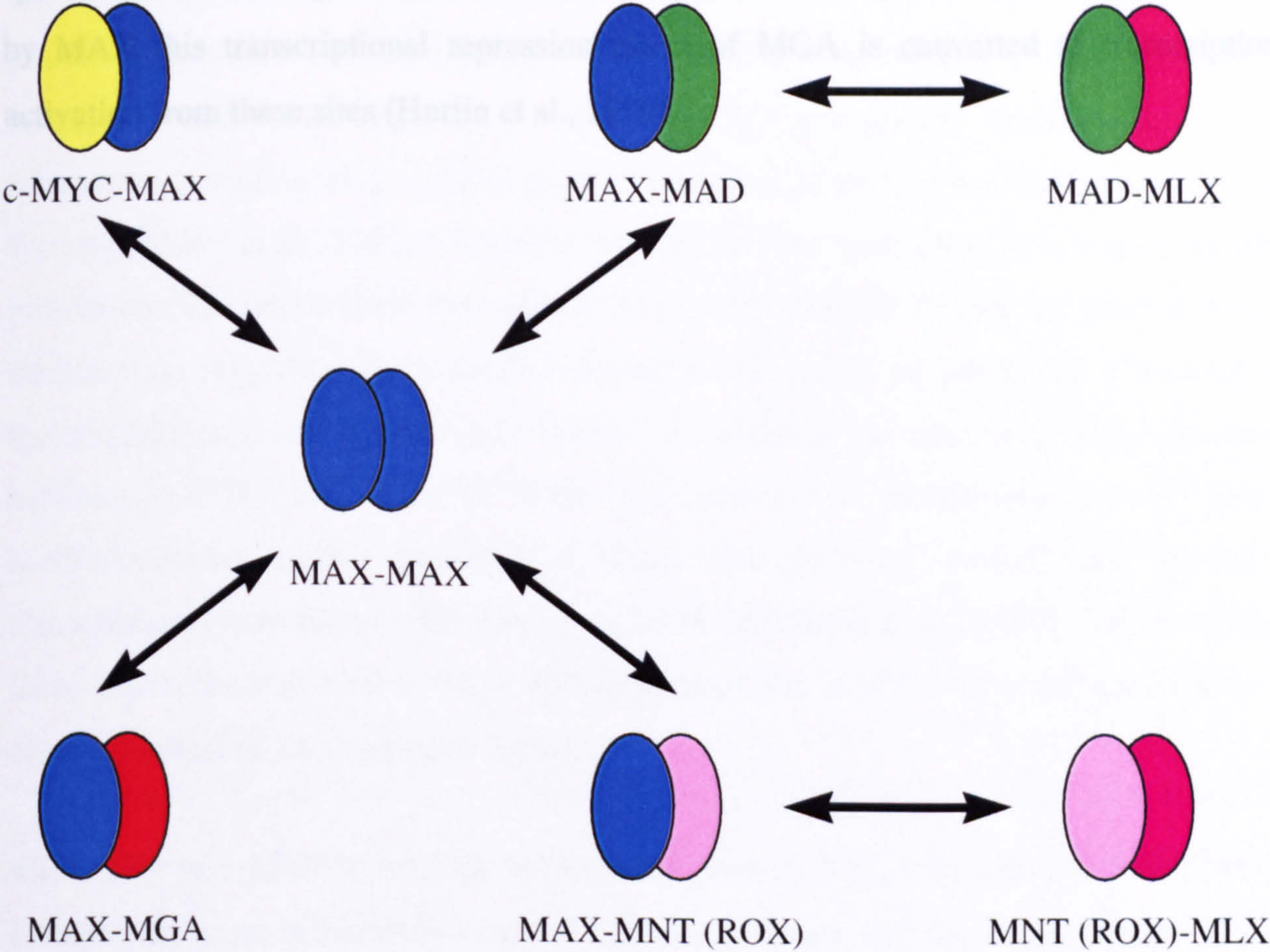
Evidence soon emerged for a network of transcription regulators with MAX acting as a central component (see Figure 4). To test for the possibility that MAX can interact with other HLH-Zip proteins, a similar cDNA expression library screen was carried out, to that which originally identified the c-MYC-MAX interaction (Ayer et al., 1993). This led to the identification of MAD, also a HLH-Zip protein. MAD, through its C-terminal half was shown to bind MAX *in vitro*. However MAD did not bind c-MYC, and like c-MYC, formed homodimers poorly. Also in common with c-MYC-MAX heterodimers, the MAD-MAX heterodimers are favoured over MAX homodimers. MAD and c-MYC can compete for binding to MAX to form DNA binding complexes with the derivative E-box sequence CACG/ATG. MAD alone did not bind this DNA binding sequence. Utilising the CAT-reporter assay described earlier, the transcriptional activity of the MAD-MAX heterodimer was also examined (Ayer et al., 1993; Kretzner et al., 1992). This assay demonstrated the opposing functions of the MAD-MAX and c-MYC-MAX heterodimers. In contrast to c-MYC-MAX, MAD-MAX acts like MAX homodimers to repress transcription of the reporter gene.

Other MAD family proteins were identified that can also interact with MAX. These include MXI1 another bHLH-Zip protein, which in complex with MAX can bind the c-MYC-MAX consensus recognition site (Zervos et al., 1993). Two more MAD family members MAD3 and MAD4 also bind MAX, and show similar characteristics to the MAD-MAX complex (Hurlin et al., 1995). However these complexes act to repress transcription at the same sites as c-MYC-MAX complexes activate transcription. This repression activity is facilitated through an association with the mSIN3 co-repressor complex, which includes for example histone deacetylases (Alland et al., 1997; Ayer et al., 1995; Schreiber-Agus et al., 1995). Unlike c-MYC-MAX the formation of these MAD-MAX complexes is closely linked to terminal differentiation (Ayer and Eisenman, 1993).

MAD and MAD4 have been demonstrated to interact with MLX (Max Like protein X) a MAX related protein (Billin et al., 1999). These dimers also have transcriptional repression activity. Two additional bHLH-Zip proteins, MNT (also known as ROX) and MGA have been shown to interact with MAX (Hurlin et al., 1999; Meroni et al., 1997).



MNT also interacts with MLX (Chen et al., 2000). MGA has an additional T-box DNA binding domain through which it represses transcription from specific sites including the c-MYC-MAX recognition binding site. However, upon binding to the c-MYC-MAX recognition binding site, MGA is converted to a transcriptional activator (Bartie et al., 1999).



**Figure 4: MAX Interacting Proteins.**  
Schematic describing the different protein-protein interactions involving MAX and MAX-like proteins.



MNT-MAX acts to repress transcription in a similar manner to the other heterodimers described above. MNT also heterodimerises with MLX (Meroni et al., 2000). MGA has an additional T-box DNA binding domain through which it represses transcription from specific sites including the c-MYC-MAX recognition binding site. However upon binding by MAX this transcriptional repression effect of MGA is converted to transcriptional activation from these sites (Hurlin et al., 1999).

## 1.9 c-MYC Function

The identification of an N-terminal transactivation domain and also a C-terminal bHLH-Zip domain (which is commonly found in a number of transcription factors) in the c-MYC protein suggested a role for this protein in transcriptional control. c-MYC through its interaction with MAX can bind a consensus DNA recognition binding sequence and activate transcription of a reporter gene in a number of *in vivo* reporter gene expression systems (Amati et al., 1992; Kretzner et al., 1992). The description of a number of novel protein-protein interactions between c-MYC and proteins known to play a role in transcription regulation gives further support to the model of c-MYC as a transcription factor (Sakamuro and Prendergast, 1999). In particular the discovery of an interaction between c-MYC and TRRAP with the subsequent recruitment of the histone acetyltransferase GCN5 provided a direct link between c-MYC and modes of transcriptional activation (McMahon et al., 1998; McMahon et al., 2000). The diversity of these interactions as well as their effects suggest that c-MYC does not conform to the classical model of a transcription factor.

The ability of c-MYC to activate transcription from both reporter genes and also putative endogenous genes is relatively weak. It has been proposed that the transactivation function of c-MYC-MAX perhaps through its association with proteins such as TRRAP is indirect. It may instead act to make target genes more accessible, through interactions with TRRAP and GCN5, to other transcription factors such as USF which are also capable of binding the E-box recognition sequence. c-MYC has also been implicated in repression of the expression of a number of genes. Again this function of c-MYC is believed to be mediated through its interactions with a number of different proteins. For example c-MYC somehow activates the repression function of the POZ domain of MIZ-1, a protein known to interact with the HLH domain of c-MYC, and inhibits the normal transactivation function of MIZ-1. Whether these two roles of c-MYC are separable or are required to act in concert to exert overall c-MYC function has not yet been elucidated.

Elevated expression of c-MYC can inhibit differentiation in some cell types whereas in cell culture inhibition of c-MYC expression can lead to induction of differentiation (Facchini and Penn, 1998). Competition for c-MYC-MAX binding sites by for example the MNT-MAX and MAD-MAX complexes is central to their antagonistic roles in transcription activation versus repression. A switch from c-MYC-MAX to these alternative complexes accompanies the switch from proliferation to differentiation (Ayer and Eisenman, 1993;



Hurlin et al., 1997). This switch leads to the sequential expression of the MAD family of transcription repressors during this differentiation process (Queva et al., 1998). *c-MYC* is also found to repress the expression of a number of genes involved in differentiation (Claassen and Hann, 1999). Therefore by blocking differentiation aberrant expression of *c-MYC* can keep cells proliferating and predispose the cells to transformation.

*c-MYC* is an immediate early gene suggesting that it plays a critical role in signalling a number of growth signal response pathways. *c-MYC* apparently plays a role in regulation of the cell cycle. The deletion of *c-MYC* through homologous recombination in Rat1 cells results in a longer cell cycle (Mateyak et al., 1997). Cell cycle regulation is through cyclins, cyclin dependent kinases and their target genes, which act as key players in inducing DNA synthesis. Induction of *c-MYC* expression e.g. through the addition of growth factors to quiescent cells, can drive cells into the cell cycle (Cole, 1986; Eilers et al., 1989). A number of candidate *c-MYC* target genes play a role in the cell cycle progression and may explain how *c-MYC* can influence this process.

The role of *c-MYC* in proliferation is unlikely to be restricted to the regulation of the cell cycle. An important aspect of the proliferative process is the ability of cells to increase in size and also to co-ordinate this growth with division. Cell growth regulation operates at a number of levels, as dictated by for example nutrient availability or changes in protein synthesis with progression through the cell cycle (Schmidt, 1999). Protein synthesis is itself regulated at two stages, ribosomal synthesis and translation initiation. The transcription factor YY1 which is known to interact with *c-MYC*, plays a major role in transcription of ribosomal protein mRNA (Chung and Perry, 1993). Translation initiation factors are then required for the assembly of ribosomes into active translation complexes. These factors are rate limiting due to the fact that they are not as abundant as the ribosomal components of these complexes. *c-MYC* has been previously implicated in the regulation of the translation initiation factor eIF4E (Jones et al., 1996). Both these roles of *c-MYC* although not yet directly tested could potentially implicate *c-MYC* as playing a role in regulation of protein synthesis and explain the growth phenotype of the *c-MYC* null cells.

*c-MYC* gene expression is clearly required for proliferation and downregulation of this gene results in growth arrest and differentiation. The discovery that the constitutive expression of *c-MYC* in both rat embryonic fibroblasts and established Rat1 fibroblasts grown in low serum can trigger apoptosis, places *c-MYC* protein at a cross-roads between two opposing pathways, proliferation versus cell death (Askew et al., 1991; Evan et al.,

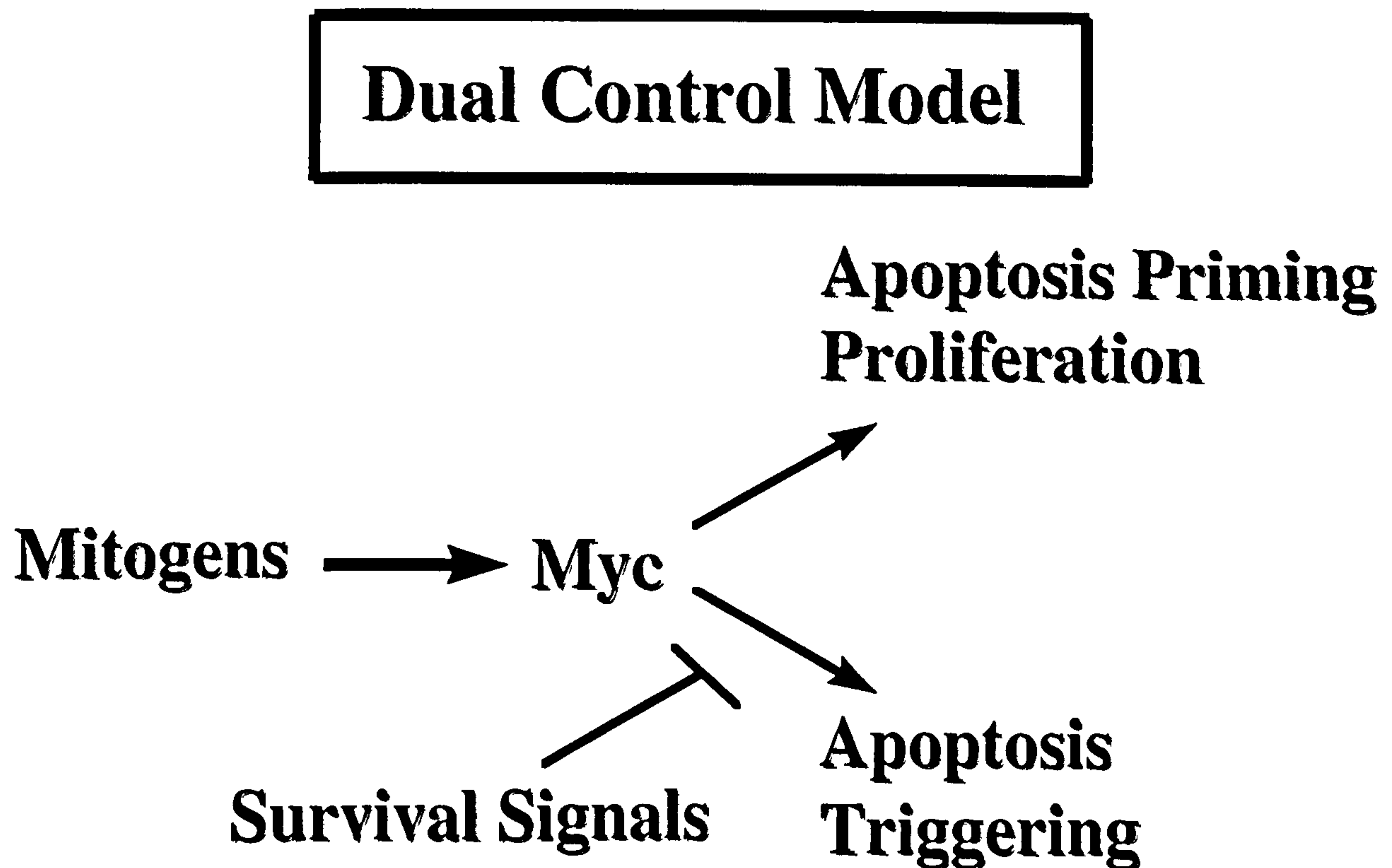


1992). Apoptosis by c-MYC was shown to be activated by a number of signals, for example amino acid deprivation, suggesting that in fact the combination of the signal for growth and the conflicting signal for arrest could be the major determinant of the death signal. In normal cells the withdrawal of growth factors is accompanied by downregulation of c-MYC expression leading to exit from the cell cycle, but in cells where c-MYC expression is constitutive, apoptosis occurs. The domains of c-MYC required for its co-transformation, transactivation and repression functions are also required for this role in apoptosis.

The requirement for c-MYC for signalling both proliferation and apoptosis, two opposing pathways, has been demonstrated. A 'dual signal model' has been proposed to explain these paradoxical roles for the protein (Harrington et al., 1994; Prendergast, 1999). This model proposes that c-MYC acts to regulate both cell division and cell death. However these functions of c-MYC cannot be separated as distinct entities. Therefore this model has been re-modelled to suggest that c-MYC can co-ordinately prime both cell death and drive proliferation (see Figure 5) (Prendergast, 1999). Priming of cell death for example could involve the transcriptional regulation of c-MYC target genes. Triggering of cell death is proposed to subsequently act through an additional pathway.

c-MYC can sensitise cells to the death receptor signals from the Tumour Necrosis Factor (TNF) transmembrane receptor family, which includes the CD95/Fas receptor (Prendergast, 1999). Evidence for the model proposed above, come from studies reporting the observation that inhibition of CD95/Fas signalling blocks apoptosis in Rat1 fibroblasts but proliferation induced by c-MYC continues. Likewise the interaction between c-MYC and the BIN1 protein is required to trigger apoptosis but is not necessary to drive proliferation or transformation (Prendergast, 1999; Sakamuro and Prendergast, 1999). The transition from 'priming' cell death to actually 'triggering' cell death could involve for example interactions between BIN1 and the c-MYC target gene products (Prendergast, 1999).





**Figure 5: Dual Control Model**

This model proposes that c-MYC can co-ordinately prime both cell death and drive proliferation (Prendergast, 1999). Priming of cell death for example could involve the transcriptional regulation of c-MYC target genes. Triggering of cell death is proposed to subsequently act through an additional pathway.

# Chapter 2

## Materials and Methods



## **2.1 Materials**

### **2.1.1 Equipment**

Routine equipment which would be an integral part of any laboratory is not listed.

Coulter Electronics Cell Counter

Millipore Electroblotting System

Kodak Film Processor

Applied Biosystems Sequencer

Hybaid Touchdown PCR Thermal Cyclers

Hybaid Omnigene PCR Thermal Cyclers

Innova 4300 Incubator shaker, New Brunswick Scientific

Innova 4000 Incubator shaker, New Brunswick Scientific

Omega Prestige Medical Autoclave

Flow laboratories CO<sub>2</sub> Incubator 220

Voss Eppendorf Rotator

MixiMatic Shaker, Jencons Scientific Ltd

MSE Soniprep 150 Sonicator

IEC Centra-7R Refrigerated Centrifuge

Sorvall Instruments - Dupont RC3C Refrigerated Centrifuge

Sorvall Instruments - Dupont RC5B Refrigerated Superspeed Centrifuge

IEC Micromax Centrifuge

Hybaid Electroporator

Invitrogen Novex Gel Apparatus

Luckham 4RT Rocking Table

Corning pH meter 240

Pharmacia Biotech Electrophoresis Power Pack Supply EPS 600

MPH Multipurpose Gel Electrophoresis Chamber

Bio-Rad Gel Doc 1000 UV box

Applied Biosystems 392 DNA/RNA Oligonucleotide Synthesiser

Pharmacia Biotech GeneQuant RNA/DNA Calculator

Synoptics Protos Colony Counter, Don Whitley Scientific

## **2.1.2 Chemicals**

All chemicals were of the highest available quality and were supplied by:

Gibco BRL Life Technologies, Paisley, Renfrewshire, UK.

Transgenomic Ltd., Crewe, Cheshire, UK.

BDH Limited Chemicals, Supplied by McQuilken & Co., Glasgow, UK.

Fisher Chemicals, Fisher Scientific, Loughborough, UK.

Sigma-Aldrich Company Ltd., Poole, Dorset, UK.

Roche Molecular Biochemicals, Lewes, East Sussex, UK.



**2.1.3            Kits**

<u>Material</u>	<u>Supplier</u>
Plasmid Maxi Kit; Qiaprep Spin Miniprep Kit; Qiaquick PCR Purification Kit RNeasy Kit	Qiagen Ltd., Dorking, Surrey England.
Promega Wizard DNA Clean-Up System	Promega Ltd, Chilworth Research Centre, Southampton, England
Nucleon Kit for Yeast DNA Extraction	Scotlab Bioscience, Coatbridge, Lanarkshire, Scotland
SUPERSCRIPT™ Preamplification System for First Stand cDNA Synthesis	Gibco BRL LifeTechnologies, Paisley, UK.

**2.1.4            General Plasticware**

<u>Material</u>	<u>Supplier</u>
Falcon tubes	Becton Dickinson Labware, Plymouth,UK.
5ml Bijous; 20ml Universals;	Bibby-Sterilin Ltd., Stone, UK.
Microcentrifuge tubes; Pipette tips	Elkay, Galway, Ireland.

**2.1.5            Microbial Host, Media and Supplies**

<u>Material</u>	<u>Supplier</u>
Bactotryptone; BactoAgar; Yeast Extract	Difco, Becton Dickinson, UK.
Competent <i>E.coli</i> DH5α cells; ElectroMAX Cells DH10B; Select Peptone	Gibco BRL LifeTechnologies, Paisley, UK.

Petri dishes	Bibby-Sterilin Ltd., Stone, UK
Ampicillin; Kanamycin	Sigma-Aldrich Company Ltd., Dorset, Yeast England
Nitrogen Base w/o Amino Acids	
Amino Acids	
Human Mammary Gland	CLONTECH Laboratories UK Ltd., Hampshire, UK
MATCHMAKER cDNA Library;	
Pretransformed Human Ovary	
MATCHMAKER cDNA Library;	
KC8 Electro-competent Cells	

**2.1.6 Electrophoresis Gels**

<u>Material</u>	<u>Supplier</u>
Agarose, electrophoresis grade;	Gibco BRL Life Technologies, Paisley, UK
Tris Base;	
Hae III digested $\phi$ X174 DNA	
Hind III digested bacteriophage $\lambda$ DNA	
1Kb DNA Ladder	
SequaGel, 19:1 acrylamide:bisacrylamide	National Diagnostics, Hull, UK
Novex Gels	Invitrogen BV, Gronigen, The Netherlands.
Rainbow <sup>TM</sup> coloured protein molecular markers	Amersham, International plc., Amersham, Bucks, England.
Redivue [ $\gamma^{32}$ P]dATP	

**2.1.7 Western Blots and Autoradiography**

<u>Material</u>	<u>Supplier</u>
3MM Filter Paper	Whatman International, Maidstone, UK.
Immobilon-P Transfer Membrane	Millipore Corporation, USA.



ECL Western Detection Agent

Amersham, International plc., Amersham,  
Bucks, England.

Fuji Super RX, X-Ray Film

Fuji Photo Film (Europe), H.A. West,  
Clydebank, Glasgow, UK.

2.1.8           Antibodies

<u>Antibody</u>	<u>Isotype</u>	<u>Supplier</u>
MLH1 (Ab-1) Mouse Monoclonal	IgG <sub>1</sub>	Oncogene Research Products
MLH1 (Ab-2) Rabbit Polyclonal	IgG	Calbiochem, UK
MSH2 (Ab-2) Mouse Monoclonal	IgG <sub>1</sub>	
PMS2 (Ab-1) Mouse Monoclonal	IgG <sub>1</sub>	
c-myc (Ab-2) Mouse Monoclonal	IgG <sub>2a</sub>	
c-MYC (C-8) Mouse Monoclonal	IgG <sub>2a</sub>	Santa Cruz Biotechnology,
c-MYC (C-19) Rabbit Polyclonal	IgG	Autogen Bioclear, Wiltshire, UK.
3/23, an anti-human c-MYC Monoclonal		Made by Karin Moelling, gift from D.Gillespie
237-6, an anti-human c-MYC Polyclonal		Made by D. Gillespie, against bacterially produced full length c- MYC
Anti-mouse IgG-horseradish peroxidase conjugate (from goat)		Santa Cruz Biotechnology, Autogen Bioclear, Wiltshire, UK.

2.1.9           Tissue Culture Media and Supplies

<u>Material</u>	<u>Supplier</u>
Foetal Bovine Serum	Autogen Bioclear, Wiltshire, UK.
L-Glutamine	Gibco BRL Life Technologies, Paisley, UK.
Trypsin (2.5%)	
Penicillin-Streptomycin	
RPMI 1640	
Dulbecco's Modified Eagle Medium,	

(with sodium pyruvate, w/o L-glutamine,  
with pyridoxine)

25, 75 and 150 cm <sup>2</sup> flasks	Iwaki, Scitech, Japan
6 and 96 well plates	

9 cm Plates	Becton Dickinson Labware, Plymouth,UK.
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**2.1.10 Buffers, Solutions and Media**

**Luria-Bertani (LB) broth**

10 g/L	Bacto-tryptone
5 g/L	Bacto-yeast extract
5 g/L	NaCl

Double distilled water was added to the mixture to a final volume of 1L. The pH was adjusted to 7 with NaOH and the mixture autoclaved and then allowed to cool and stored at 4°C.

**S.O.C. Medium**

2.0%	Bactotryptone
0.5%	Yeast Extract
10 mM	NaCl
2.5 mM	KCl
10 mM	MgCl <sub>2</sub>
10 mM	MgSO <sub>4</sub>



20 mM          Glucose

A 50X stock solution of the sodium, potassium and magnesium salts in distilled water was prepared. The Bactotryptone/Yeast Extract solution was prepared and the pH was adjusted to 7. The three solutions were autoclaved separately and allowed to cool. Appropriate volumes of the salts solution and glucose solution were added to the Bactotryptone/Yeast Extract solution.

### **S.O.B. Medium**

SOB contains the same nutrients and salts as SOC, but it lacks glucose.

### **M9 Minimal Medium**

200 ml          5X M9 Salts

100 ml          of the appropriate sterile 10X dropout solution (Leu<sup>-</sup>)

18 g/L          Bacto-agar

The solution was made up to a final volume of 980ml with sterile double distilled water and autoclaved. 20ml of sterile 20% Glucose solution was added to the medium.

### **M9 Salts**

64 g/L          Na<sub>2</sub>HPO<sub>4</sub>·7H<sub>2</sub>O

15 g/L          KH<sub>2</sub>PO<sub>4</sub>

2.5 g/L          NaCl

5 g/L          NH<sub>4</sub>Cl

The salts were dissolved in a final volume of 1 L of double distilled water and autoclaved.

### **YPD Medium**

20 g/L          Difco Peptone

- 10 g/L        Yeast Extract
- 18 g/L        Bacto-Agar (for plates only)

Distilled water was added to the mixture to 950 ml. The pH was adjusted to 5.8 using HCl and the mixture autoclaved and then allowed to cool to approximately 55°C. Glucose was added to 2% before use.

**10X Dropout Solution**

Dropout solution was made up with the following concentrations of amino acids. When yeast were grown under nutrient selection, the appropriate amino acids were left out of the mixture.

<u>Amino acids</u>	<u>mg/L</u>	<u>Sigma Catalogue No.</u>
L-Adenine hemisulfate salt	200	A-9126
L-Arginine	200	A-5131
L-Histidine HCl monohydrate	200	H-9511
L-Leucine	1000	L-1512
L-Lysine HCl	300	L-1262
L-Methionine	200	M-9625
L-Phenylalanine	500	P-5030
L-Threonine	2000	T-8625
L-Tryptophan	200	T-0254
L-Tyrosine	300	T-3754
L-Uracil	200	U-0750



L-Isoleucine	300	I-7383
L-Valine	1500	V-0500

**SD Medium**

6.7 g/L	Yeast Nitrogen Base
18 g/L	Bacto-Agar
850 ml	H <sub>2</sub> O
100 ml	of the appropriate sterile 10X dropout solution

The pH was adjusted to 5.8 using NaOH and the mixture autoclaved and then allowed to cool to approximately 55°C. Glucose was added to 2% before use.

**10X TE Buffer**

0.1 M	Tris- HCl
10 mM	EDTA

The pH of the solution was adjusted to 7.5 and the solution autoclaved.

**10x LiAc**

1 M	Lithium Acetate.
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The pH of the solution was adjusted to 7.5 and the solution autoclaved.

**1X TE/LiAc Solution**

Prepared fresh immediately prior to use from stocks of 10X TE buffer and 10X LiAc diluted in sterile water.

**PEG/LiAc Solution**

8 ml            50% PEG4000

1 ml            10X TE

1 ml            10X LiAc

### **Z buffer**

16.1 g/L         $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$

5.5 g/L          $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$

0.75 g/L        KCl

0.246 g/L        $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$

The pH of the solution was adjusted to 7 and autoclaved.

### **X-gal Stock Solution**

5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside (X-GAL, Sigma #8060-1) was re-suspended in N,N-dimethylformamide (DMF) at a concentration of 20mg/ml. The solution was stored in the dark at -20°C.

### **Z buffer/X-gal Solution**

100 ml         Z buffer

0.27 ml          $\beta$ -mercaptoethanol

1.67 ml         X-gal stock solution

### **Whatman #5 Filters**

75 mm diameter filters were used.



**Yeast Protein Extraction Buffer**

50 mM	Tris-HCl pH7.4
100 mM	NaCl
2 mM	EDTA

**Protease Inhibitor Cocktail**

0.1 mg/ml	Chymostatin
0.1 mg/ml	Leupeptin
0.05 M	PMSF
0.1 mg/ml	Pepstatin
0.05 M	Benzamidine
0.1 mg/ml	Aprotonin

**DNA Loading Buffer**

30% w/v	Glycerol
0.25% w/v	Bromophenol blue
0.25% w/v	Xylene cyanol

**10X TBE**

108 g/L	Tris
55 g/L	Boric Acid
8.45 g/L	EDTA

**Phosphate Buffered Saline (PBS)**

- 0.8% w/v      NaCl
- 0.115% w/v   Na<sub>2</sub>HPO<sub>4</sub>
- 0.02% w/v     KCl
- 0.02% w/v     KH<sub>2</sub>PO<sub>4</sub>

**3X Protein Sample Buffer**

- 1 ml            1M Tris (pH8)
- 20% w/v       SDS
- 0.5 ml          β-mercaptoethanol
- 1 ml            Glycerol
- 0.25% w/v     Bromophenol blue

**10X Blotto**

- 500 mM        Tris pH7.5
- 500 mM        NaCl
- 10 mM          EDTA

**Blocking Solution**

- 1X               Blotto
- 5% w/v         Marvel
- 0.01% v/v      Tween-20



**5X Annealing Buffer**

335 mM      Tris pH 7.6

65 mM        MgCl<sub>2</sub>

33.5 mM      DTT

6.5 mM        Spermidine

6.5 mM        EDTA pH 8

**2X Salt Binding Buffer**

1.12 ml       4 M            NaCl

1 ml           1 M            Tris pH7.9

3 ml                            Glycerol (100%)

16 ml          250 mM        EDTA

20 ml          1 M            DTT

4.88 ml                        dH<sub>2</sub>O

**2X No Salt Binding Buffer**

1 ml           1 M            Tris pH 7.9

3 ml                            Glycerol (100%)

0.5 ml                        Salmon Sperm DNA (2.5mg/ml sonicated)

16 ml          250 mM        EDTA

20 ml          1 M            DTT

5.5 ml  $\text{dH}_2\text{O}$

### 10X Binding Buffer

100 mM      Hepes pH 7.9

2 mM EDTA

1 mg/ml      BSA

40% Glycerol

## Gel Fix Solution

10% Methanol

**10%                      Glacial Acetic Acid**

**Made up to a final volume of 100 ml with ddH<sub>2</sub>O**

## Non-Denaturing Polyacrylamide Gels for EMSA

**8 ml      30% Acrylamide/Bisacrylamide (19:1 sequencing grade)**

6 ml                      10X TBE

46 ml                      ddH<sub>2</sub>O

300  $\mu$ l      10% APS (freshly made)

30  $\mu$ l                      TEMED

### Low Stringency Lysis Buffer

1% v/v      NP40

0.2 mM PMSF



0.7 mg/ml      Pepstatin

0.5% w/v      Aprotinin

10 mM          NaF

50 mM          β-glycerophosphate

**Nuclear Extraction Buffer**

25 mM          Hepes. KOH pH 8.0

1 mM           EDTA

1 mM           Benzamidine

2 mM           β-mercaptoethanol

0.5 mM          Spermidine

0.1 mM          Spermine

**Mismatch Retard Buffer**

25 mM          Hepes.KOH pH8

0.5 mM          EDTA

10% v/v        Glycerol

0.1 mM          ZnCl<sub>2</sub>

0.5 mM          DTT

**Complete Mismatch Gel Retard Mix**

200 ml          Mismatch Retard Buffer

10 µl	(10mg) Poly (dI.dC) <sub>2</sub> (Sigma) - 1mg/ml in TE or ddH <sub>2</sub> O
4 ml	G:C 34mer @100fmol/ml (40fmol/tube) ds complementary oligo competitor

**6% Polyacrylamide Non-denaturing Gel**

6 ml	30% Acrylamide/Bisacrylamide (19:1 sequencing grade)
20.4 ml	ddH <sub>2</sub> O
600 µl	10% ammonium persulphate solution (fresh made)
3 ml	10X TBE
15 µl	TEMED

**Reagent B**

400 mM	Tris-HCl pH8.0
60 mM	EDTA
150 mM	NaCl
1% w/v	SDS (added after the solution is autoclaved)

**20X MOPS SDS Running Buffer**

1 M	MOPS
1 M	Tris Base
69.3 mM	SDS
20.5 mM	EDTA



**20X MES SDS Running Buffer**

- 1 M            MES
- 1 M            Tris Base
- 69.3 mM       SDS
- 20.5 mM       EDTA

**2.1.11            Oligonucleotides**

**Oligonucleotides for Electrophoretic Mobility Shift Assays**

Myc oligo with the ‘E box’ sequence.

- GATCAATTCGACACGTGGTCGGATC            MycF-band
- CTAGTTAAGCTGGTGCACCAGCCTAG            MycR-band

EMSA Mismatch Substrate Oligonucleotide

5’-AGCTTGGCTGCAGGTNGACGGATCCCCGGGAATT-3’

EMSA SP1 Consensus Binding Site Oligonucleotide

5’-ATTCGATCGGGGCGGGGCGAGC-3’

**Oligonucleotide Primers for PCR and Sequencing**

<u>PMS2-F1</u> : 5’-CTAGGTGAATTCATGGAGCGAGCTGAG-3’
<u>PMS2-R1</u> : 5’-ACCTAGGGATCCTTAATGCAGAGCATCCGAACAGCT-3’
<u>PMS2-F2</u> : 5’-TCGCCGGAATTCCTAGTGACTCCGTGTGT-3’
<u>PMS2-R2</u> : 5’-TCGCCGGGATCCTTATCCTAGAGGGCTCCTTCT-3’

<u>PMS2-F3</u> : 5'-TCGCCGGAATTCGAGAACAAGCCTCACAGC-3'
<u>PMS2-R3</u> : 5'-TCGCCGGGATCCTTACTTCCTGTAATTCTGTTCCCC-3'
<u>PMS2-F4</u> : 5'-TCGCCGGAATTCCAGTTACATCATGAAGCACAGCAA-3'
<u>PMS2-R4</u> : 5'-ACCTAGGGATCCTCAGTTCTGAGAAATGACACC-3'
<u>PMS2-R3-9</u> : 5'-TCGCCGGGATCCTTAATTCTGTTCCCCTTCACT-3'
<u>PMS2-R3-24</u> : 5'-TCGCCGGGATCCTTAACTTTGCTGTGCTTCATG-3'
<u>PMS2-R3-51</u> : 5'-TCGCCGGGATCCTTACTTTATTCGTTTAGCTAA-3'
<u>PMS2-R3-102</u> : 5'-TCGCCGGGATCCTTACTTATTAATTTTCAC-3'
<u>PMS2-R3-300</u> : 5'-TCGCCGGGATCCTTATGAAAAGAGTCGTCAGT-3'
<u>pGAD424-PMS2-F3</u> : 5'-GAGATCGAATTCGAGAACAAGCCTCACAGC-3'
<u>pGBT9-PMS2-F3</u> : 5'-TCGCCGGAATTCGAGAACAAGCCTCACAGC-3'
<u>MLH1-F1</u> : 5'-TCGCCGGAATTCATGTCGTTTCGTGGCAGGG-3'
<u>MLH1-R1</u> : 5'-AGGATGGATCCTTACAGTTCTCGACTAACAGCATTTC-3'
<u>MLH1-F2</u> : 5'-TCGCCGGAATTCGGCAGGTATTCAGTACAC-3'
<u>MLH1-R2</u> : 5'-AGGATGGATCCTTACATCCTGGAGGAATTGGA-3'
<u>MLH1-F3</u> : 5'-TCGCCGGAATTCCTCCACAAAGCATGAAGTTCAC-3'
<u>MLH1-R3</u> : 5'-AGGATGGATCCTTATGGCTCCGATAACCTGAG-3'
<u>MLH1-F4</u> : 5'-TCGCCGGAATTCGAGCAGGGACATGAGGTT-3'



<u>MLH1-R4</u> : 5'-AGGATGGATCCTTAACACCTCTCAAAGACTTTGTATAG-3'
<u>pGBT9-F</u> : 5'-GCATTGTTAACAGGATTATTTGTAC-3'
<u>pGBT9-R</u> : 5'-TACAGGAAAGAGTTACTCAAGAA-3'
<u>pGAD424-F</u> : 5'-ACCAAACCTGCGTATAACGCGTTT-3'
<u>pGAD424-R</u> : 5'-TGGCCAAGATTGAAACTTAGAGG-3'
<u>pACT2-F-LAST</u> : 5'-CGATGCACAGTTGAAGTGAACCTTG-3'
<u>pACT2-F-LAST</u> : 5'-ACGATGTTCCAGATTACGCTAGCT-3'
<u>pGBKT7-F</u> : 5'-TCATCGGAAGAGAGTAG-3'
<u>pGBKT7-R</u> : 5'-TAAAACCTAAGAGTCACT-3'
<u>myc-F</u> : 5'-GCATACATCCTGTCCGTC-3'
<u>myc-R</u> : 5'-GACGGACAGGATGTATGC-3'
<u>M1</u> : 5'-ATCGCGAATTCCGCCCACGACCAGCAGCGAC-3'
<u>M2</u> : 5'-ACGTCTCGAGCTATGCACGAGAGTTCCTTAG-3'
<u>HPRT-F</u> : 5'-GACTGAACGTCTTGCTCGAGATG-3'
<u>HPRT-R</u> : 5'-AATCTACAGTCATAGGAATGGA-3'

**2.1.12            Cell Lines**

**A2780**

A human ovarian adenocarcinoma cell line derived from an untreated patient received from R. F. Ozols and T. C. Hamilton, Fox Chase Cancer Centre, Philadelphia.

**K562**

Human erythroleukaemia cell line, derived from Chronic Myeloid Leukaemia in blast crisis.

**Rat-1**

Rat fibroblast cell line.

**Rat-1 MYCER™**

Rat-1 fibroblasts expressing c-MYCER™(Littlewood et al., 1995).

**2.1.13            Yeast Strains**

**Y190**

<u>Genotype</u>	<i>MATa, gal4Δ, gal80Δ, his3, trp1-901, ade2-101, ura3-52, leu2-3, 112, URA3::GAL-lacZ, LYS::GAL<sub>(UAS)</sub>-HIS3, cyh.r</i>
<u>Reporters</u>	<i>HIS3, lacZ</i>
<u>Transformation Markers</u>	<i>trp1, leu2</i>



**PJ69-2A**

Genotype *MATa, trp1-901, leu2-3, 112, ura3-52, his3-200, gal4Δ, gal80Δ, LYS2::GAL1<sub>UAS</sub>-GAL1<sub>TATA</sub>-HIS3, GAL2<sub>UAS</sub>-GAL2<sub>TATA</sub>-ADE2*

Reporters *HIS3, ADE2*

Transformation Markers *trp1, ura3, leu2*

**Y187**

Genotype *MATα, ura3-52, his3-200, ade2-101, trp1-901, leu2-3, 112, gal4Δ, gal80Δ, met<sup>-</sup>, URA3::GAL1<sub>UAS</sub>-GAL1<sub>TATA</sub>-lacZ*

Reporters *lacZ*

Transformation Markers *trp1, leu2*

**2.1.14 Plasmids**

GAL4 DNA-BD Vectors	Description	Size	Unique Cloning Sites
pGBT9	GAL4 <sub>(1-147)</sub> DNA-BD, TRP1, amp <sup>r</sup>	5.4 Kb	BamHI, EcoRI, PstI, Sall, SmaI, XmaI
pGBKT7	GAL4 <sub>(1-147:762-1202)</sub> DNA-BD, TRP1, kan <sup>r</sup>	7.3 Kb	NdeI, NcoI, SfiI, EcoRI, SmaI, XmaI, BamHI, Sall, PstI

GAL4 DNA-AD Vectors	Description	Size	Unique Cloning Sites
pGAD424	GAL4 <sub>(768-881)</sub> DNA-AD, <i>LEU2</i> , amp <sup>r</sup>	6.6 Kb	<i>EcoRI</i> , <i>SmaI</i> , <i>BamHI</i> , <i>Sall</i> , <i>PstI</i>
pACT2	GAL4 <sub>(768-881: 5081-5419)</sub> DNA-AD, <i>LEU2</i> , amp <sup>r</sup>	8.1 Kb	<i>NcoI</i> , <i>SfiI</i> , <i>SmaI</i> , <i>XmaI</i> , <i>BamHI</i> , <i>EcoRI</i> , <i>SacI</i> , <i>XhoI</i> , <i>BglII</i>

Control Plasmids	Description	Size
pCL1	wild-type full length <i>GAL4</i> gene in a Ycp50 derivative, <i>LEU2</i> , amp <sup>r</sup>	15.3 Kb
pVA3	murine p53 <sub>(72-390)</sub> in pGBT9, <i>TRP1</i> , amp <sup>r</sup>	6.4 Kb
pTD1	SV40 large T-antigen <sub>(84-708)</sub> in pGAD3F, <i>LEU2</i> , amp <sup>r</sup>	15 Kb
pLAM5'	Human lamin C <sub>(66-230)</sub> in pGBT9, <i>TRP1</i> , amp <sup>r</sup>	6.0 Kb



## **2.2 Methods**

### **2.2.1 Bacterial Protocols**

#### **2.2.1.1 Bacterial Culture**

Bacteria were maintained either in Luria-Bertani (LB) broth liquid culture or on LB agar plates under selective conditions. Cells were stored for long periods in 15% glycerol in LB broth at -70°C.

#### **2.2.1.2 Preparation of Electrocompetent Bacterial Cells.**

50 ml of S.O.B. media was inoculated with a freshly grown single colony of the KC8 bacterial strain. The culture was incubated overnight (12-16 hours) at 37°C in a shaking incubator. 5 ml of the overnight culture was used to inoculate 500 ml of S.O.B media in a 2 L flask.. This culture was incubated at 37°C in a shaking incubator until the cell density reached an optical density of 0.8 at 550 nm (2-3 hours). The culture was transferred into chilled centrifuge bottles and the cells collected by centrifugation in the Sorvall RC3C, H-6000A rotor at 1000 g. The cell pellet was re-suspended in ice-cold sterile distilled water to wash the cells and collected again by centrifugation. This wash step was repeated. The cells were then washed twice in ice-cold sterile 10% glycerol. The cell pellet was re-suspended in 10% glycerol and aliquots were dispensed into chilled microcentrifuge tubes and stored at -70°C.

#### **2.2.1.3 Transformation of Bacterial Cells with Plasmid DNA by Electroporation**

10-50 ng of plasmid DNA was added to 40 µl of electrocompetent bacterial cells in pre-chilled microcentrifuge tubes. This mixture was then transferred into a standard electroporation cuvette with a 1mm gap. The loaded cuvette was placed into the Hybaid electroporator cuvette holder and the safety cover closed. A pulse was delivered through the cuvette and 1 ml of S.O.C media was added immediately to the cuvette to wash the cells out of the electrode gap and transferred to a culture tube. The cells were incubated at 37°C in a shaking incubator for 1 hour. Appropriate dilutions were plated on selective

medium (1.5% LB-agar plates, 100 µg/ml ampicillin or 50 µg/ml kanamycin) using a sterile glass loop. Plates were incubated overnight at 37°C.

#### **2.2.1.4 Transformation of Bacterial Cells with Plasmid DNA**

10-20 ng of plasmid DNA was added to 50 µl of Library Efficiency DH5α competent cells (Gibco) and left on ice for 30 minutes. After a 45 sec heat-shock at 42°C, 900 µl of S.O.C media was added and the samples vortexed before incubation at 37°C for 1 h.. Appropriate dilutions were plated on selective medium (1.5% LB-agar plates, 100 µg/ml ampicillin or 50 µg/ml kanamycin) using a sterile glass loop. Plates were incubated overnight at 37°C.

#### **2.2.1.5 Plasmid DNA Mini-Preparation.**

Cultures for plasmid preparation were grown from a single colony picked with a sterile loop from a freshly streaked selective plate. This single colony was used to inoculate 5 ml of Luria-Bertani (LB) broth and the culture incubated overnight at 37°C in a shaking incubator. Antibiotic selection was used at every stage of growth, ampicillin 100 mg/ml or kanamycin 50 mg/ml depending on the plasmid being used. The Qiaprep Spin Miniprep kit was used according to the manufacturers recommendations to obtain plasmid DNA.

#### **2.2.1.6 Plasmid DNA Maxi Preparation.**

A 5 ml culture was used to inoculate 500 ml of LB-broth in a 1 L flask containing 100 µg/ml ampicillin or 50 µg/ml kanamycin (for selection) and this culture was grown to stationary phase overnight in a 37°C shaking incubator. The Qiagen Maxi Preparation kit was used according to the manufacturers recommendations to obtain plasmid DNA. Both Qiagen Mini and Maxi Preparation Kits utilise a modified alkaline lysis protocol. The lysate is then cleared by centrifugation and the plasmid DNA bound to an anion-exchange resin. Any RNA, protein etc. are removed by a medium-salt wash through this resin. Plasmid DNA is subsequently eluted from the resin in a high salt buffer and concentrated and desalted by isopropanol precipitation.



## **2.2.2 Yeast Protocols**

### **2.2.2.1 Yeast Culture.**

Yeast were maintained either in YPD liquid culture or on YPD agar plates. Cells were stored for long periods in 15% glycerol in YPD at -70°C.

Synthetic dropout (SD) minimal medium was used for yeast transformations to select and test for specific phenotypes. SD medium was prepared with a minimal SD base with a stock of 'dropout' solution containing a specific mixture of amino acids and nucleosides.

### **2.2.2.2 Yeast Strain Phenotype Testing**

The phenotypes of each yeast strain were verified before any experiments were carried out. Yeast strains used were supplied as frozen glycerol stocks. Fresh working stock plates were prepared for each, by scraping a small amount of cells with a sterile loop from the frozen stock and streaking these onto YPD plates. The yeast strains used in these studies are all deficient for Leu and Trp i.e. they cannot grow on SD minimal medium lacking these nutrients. Therefore the phenotype of the yeast strains was tested by streaking them out onto SD medium plates selecting for a number of different essential amino acids.

### **2.2.2.3 Preparation of Yeast Competent Cells**

A single yeast colony was inoculated into 20 ml of YPD medium (adenine supplemented 2 g/L). The inoculates were incubated at 30°C for 16-18 hours or until the culture was at stationary phase ( $OD_{600} > 1.5$ ). This overnight culture was transferred into a final volume of 300 ml YPD. This was incubated at 30°C with shaking for 3 hours. The cells were collected by centrifugation in the Sorvall RC3C, H-6000A rotor at 1000 g for 5 min at room temperature (20-21°C). The supernatant was discarded and the cell pellet was re-suspended in 50 ml of water. The cells were centrifuged again for 5 min at 1000 g at room temperature. The pellet was re-suspended in 1.5 ml of freshly prepared, sterile 1X TE/LiAc. Competent cells were stored at -70°C in 15% glycerol.

#### **2.2.2.4 Lithium/Acetate Transformation of Yeast Cells with Plasmid DNA**

1.5 µg of each plasmid DNA was mixed together with 100 µg of herring testes DNA (20 µg/µl, denatured by placing on a 100°C heating block for 20 min and cooling rapidly on ice) in a 1.5 ml microcentrifuge tube. 100 µl of yeast competent cells was added to the DNA and mixed well. 600 µl of sterile PEG/LiAc solution was added to each tube and vortexed to mix. The tubes were incubated at 30°C for 30 min, with shaking (200 rpm). 70 µl of DMSO was added and mixed gently by inversion. The cells were heat shocked for 15 min in a 42°C water bath. To pellet the cells, the tubes were chilled on ice and centrifuged for 5 sec at 18,300 g in a microcentrifuge. The supernatant was removed and the cells were re-suspended in 0.5 ml of 1X TE buffer. 100 µl of the transformation mixture was spread onto each 10 cm plate containing the appropriate selection medium. The plates were incubated at 30°C for 2-4 days (until colonies appeared). Master plates were sealed with parafilm and stored at 4°C for 3-4 weeks.

#### **2.2.2.5 Large Scale Transformation Protocol for Library Screen 1**

A 5 ml culture of Trp SD liquid media was inoculated with a single colony of Y190 transformed with pGBT9 MLH1. This was allowed to grow at 30°C for 8 hours. The 5ml culture was made up to a 50 ml culture and was grown overnight in Trp dropout media. One litre of cells was grown by adding half of this overnight culture to each of two flasks with 500 ml YPD in each. These cells were allowed to grow at 30°C with shaking for 4 hours. The cells were collected by centrifugation at 1000 g for 5 min at room temperature. The supernatant was decanted and the pellet re-suspended in 500 ml sterile water and centrifuged as above. The supernatant was decanted and the pellet re-suspended in 8 ml PEG/LiAc solution. The library DNA (Human Mammary Gland MATCHMAKER cDNA Library, Clontech Cat.#HL4036AH) was mixed with 0.25 ml of 20 mg/ml denatured herring testes DNA and 30 µg of library DNA. This mixture was then added to the cell suspension and mixed. 60 ml of PEG/LiAc solution was also added and this mixture was incubated at 30°C for 30 mins with shaking. 7.6 ml of DMSO was then added and swirled to mix. This mixture was heat shocked for 15 min at 42°C with occasional swirling to facilitate heat transfer. The cells were collected by centrifugation at 1000 g for 5 min at room temperature. The pellet was re-suspended in 50 ml of TE buffer and centrifuged as above. The supernatant was decanted and the pellet re-suspended in 10 ml of TE buffer.



500 µl of this mixture was plated out onto Leu<sup>-</sup>/Trp<sup>-</sup>/His<sup>-</sup> 23 cm x 23 cm plates with 25 mM 3-AT (3-amino-1,2,4-triazole, SIGMA).

#### **2.2.2.6 Large Scale Transformation Protocol for the Yeast Two-Hybrid Pretransformed Library Mating Screen**

hMLH1 was cloned into a GAL 4 binding domain (BD) vector, pGBKT7 and transformed into the PJ69-2A strain. The pGBKT7 hMLH1 construct, like pGBT9 MLH1 did not autonomously activate the reporter genes, was not toxic when expressed in yeast and gave a positive interaction when co-transformed into Y190 with pGAD424 PMS2 F3. One large pGBKT7 hMLH1 transformed PJ69-2A colony was used to inoculate 50 ml of Trp SD liquid media. This culture was incubated at 30°C with shaking for 24 hours. The cells were collected by centrifugation at 1000 g for 5 minutes. The supernatant was decanted off and the pellet re-suspended in the residual liquid by vortexing. A 1 ml aliquot of the pre-transformed library was allowed to thaw in a room temperature water bath. 10 µl of this aliquot was set aside. The bait containing cells and the 1 ml library aliquot were mixed with 45 ml YPDA/Kanamycin (15 mg/L Kanamycin) in a 2 L conical flask and mixed gently by swirling. The flask was incubated at 30°C in a shaking incubator (50 rpm) for 24 hours. This mating culture was transferred into two 50 ml Falcon tubes and the cells collected by centrifugation at 1000 g for 10 minutes. The pellet was re-suspended in 10 ml of YPDA/Kan giving a total volume of 11 ml. 100 µl of a 1:10, 1:100, 1:1000 and a 1:10000 dilution of this mixture was plated out on 10 cm Leu<sup>-</sup>, Trp<sup>-</sup> and Leu<sup>-</sup>/Trp<sup>-</sup> SD plates. 200 µl of the remaining mixture was plated out onto 52 QDO (quadruple dropout) plates and incubated at 30°C for 16 days.

#### **2.2.2.7 Library Titre**

The 10 µl aliquot of the library set aside was mixed with 1 ml YPDA/Kan in a 1.5 ml microcentrifuge tube. This was labelled dilution A (dilution factor of 10<sup>-2</sup>). 10 µl of dilution A was mixed with 1 ml YPDA/Kan and labelled dilution B (dilution factor 10<sup>-4</sup>). 10 µl of dilution A was mixed with 50 µl YPDA/Kan and plated out onto a Leu<sup>-</sup> SD plate. 50 µl and 100 µl of dilution B was plated out onto Leu<sup>-</sup> SD plates. The plates were incubated at 30°C until colonies appeared.



### **2.2.2.8 Colony Lift Filter Assay**

The colony lift filter assay can be performed at any time after colonies are visible. A sterile Whatman #5 filter was placed over the surface of the agar plate with the transformant colonies. To orient the filter on the agar, holes were poked through the filter into the agar, in three asymmetric locations, with a sterile needle. The filter was carefully lifted off the agar plate with a sterile forceps and transferred (colonies facing up) to a pool of liquid nitrogen. Using the forceps, the filters were completely submerged for 10 sec. After the filter had frozen completely, it was removed from the liquid nitrogen and allowed to thaw at room temperature. This freeze/thaw treatment serves to permeabilise the cells. The filter was carefully placed, colony side up, on another filter that had been pre-soaked in Z buffer/X-gal solution. The filters were incubated at 30°C and checked periodically for the appearance of blue colonies. The time for blue colonies to appear varied between 30mins and up to 8 hours (blue colonies after 8 hours taken as false positives). Certain strains, e.g. those transformed with pCL1 (a wild-type GAL4 control), turned blue more quickly (20-30 min). For the c-DNA library screen  $\beta$ -galactosidase-producing colonies were identified by aligning the filter to the agar plate using the orienting marks. Positives were isolated by picking the corresponding colonies from the original plates to fresh media.

### **2.2.2.9 Plasmid DNA Isolation from Yeast**

Cultures for plasmid preparation were grown from a single colony, picked from a freshly streaked selective plate, with a sterile loop. A single colony was used to inoculate 5 ml of the appropriate SD medium and allowed to grow overnight in a 30°C shaking incubator. The Nucleon Biosciences Mini Preparation (Scotlab Biosciences) kit was used according to the manufacturers recommendations to obtain plasmid DNA.

### **2.2.2.10 Preparation of Protein Extracts from Yeast**

Fresh single yeast colonies were used to grow up 300 ml yeast cultures as described above. The yeast cells were collected by centrifugation at 1000 g and the pellet washed in sterile distilled water. The cell pellet was re-suspended in 10 ml of sterile distilled water and transferred to a 15 ml Falcon-2059 tube. The cells were again collected by centrifugation and the pellet re-suspended in 0.5 ml of ice cold yeast protein extraction buffer which contains a protease inhibitor cocktail. Glass beads (425-600 microns, Sigma) were added to two thirds the height of the meniscus. The tubes were vortexed at 4°C for 10 min. The



cell extract was recovered from the glass beads using a fine-tipped plastic Pasteur pipette. To recover as much extract as possible the beads were re-extracted with 0.5 ml of buffer. Total protein concentration was estimated for the extracts using the BCA method.

### **2.2.3 DNA Protocols**

#### **2.2.3.1 Preparation of Herring Testes Carrier DNA**

Herring testes DNA for use as a carrier DNA in yeast transformations was prepared by dissolving herring testes DNA in 1X TE buffer and sonicating the mixture to reduce its viscosity. The DNA was extracted with phenol/chloroform, and precipitated with ethanol. The DNA was re-suspended at a concentration of 20 mg/ml in 1X TE buffer. The DNA was placed in a tube in a boiling water bath for 20 min and immediately cooled on ice. The carrier DNA was re-denatured just prior to use.

#### **2.2.3.2 Phenol/Chloroform Extraction of DNA**

An equal volume of phenol:chloroform was added to the nucleic acid sample in a polypropylene tube. The contents of the tube were mixed until an emulsion formed. The mixture was centrifuged at 2000 g in a Sorvall RC3C, H-6000A rotor for 3 min at room temperature. A pipette was used to transfer the aqueous phase to a fresh tube. The interface and organic phase were discarded. An equal volume of chloroform was added to this and again the mixture was centrifuged at 2000 g for 3 min at room temperature. The nucleic acid was recovered by precipitation with ethanol.

#### **2.2.3.3 Ethanol Precipitation of DNA**

3 M Na Acetate (pH 8) was added to the DNA sample (final concentration of 0.3 M i.e. a 1/10 volume) followed by 2 ½ volumes of ice-cold ethanol. The mixture was placed at -20°C for 30 min to allow the DNA precipitate to form. The samples were centrifuged at 15,800 g for 15 min in a microcentrifuge. The supernatant was removed, 1 ml of 70% ethanol was added to wash the pellet and the tube was placed at -20°C for 30 minutes. The tubes were again centrifuged at 15,800 g for 15 min. The supernatant was removed and the pellet was allowed to air dry before re-suspending in sterile water.

#### **2.2.3.4 Oligonucleotide Synthesis**

Oligonucleotides were synthesised by the Beatson Institute technical services staff on an Applied Biosystems 392 DNA/RNA oligonucleotide synthesiser, using the manufacturers protocol and Cruachem reagents. After de-protection in ammonia at 55°C overnight, the oligonucleotides were precipitated and used without further purification.

#### **2.2.3.5 Quantitation of DNA Concentrations**

DNA was quantified by spectrophotometric determination of its UV light absorbency. A Pharmacia Biotech GeneQuant machine was used for this measurement. This instrument measures absorbency of the input solution at 260 nm and 280 nm in a quartz capillary tube. dH<sub>2</sub>O was used as a blank. Calculation of the solution concentration utilises de Beer's law which takes an optical density of 1.0 at 260 nm as corresponding to a concentration of 50 µg/ml for double stranded DNA and 37 µg/ml for single stranded oligonucleotides. A<sub>260</sub>/A<sub>280</sub> ratios are 1.8 and 2.0 for pure DNA and RNA preparations respectively. The GeneQuant gives out a direct concentration reading for the DNA.

#### **2.2.3.6 Restriction Digests**

All restriction endonucleases used were supplied by Gibco BRL Life Technologies. Restriction digests were carried out according to the manufacturer's instructions, with the total enzyme concentration in the reaction not exceeding 10%. 50 µl reactions were used with 500-750 ng of plasmid DNA per reaction. Reactions were incubated on a 37°C heating block for 1-2 hours. When a double digest was required with two enzymes that required different enzyme buffers, sequential digestions were carried out. The first enzyme digestion was completed. This reaction was cleaned up using a Promega Wizard DNA Clean-Up System according to the manufacturers recommendations to remove the first enzyme used and small nucleotides resulting from digestion. This cleaned up linear DNA was subsequently digested with the second enzyme.

#### **2.2.3.7 De-Phosphorylation Reactions**

De-phosphorylation of restriction digested plasmids was performed with CIP (calf intestinal phosphatase). The protocol detailed in Maniatis suggests the use of 1 unit of CIP per 100 pmoles of DNA, however CIP was used in excess of this concentration. In the case



of double digests CIP was added in excess to the second digest reaction and incubated with this reaction at 37°C on a heating block for 1-2 hours. The digested DNA was again cleaned up using the Promega Wizard DNA Clean-Up System.

### **2.2.3.8 Ligation Reactions**

Ligation reactions were carried out in a final volume of no greater than 15 µl so as to maintain a high concentration of 5' and 3' DNA ends. T4 DNA Ligase (Promega) catalyses the joining of two strands of DNA between the 5'-phosphate and the 3'-hydroxyl groups of adjacent nucleotides in either a sticky-ended or blunt-ended configuration. The reactions were performed using a 1:1 and 1:3 molar ratio of vector:insert DNA when cloning a fragment into a plasmid vector. Plasmid DNA was treated with CIP before the ligation reactions were carried out. Reactions consisted of 100 ng of plasmid DNA, insert DNA, 1 Weiss Unit of T4 DNA ligase in 1X ligase buffer (300 mM Tris-HCl, pH 7.8, 100 mM MgCl<sub>2</sub>, 100 mM DTT and 10 mM ATP) in a final volume of 10-15 µl. The ligated products were used to transform DH5α library efficiency competent cells as described.

### **2.2.3.9 Preparation of cDNA**

RNA was prepared from  $1 \times 10^7$  A2780 cells using a Qiagen RNeasy Kit according to the manufacturers protocol. cDNA was subsequently prepared from this using a SUPERScript™ Preamplification System kit for first strand cDNA synthesis (Gibco BRL) again according to the manufacturers protocol.

### **2.2.3.10 Polymerase Chain Reaction (PCR)**

The following conditions were used to perform the majority of PCR amplifications. Slight alterations were made to the annealing temperature or concentration of magnesium ions where required for optimal results. Each standard PCR reaction consisted of 100-300 ng template DNA, 1X PCR reaction buffer (contains MgCl<sub>2</sub>), 0.18 mM dNTP, 0.16 µM of each primer, 2.5 U Taq DNA polymerase (Roche) or 1.75 U Expand Taq (Roche) with the volume adjusted to 50 µl with ddH<sub>2</sub>O. ddH<sub>2</sub>O instead of template was added to control reactions. The thermal cycling parameters consisted of 30 cycles of 1 min denaturation at 94°C, 1 min annealing at 55°C, and 1 min extension at 72°C using a Hybaid Touchdown or Omnigene PCR Thermal Cycler.

### **2.2.3.11 Removal of Primers from PCR Products**

Primer dimers were removed from PCR products using the Qiaquick PCR Purification Kit (Qiagen).

### **2.2.3.12 Automated DNA Sequencing**

Samples were sequenced using the PRISM BigDye™ terminator cycle sequencing ready reaction kit (ABI). Purified PCR products were mixed with 3.2 pmoles of primer and the volume made up to 6 µl with H<sub>2</sub>O. 4 µl of the BigDye™ terminator ready reaction mix was added to each sample before undergoing the following PCR protocol with Perkin Elmer 9600. Preheat to 96°C followed by 25 cycles; 96°C for 15 sec, 50°C for 1 sec, 60°C for 4 min, and left to soak at 4°C.

Excess dye terminators were removed using ethanol precipitation and samples were loaded in appropriate buffer on an automated DNA sequencer and run overnight. Sequence information was examined using an ABI data analysis program (version 1.2.1) by technical services staff of the Beatson Institute.

### **2.2.3.13 Isolation of Genomic DNA from Cells**

The cell pellet ( $1.5 \times 10^6$  cells) was re-suspended in 340 µl Reagent B. 100 µl of 5 M sodium perchlorate was added to this mixture. The tubes were incubated at 37°C for 20 minutes followed by a 20 min incubation on a 70°C heating block. The DNA was extracted using a phenol/chloroform extraction procedure and precipitated with ethanol, in the absence of salt.

### **2.2.3.14 Agarose Gel Electrophoresis**

DNA samples and size markers, both in DNA loading buffer, were electrophoresed through 1-2% agarose gels containing 0.5 mg/ml ethidium bromide. Gels were made by dissolving the appropriate amount of electrophoresis grade agarose in 1X TBE. The gels were submerged in TBE and run at 3 V/cm. Electrophoresed DNA was visualised using a UV transilluminator and a record of the gel was made using a digital imaging system.



## **2.2.4 Electrophoretic Mobility Shift Assays**

### **2.2.4.1 Annealing Reaction for Re-annealing of Complementary Oligonucleotides**

Equal amounts of oligonucleotides were incubated together with 20 µl of 5X Annealing Buffer, 1.4 µl of 0.5 M DTT and made up to 100 µl total volume with ddH<sub>2</sub>O. The annealing reaction was incubated in a boiling water bath (95°C) for two minutes and then left in the water until the water bath had reached room temperature.

### **2.2.4.2 $\gamma^{32}\text{P}$ -dATP Labelling Reaction for Double Stranded Oligonucleotide**

Approximately 200 ng of the re-annealed oligonucleotide was mixed with 2 µl kinase buffer (Transgenomic), 5 µl  $\gamma^{32}\text{P}$ -dATP (specific activity 10 mCi/ml) and 2 µl T4 polynucleotide kinase (Transgenomics) made up to 20 µl total volume with ddH<sub>2</sub>O. The reaction was incubated at 37°C for 60 min after which time the volume of the reaction was made up to 100 µl with ddH<sub>2</sub>O. This 100 µl was put through a Biorad Micro Bio-Spin chromatography column to clean up and purify the double stranded labelled oligo.

For the mismatch gel retards the probes were gel purified by resolving the labelling reaction on a 15% acrylamide/TBE gel. The bromophenol blue dye band was allowed to run half way down the gel. The DNA was located by autoradiography of the wet gel (film exposed for 2 minutes) and the band excised. The DNA was eluted by soaking the macerated gel slice in TE (200 µl) overnight at room temperature. The eluate was filtered through glass wool and stored at -20°C.

### **2.2.4.3 MYC/MAX/MLH1 Electrophoretic Mobility Shift DNA Binding Assays.**

The binding reaction included extract at a range of concentrations (purified GST-MYC, GST-MLH1 and HIS-MAX), 8 µl of no salt binding buffer, 2 µl salt binding buffer, and 1 µl of the probe (added later), made up to a final volume of 20 µl. The reaction was pre-incubated for 10 min at 30°C. Probe was added to each reaction and incubated at 30°C for another 10 min. Samples were loaded onto a non-denaturing polyacrylamide gel and run at 150 V for 1.5 hours. The gels were fixed in gel fix solution for 30 min with gentle shaking and dried using a vacuum pump system. The gel was exposed to film overnight at -70°C.

#### 2.2.4.4 Preparation of Nuclear Cell Extracts

$2 \times 10^8$  cells were grown up in T150 flasks and collected by centrifugation at 700 g. The pellet was washed in PBS and re-suspended in 1 ml of freshly made extraction buffer (25 mM Hepes. KOH pH 8.0, 1 mM EDTA, 1 mM Benzamidine, 2 mM  $\beta$ -mercaptoethanol, 0.5 mM Spermidine, 0.1 mM Spermine). The extraction procedure was carried out as described previously (Jiricny et al., 1988; Stephenson and Karran, 1989). 50  $\mu$ l aliquots of extract were placed in pre-cooled microcentrifuge tubes and stored at  $-70^\circ\text{C}$ .

#### 2.2.4.5 SP1 Electrophoretic Mobility Shift DNA Binding Assays.

These gel shift assays were carried out as for the MYC/MAX/MLH1 assays except for the binding reaction which varied slightly. A binding master mix was prepared, 45  $\mu$ l 10X Binding Buffer, 7.5  $\mu$ l poly dI.dC (1 mg/ml), 2  $\mu$ l DTT (1 M), 320  $\mu$ l ddH<sub>2</sub>O. 25  $\mu$ l of master mix was put in each reaction tube on ice followed by the addition of 20  $\mu$ g of nuclear extract and made up to a final volume of 30  $\mu$ l with ddH<sub>2</sub>O. The binding assay was initiated by addition of the  $^{32}\text{P}$  labelled oligo probe and incubated on ice for 30 min. Reaction samples were treated as before.

#### 2.2.4.6 EMSA with Mismatched Substrate

Experiments were performed using 34mer oligonucleotides:

5'-AGCTTGGCTGCAGGTNGACGGATCCCCGGGAATT-3'

where  $N=G$ , annealed to a complementary strand which was either an exact match or contained a single non-complementary T base at the position corresponding to  $N$  (position 16) (Jiricny et al., 1988; Stephenson and Karran, 1989). Reactions were carried out at  $20^\circ\text{C}$  in a total volume of 30  $\mu$ l with 20  $\mu$ l of Complete Mismatch Gel Retard Mix which contains poly (dI.dC).poly(dI.dC) at a concentration of 50  $\mu$ g/ml. In this standard assay, cell extracts (30  $\mu$ g of protein in  $\leq 5$   $\mu$ l) were pre-incubated for 5 minutes with 200 fmol of standard duplex 34-mer oligonucleotide containing a G.C base pair at position 16 (included in the Complete Mismatch Gel Retard Mix) before addition of the radioactive substrate. Incubation with the radioactive substrate (20 fmol) was for 20 min at  $20^\circ\text{C}$ . Samples were loaded onto 6% polyacrylamide gels (2 mm thick). Electrophoresis was carried out at 150 V in 1X TBE buffer. The gels were dried and exposed to X-ray film.



## **2.2.5 Protein Protocols**

### **2.2.5.1 Protein Concentration Estimation**

Estimation of protein concentrations was performed using the bicinchoninic acid (BCA) method. BCA reagent (Sigma) was prepared fresh by the addition of 0.02 volumes of 4% (w.v) copper (II) sulphate (Sigma) to BCA solution. Protein standards, 10µl of 80-2000µg/ml BSA, were added to 200µl of BCA reagent per well of a 96 well micro-titre plate. 10µl of extract was also added to 200µl of the BCA reagent in triplicate. The plate was incubated at 37°C for 45 min and then the absorbance at 562 nm read using a micro-titre plate reader. From this reading the average concentration for each sample was calculated.

### **2.2.5.2 Co-immunoprecipitation Assays**

Low-stringency immunoprecipitations were carried out as described previously (Blackwood et al., 1992). Cells ( $1 \times 10^7$ ) were lysed in low stringency lysis buffer (1% NP-40, 0.2 mM PMSF, 0.7 mg/ml Pepstatin, 0.5% Aprotinin, 10 mM NaF, 50 mM  $\beta$ -glycerophosphate), sonicated on ice, clarified by ultracentrifugation (twice at 304,000 g) and subjected to immunoprecipitation with saturating amounts of antibody (all steps at 4°C). Immunoprecipitates were collected on protein A-Sepharose CL4B (Sigma) beads. Low stringency buffer was used to wash the precipitate three times. All samples were re-suspended in sample buffer and resolved on 10% SDS PAGE and western blotted.

### **2.2.5.3 Polyacrylamide Gel Electrophoresis (PAGE) for Protein Samples**

All PAGE for Western blots was carried out using the Novex NUPAGE Bis-Tris system. Samples were loaded onto 10% or 4-12% acrylamide pre-poured polyacrylamide gels in 1X sample buffer. The gels were run with either 1X MES SDS or MOPS SDS running buffer with NuPAGE Running Buffer Antioxidant added to the buffer in the upper chamber for reducing conditions.

### **2.2.5.4 Western Transfer of Proteins by Electroblotting**

Electroblotting was performed using a semi-dry electroblotter (Millipore). Immobilon-P membrane was immersed in methanol and then Transfer Buffer. Six sheets of 3M

Whatman filter paper pre-soaked in transfer buffer was sandwiched adjacent to the anode and cathode with the membrane and gel layered in between. Transfer took place over 45 min at 30 mA (0.65 mA per cm<sup>2</sup> of the gel). The gel was stained in Coomassie stain overnight and destained as described. This allowed a visual assessment to be made of the integrity of the proteins and how well they transferred onto the membrane.

#### **2.2.5.5 Coomassie Staining of Acrylamide Gels**

Gels were stained with Coomassie stain (0.2% Coomassie brilliant blue R250 in a 50:50:7 v/v ratio of methanol:H<sub>2</sub>O:glacial acetic acid) for 4 hours and then de-stained using a 25:68:7 v/v ratio of methanol:H<sub>2</sub>O:glacial acetic acid overnight.

#### **2.2.5.6 Immunological Detection of Protein**

Membranes were incubated with blocking solution at 4°C for 4 hours, probed overnight in the same buffer with primary antibody and washed with 0.1% Tween-20 in PBS. Blots were incubated in blocking solution with anti-mouse IgG HRP-linked rabbit antibody (Santa-Cruz), then washed again in 0.1% Tween-20 in PBS, after which bound complexes were visualised by enhanced chemiluminescence (Amersham).

#### **2.2.5.7 Autoradiography**

Membranes and gels were exposed to X-ray film (Fuji) and were developed using a Kodak X-ray developer. Radioactive gels were exposed to X-ray film for a few hours or overnight in a -70°C freezer.

#### **2.2.5.8 GST Fusion Proteins (work done in collaboration with Dr. E. Homer)**

Full length and the four overlapping fragments of hMLH1 (described ) were cloned into pGex4T. From these plasmids, the proteins are expressed as fusion proteins with GST (glutathione S-transferase). GST-MAX (expressing full length MAX) was also used, a gift from Prof. D. Gillespie (Beatson Labs, Glasgow). The pGEX constructs were transformed into BL21 DE3, *E.coli* competent cells. The transformants were grown up in 10 ml of LB media with ampicillin selection overnight at 37°C overnight in a shaking incubator. 1 ml of this overnight culture was added to 100 ml LB media with ampicillin and incubated in a shaking incubator for 3 hours, A<sub>600</sub> 0.5-2. Expression of the fusion proteins was induced



by addition of 100  $\mu$ l IPTG (100 mM) to this culture. The culture was incubated for 1.5 hours at 30°C. The cells were collected by centrifugation at 4°C for 10 mins at 2000 g. The pellets were re-suspended in 5 ml of ice cold PBS and this mixture was sonicated appropriately to disrupt the bacteria but not destroy the proteins, ten second pulses twice with a probe sonicator was sufficient. 250  $\mu$ l of 20% Triton X100 in PBS was added to every 5ml of sonicate (i.e. 1%). This was mixed at room temperature for 30 minutes. The mixture was then centrifuged at 4°C for 10 min at 9300 g. The supernatant was collected.

The GST fusion proteins were collected from the supernatant using glutathione sepharose beads. 250  $\mu$ l of 50% glutathione sepharose bead slurry was added to each 5 ml of sonicate. This mixture was placed on a gently moving rocking table to mix for 30 min at room temperature. The mixture was subsequently centrifuged at 2000 g for 10 min and the supernatant carefully removed. The pellet was washed in PBS. The spin was repeated and the supernatant discarded. This wash step was repeated twice. The final pellet with GST-fusion protein linked to beads was stored at -20°C in PBS 20% glycerol.

#### **2.2.5.9 GST Pull Down Assay (work done in collaboration with Dr. E. Homer)**

Mammalian cell extracts were prepared as described for the co-immunoprecipitation assays. The stored GST-fusion protein linked to beads was thawed and washed twice in PBS and re-suspended in the same extract buffer used for making the mammalian cell extracts. 1  $\mu$ g of fusion protein was added to 500  $\mu$ g of cell extract. These mixtures were placed on a rotating wheel for 1 hour at 4°C. The beads were precipitated by centrifugation for 5 min at 21,000 g at 4°C and washed six times in 500  $\mu$ l wash buffer. The final pellet of beads plus proteins bound to the beads was re-suspended in sample buffer, denatured, and resolved by SDS-PAGE. Proteins bound to the various GST fusion proteins were detected by western blot.

### **2.2.6 General Tissue Culture Techniques**

#### **2.2.6.1 Cell Culture**

Aseptic manipulations were performed using sterilised glassware in a class II microbiological safety cabinet with vertical airflow. Cells were grown in 25, 75 or 150 cm<sup>2</sup> flasks (Iwaki) or 9 cm dishes (Falcon) at 37°C as monolayers in supplemented RPMI or

DMEM medium (Rosswell Park Memorial Institute) in the presence of 5% or 10% CO<sub>2</sub> respectively.

Cells were frozen at a concentration of 1 x 10<sup>6</sup>/ml with 10% di-methyl sulphoxide (DMSO) at -70°C. After 24 h, samples were transferred to liquid nitrogen.

**2.2.6.2 Cell Line Maintenance**

<u>Cell Line</u>	<u>Growth Conditions</u>
A2780	RPMI (500 ml), 10% FCS, 5 ml, 2 mM L-Glutamine, 1 mM Sodium Pyruvate, 500 iu/ml/500 µg/ml Pen./Strep., 5% CO <sub>2</sub>
K562	RPMI (500 ml), 10% FCS, 1 ml 1 M NaOH, 2 mM L-Glutamine,. 500 iu/ml/500 µg/ml Pen./Strep., 5% CO <sub>2</sub>
Rat-1	DMEM (500 ml), 2 mM L-Glutamine, 500 iu/ml/500µg/ml Pen./Strep, 10% CO <sub>2</sub>
Rat-1 MYCER <sup>TM</sup>	DMEM (500 ml), 2 mM L-Glutamine, 500 iu/ml/500µg/ml, 2.5 µg/ml Puromycin, 10% CO <sub>2</sub>

**2.2.6.3 Treatment of Cells with Cisplatin**

Cell cultures were treated with cisplatin from a stock dissolved in DMSO. The final concentration of DMSO in culture was 0.01%. Cells were treated for one hour with cisplatin and then incubated for 24 hours in media without cisplatin and harvested.

**2.2.6.4 Staining Colonies on Culture Dishes**

PBS was added to the media on the culture plates and this mixture was poured off. A 50:50 mix of methanol and PBS was poured onto the plates and left for 10 minutes. This mixture was poured off the plates and methanol was poured onto the plates. Again the plates were left in methanol for 10 minutes and poured off. The plates were allowed to dry for 1 hour at 37°C. Crystal violet stain was poured onto the plates and left on for 10 minutes. The crystal violet was poured off and the plates gently rinsed with tap water. The



plates were allowed to dry at room temperature overnight and the stained colonies were counted using a colony counter.

# Chapter 3

## Results



### 3.1 Introduction

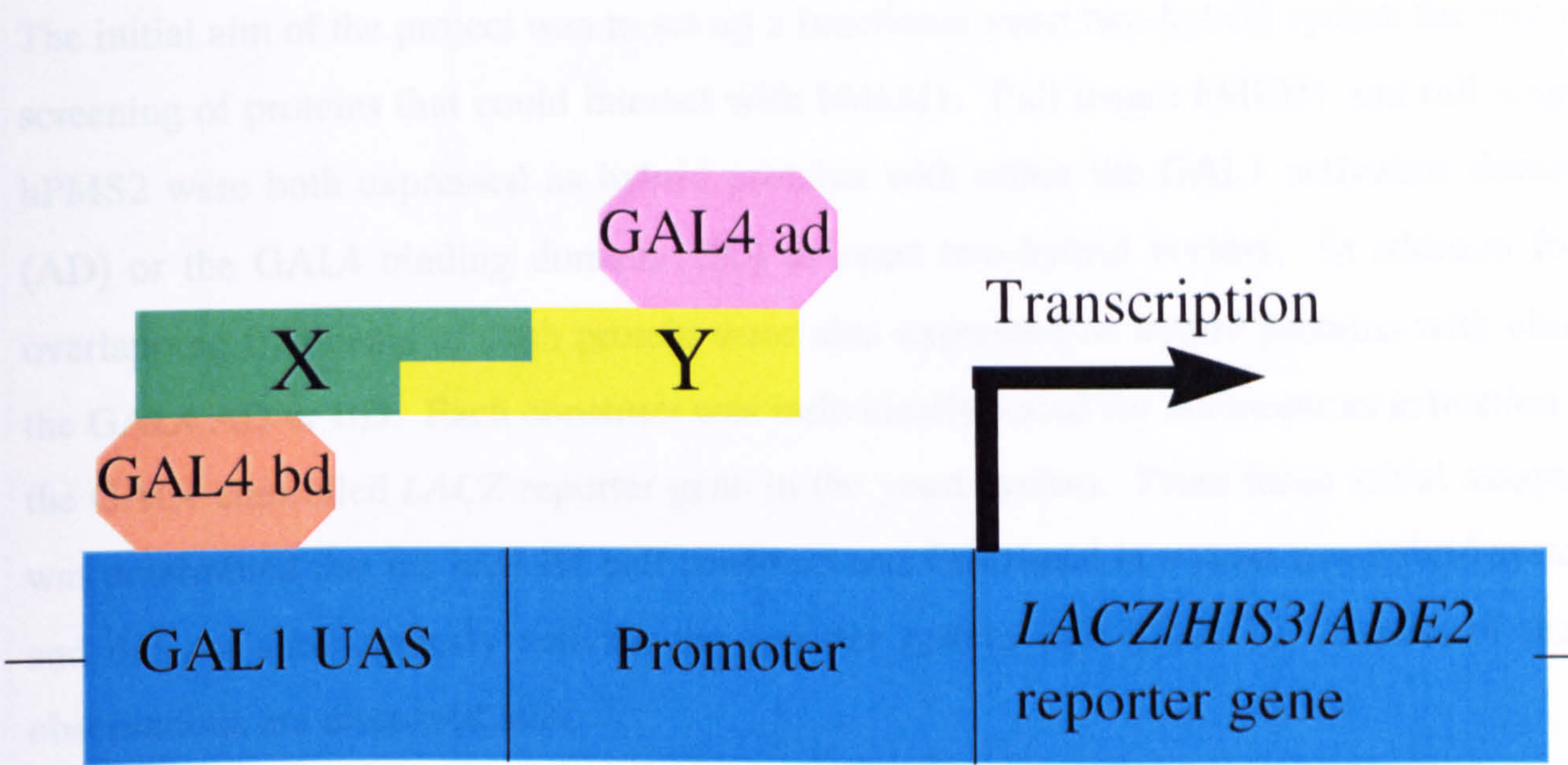
Mismatch Repair (MMR) recognises and repairs mismatched nucleotides that result from the mis-incorporation of bases during replication, recombination between partially homologous sequences and physical damage to DNA. hMLH1 protein co-purifies with another MMR protein, hPMS2, as a heterodimer (Umar et al., 1996). Their exact function in MMR is unknown, although they have been shown to be essential for *in vitro* MMR activity. Loss of hMLH1 expression is seen in a number of tumour cell lines resistant to the cytotoxic drug cisplatin, and restoration of MMR leads to restoration of drug sensitivity (Brown et al., 1997). This has led to the hypothesis that MMR is involved in coupling DNA damage to an apoptotic response. Indeed loss of apoptotic signalling responses (c-Abl, p53 and p73) to DNA damage is seen in MMR deficient cells (Duckett et al., 1999; Gong et al., 1999; Nehme et al., 1997). Exactly how MMR couples to these apoptotic pathways is unclear. The aim of this study was to identify other proteins that interact with hMLH1 to attempt to further elucidate its role in MMR and the engagement of downstream damage response pathways.

The yeast two-hybrid system was used to identify proteins interacting with hMLH1. This is an *in vivo* system to identify protein-protein interactions (Fields and Song, 1989). The yeast two-hybrid system utilises the fact that transcription factors have two physically separable modular domains, a DNA binding domain and a transcription activation domain. The DNA binding domain targets the transcription factor to specific promoter sequences (UAS, upstream activator sequence), and the activation domain facilitates the assembly of the transcription complex allowing transcription initiation (see Figure 6). Therefore the basis of the yeast two-hybrid system is that, through the non-covalent interaction of two independent hybrid proteins containing either the DNA-binding domain or activation domain, a functional transcription factor can be reconstituted.



3.2 The interaction of hMLH1 and hPMS2 in the yeast two-hybrid system

Hybrid System.



From Clontech Matchmaker Yeast Two-Hybrid System Handbook

**Figure 6: Schematic diagram demonstrating the basis of the two-hybrid system.**

The hybrid of the GAL4 DNA-binding domain (bd) and protein X binds to the GAL1 UAS (upstream activating sequence) but cannot activate transcription without the activation domain (ad). Likewise the hybrid of the activation domain and protein Y cannot localise to the UAS and therefore does not activate transcription. When the X and Y parts of the hybrid proteins interact *in vivo*, e.g. hMLH1 and hPMS2, this reconstitutes the GAL4 function and results in expression of the reporter gene. The various yeast strains used for the two-hybrid analysis carry *LACZ* and/or the nutritional reporters *HIS3* and *ADE2* under the control of GAL4-binding sites.



### 3.2 The Interaction of hMLH1 and hPMS2 in the Yeast Two-Hybrid System.

The initial aim of the project was to set up a functional yeast two-hybrid system for routine screening of proteins that could interact with hMLH1. Full length hMLH1 and full length hPMS2 were both expressed as hybrid proteins with either the GAL4 activation domain (AD) or the GAL4 binding domain (BD) in yeast two-hybrid vectors. In addition four overlapping fragments of each protein were also expressed as hybrid proteins with either the GAL4 AD or BD. Each construct was individually tested for autonomous activation of the GAL4 controlled *LACZ* reporter gene in the yeast system. From these initial assays it was determined that the hMLH1 bait construct was functional in a yeast two-hybrid system and did not autonomously activate the reporter system (see Table 7). Details of these observations are discussed later.

Full length and fragments of hMLH1 and hPMS2 were amplified from A2780 cDNA by PCR and cloned in frame into yeast two-hybrid vectors (see Table 3; Figure 7). The sequences of the *hMLH1* and *hPMS2* genes, and the primers used for the PCR reactions are listed in Appendix 1-4. The oligo primers used for PCR are listed in section 2.1.11, the forward primers (5' end of fragment) used had an *EcoRI* site at the 5' end of the sequence and the reverse primers (3' end of the fragment) had a *BamHI* site allowing restriction digestion of the ends of the PCR products. This allowed cloning of the product into the yeast two-hybrid vectors which both have unique *EcoRI* and *BamHI* sites within their multiple cloning sites. The yeast two-hybrid plasmids utilised were the Clontech plasmid vectors, pGAD424 (carries the GAL4 activation domain) and pGBT9 (carries the GAL4 DNA-binding domain). These plasmids were subsequently transformed into *E.coli*, DH5 $\alpha$  competent cells and the plasmid DNA prepared from the transformant colonies.

Each insert was checked by restriction enzyme digestion and also by sequencing of the inserts using vector primers for both vectors (pGAD424-F, pGAD424-R, pGBT9-F, pGBT9-R) and internal primers (those used to clone the fragments of hMLH1 and hPMS2). The restriction enzyme digestions were carried out utilising the *SpeI*, *ClaI* and *HindIII* restriction enzymes. The digestion products were examined on an agarose gel. The predicted fragment sizes for digestion of both full length and fragments of hMLH1 and hPMS2 expressed in pGAD424 and pGBT9 were observed (see Table 4 and Table 5).

Gene	Fragment	Region (bp)	Size (bp)	Region (αα)	Size (αα)
hMLH1	F1	42-725	683	1-228	228
	F2	582-1067	485	180-342	163
	F3	966-1777	811	308-579	272
	F4	1584-2309	725	514-756	243
	Full Gene	42 (start codon)-2309	2267	1-756	756
hPMS2	F1	3-794	791	1-264	264
	F2	741-1346	605	246-448	203
	F3	1293-1943	650	430-647	218
	F4	1893-2588	695	630-862	233
	Full Gene	3 (start codon)-2588	2585	1-862	862

**Table 3: *hMLH1* and *hPMS2* sequences cloned into pGBT9 and pGAD424**

Full length and four overlapping fragments of *hMLH1* and *hPMS2* were cloned into both pGBT9 and pGAD424. This table lists the regions of the genes included in each fragment and the size of each fragment in both bp (base pairs) and αα (amino acids).





**Figure 7: Schematic diagram representing the fragments of hMLH1 and hPMS2 cloned into pGBT9 and pGAD424**

This diagram represents the overlapping nature of hMLH1 and hPMS2 protein fragments expressed as hybrid proteins with the GAL4 DNA binding domain and activation domain.



Vector	Insert	<i>SpeI</i>	<i>ClaI</i>	<i>HindIII</i>
pGAD424-hMLH1	Full length	1300bp, 7.7 Kb	Linear	450 bp, 700 bp 900 bp, 1 Kb, 5.4 Kb
	F1	Linear	Linear	1 Kb, 5.4 Kb
	F2	-	Linear	1.2 Kb, 5.4 Kb
	F3	Linear	Linear	300 bp, 450 bp, 1 Kb, 5.4 Kb
	F4	-	Linear	1.4 Kb, 5.4 Kb
pGBT9-hMLH1	Full length	1.3 Kb, 7 Kb	-	450 bp, 700 bp, 900 bp, 1.3 Kb, 4.6 Kb
	F1	Linear	-	700 bp, 900 bp, 4.6 Kb
	F2	-	-	1.4 Kb, 4.6 Kb
	F3	Linear	-	500 bp, 600 bp, 700 bp, 4.6 Kb
	F4	-	-	1.4 Kb, 4.6 Kb

**Table 4: Predicted Restriction Fragments from Digesting pGAD424-hMLH1 and pGBT9-hMLH1 Constructs**

Digestion with *SpeI*, *ClaI* and *HindIII* of the various pGBT9 and pGAD424 hMLH1 constructs gave the fragment sizes listed when digest were run out on agarose gels.



Vector	Insert	<i>SpeI</i>	<i>ClaI</i>	<i>HindIII</i>
pGAD424-hPMS2	Full length	Linear	1 Kb, 7.3 Kb	600 bp, 875 bp, 1.9 Kb, 5.9 Kb
	F1	-	Linear	613 bp, 869 bp, 5.9 Kb
	F2	-	Linear	540 bp, 750 bp, 5.9 Kb
	F3	-	Linear	1.35 Kb, 5.9 Kb
	F4	Linear	Linear	1.9 Kb, 5.9 Kb
pGBT9-hMLH1	Full length	Linear	-	850 bp, 880 bp, 1.7 Kb, 4.6 Kb
	F1	-	-	643 bp, 1 Kb, 4.6 Kb
	F2	-	-	720 bp, 890 bp, 4.6 Kb
	F3	-	-	1.5 Kb, 4.6 Kb
	F4	Linear	-	1.5 Kb, 4.6 Kb

**Table 5: Predicted Restriction Fragments from Digesting pGAD424-hPMS2 and pGBT9-hPMS2 Constructs**

Digestion with *SpeI*, *ClaI* and *HindIII* of the various pGBT9 and pGAD424 hPMS2 constructs gave the fragment sizes listed when digest were run out on agarose gels.

Construct	Fragment	Region (bp)	Mutations	Amino Acid (αα) Change
pGAD424-hMLH1	F1	42-725	AAC→AGC @154 bp  GTC→ATC @ 696 bp	Asn→Ser @ αα38  Val→Ile @ αα219
	F2	582-1067	GGA→GGG @ 635 bp  GTC→ATC @ 696 bp  TCA→TCG @ 797 bp	Gly→Gly @ αα198  Val→Ile @ αα219  Ser→Ser @ αα252
	F3	966-1777	-	
	F4	1584-2309	-	
	Full Length	42 -2309	GTC→ATC @ 696 bp	Val→Ile @ αα219
pGBT9-hMLH1	F1	42-725	GAG→AAG @ 198 bp  GTC→ATC @ 696 bp	Glu→Lys @ αα53  Val→Ile @ αα219
	F2	582-1067	GTC→ATC @ 696 bp  GCC→GTC @ 865 bp	Val→Ile @ αα219  Ala→Val @ αα275
	F3	966-1777	-	
	F4	1584-2309	-	
	Full Length	42 -2309	ATC→ATT @116 bp  GGA→GGG @ 203 bp  GTC→ATC @ 696 bp  AGA→GGA @ 858 bp	Ile→Ile @ αα25  Gly→Gly @ αα54  Val→Ile @ αα219  Arg→Gly @ αα273

**Table 6: Summary of Sequencing of pGAD424-hMLH1 and pGBT9-hMLH1 constructs. (- = no changes)**



Construct	Fragment	Region (bp)	Mutations	Amino Acid (αα) Change
pGAD424- hPMS2	F1	3-794	-	
	F2	741-1346	-	
	F3	1293-1943	AAA→AGA@ 1615	Lys→Arg @ αα538
	F4	1893-2588	AAG→AGG @ 1954	Lys→Arg @ αα651
pGBT9- hPMS2	F1	3-794	-	
	F2	741-1346	-	
	F3	1293-1943	TCT→CCT @ 1410	Ser→Pro @ αα470
	F4	1893-2588	-	

**Table 7: Summary of Sequencing of pGAD424-hPMS2 and pGBT9-hPMS2 constructs. (- = no changes)**

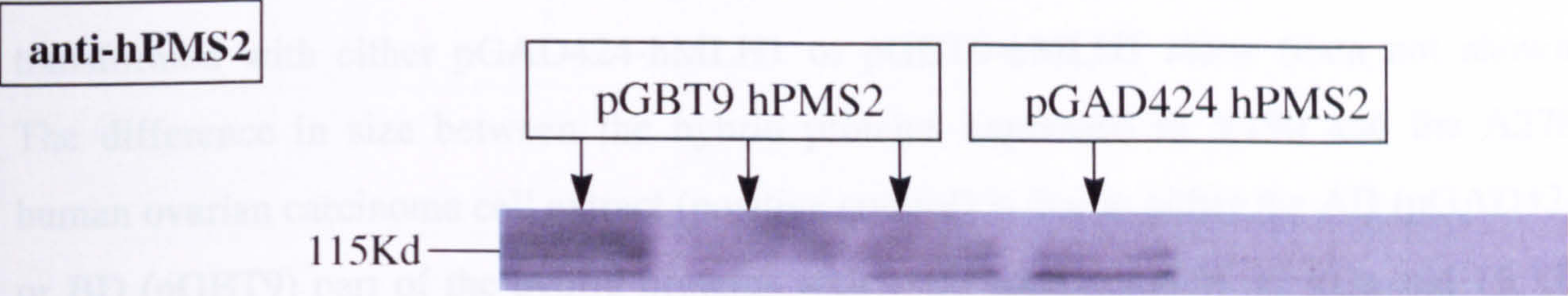
Sequencing of the different construct inserts demonstrated a number of mutations in some of the constructs (see Table 6 and Table 7). A single mutation at base pair 696 was seen consistently in hMLH1, leading to a neutral amino acid change. It is assumed this is a polymorphism as the various hMLH1 fragments were cloned individually by PCR from A2780 cDNA. This mutation was also seen in pGBKT7-hMLH1 (used later) which was cloned from a completely different source. No mutations were seen in fragment 3 or 4 of hMLH1. No mutations were seen in F1 and F2 of hPMS2 either. Two neutral amino acid changes were shown in both F3 and F4 of pGAD424-hPMS2 which would suggest this should not effect these fragments, which will be borne out by the demonstration of an interaction between these fragments and hMLH1 later. The original full length hPMS2 constructs had too many point mutations to be used in this system. Recently new constructs were made and sequenced. No mutations were found in the sequence examined.

The Y190 yeast strain was used for the yeast two-hybrid analysis. Before starting any of the transformations the nutritional requirement phenotype of Y190 was checked. The Y190 yeast strain is auxotrophic for Trp (tryptophan) and Leu (leucine), allowing transformation selection, and has two reporter genes *LACZ* and *HIS3* both of which are under the control of GAL4-responsive sequences (UAS). The hMLH1 and hPMS2 constructs were transformed into Y190 competent cells using a lithium acetate based transformation protocol.

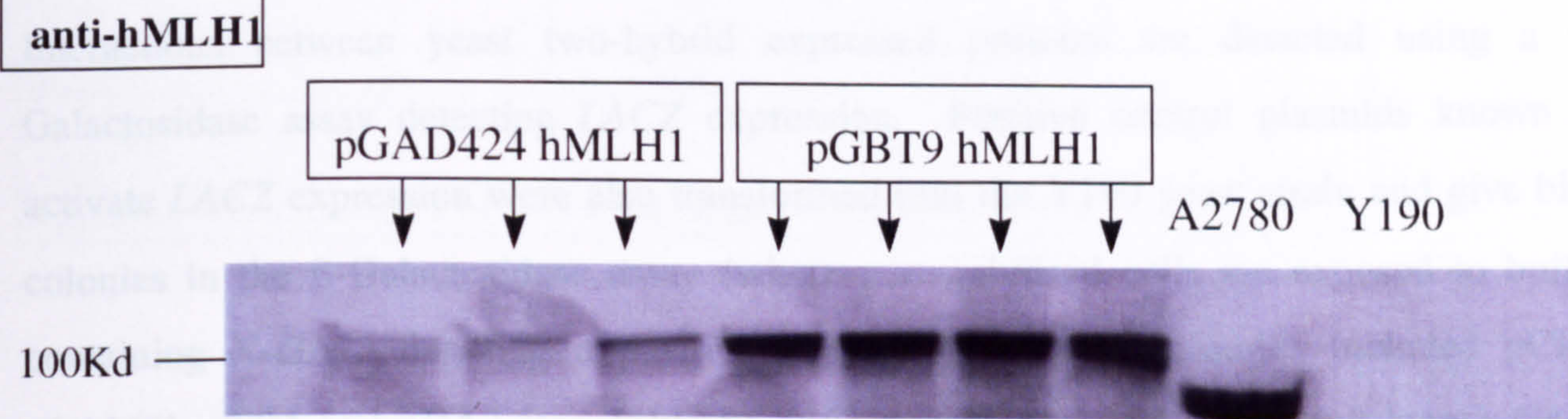
Yeast protein extracts were made from Y190 yeast and from Y190 transformed with the hMLH1 and hPMS2 constructs alone or from co-transformations of hMLH1 and hPMS2. These extracts were denatured and loaded onto SDS-polyacrylamide gels which were subsequently western blotted and probed with anti-hMLH1 and anti-hPMS2 antibodies (see Figure 8). In western blot A. a hPMS2 hybrid protein has been clearly detected by probing with anti-hPMS2. These extracts were prepared from Y190 cells co-transformed with a full length hMLH1 yeast two-hybrid vector (pGAD424-hMLH1 or pGBT9-hMLH1). However, in un-transformed Y190 extracts or extracts from Y190 transformed with vectors expressing only hPMS2 no hPMS2 was detected (lanes not shown). This suggests that when transformed alone into Y190 the hPMS2 hybrid protein is not stable. The hPMS2 protein is not detected in human cells defective for hMLH1 expression. It has been suggested that hMLH1 binding to hPMS2 is required to stabilise the hPMS2 protein. The lack of detectable hPMS2 when transformed alone into yeast, but presence when co-transformed with hMLH1 is consistent with these observations. In western blot B. hMLH1 hybrid protein is also clearly expressed from both pGBT9-hMLH1 and pGAD424-hMLH1.



A.



B.



**Figure 8: anti-hPMS2 (A) and anti-hMLH1 (B) Western Blots of Transformed Yeast Extracts**

Protein extracts were prepared from Y190 yeast alone and Y190 transformed with constructs expressing both hMLH1 and hPMS2 hybrid proteins and analysed by SDS-PAGE. The gels were western blotted and probed with antibodies against both hPMS2 (A) and hMLH1 (B).



These extracts again are from Y190 co-transformed with vectors expressing both hMLH1 and hPMS2. However unlike hPMS2, hMLH1 is detected in extracts from Y190 transformed with either pGAD424-hMLH1 or pGBT9-hMLH1 alone (data not shown). The difference in size between the hybrid proteins expressed in Y190 and the A2780 human ovarian carcinoma cell extract (positive control) is due to either the AD (pGAD424) or BD (pGBT9) part of the hybrid proteins which are approximately 12 kDa and 16 kDa respectively. From these western blots it is concluded that the hybrid proteins are being expressed at detectable levels and in a form recognised by the anti-hPMS2 and anti-hMLH1 antibodies.

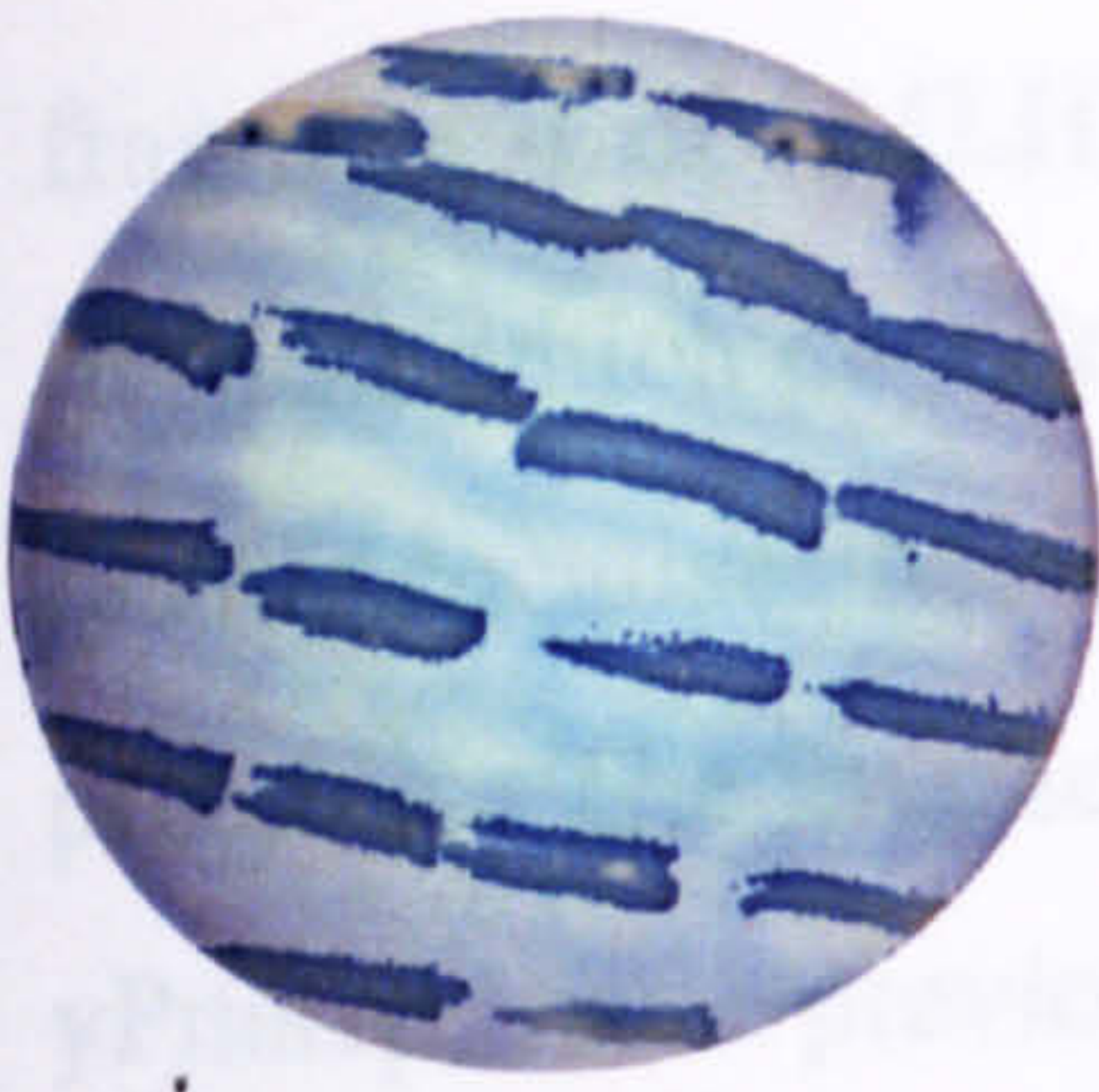
Interactions between yeast two-hybrid expressed proteins are detected using a  $\beta$ -Galactosidase assay detecting *LACZ* expression. Positive control plasmids known to activate *LACZ* expression were also transformed into the Y190 yeast strain and give blue colonies in the  $\beta$ -Galactosidase assay (where permeabilised cells are exposed to buffer containing X-GAL) denoting a positive interaction. These plasmids included pCL1, pLAM5', pTD1 and pVA3 (see Table 8). pCL1 expresses a wild-type full length *GAL4* gene, and therefore yeast transformed with this plasmid alone will express a full length *GAL4* transcription factor, capable of activating transcription of the *LACZ* gene which is under transcriptional control of a *GAL4* UAS. Transformation of Y190 with this plasmid therefore provides an excellent positive control for the  $\beta$ -Galactosidase assay. Co-transformation of pCL1 with pLAM5' (Lamin C protein expressed in pGBT9) allows growth of these transformed cells on Leu<sup>-</sup>/Trp<sup>-</sup> selection plates, so the yeast are growing under the same conditions as Y190 co-transformed with pGBT9 and pGAD424 constructs. Lamin C has been found not to interact with, or form complexes with most proteins (Bartel et al., 1993). pVA3 expresses a murine p53 hybrid protein in pGBT9 and pTD1 expresses an SV40 T-antigen hybrid protein in pGAD3F. Both these proteins are known to interact with each other and therefore, upon co-transformation of these two constructs into Y190, activation of transcription of the *LACZ* gene is achieved. Together transformation of Y190 with these combinations of plasmids serve as strong positive controls for *GAL4* mediated activation of transcription of the *LACZ* gene.

hMLH1 and hPMS2 constructs were transformed into Y190 alone and also co-transformed in a variety of combinations. None of the constructs autonomously activated *LACZ* expression. From these transformations it was shown that hMLH1 full length and fragment 4 both clearly interact with hPMS2 fragment 3 and 4 as shown by the  $\beta$ -Galactosidase assay (see Table 9; Figure 9). Full length hMLH1 encodes amino acids 1-756 and

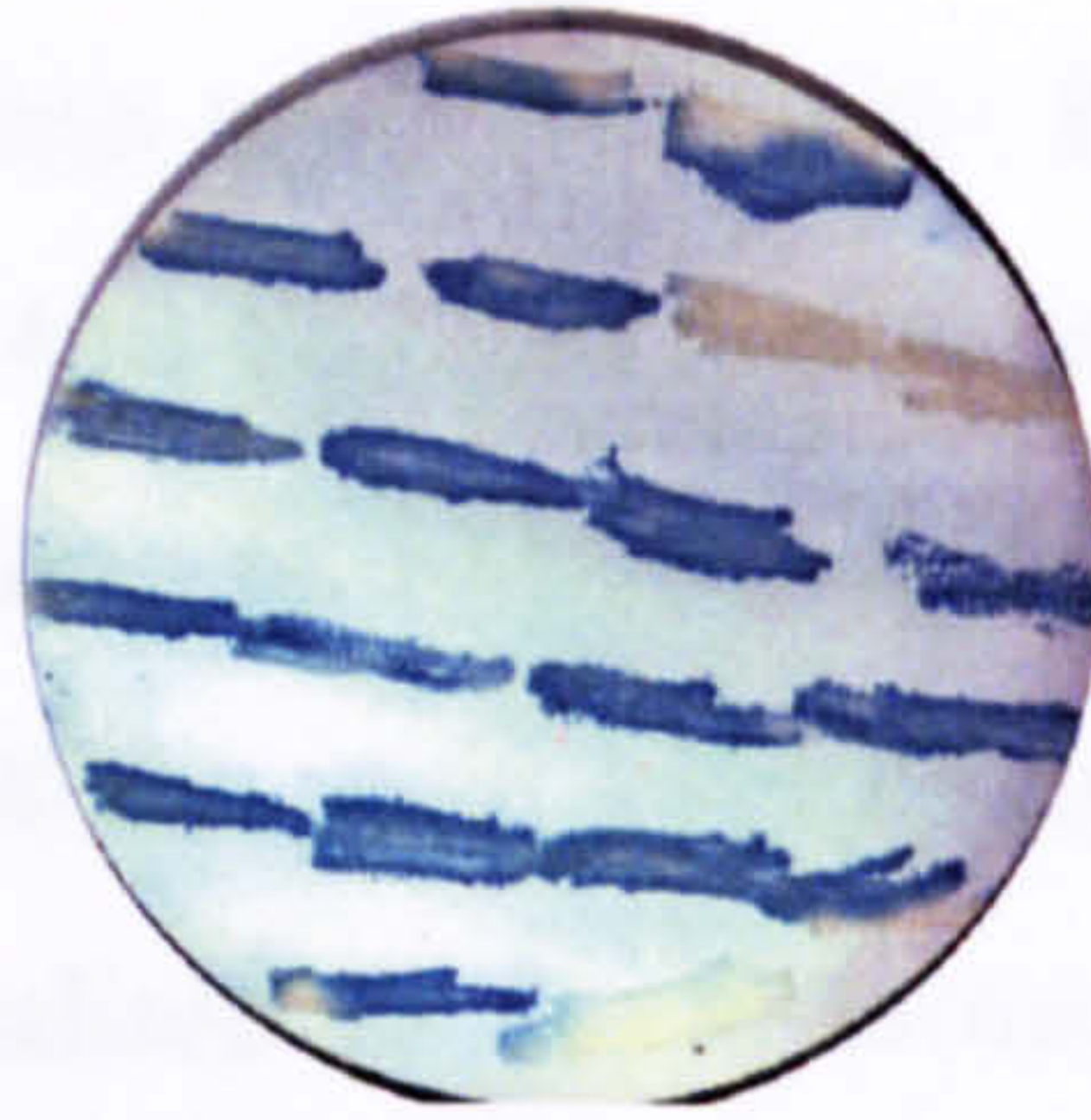




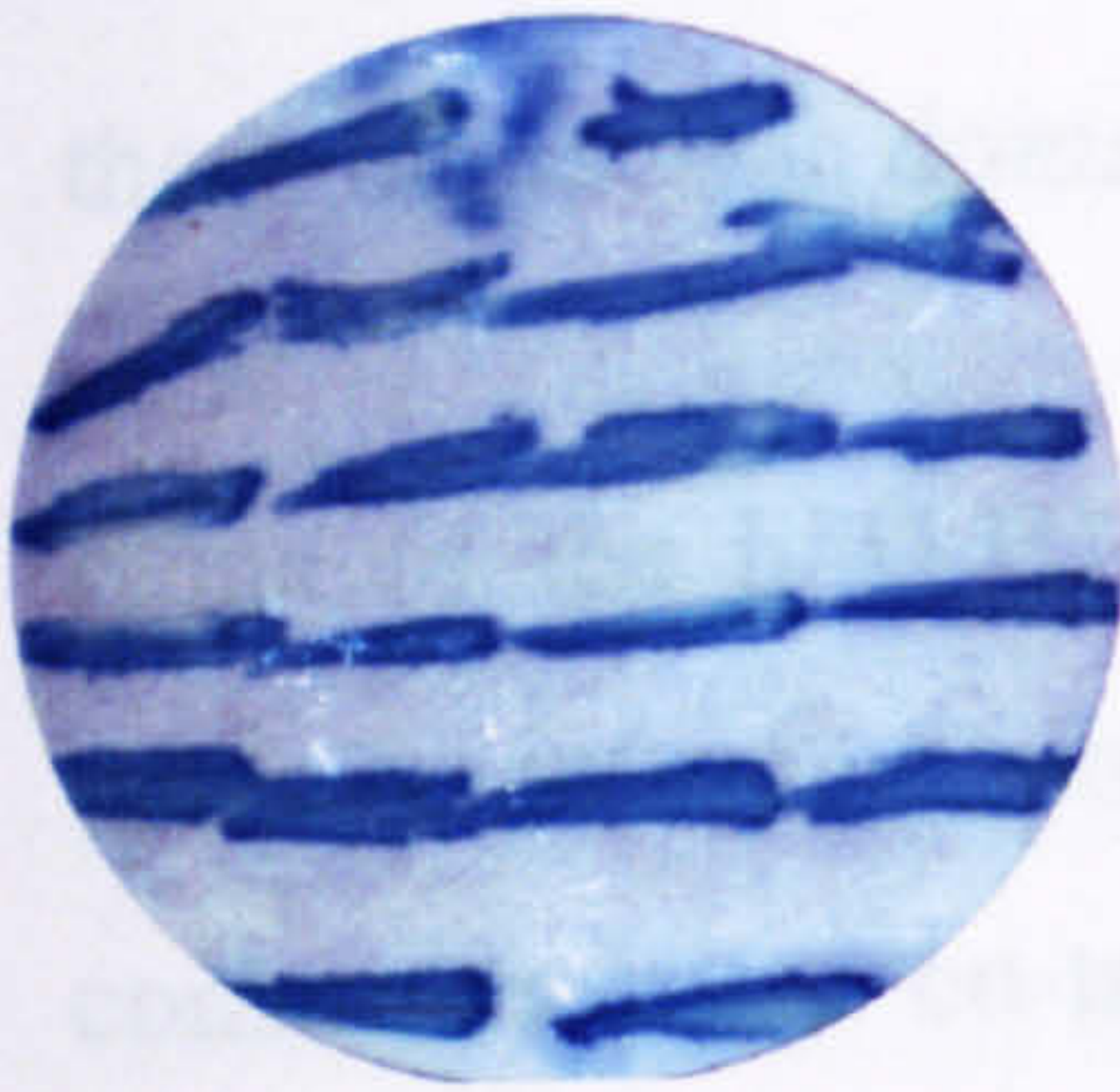




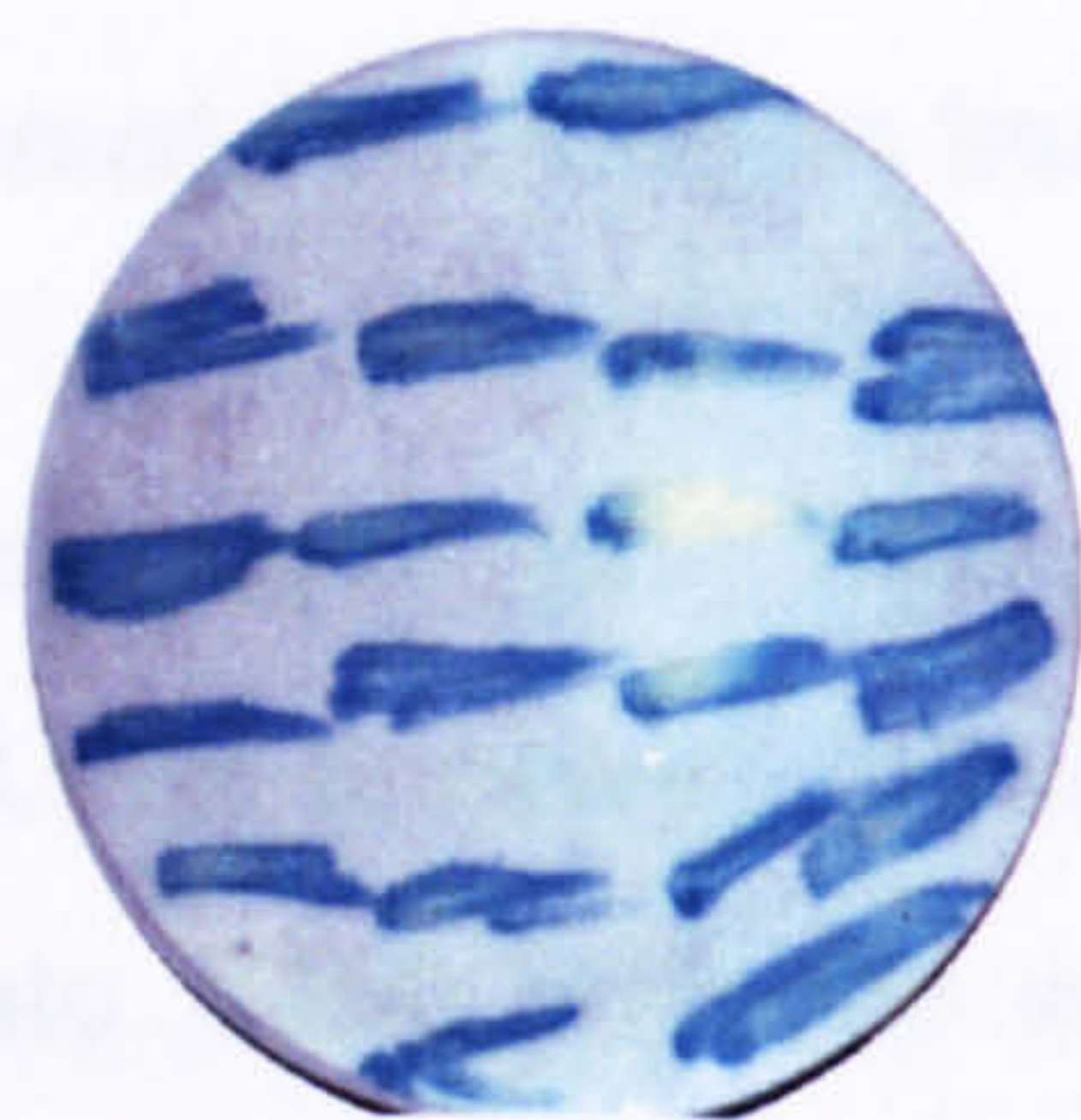
A. pCL1



B. pVA3+pTD1



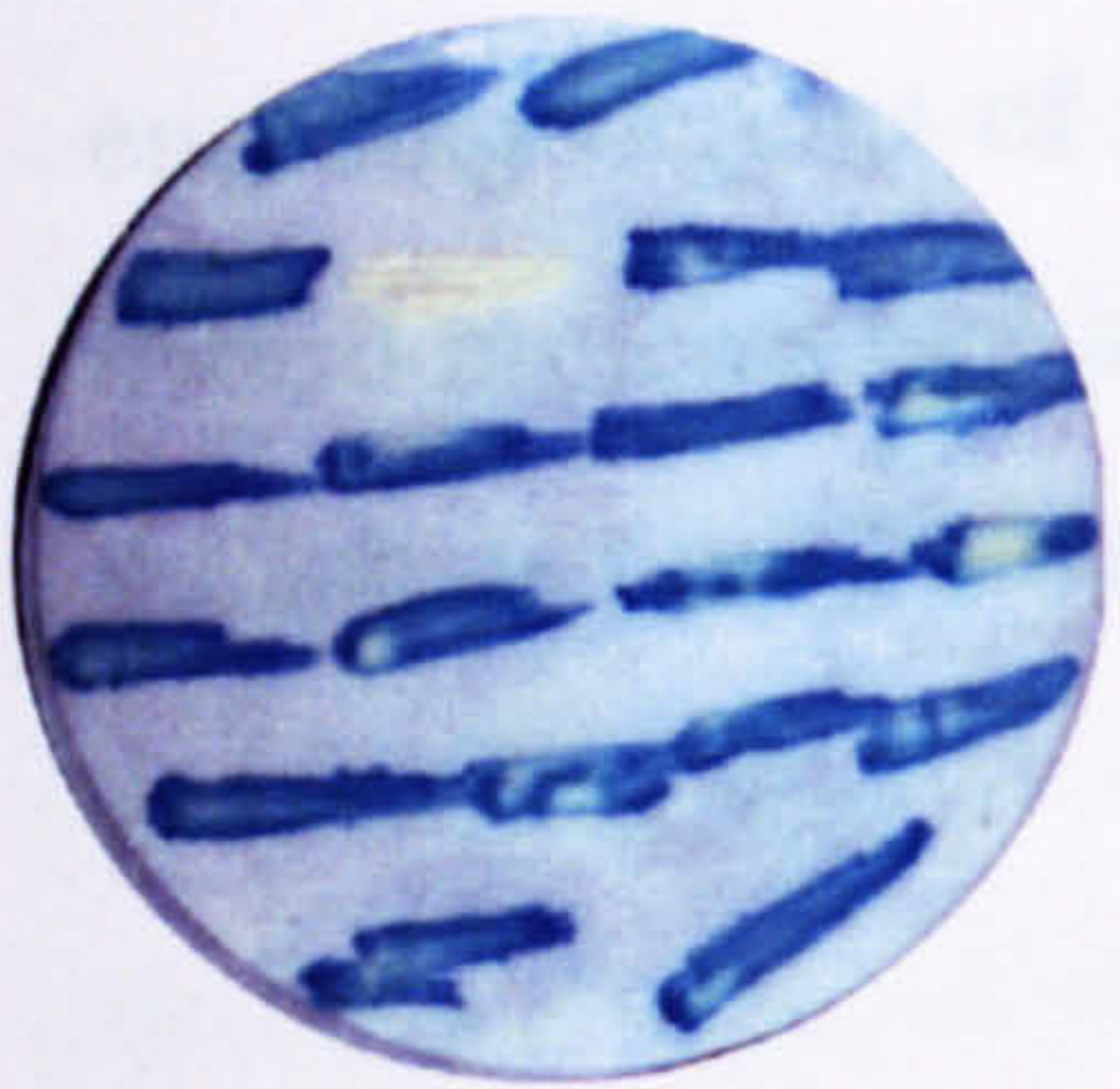
C. pGBT9 MLH1+



D. pGAD424 MLH1+

pGAD424 PMS2 F3

pGBT9 PMS2F4

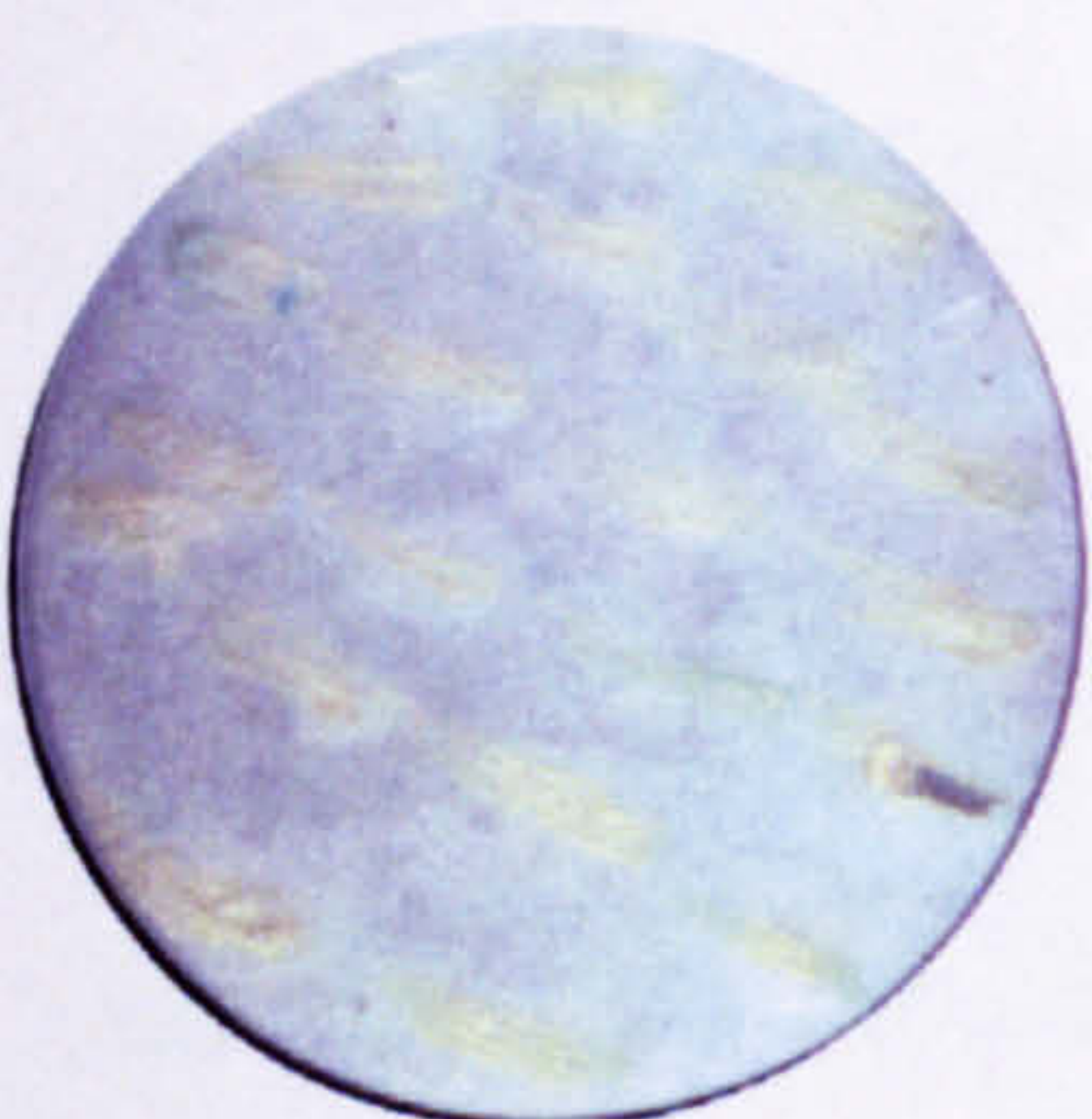


E. pGAD424 MLH1 F4+

F. pGAD424 MLH1 F4+

pGBT9 PMS2 F3

pGBT9 PMS2 F3

G. pGAD424 MLH1 F2+  
pGBT9 PMS2 F4

**Figure 9: GAL4 Activation.** Blue colonies in the  $\beta$ -galactosidase assay denote a positive interaction (A-F), white colonies denote no interaction (G).



fragment 4 of hMLH1 encodes amino acids 514-756. Both bind to fragment 3 and 4 of hPMS2 which encode amino acids 430-647 and amino acids 630-862 respectively. These results localise the interaction regions of hMLH1 and hPMS2 to the C-termini of both proteins. The interaction regions of the yeast homologues of these proteins, yMlh1p and yPms1p, have previously been localised to the C-termini of these proteins (Pang et al., 1997). Subsequent to our studies, the hMLH1 interaction with hPMS2 has been shown to occur through the C-termini of both proteins (Guerrette et al., 1999). Overall the regions of interaction between hMLH1 and hPMS2 localise to the C-termini of both proteins and these interaction domains are conserved from yeast to humans.

None of the hMLH1 or hPMS2 constructs gave positives in the  $\beta$ -Galactosidase assay when transformed alone into Y190. No positive activation of *LACZ* was seen when these constructs were co-transformed into Y190 with the empty reciprocal yeast two-hybrid plasmid, with constructs expressing regions of the ATR kinase gene or the RPA70 gene and finally with the pLAM5' plasmid. More specifically pGBT9 constructs were co-transformed into Y190 with pACT2 (an AD expressing yeast two-hybrid vector) expressing RPA70. The pGAD424 constructs were co-transformed into Y190 with both pLAM5' and pAS2-1 (a BD expressing yeast two-hybrid vector) expressing hybrid proteins encoding regions of ATR. No interactions were seen between the hMLH1 and hPMS2 hybrid proteins and any of these hybrid proteins described. Therefore it is concluded that the interactions between hMLH1 and hPMS2 observed are true positive interactions.

### 3.3 Deletion Analysis of hPMS2 Fragment 3.

hMLH1 full length (amino acids 1-756) and fragment 4 (amino acids 514-756) hybrid proteins both interacted with hPMS2 fragment 3 (F3, amino acids 430-647) and 4 (F4, amino acids 630-862) in the yeast two-hybrid system. Because of the overlapping nature of hPMS2 F3 and F4, deletion analysis of the 3' end of F3 was carried out, to further define the hMLH1 interaction domain of hPMS2. Constructs were made as before, by PCR from the PMS2 F3 previously cloned into pGAD424 and pGBT9. pGAD424-F3 and pGBT9-F3 along with PMS2-R3-9, -24, -51, -102, and -300 primers were used for the PCR. The different products expressing F3 of hPMS2 with gradual deletions from its 3' end, of 9, 24, 51, 102 and 300 bp were cloned into both pGAD424 and pGBT9. These fragments of hPMS2 were each sequenced and found not to carry any point mutations (other than those listed in F3 earlier) and are expressed in frame with the activation or binding domain of GAL4. Once again these constructs were transformed alone into Y190 competent cells and also co-transformed with both full length hMLH1 and hMLH1 F4 (see Table 10).

hPMS2 F3 constructs with between 9 and 102 bp deleted from the 3' end of hPMS2 fragment 3 gave positive interactions with both full length and F4 of hMLH1. However with 300bp deleted from the 3' end of F3 a loss of interaction was seen with both full length and F4 of hMLH1. This demonstrates that a crucial domain of hPMS2 for the interaction with hMLH1 lies between amino acids 547 and 613. The interaction of the overlapping fragments of hPMS2 F3 and F4 was not further characterised as these two deletion fragments of F3 encode a region of hPMS2 upstream of the overlapping region of F3 and F4. Therefore in addition to this defined region of hPMS2 required for the interaction with hMLH1, additional regions of hPMS2 involved in the hPMS2-hMLH1 interaction could lie between amino acids 613-862. In the study mentioned above, the hPMS2 interaction region of hMLH1 has been shown to lie between amino acids 506 and 675, and in hPMS2, the hMLH1 interaction region was shown to lie between amino acids 675 and 850 (Guerrette et al., 1999).

From these studies of the interaction between hMLH1 and hPMS2, the C-termini of both proteins are clearly responsible for this protein-protein interaction. In particular the C-terminal 243 amino acids of hMLH1 can facilitate an interaction with the C-terminal fragments of hPMS2 encoding amino acids 430-647 and amino acids 630-862. A specific



Region of PMS2	Full Length MLH1	MLH1-F4
$\alpha\alpha$ =amino acids	$\alpha\alpha$ 1-756	$\alpha\alpha$ 514-756
PMS2-F3	+	+
$\alpha\alpha$ 430-647		
hPMS2-F3-9	+	+
$\alpha\alpha$ 430-644		
hPMS2-F3-24	+	+
$\alpha\alpha$ 430-639		
hPMS2-F3-51	+	+
$\alpha\alpha$ 430-630		
hPMS2-F3-102	+	+
$\alpha\alpha$ 430-613		
hPMS2-F3-300	-	-
$\alpha\alpha$ 430-547		

**Table 10: Deletion analysis of the 3’ end of hPMS2 fragment 3.**

hPMS2 F3 constructs with between 9 and 102 bp deleted from its 3’ end gave positive interactions with both full length and F4 of hMLH1 (blue colonies in the  $\beta$ -Gal assay). However with 300bp deleted from the 3’ end of F3 this interaction was lost.

domain on hPMS2 that lies between amino acids 547 and 613 is required for the interaction of this F3 fragment of hPMS2 with hMLH1. This has been demonstrated by the examination of interactions between hMLH1 and fragments of hPMS2 which are derived from gradual deletions of amino acids from the C-terminal end of hPMS2-F3. hPMS2-F3-102 (αα 430-613) and hPMS2-F3-300 (αα 430-547) differ by only 66 amino acids at their C-termini. Only hPMS2-F3-102 interacts with hMLH1 demonstrating that a domain facilitating this interaction lies within this 66 amino acid sequence. Overall the demonstration of interactions between hMLH1 and hPMS2 in the yeast two-hybrid system suggests the constructs made and utilised are suitable for looking for new proteins interacting with full length hMLH1 utilising this system.



### 3.4 Yeast Two-Hybrid cDNA Library Screen with pGBT9 hMLH1

As described in the previous sections, the yeast two-hybrid system was fully operational; with the transformation and  $\beta$ -galactosidase assay protocols optimised. A number of problems had materialised when setting up the system. For example a higher concentration of DNA was used in each transformation than proposed in the Clontech manufacturers protocol. This may be due to the fact that Y190 competent cells were made in bulk batches and stored at  $-70^{\circ}\text{C}$ . The volume of transformation mixtures was also adjusted, so that not too much liquid culture was left on the plate surface, giving rise to a higher probability of contamination.  $\beta$ -galactosidase assays were also performed on colonies that were not overgrown as Y190 has an *ade1* mutation. This means that after a few days growth as nutrients are depleted these colonies turn pink-red which makes the blue colour of positive colonies more difficult to decipher. Overall the system was now optimised for characterisation of any positive interactions resulting from a library screen with pGBT9-hMLH1.

pGBT9-hMLH1 has been shown not to autonomously activate transcription from the *LACZ* gene when transformed into Y190. This plasmid, when co-transformed into Y190 with pGAD424 or with pACT2-RPA70 did not activate transcription of this gene either. These results suggested that this construct is suitable for use in the yeast two-hybrid system. pGBT9-hMLH1 also gave a clearly positive interaction with fragments of hPMS2 in the yeast two-hybrid system. This would suggest that this hybrid hMLH1 protein is still able to participate in protein-protein interactions inherent to the naturally expressed protein. Finally protein extracts made from Y190 transformed with pGBT9-hMLH1 were shown by western blot to express a hMLH1 protein detectable by probing with an anti-hMLH1 antibody. It was concluded therefore that pGBT9-hMLH1 is suitable for use in a yeast two-hybrid cDNA library screen. Therefore this construct was used as the bait hybrid protein in a yeast two-hybrid cDNA library screen to identify new proteins that interact with this hMLH1 protein.

A pre-made Clontech MATCHMAKER Normal Mammary Gland cDNA library was used in this screen. A cDNA library made from normal tissue was chosen, rather than one from a tumour tissue source, as it was hoped to identify proteins interacting with hMLH1 that are either involved in MMR or play a role in mediating signals from the MMR system to

other cellular response pathways. If a library from tumour tissue was used it is possible that normal protein-protein interactions involving hMLH1 could be disrupted through down-regulation or amplified expression of certain proteins associated with tumour development. The library is built into the GAL4 activation domain vector pACT2 (priming method: *Xho*I-(dT)<sub>15</sub>) such that random proteins fused to the activation domain will be expressed.

pGBT9 MLH1 full length was transformed into Y190 competent cells (see Figure 10). A single transformed colony was grown and prepared for large scale transformation with the yeast two-hybrid library DNA. The co-transformed Y190 was subsequently plated out on Leu<sup>-</sup>/Trp<sup>-</sup>/His<sup>-</sup> plates. The basal level of *HIS3* expression from the reporter construct in which the *HIS3* UAS sequence has been replaced by the GAL4 UAS is sufficient to allow growth on His<sup>-</sup> media. Therefore 3-AT (3-amino-1,2,4-triazole, SIGMA), an inhibitor of *HIS3*-encoded IGP-dehydratase was also added to the plates at a concentration sufficient to inhibit low levels of His3p expressed in a leaky manner and suppress background growth on media lacking His (Durfee et al., 1993). By testing different concentrations of 3-AT it was found that 25mM 3-AT was sufficient for this purpose.

The plates were incubated at 30°C for eight days. At this point, the number of colonies on each plate were counted. 1 x 10<sup>5</sup> colonies were counted in total. A β-Galactosidase assay was carried out using a colony lift protocol from each plate. Any colonies that gave a blue positive result in this assay were re-streaked out on Leu<sup>-</sup>/Trp<sup>-</sup>/His<sup>-</sup> plates. 131 colonies were blue in the initial β-Galactosidase assay. The 131 colonies were once again tested for *LACZ* activity utilising the β-Galactosidase assay. Of these, 39 confirmed positives (blue colonies) were identified.

These 39 clones that gave a positive interaction in both assays were grown and yeast DNA mini preps were performed using a Nucleon Kit (Scotlab Bioscience). The yeast DNA was subsequently transformed by electroporation into KC8 *endA*<sup>+</sup> *E.coli* electro-competent cells. Up to 50 ng of DNA was required for these transformations as the transformation efficiency into the KC8 cells was not very high. KC8 cells have a defect in *leuB* which can be complemented by *LEU2* from yeast. Therefore through transformation into these KC8 cells the library plasmids pACT2-X (which carry *LEU2*) can be directly rescued from the yeast DNA mixture by plating transformants onto M9 minimal medium lacking leucine. DNA was prepared from these transformants with a mini-prep kit following the manufacturers directions for preparing DNA from *endA*<sup>+</sup> bacterial cells.



**Figure 10: Schematic describing the Methods for the Yeast Two-Hybrid Normal Mammary Gland cDNA Yeast Two-Hybrid Library Screen**

pGBT9-hMLH1  
transformed into Y190

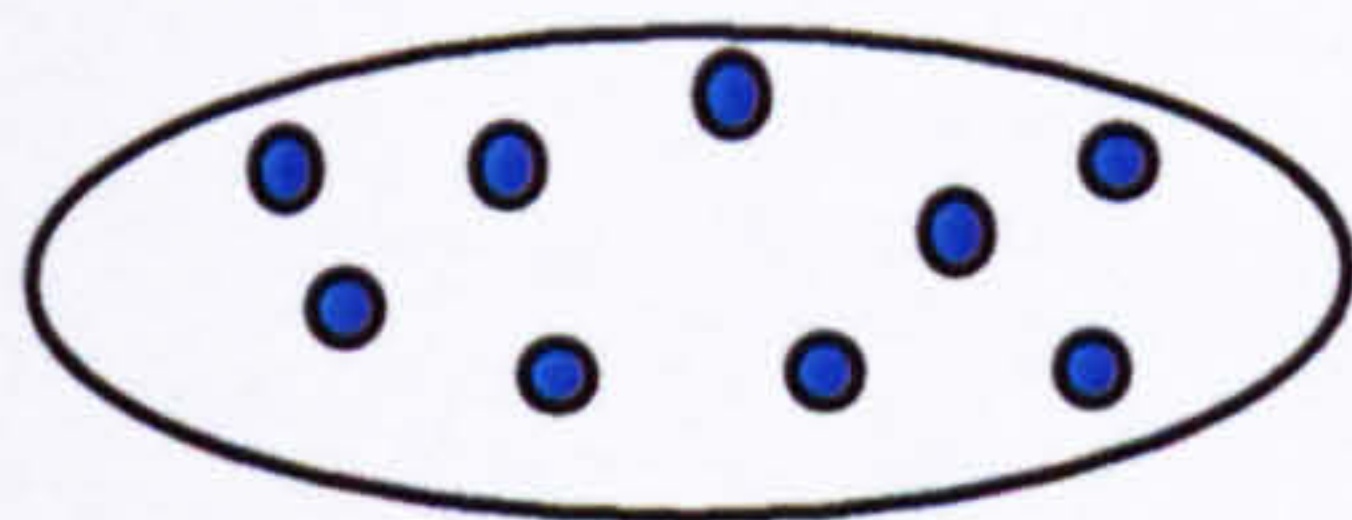


pACT2 (with leucine marker)  
Normal Mammary Gland yeast  
two hybrid cDNA library

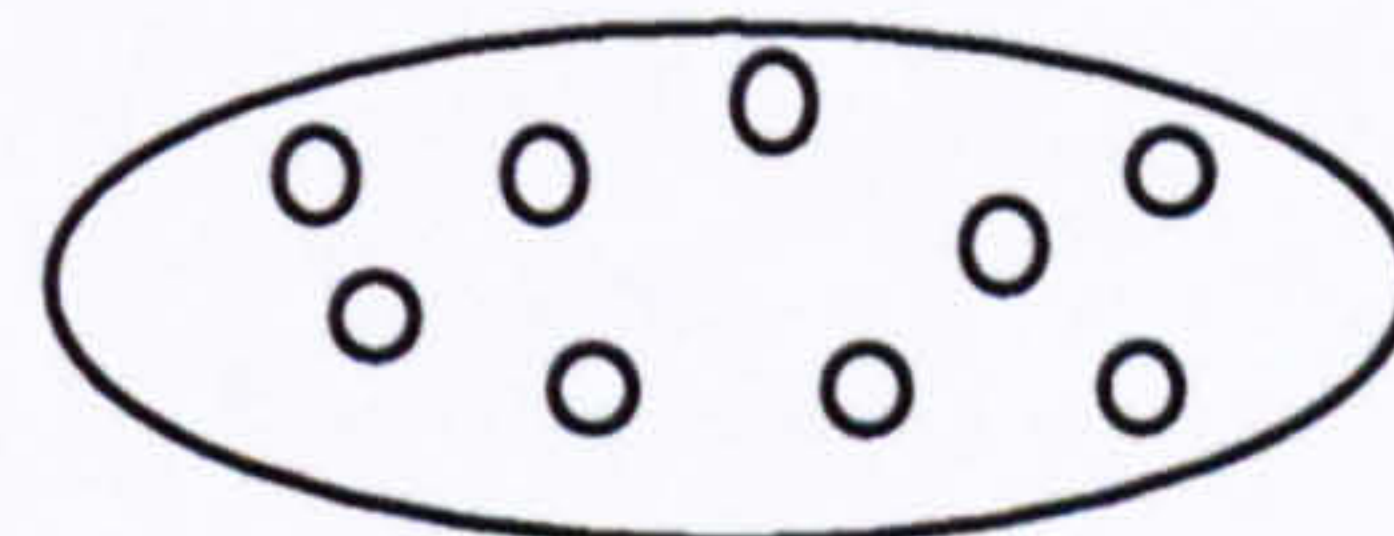
$1 \times 10^5$  yeast transformants screened for *lacZ* activity in the  $\beta$ -galactosidase assay  
131 blue colonies in the  $\beta$ -galactosidase assay



131 colonies streaked onto  $\text{Leu}^-/\text{Trp}^-$   
 $/\text{His}^-$  plates,  $\beta$ -galactosidase filter  
assay was repeated.



39/131 clones gave blue colonies

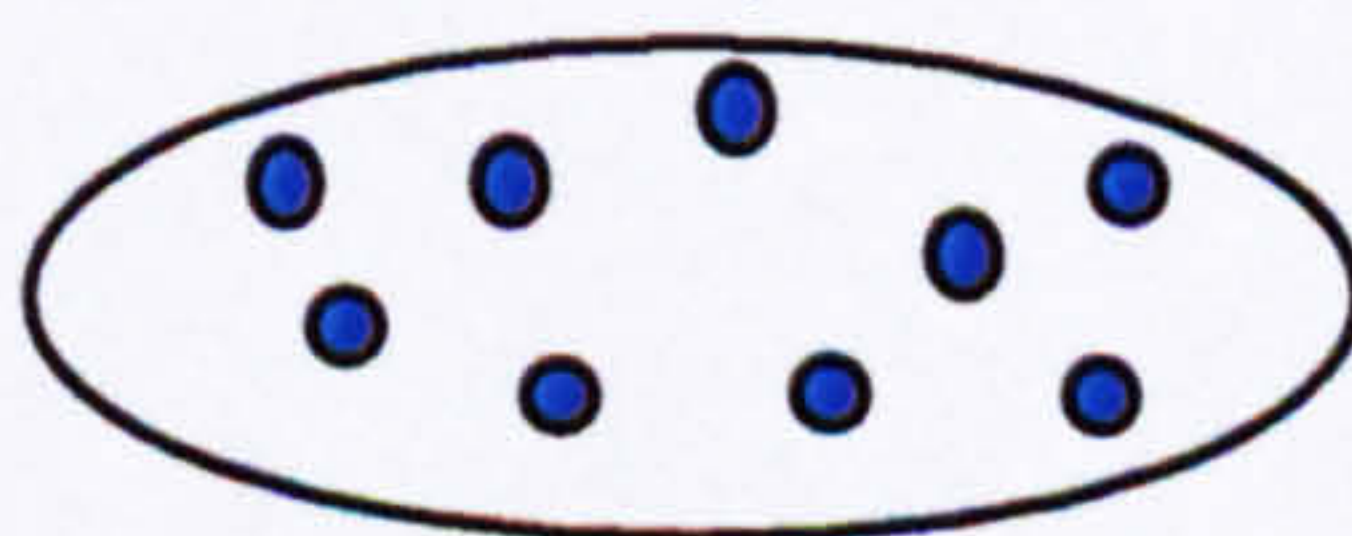


92/131 clones gave white colonies

**False Positives**

Plasmid DNA was prepared from the 39 clones and transformed into KC8, a *leuB*<sup>-</sup> *E. coli* strain to allow selection for bacteria transformed with the library plasmid pACT2-X

pACT2-X plasmid DNA was prepared from colonies that grew on M9 minimal medium plates and was co-transformed into Y190 with pGBT9-hMLH1, followed by a  $\beta$ -Gal assay



25/39 of these clones interacted with hMLH1  
as demonstrated by blue colonies in this assay.

These 25 'True Positives' were identified by sequencing



This DNA was subsequently transformed into DH5 $\alpha$  competent cells and plasmid DNA prepared from the transformants. This is because the DNA prepared from *endA*<sup>+</sup> cells is more difficult to use for PCR and sequencing. Library plasmid insert sizes were checked by PCR.

The plasmid DNA from the 39 clones was co-transformed into Y190 with pGBT9-hMLH1 to confirm the positive interaction. Each library plasmid was also transformed alone, and co-transformed with pGBT9 and with pLAM5' into Y190. Any clone which gave blue colonies in a  $\beta$ -Galactosidase assay when co-transformed into Y190 with pGBT9-hMLH1, but not when transformed alone, with pGBT9 or pLAM5', was designated a true positive interactor. 25 'true positive' clones were identified and these clones were sequenced to identify the proteins interacting with hMLH1. These clones were identified as true positive interacting proteins with hMLH1(see Table 11).



Positive Interacting Clone	Identity	Accession Number
9B1; 13C1	c-Myc	V00568
14E1	Polypeptide chain elongation factor 1-alpha	X16869
3H1; 16B23; 16A21	Ribosomal protein L26	X69392
3D1	U4/U6 small nuclear ribonucleoprotein (hPrp3)	AF016370
14G21; 19A21; 18B23	Protein phosphatase methylesterase 1 (PME1)	AF157028
3C2; 5A1; 11D21	Endothelial PAS domain protein 1 (EPAS1)	U81984
9C1	Alpha-L-iduronidase gene	M95740
4A21	DC3	AF201938
13E1	RIGUI putative mammalian ortholog of PER	AF022991
4C21; 15D23	Uncoupling protein 2 (UCP2)	AF208500
8D1	KIAA09134	AB020721
8B1; 18C1	KIAA0167	D79989
7C1	mRNA from chromosome 5q 21-22	AB002439
4A22; 2K1	mRNA full length insert cDNA clone	AL079296
12A1	cDNA DKFZp434E2321	HSM80144

**Table 11: Positive Interacting Clones from a Yeast Two-Hybrid Screen with pGBT9-hMLH1 as bait and a Clontech Matchmaker Normal Mammary Gland cDNA library**

### 3.5 Yeast Two-Hybrid Library Screen with hMLH1 using a Pre-transformed cDNA Library.

A second yeast two-hybrid screen was performed using hMLH1 as the bait. Because of the relatively low number of clones screened in the original screen ( $1 \times 10^5$ ) it was decided that to carry out a more representative screen would be useful. This in no way invalidates the first screen but merely adds to it as a method of identification of new protein-protein interactions between hMLH1 and other proteins. The development of a new system by Clontech, MATCHMAKER III, made the second screen much easier, with a substantially lower level of false positives allowing rapid analysis of results.

A Clontech pre-transformed MATCHMAKER III Ovarian cDNA library was utilised. This is a high complexity cDNA library cloned into a yeast GAL4 activation domain (AD) vector, pACT2, (priming method: *Xho*I-(dT)<sub>15</sub>) and pre-transformed into the *Saccharomyces cerevisiae* host strain Y187. Y187 is transformed with an entire pre-made MATCHMAKER library ( $>1 \times 10^6$  independent clones). This library is screened by yeast mating rather than library-scale transformation. The yeast mating partner for Y187 (MAT $\alpha$ ) used was the PJ69-2A (MATa) strain. PJ69-2A unlike most other reporter strains contains two nutritional reporter genes, *HIS3* and *ADE2* which both carry a GAL4 UAS. This additional selection for *ADE2* allows a more stringent selection for false positives.

hMLH1 was cloned into a GAL 4 binding domain (BD) vector, pGBKT7 and transformed into the PJ69-2A strain. pGBKT7 was used this time, instead of pGBT9, because it carries a kanamycin selection gene which will allow easier isolation of the pACT2 library plasmid later, which has ampicillin selection. The pGBKT7-hMLH1 construct did not autonomously activate the reporter genes, was not toxic when expressed in yeast and gave a positive interaction when co-transformed into Y190 with pGAD424-PMS2-F3 and pGAD424-PMS2-F4. PJ69-2A transformed with this pGBKT7-hMLH1 construct was also mated with Y187 transformed with pTD1. The mating efficiency with the two strains was not effected by the pGBKT7-hMLH1 construct and no *LACZ* activation was seen in the diploid yeast that resulted. Therefore it was concluded that this pGBKT7-hMLH1 construct is suitable for use in this system.

To screen the pre-transformed library, it was simply mated with the bait strain, PJ69-2A transformed with pGBKT7-hMLH1, for 24 hours and the mixture plated onto quadruple drop out (QDO) selection media (Leu<sup>-</sup>/Trp<sup>-</sup>/His<sup>-</sup>/Ade<sup>-</sup>). The double nutritional selection



results in fewer false positives than seen with the earlier screen which used only a single reporter gene (see Figure 11). The plates were incubated at 30°C for 21 days. The 29 large colonies growing on the QDO media were streaked out onto fresh QDO plates and incubated for 3 days at 30°C. A  $\beta$ -Galactosidase assay was performed on these plates using a filter lift assay. After lifting the colonies for the  $\beta$ -Galactosidase assay the master plates were placed at 30°C for 2 days to allow the colonies to re-grow. The master plates were wrapped in parafilm and incubated at 4°C. The positive colonies were re-streaked on Leu<sup>-</sup>/Trp<sup>-</sup> plates three times to allow segregation of some of the AD/library plasmids to take place while maintaining selective pressure on the two plasmid types (i.e., DNA-BD/target, and AD/library). Single colonies from these plates were re-streaked onto QDO medium to verify the correct growth phenotype is maintained and the colonies re-assayed for  $\beta$ -Gal activity using the colony lift filter assay.

The viability (colony forming units (cfu)/ml) of the Y187 partner, the PJ69-2A partner, and the viability of the diploids was calculated from control plates (100 $\mu$ l of a 1:10, 1:100, 1:1000, 1:10000 on 10cm plates) set up by spreading dilutions of the library mixture onto Leu<sup>-</sup>, Trp<sup>-</sup> and Leu<sup>-</sup>/Trp<sup>-</sup> plates. An estimate of the number of clones screened was made:

$$\# \text{ cfu/ml of diploids} \times \text{re-suspended volume} = \# \text{ of clones screened}$$

An estimated  $5.3 \times 10^6$  clones were screened.

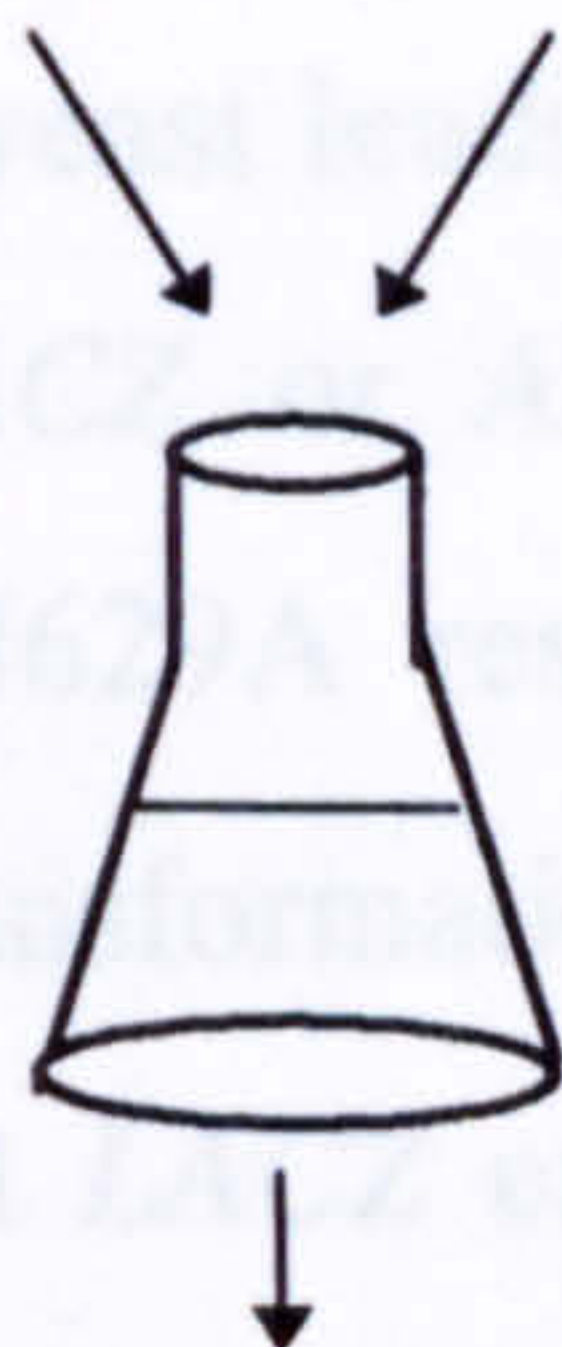
Yeast plasmid DNA mini-preps were performed on the 29 clones. This DNA was transformed into electro-competent DH10B cells (Gibco BRL) and plated onto LB-broth plates with 100  $\mu$ g/ml ampicillin therefore selecting specifically for cells transformed with the library plasmid (pACT2 has an amp<sup>r</sup> marker, PGBKT7 has a kan<sup>r</sup> marker). These transformations and the subsequent selection were more efficient than utilising the KC8 strain used in the previous screen. Plasmid DNA was prepared from the transformants and duplicates identified by digesting the DNA with *Alu* 1.

False positives were eliminated by co-transforming the newly isolated library plasmid DNA with pGBKT7-hMLH1 into Y190 and performing a  $\beta$ -Galactosidase assay on the transformants. Additionally both plasmids were also co-transformed into PJ69-2A and plated out onto QDO plates testing for the correct growth phenotype. Library plasmids were co-transformed with pLAM5' and also pGBKT7 into Y190 and tested for *LACZ* activation and into PJ69-2A and tested for the ability to grow on QDO plates. It was found that 23/29 clones were 'true positives'.

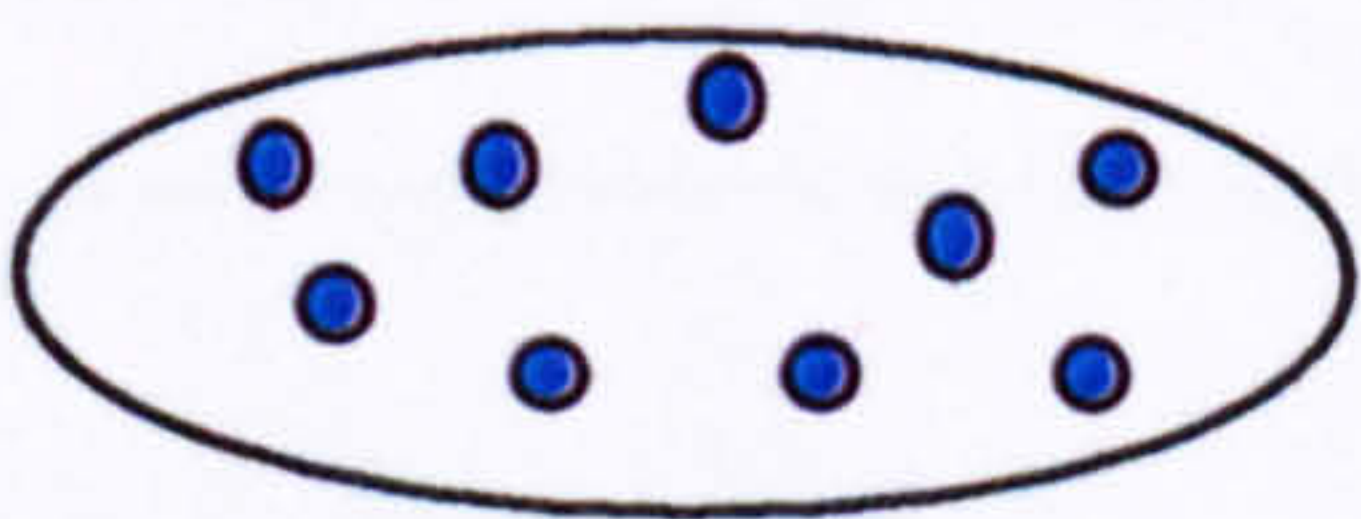


**Figure 11: Schematic for Yeast Two-Hybrid Pre-Transformed Library Screen**

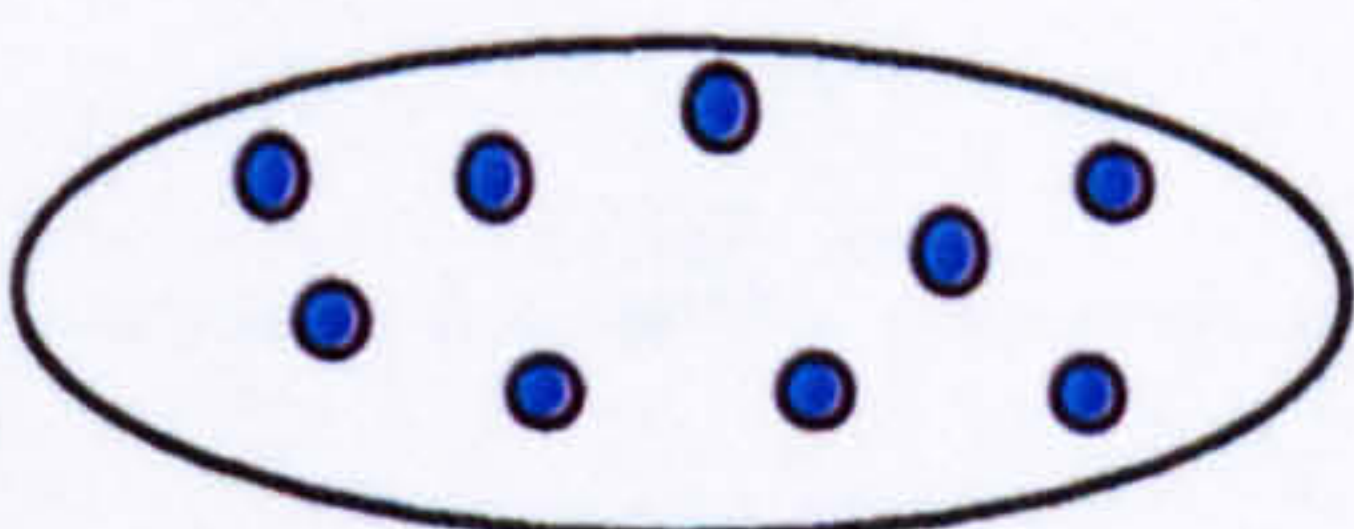
pGBKT7-hMLH1 in PJ69-2A, MATa + Pre-transformed Ovarian Yeast Two-Hybrid cDNA library, in Y187, MATα



29 large colonies had grown after three weeks on Leu<sup>-</sup>/Trp<sup>-</sup>/His<sup>-</sup>/Ade<sup>-</sup>, QDO plates. The 29 colonies were re-streaked out onto QDO plates and a colony lift filter β-Galactosidase assay carried out on these plates. 29/29 plates gave blue colonies.



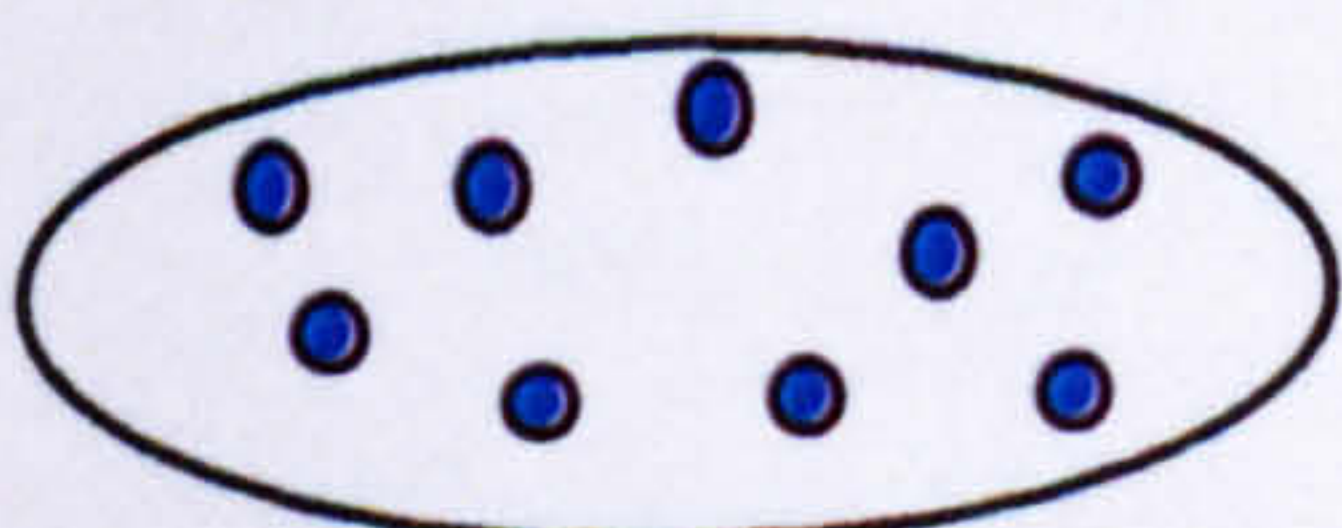
Positive clones were streaked out onto consecutive Leu<sup>-</sup>/Trp<sup>-</sup> plates three times and then onto QDO plates. Colony lift filter β-Galactosidase assays were carried out on these plates. 29/29 plates gave blue colonies



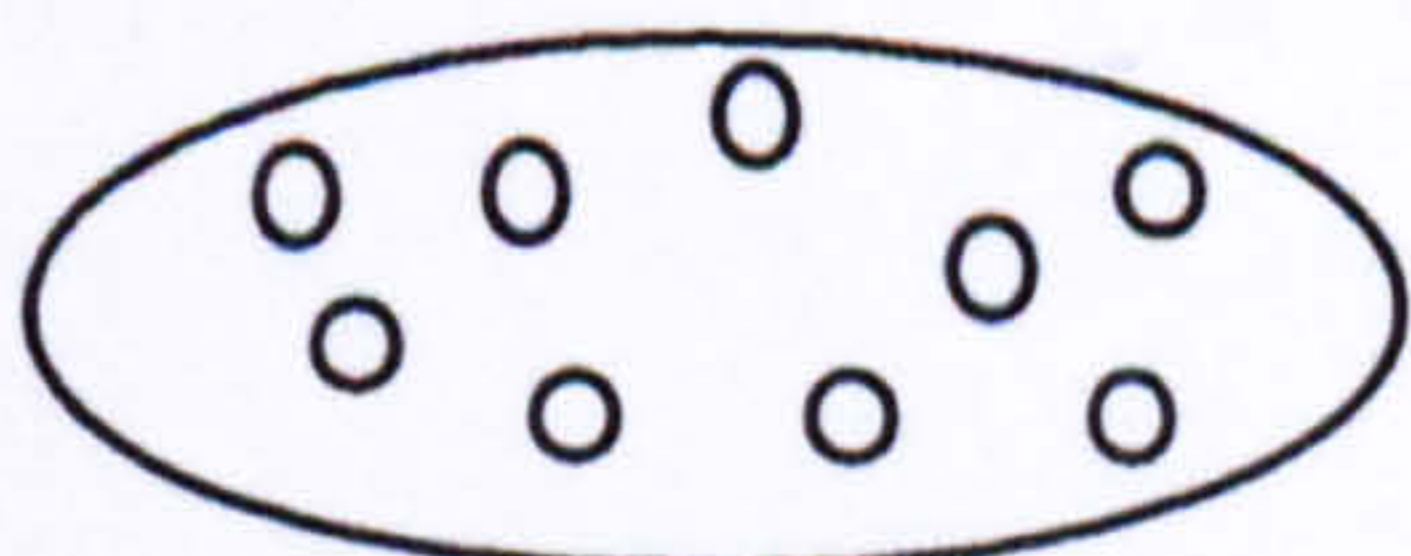
Plasmid DNA was prepared from the 29 clones and transformed into DH10B *E.coli*, the transformations were plated onto plates with ampicillin to allow specific selection of cells with the library pACT2-X plasmids as pGBKT7 does not have an ampicillin marker.

pACT2-X plasmid DNA was prepared from these colonies and was co-transformed into Y190 with pGBKT7-hMLH1, followed by a β-Gal assay

23/29 plates gave blue colonies ← 6/29 plates gave white colonies



**These 23 'True Positives' were identified by sequencing**



**6 'False Positives'**



The inserts of all true positive interacting clones were amplified by PCR and sequenced to identify the interacting proteins (see Table 12). In summary 'true positives' are defined as proteins expressed from the library constructs that interact with the hMLH1 protein in the yeast two-hybrid system to give blue colonies in a  $\beta$ -galactosidase assay. Subsequent re-isolation of these plasmids from the yeast leads to further verification of a 'true positive'. These plasmids do not activate *LACZ* or *ADE2* activity autonomously or when co-transformed into yeast (Y190 or PJ629A respectively) with control plasmids such as pLAM5' or pGBKT7. Upon co-transformation of the 'true positives' into Y190 with pGBT9-hMLH1 or pGBKT7-hMLH1 *LACZ* expression is clearly activated. In the latter screen, mating of PJ69-2A transformed with pGBKT7-hMLH1 and Y187 with the library plasmid allowed the growth phenotype of the diploids to be tested. Once again, only 'true positives' were able to grow on QDO plates.

Positive Interacting Clone	Identity	Accession Number
2.1.1	Polypeptide chain elongation factor 1-alpha	E02628
13.1.1; 20.1.1	Polypeptide chain elongation factor 1-alpha	E02628
8.2.1; 6.3.1; 3.1.1  19.2.1;10.1.3;  15.1.1; 14.1.3	Polypeptide chain elongation factor 1-alpha	E02628
18.1.1; 14.1.3	Polypeptide chain elongation factor 1-alpha	E02628
12.1.1	ELPIN	AF198455
7.2.2; 7.1.1; 21.3.1  9.2.1; 20.2.1; 11.1.1	PMS1	U13695
6.2.1	MLH3	AB039667
14.3.1	MED1	AF114784
1.1.1	FHL2	U29332

**Table 12: Positive Interacting Clones from a Yeast Two-Hybrid Screen with pGBKT7 hMLH1 as bait and pre-transformed MATCHMAKER Ovarian cDNA library**



### 3.6 c-MYC-hMLH1 Yeast Two-Hybrid Interaction.

Further examination of the c-MYC-hMLH1 interaction identified from the first yeast two-hybrid library screen was carried out. The particular aim of this project was to identify novel protein-protein interactions, between hMLH1 and other cellular proteins, to help further elucidate the role of hMLH1 and the MMR system in signalling cellular DNA damage response pathways. Because of the role of c-MYC in a variety of cellular processes, including signalling progression through the cell cycle and apoptosis, this represented the most intriguing of the interactions identified. A specific link between DNA damage recognition by the MMR system to possible downstream pathways represents the 'holy grail'.

From the yeast two-hybrid library screen with the mammary gland cDNA library it has been shown that hMLH1 interacts with the C-terminal half of c-MYC ( $\alpha$ 245-439, stop codon). The full sequence of the insert (1291-1878bp) was established by sequencing (myc-F and myc-R primers were used). This region of c-MYC includes a basic/helix-loop-helix/leucine zipper domain. MAX a c-MYC binding partner is known to bind to this region of c-MYC. The yeast two-hybrid system was further utilised to verify whether this interaction between c-MYC and hMLH1 is real. Elucidation of domains facilitating this interaction could perhaps hint at the functional significance of this protein-protein interaction.

The construct pulled out of the yeast two-hybrid library screen expressing the C-terminal half of c-MYC was co-transformed into Y190 with pGBT9 expressing fragments of hMLH1 (F1-F4). From this it is clear that c-MYC specifically interacts with fragment 4 ( $\alpha$ 514-756) of hMLH1, which also includes the hPMS2 interaction domain of this protein (see Table 13). To further elucidate the hMLH1 interaction domains of c-MYC the interaction of avian c-MYC mutants with hMLH1 was examined, again using the yeast two-hybrid system (see Table 14). The C-terminal half of avian c-MYC is highly homologous to that of human c-MYC and therefore deemed suitable for use in this experiment (see Figure 12). These mutants were available from Dr. D. Crouch (University of Dundee) and had also been previously characterised in terms of their effect on the interaction of c-MYC with MAX (Crouch et al., 1993; Crouch et al., 1990). Full length c-MYC was not used in the two-hybrid system to further verify this interaction as it has an N-terminal transactivation domain making it unsuitable for use in this system.

	pACT2 c-MYC  αα245-stop codon
MLH1 Full Length αα1-756	+
MLH1 F1 αα1-228	-
MLH1 F2 αα180-342	-
MLH1 F3 αα308-579	-
MLH1 F4 αα514-756	+

**Table 13: Interactions of the 9B1 (c-MYC αα245-stop codon) clone identified from the yeast two-hybrid library screen with full length and fragments 1-4 of pGBT9-MLH1.**

Both full length and fragment 4 of hMLH1 interact with the C-terminal half of c-MYC expressed by the 9B1 plasmid identified from the first two-hybrid library screen.



Mutant	Domain Mutated	Amino Acids Mutated	Mutation	MYC-MAX Interaction
C/T	Basic Region	$\alpha\alpha 339$	L→FR	+
H1	Helix 1	$\alpha\alpha 353$	A→P	+
H2	Helix 2	$\alpha\alpha 377$	T→P	-
C $\delta$ 10	Leucine Zipper	deleted $\alpha\alpha 407-416$	N→STOP	-
C $\delta$ 28	Leucine Zipper	deleted $\alpha\alpha 389-416$	R→STOP	-

**Table 14: List of mutants of avian c-MYC used to localise the region of interaction of c-MYC with hMLH1.**

The C-terminal half of avian C-MYC , V-MYC and the mutants listed were cloned into the yeast two-hybrid vector pACT2 and tested for an interaction with pGBT9-MLH1. This table also lists the effect of these mutations on the c-MYC-MAX interaction.





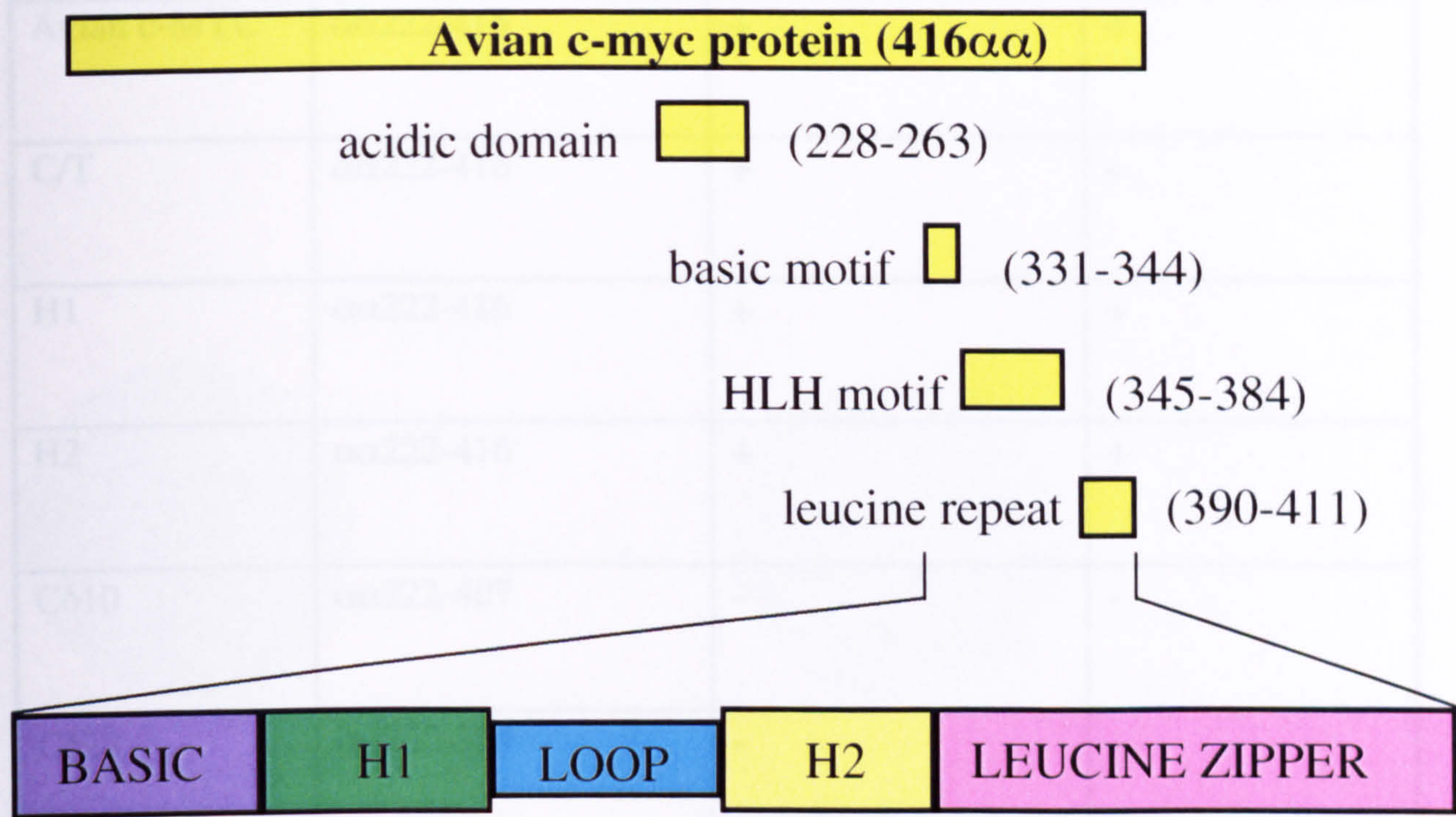


From these constructs the C-terminal half (equivalent to the C-terminal region of human c-MYC identified in the Y2H library screen) of each was amplified by PCR (using primers M1 and M2) and cloned into the yeast two-hybrid vector pACT2. The C-terminal half of avian c-MYC includes a number of defined motifs (see Figure 13). The vector inserts were checked by sequencing and each was found to be in frame and no point mutations had been incurred. Each of these constructs were co-transformed with pGBT9 hMLH1 (full length and fragment 4), with pGBT9 alone and also transformed alone into Y190 (see Table 15).  $\beta$ -Galactosidase assays were again performed on the transformant colonies (see Figure 14). None of the MYC-hybrid proteins activated *LACZ* alone or when co-transformed with pGBT9.

Initial results suggested the leucine zipper domain of c-MYC was required for the interaction with hMLH1. However these results were initially somewhat complicated. The C $\delta$ 10 mutant of c-MYC did not interact with hMLH1, but the C $\delta$ 28 mutant retained the ability for this interaction. Thus it appeared that deleting the last 10 amino acids abolished activity, while deleting the last 28 amino acids did not. These conflicting results could not initially be explained. The only difference between these two mutants was the stop codon mutation used for mutagenesis. In C $\delta$ 10 TAA encoded the stop codon, whereas in C $\delta$ 28 TGA was used. It has been suggested previously that stop codon suppressors exist in yeast, which could possibly explain this different result in the interaction with hMLH1. It was proposed that TGA stop codon suppressors exist in Y190. Therefore the stop codon was changed in C $\delta$ 28 to TAA as well. This change resulted in the loss of a positive interaction between this mutant form of c-MYC and hMLH1. Thus deleting 10 and 28 amino acids from the C-terminal end was consistent. From these results it was concluded that the region of c-MYC required for the interaction with hMLH1 is the leucine zipper domain.

In summary a C-terminal fragment (F4) of hMLH1 (amino acids 514-756) was found to interact with c-MYC. This region of hMLH1 has also previously been found to contain a hPMS2 interaction domain. The leucine zipper domain of c-MYC is required for this interaction with hMLH1. This region of c-MYC is involved in the binding of c-MYC to other proteins. In particular it is required for the c-MYC-MAX interaction. However a mutation in the second helix of the helix-loop-helix domain of c-MYC also knocks out the interaction of this protein with MAX but has no effect on the hMLH1 interaction. This would suggest that the interaction domains for this protein with MAX and hMLH1 differ slightly. The demonstration of the ability of hMLH1 full length and fragment 4 to interact





**Figure 13:** Schematic diagram representing the domains incorporated by the C-terminal half of avian c-MYC.

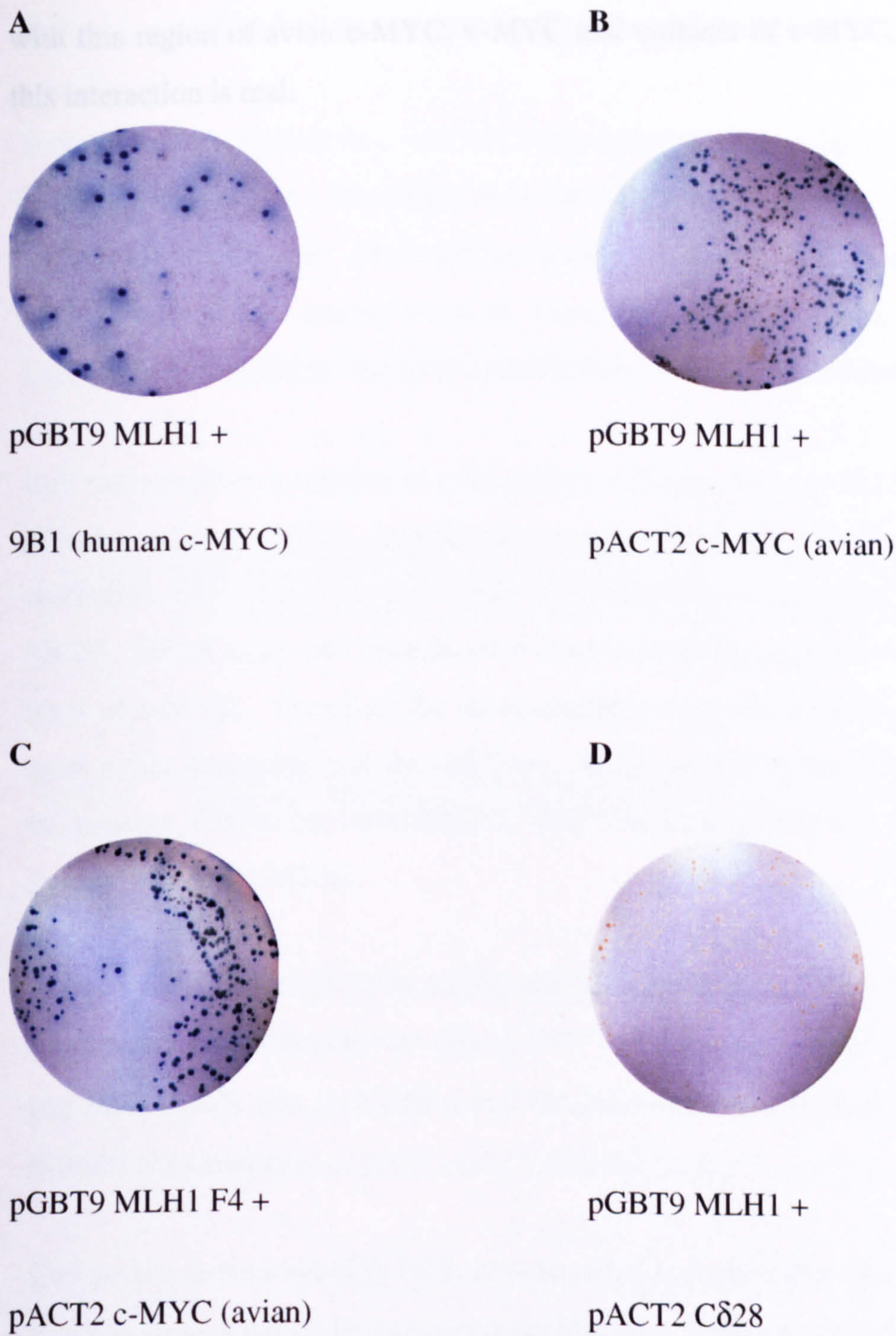


	Region (amino acids)	Full Length MLH1  $\alpha\alpha$ 1-756	MLH1-F4  $\alpha\alpha$ 514-756
Human c-MYC	$\alpha\alpha$ 245-439	+	+
Avian v-MYC	$\alpha\alpha$ 244-438	+	+
Avian c-MYC	$\alpha\alpha$ 222-416	+	+
C/T	$\alpha\alpha$ 222-416	+	+
H1	$\alpha\alpha$ 222-416	+	+
H2	$\alpha\alpha$ 222-416	+	+
C $\delta$ 10	$\alpha\alpha$ 222-407	-	-
C $\delta$ 28	$\alpha\alpha$ 222-389	-	-

**Table 15: Transformation of pGBT9-MLH1 and pGBT9-MLH1 F4 with pACT2 expressing the C-terminal half of avian c-MYC, v-MYC and mutants.**

$\beta$ -Galactosidase assays carried out on the transformed Y190 cells showed that both full length and fragment 4 ( $\alpha\alpha$ 514-756) of hMLH1 could interact with avian c-MYC and v-MYC and that this interaction requires the leucine zipper region of c-MYC. C $\delta$ 10 and C $\delta$ 28 are leucine zipper domain mutants (10 and 28 amino acids deleted at the C-terminal of c-MYC leucine zipper domain respectively) and do not interact with hMLH1.





**Figure 14:  $\beta$ -Galactosidase assays on Y190 co-transformed with pGBT9-hMLH1 full length and fragment 4 with wild type and mutant forms of avian c-MYC and v-MYC.**

<b>Positive Interaction</b> <b>Blue Colonies</b>	<b>A</b>	pGBT9 MLH1 +9B1(human c-MYC), from original screen
	<b>B</b>	pGBT9 MLH1 + pACT2 c-MYC (avian)
	<b>C</b>	pGBT9 MLH1 F4 + pACT2 c-MYC (avian)
<b>No Interaction</b> <b>White Colonies</b>	<b>D</b>	pGBT9 MLH1 + pACT2 Cδ28



with this region of avian c-MYC, v-MYC and mutants of c-MYC, would further support this interaction is real.

### 3.7 Co-Immunoprecipitation of hMLH1 and c-MYC.

Protein-protein interactions identified by the yeast two-hybrid system may not be biologically relevant. Therefore it is essential that secondary evidence for a physical association is obtained. From the yeast two-hybrid data the question must be asked, do hMLH1 and c-MYC interact *in vivo*? Co-immunoprecipitation assays were carried out to look for *in vivo* evidence for an interaction between c-MYC and hMLH1.

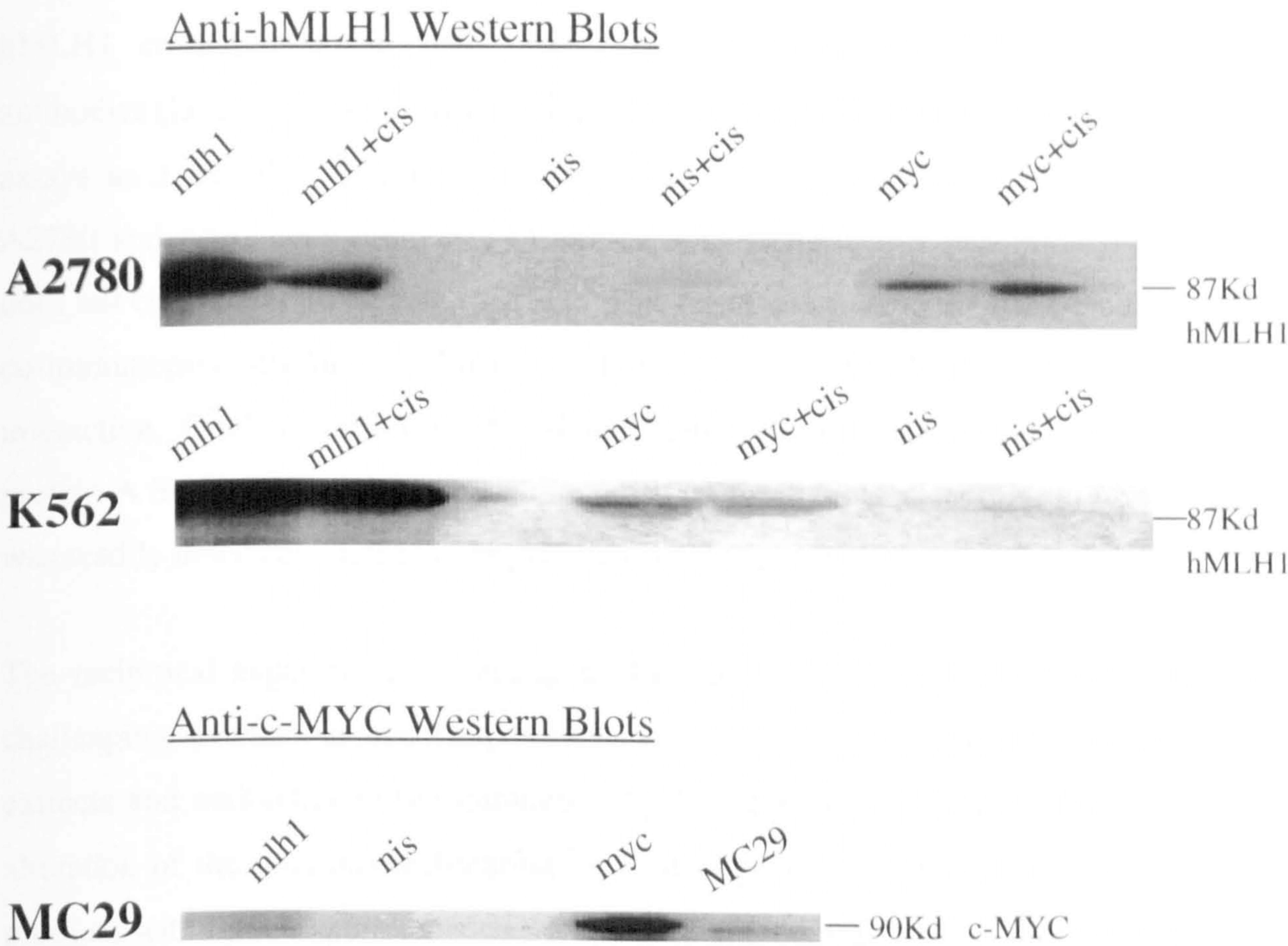
Cell extracts from a number of mammalian cell lines and an avian cell line were prepared (Blackwood et al., 1992). Specifically extracts were made from the human A2780 ovarian carcinoma cell line, from the K562 erythroleukaemia cell line and the avian cell line MC29. MC29 avian cell extracts were used because they are known to express an elevated level of c-MYC. Therefore the co-immunoprecipitation of these two proteins should be more easily detectable in these cell lines. MLH1 was detected in MC29 crude cell extracts by western blot using anti-hMLH1, thus confirming that the MLH1 antibodies cross reacted with avian MLH1.

Extracts were also made from A2780 and K562 cells treated with the DNA damaging agent cis-diamminedichloroplatinum (II), (CDDP). Cells were treated with CDDP for one hour and fresh media was then added and the cells left for a further 24 hours at 37°C before extracts were made.

Cell pellets were washed in PBS, re-suspended in lysis buffer at 4°C and sonicated on ice. Extracts were clarified by two ultracentrifugation steps of 30 minutes at 304,000 g at 4°C. Extracts were immunoprecipitated with anti-c-MYC polyclonal antibody, anti-hMLH1 polyclonal antibody and non specific rabbit serum (NIS) and collected on protein A-Sepharose beads. The beads were collected by centrifugation and washed a number of times in low stringency buffer and denatured samples were run out on SDS-PAGE. Western blots were performed on the gels and probed with anti-c-MYC and anti-hMLH1 monoclonal antibodies to look for co-immunoprecipitation of the two proteins (see Figure 15).

It is clear from these blots that hMLH1 and c-MYC co-immunoprecipitate providing evidence for an *in vivo* interaction between these two proteins. The first two western blots represent blots probed with anti-hMLH1, with co-immunoprecipitation assays from both A2780 and K562 extracts. Polyclonal anti-hMLH1 antibody is clearly able to





**Figure 15: Western Blots of Co-Immunoprecipitation Experiments with polyclonal anti-c-MYC and polyclonal anti-hMLH1**

Co-immunoprecipitation experiments were carried out with extracts from two human cell lines, K562 (an erythroleukaemia cell line) and A2780 (an adenocarcinoma cell line), and also from MC29 an avian cell line which expresses c-MYC at elevated levels. Using polyclonal anti-c-MYC and anti-hMLH1 it is clear that the two proteins are co-immunoprecipitating.

immunoprecipitate hMLH1 from A2780 and K562 extracts (lane: mlh1). Polyclonal anti-c-MYC also clearly co-immunoprecipitates hMLH1 as shown by the distinct hMLH1 band on both blots (lane: myc). The level of hMLH1 protein seen in these lanes are clearly not as strong as those with polyclonal anti-hMLH1. This is an expected result as c-MYC is not an abundant protein, forms complexes with other cellular proteins and has a short half life. The treatment of the A2780 and K562 cells with CDDP does not effect the abundance of hMLH1 co-immunoprecipitated with either polyclonal anti-hMLH1 or anti-c-MYC antibodies (lanes: mlh1 + cis, myc + cis). Non-specific rabbit serum was also used in these assays as a negative control for non-specific immunoprecipitation of the proteins. In A2780 and K562 cells (untreated or treated with CDDP), this pre-immune rabbit serum does not co-immunoprecipitate hMLH1. This observation supports the conclusion that the co-immunoprecipitation of hMLH1 with polyclonal anti-c-MYC represents a specific interaction. Crude extracts from A2780 and K562 cells as well as supernatant taken off the protein-A Sepharose beads were also included on these blots as positive controls. hMLH1 was readily detectable in these samples (lanes not shown).

The reciprocal experiments, detecting c-MYC on the western blots proved much more challenging. Numerous repeat experiments were carried out, varying the amount of protein extracts and antibodies in the immunoprecipitation assays. However these attempts and alteration of the immunoprecipitation conditions proved unsuccessful. The fundamental problem with these experiments was the lack of a good anti-c-MYC monoclonal antibody. This factor along with the relative instability of the c-MYC protein are the most likely explanation for the difficulties encountered. Problems detecting c-MYC by western blot techniques was not limited to the co-immunoprecipitation assays but also in crude extracts from A2780 and K562 cell extracts. Therefore extracts were made from the MC29 avian cell line. hMLH1 was shown to interact with avian c-MYC in the yeast two hybrid system, and both MLH1 and c-MYC were detected with anti-hMLH1 and anti-c-MYC from MC29 cell extracts. These co-immunoprecipitation experiments with MC29 cell extracts and subsequent probing of the western blot with anti-c-MYC proved successful. Polyclonal anti-hMLH1 immunoprecipitates avian c-MYC (lane: mlh1) and polyclonal c-MYC also immunoprecipitates avian c-MYC (lane: myc). No avian c-MYC is seen in the pre-immune rabbit serum immunoprecipitate (lane: nis). c-MYC is clearly detected in the MC29 crude cell extract. Overall these results provide evidence for an *in vivo* interaction between hMLH1 and c-MYC.



### 3.8 GST Pull Down Assays ( work done in collaboration with Dr. E. Homer)

A criterion for biological relevance for the hMLH1-c-MYC interaction described, is the demonstration of an interaction between these proteins using other systems. GST fusion proteins were prepared with full length hMLH1 and the four overlapping fragments 1-4 described for the yeast two-hybrid experiments. GST-MYC and GST-MAX were also prepared. However the GST-MYC fusion proteins degraded rapidly upon expression and were therefore not available for these assays. The original aim of these experiments was to demonstrate a specific interaction between hMLH1 in cell extracts and the full length GST-c-MYC fusion protein, as the interaction studies in the yeast two-hybrid experiments involve the C-terminal half of c-MYC. Unfortunately this was not possible. These assays however provide important evidence corroborating the yeast two-hybrid observations.

As described, GST fusion proteins were expressed from pGex4T plasmids in BL21 *E.coli* cells. The fusion proteins were collected on glutathione sepharose beads. The pull down assays were carried out by incubating the GST fusion proteins on the beads with cell extracts from both the human A2780 ovarian carcinoma cell line, and from the K562 erythroleukaemia cell line. Following this incubation, the beads bound to the GST fusion proteins along with any proteins from the cell extracts that bound the fusion proteins were precipitated. These samples were denatured and resolved by SDS-PAGE. Western blots of these gels were subsequently probed with anti-hMLH1 and anti-c-MYC antibodies.

c-MYC clearly binds the GST fusion proteins with full length hMLH1, fragment 4 (F4) of hMLH1 and MAX. An example of a western blot gel demonstrating an interaction between hMLH1 and c-Myc is shown ( see Figure 16). From this gel, it is demonstrated that GST alone and GST fusion proteins with hMLH1-F1 (GST F1), hMLH1-F2 (GST-F2) and hMLH1-F3 (GST-F3) do not interact and hence do not pull down cellular c-MYC from cell extracts. However both fragment 4 ( $\alpha$ 514-756) of hMLH1, hMLH1-F4 (GST-F4) and full length hMLH1 (GST MLH1) clearly interact with and pull down cellular c-MYC from the K562 cell extracts. As a positive control, MAX a known c-MYC binding partner was expressed as a GST-fusion protein, GST-MAX , which clearly binds and pulls down cellular c-MYC in this assay system. The same binding patterns have been shown with this assay using cell extracts from the A2780 cell line.



Anti-c-Myc Western Blot

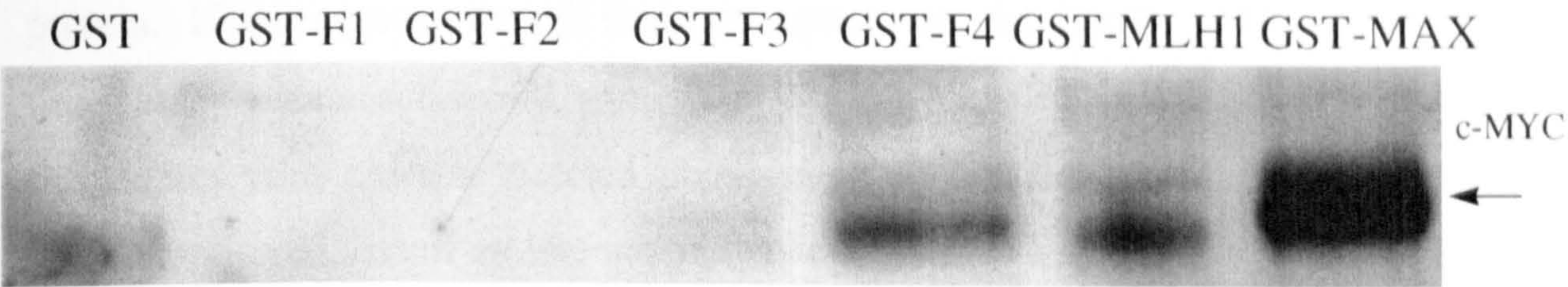


Figure 16: GST Pull Down Assays

GST pull downs with the GST fusion proteins listed in the gel lanes above, and extracts from K562 cells were resolved by SDS-PAGE and the subsequent western blot probed with anti-c-MYC. GST-MAX, GST-MLH1 and GST-F4 each bind c-MYC from K562 extracts as shown by probing the western blot with anti-c-MYC.



This study provides further support to the observations from the yeast two-hybrid experimental results and the co-immunoprecipitation studies. c-MYC and hMLH1 have been shown in a second independent assay to bind. Using these two assays to detect an interaction however does not define whether this hMLH1-c-MYC interaction is specific. Demonstrating that this interaction is specific is a significant obstacle, due to the fact that c-MYC is such an unstable protein and difficult to express for example as a GST fusion protein. However, even though these attempts to use full length c-MYC in this GST-pull down assay were unsuccessful, some hints that this is a specific interaction have come from the studies with the site directed mutagenesis of avian c-MYC. In these studies the demonstration of loss of an interaction between hMLH1 and specific mutants of the avian c-MYC protein have been shown in the yeast two-hybrid system and identify the leucine zipper domain of c-MYC as being crucial for this interaction.

Recent studies have demonstrated an interaction between hMSH2 and MAX (pers. comm. Dr. E. Homer). GST pull down assays with GST-MAX and extracts from A2780 cells were carried out, and the western blot on assay samples probed with an anti-hMSH2 antibody. These experiments clearly demonstrated that hMSH2 in A2780 cells is binding and therefore pulled down with the GST-MAX fusion protein.

### 3.9 MYC/MAX/MLH1 Electrophoretic Mobility Shift DNA Binding Assays.

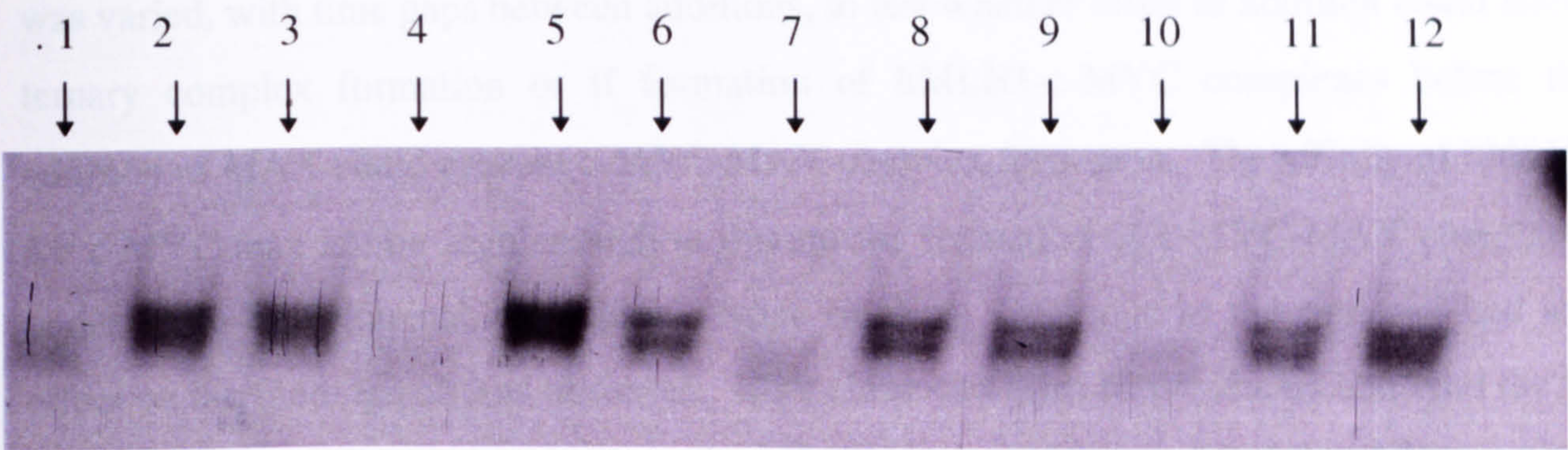
c-MYC and MAX proteins dimerise and bind DNA through their basic-helix-loop-helix-leucine zipper motifs (b-HLH-LZ) (Blackwood and Eisenman, 1991). c-MYC-MAX heterodimer binds with highest affinity to the consensus sequence CACG/ATG, the E-box sequence (Prendergast and Ziff, 1991). c-MYC-MAX and MAX-MAX complexes can form and bind to this E-box DNA core sequence. However c-MYC does not form homooligomers or bind this DNA sequence except at very high concentrations *in vivo* (Dang et al., 1991). This binding of c-MYC-MAX heterodimers can be observed using electrophoretic mobility shift DNA binding assays (EMSA).

The gel shift system has been used in the past to identify proteins which bind to specific sequences involved in control of gene transcription. This system is based on the fact that the movement of DNA through a non-denaturing polyacrylamide gel is retarded when a protein binds to it. The DNA is  $^{32}\text{P}$  labelled so the complexes formed can be visualised by autoradiography. Using electrophoretic mobility shift DNA binding assays it was investigated whether hMLH1 can form a ternary complex with the c-Myc-Max heterodimer or if indeed it can compete with Max for binding to c-Myc. The requirement for the leucine zipper domain of c-MYC for an interaction with both MAX and hMLH1 (yeast two-hybrid studies) would suggest that the hMLH1 interaction with c-MYC could affect c-MYC-MAX binding.

Purified GST-MYC, GST-MLH1 and HIS-MAX were used to test the effect of hMLH1 on Myc-Max heterodimerisation and DNA-binding function. Using a  $^{32}\text{P}$  radiolabelled oligonucleotide incorporating the consensus DNA binding sequence, the E-box sequence, a shift in mobility of the oligonucleotide using gel electrophoresis is visible when purified Max is added to the reaction (at a concentration of  $0.15\mu\text{g}$ ). A further shift is seen upon the addition of both c-MYC (in excess at a concentration of  $1.2\mu\text{g}$ ) and MAX ( $0.15\mu\text{g}$ ) to the reaction (see Figure 17). However the addition of hMLH1 had no effect on the formation of this complex.

Numerous repeat experiments were carried out to try and determine whether hMLH1 could effect the formation or DNA binding ability of the c-MYC-MAX heterodimer. hMLH1 was added, to the reactions with c-MYC and MAX, at a range of concentrations





Lanes 1-12

1. MAX	5. MAX, MYC1:2	9. MAX, MYC 1:4, MLH1
2. MAX, MYC	6. MAX, MYC 1:2, MLH1	10. MAX
3. MAX, MYC, MLH1	7. MAX	11. MAX, MYC 1:8
4. MAX	8. MAX, MYC 1:4	12. MAX, MYC 1:8, MLH1

MAX (0.15µg), MYC (1.2µg and diluted 1:2, 1:4 and 1:8), MLH1 (1.5µg)

Figure 17: EMSA with purified GST-MYC, HIS-MAX and GST-hMLH1

MAX binds the E-box oligo causing a mobility shift as seen from the complex formed. MYC and MAX binding as a heterodimer to the oligo cause a further shift. hMLH1 had no effect on complex formation by MYC and MAX nor did it form a ternary complex with these proteins under these conditions. Gel shifts were also performed with a range of hMLH1 and MAX concentrations.



(from 5 $\mu$ g down to 0.5 $\mu$ g ). Addition of hMLH1 to reactions with a range of concentrations of c-MYC and MAX also had no effect on the formation of the c-MYC-MAX heterodimer. hMLH1 and c-MYC were added to the reactions alone to test whether under these conditions a heterodimer of these two proteins could itself bind the E-box recognition sequence. No binding was observed. The order of additions of each protein was varied, with time gaps between additions, to test whether order of addition could affect ternary complex formation or if formation of hMLH1-c-MYC complexes before the addition of MAX could prevent c-MYC-MAX complex formation. The affinity of hMLH1 for c-MYC may not be high enough to disrupt the formation of c-MYC-MAX complexes under these experimental conditions. None of these variations in the protocol had any effect on the binding patterns observed. Max alone can form heterodimers and bind the E-box oligonucleotide. Upon addition of c-MYC to the reaction, a shift in mobility is seen, demonstrating the formation of a c-MYC-MAX heterodimer capable of binding this E-box oligonucleotide. Addition of hMLH1 to the reaction however had no effect on these observations. No shift in mobility of the complex band or indeed loss of the band on any of the gels was observed upon addition of hMLH1.

From these *in vitro* studies hMLH1 does not appear to form a ternary complex with the c-MYC-MAX heterodimer or effect the ability of this heterodimer to bind the E-box recognition sequence. However as these are *in vitro* studies carried out with fusion proteins, a negative result is somewhat difficult to interpret. There is a possibility that under these conditions the native conformation of the protein could be effected through expression of the individual proteins as fusion proteins. Although this does not seem to effect the c-MYC-MAX interaction and binding to DNA, this does not rule out the possibility in terms of formation of a ternary complex. These *in vitro* studies may also lack other factors that could facilitate the formation of such a complex or indeed a role for hMLH1 in disrupting binding of the heterodimer to DNA. In conclusion, the addition of hMLH1 to these assays at a range of concentrations, does not affect the ability of c-MYC-MAX heterodimer formation or binding to DNA under these *in vitro* conditions.



### 3.10 Electrophoretic Mobility Shift Assays with a Mismatched Substrate ( In Collaboration with Prof. D. Gillespie, Beatson Institute).

DNA mismatch repair proteins recognise and bind mismatched DNA substrates. A number of studies utilising gel shift assays have shown proteins contained within nuclear cell extracts can bind single mismatched base pairs on short fragments of DNA causing the delayed migration of these oligos through polyacrylamide gels (Jiricny et al., 1988; Stephenson and Karran, 1989). Purified yeast proteins yMSH2, yMLH1 and yPMS1 (homologous to human PMS2) have been shown by gel shift analysis to interact with heteroduplex DNA (Prolla et al., 1994). hMLH1 has also been shown to be able to co-immunoprecipitate heteroduplex DNA (Matton et al., 2000).

The aim of this experiment was to elucidate whether c-MYC expression could affect mismatch recognition and binding. The experiments utilised nuclear extracts from Rat-1 fibroblasts expressing c-MYCER<sup>TM</sup> in gel shift assays with an oligonucleotide carrying a GT mismatch (Littlewood et al., 1995). These constructs were first made to elucidate the biochemical function of the human proto-oncogene *c-MYC* (Eilers et al., 1989). A number of proteins have been rendered functionally hormone-dependent by fusing them with the hormone binding domain (HBD) of steroid receptors such as the human oestrogen receptor (ER) (Picard, 1993). The original c-MYCER construct made was induced by addition of either oestrogen or 4-hydroxytamoxifen (4-OHT) (Eilers et al., 1989), but as most *in vitro* experimental systems use media containing phenol red, a weak agonist of ER and also serum which usually contains oestrogens, this system had a number of practical drawbacks. To overcome these drawbacks a mutant murine oestrogen receptor(G525R) was utilised, that no longer binds oestrogen, but continues to be responsive to activation by the synthetic steroid 4-OHT (Danielian et al., 1993). The mitogenic effects of this conditionally expressed allele of c-Myc are dependent on the presence of 4-OHT but in no way respond to oestrogen.

The gel shift experiments were performed with nuclear extracts prepared as described (Jiricny et al., 1988), from Rat-1 c-MYCER<sup>TM</sup> cells grown in the absence of 4-OHT or grown in the presence of 4-OHT for between 2 and 24 hours. The first experiment used extracts (made from  $2 \times 10^8$  cells) from cells at 0 hours (no 4-OHT added), 2 and 24 hours (plus 4-OHT). The second experiment used extracts from 0, 2, 4, 8 and 24 hours. A2780



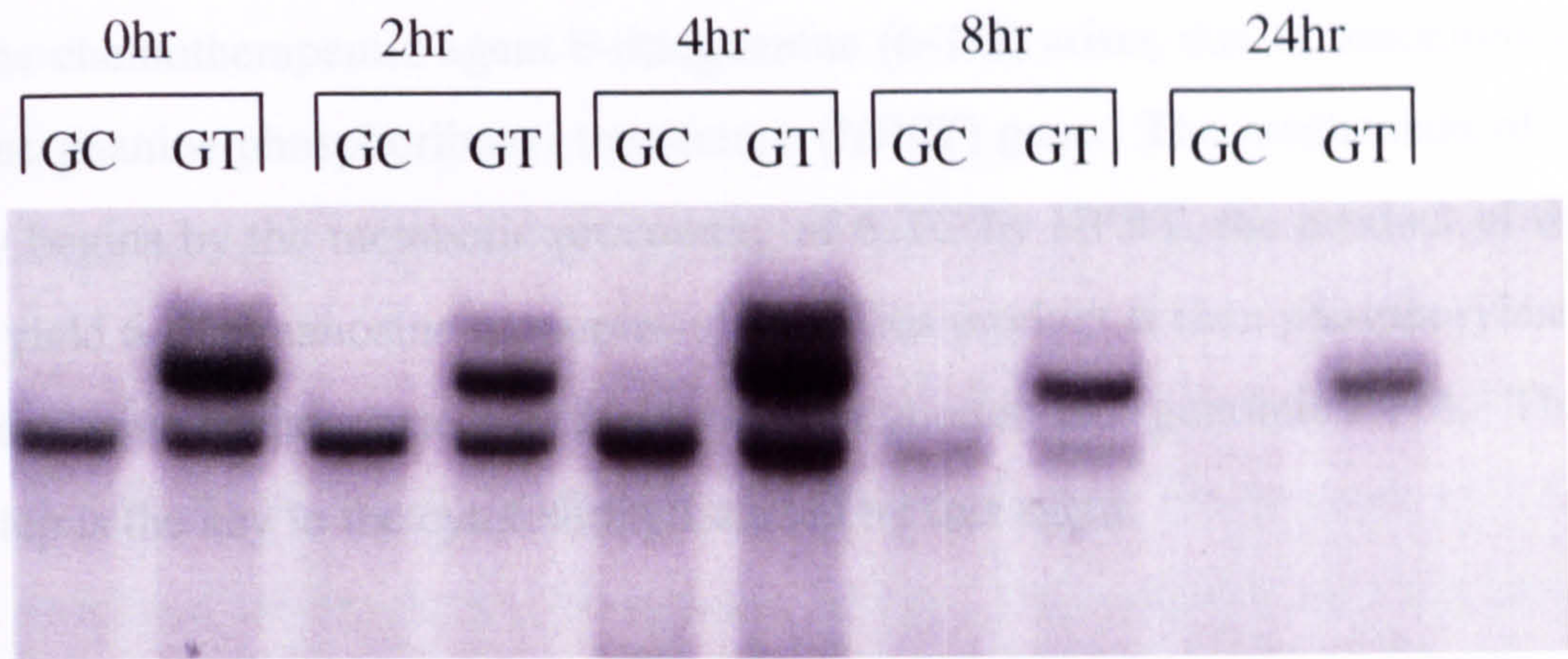
nuclear cell extracts were used as a positive control for the assay. Experiments were performed using a 34 base oligonucleotide, with a G at position 16, annealed to a complementary strand, a C at position 16, or a non-complementary strand with a T at position 16, giving a GT mismatch.

Specific binding of the mismatch recognition complex to the mismatched substrate was clearly demonstrated in these extracts. However no significant difference in this specific binding ability was observed in cell extracts after the addition of 4-OHT to the Rat-1 c-MYCER<sup>TM</sup> cells over a range of time points (see Figure 18). In gel A extracts from 0, 2, 4, 8 and 24 hours were incubated with oligonucleotides with either GC at position 16 or GT. In both cases i.e. GC and GT a non-specific band is observed. However in the case of the mismatch, GT, a clear specific bandshift is observed. These results were observed in the first experiment as well, where extracts were made at 0, 2 and 24 hours. This pattern was also seen in the A2780 extracts (data not shown). No bands were observed where the oligonucleotide probe was incubated alone in the absence of cell extract (data not shown). It is concluded therefore that this specific bandshift represents a shift in mobility of the oligonucleotide due to mismatch recognition by an MMR protein complex.

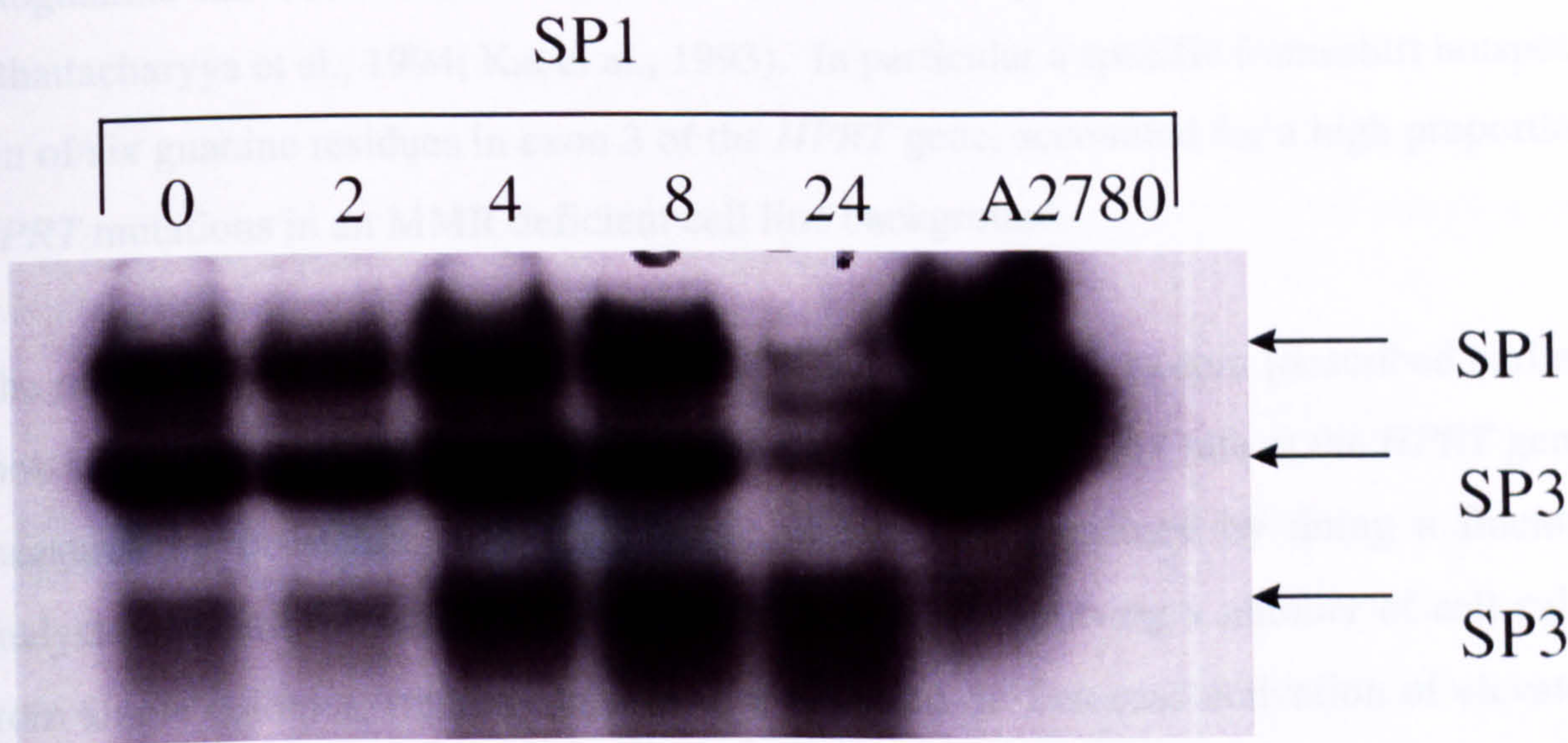
SP1 (Specificity protein 1) is a transcription factor which can bind and act through GC-boxes, which are G-rich elements found in the regulatory region of many genes (Suske, 1999). SP1 belongs to a family of proteins which include SP3. SP3 exists in a number of isoforms and as can be seen in gel B can recognise and bind the same DNA recognition sequence as SP1(see Figure 18). These proteins recognise these GC-box sequences in a wide variety of genes and therefore an oligonucleotide incorporating such a sequence was used for EMSA experiments with the same Rat1 c-MYCER<sup>TM</sup> and A2780 extracts. The reason for this was to identify whether there was any difference in binding intensity between the different extracts. By comparison to the experiments with the mismatch oligonucleotide it is concluded that any differences in band intensity between extracts is purely due to extract quality. As seen from these two gels any differences in intensity for a particular extract is seen in both gels. Therefore the elevated expression of c-MYC in these cells does not affect the binding activity of an MMR protein complex to the mismatched DNA.



A.



B.



**Figure 18:** A. **EMSA with a Mismatched Substrate**  
Extracts from Rat1 c-MYCER<sup>TM</sup> plus 4-OHT at 0, 2, 4, 8 and 24 hr in the gel shift assay with the 34mer complementary oligonucleotide (GC) or that with a GT mismatch at position 16.

B. **EMSA with an Oligo carrying an SP1 Consensus Binding Site.**  
Extracts from Rat1 c-MYCER<sup>TM</sup> plus tamoxifen at 0, 2, 4, 8 and 24hr in the gel shift assay with an oligo carrying an SP1 consensus binding site.



### 3.11 Mutator Phenotype Assay

Resistance to the chemotherapeutic agent 6-thioguanine (6-TG) arises due to mutations in the hypoxanthine-guanine phosphoribosyl transferase (*HPRT*) gene. The mechanism of 6-TG cytotoxicity begins by the metabolic processing of 6-TG by HPRT, the product of the *HPRT* gene, to yield 6-thioguanosine monophosphate. This product is then phosphorylated to give 6-thioguanosine triphosphate which can be incorporated into genomic DNA. This incorporation step is the key to the cytotoxicity induced by this agent.

Loss of mismatch repair is known to lead to an increased mutation rate at *HPRT* and consequently increased resistance to 6-TG (Bhattacharyya et al., 1994; Eshleman et al., 1995; Glaab et al., 1998). In an MMR deficient background acquired resistance to 6-thioguanine has been found to be associated with hotspot mutations in the *HPRT* gene (Bhattacharyya et al., 1994; Kat et al., 1993). In particular a specific frameshift hotspot at a run of six guanine residues in exon 3 of the *HPRT* gene, accounted for a high proportion of *HPRT* mutations in an MMR deficient cell line background.

The aim of this experiment was to use the Rat-1 MYCER<sup>TM</sup> system (described earlier) to look at the effect of elevated c-MYC expression on the mutation rate at the *HPRT* gene, as measured by resistance to 6-TG. Mutation rate was measured by doing a fluctuation analysis. Luria-Delbruck fluctuation analysis involves growing a number of cell cultures from single colonies, treating these with a mutagen, in this case activation of elevated c-MYC expression by addition of 4-OHT to Rat-1 MYCER<sup>TM</sup> cells, and estimating the number of mutants that arise in each culture replicate by selecting for resistance to the anti-metabolite 6-TG (Delbruck, 1943). It is the number of plates with zero colonies that is used to determine the mutant number, thus avoiding the complication of daughter colonies in mutant frequency determinations.

Rat-1 MYCER<sup>TM</sup> cells were plated out on 9cm plates at a density of  $1 \times 10^3$  cells/plate and left in a 10% CO<sub>2</sub> incubator at 37°C for two weeks to allow growth of colonies. Individual colonies were picked from these plates onto 6 well plates. These single colonies were allowed to grow up and expanded up into T75 flasks. Flasks grown from one single original colony were split into two halves, one half grown in the presence and one half in the absence of 4-hydroxy tamoxifen (4-OHT). Cells were exposed to fresh media +/- 4-OHT twice, after 48hr intervals.



At this point the cells were collected and plated out onto 10cm plates at a density of  $2 \times 10^6$  cells per plate in media containing 5  $\mu\text{g/ml}$  6-TG. Based on preliminary experiments and on other studies this concentration of 6-TG was deemed appropriate for use. Control plates were also set up from these flasks, plating out  $1 \times 10^3$  cells per plate in media without 6-TG. The plates were incubated again in a 10%  $\text{CO}_2$  incubator at  $37^\circ\text{C}$  for two-three weeks.

The number of colonies were counted on the control plates (taken and stained after 2 weeks) for calculation of the plating efficiency. All plates grown in the presence of 6-TG were carefully examined for the growth of resistant clones. Any colonies on these plates were picked as before into 6-well plates and grown up.

From this experiment the mutation rate was calculated. To measure the induced mutation frequency in these cultured mammalian cells the population was treated with the proposed mutagen as described, in this case elevated expression of c-MYC induced by the addition of 4-OHT. The clones were then grown in selective media (media + 6-TG) to estimate the number of variants. Survival was measured by growing the clones in non selective media (control plates).

Luria-Delbruck fluctuation analysis was used to estimate the spontaneous mutation rate per viable cell in cultured cells (Delbruck, 1943). Because this is a non-Poisson distribution of data, tests based upon actual values for the number of mutants per clone are not used. Instead the appropriate analysis utilised rank ordering of the number of mutants per plate (replicate). The lowest rank was given to the replicate with the highest number of resistant colonies, where there were tied ranks a mean rank was given. The significance of the difference between the two groups, replicates from cells +/- 4-OHT were analysed using a t-test.

The mutation rates were calculated from the combined results of four individual experiments carried out as described. Rat-1 MYCER<sup>TM</sup> cells grown in the absence of 4-OHT will be described first. In total 482 replicates (plates) were available for the fluctuation analysis, originating from 31 single clones. The plating efficiency of each clone was calculated by dividing the average number of colonies on the control plates for each clone by the number of cells plated ( $1 \times 10^3$ ). The number of colonies on the selective plates (mutants) were multiplied by the plating efficiency and these numbers were used for the fluctuation analysis. Of the 482 replicates 218 had mutant colonies growing, 264 had

none. The mutation rate was calculated with the following equation, the  $P_0$  method of Luria and Delbruck (Delbruck, 1943);

$$m = \frac{\log_2(-\log_2 P_0)}{N_t}$$

$m$  = mutation rate,  $N_t$  = number of cells plated,  
 $P_0$  = number of replicates with no mutants/total number of replicates.

For the Rat-1 MYCER<sup>TM</sup> cells grown in the absence of 4-OHT the calculation is:

$$m = \frac{\log_2(-\log_2 264/482)}{2 \times 10^6} = 1.52 \times 10^{-6} \text{ mutants per viable cell}$$

There were 310 replicates from Rat-1 MYCER<sup>TM</sup> cells grown in the presence of 4-OHT, therefore with increased c-MYC expression, originating from 29 individual clones. These were used for the mutation rate and fluctuation analysis calculations. Of these 310 replicates 171 had mutant colonies growing on them, 139 had none. The mutation rate,  $m$ , was calculated with the equation shown and found to be  $3.47 \times 10^{-6}$  mutants per viable cell.

Cells and Treatment	Phenotype	Mutation Rate (m)  mutants/viable cell	t-Test on the numbers from the Fluctuation Analysis
Rat-1 MYCER <sup>TM</sup>	no elevated c-MYC	$1.52 \times 10^{-6}$	p-value<0.001
Rat-1 MYCER <sup>TM</sup> + 4-OHT	elevated c-MYC	$3.47 \times 10^{-6}$	

A t-test was carried out on the two groups of rank numbers from the fluctuation analysis and a p-value<0.001, a highly significant number was shown. This result demonstrates that the difference in the mutation rates between cells exposed to elevated c-MYC expression through addition of 4-OHT to the Rat-1 MYCER<sup>TM</sup> cells and cells grown without 4-OHT



i.e. no elevated c-MYC expression is significant. Therefore elevated c-MYC expression results in a mutator phenotype in this cell system.

A control experiment was carried out with both Rat-1 cells (without the MYCER<sup>TM</sup> construct) and Rat-1 MYCER<sup>TM</sup> cells both treated as described above and the mutation frequency measured. Mutation frequencies were calculated because in the Rat-1 cells no replicates had zero colonies and therefore fluctuation analysis was impossible. The mutation frequencies were measured as follows:

$$\frac{1}{\text{plating efficiency}/1000 \times (2 \times 10^6)} \times \text{average no. of colonies on replicates from a clone}$$

A t-test was used to compare the mutations frequencies from Rat-1 with or without tamoxifen and also to compare the mutation frequencies from Rat-1 MYCER<sup>TM</sup> with or without 4-hydroxy tamoxifen. In all, mutation frequencies were calculated from 44 replicates from 10 original clones for Rat-1 cells and from 50 replicates from 8 original clones for Rat-1 cells plus 4-OHT. The p-value from the t-test comparing the two groups of mutation frequencies was found to be 0.34, which shows that the mutation frequencies between the two groups are not significantly different. For the Rat-1 MYCER<sup>TM</sup> cells mutation frequencies were calculated from 89 replicates from 10 original clones and from 78 replicates from 10 original clones for Rat-1 MYCER<sup>TM</sup> cells plus 4-OHT. The p-value from this t-test comparing the two groups of mutation frequencies was found to be 0.001, which shows that the mutation frequencies between these two groups are significantly different. These results demonstrate that the 4-hydroxy tamoxifen (4-OHT) does not have a mutagenic effect, as treating Rat-1 cells in the same manner with 4-OHT does not produce a mutator phenotype in these cells.

To associate this discovery of a c-MYC induced mutator phenotype specifically to a defect in MMR (for example elevated c-MYC expression having an inhibitory effect on MMR), genomic DNA was prepared from resistant clones that were isolated and grown up from these experiments. A specific frameshift hotspot at a run of six guanine residues in exon 3 of the *HPRT* gene, was previously shown to account for a high proportion of *HPRT* mutations in an MMR deficient cell line background (Bhattacharyya et al., 1994). PCR across a 160bp fragment of exon 3 of the *HPRT* gene was carried out using specifically designed oligonucleotide primers (HPRT-F and HPRT-R). These PCR products were sequenced and examined for frameshift mutations at a run of six G residues at position 206



bp of the *HPRT* gene. DNA from 22 mutant clones from Rat-1 MYCER<sup>TM</sup> cells grown in the absence of 4-OHT and DNA from 25 Rat-1 MYCER<sup>TM</sup> mutant clones from cells grown in the presence of 4-OHT was examined by this method. None of the 21 mutant clones from Rat-1 MYCER<sup>TM</sup> cells grown without 4-OHT had any frameshift mutations at this run of guanines. However 4 out of the 25 mutants clones from Rat-1 MYCER<sup>TM</sup> cells grown in the presence of 4-OHT each had a frameshift mutation, 5 instead of 6 guanines at this position in the *HPRT* gene. The significance of these 4 mutant clones from Rat-1 MYCER<sup>TM</sup> cells grown in the presence of 4-OHT with a frameshift mutation at this run of six guanines was tested using Fisher's Exact Test. From this a p-value of,  $p=0.071$ , was obtained. A p-value of  $p=0.05$  and below is taken as significant. Therefore this small number of frameshift mutants observed is not significant.

The large numbers of replicates used for these experiments and the relatively lengthy incubation time led to a number of problems with these experiments. In particular a large proportion of replicates were lost in each experimental set due to contamination. Picking the mutant colonies and growing them up from six well plates into flasks also proved difficult. However from the replicates which survived, it is shown that elevated expression of c-MYC in this cell system clearly leads to a mutator phenotype. From the 47 mutants grown up, genomic DNA was isolated and a previously identified mutation hotspot in the *HPRT* gene in MMR deficient cell lines was examined for mutations in a run of 6 guanine residues. In DNA from 22 mutant clones from Rat-1 MYCER<sup>TM</sup> cells grown in the absence of 4-OHT no frameshifts at this mutation hotspot were observed. In DNA from mutant clones from Rat-1 MYCER<sup>TM</sup> cells grown in the presence of 4-OHT, i.e. with elevated c-MYC expression, 4 out of the 25 mutants had frameshift mutations at this mutation hotspot. Although this number of frameshift mutants in the Rat-1 MYCER<sup>TM</sup> cells grown in the presence of 4-OHT is not significant, it does strongly suggest a possible link between elevated c-MYC expression and loss of functional MMR. Because of the small numbers of mutants examined, due to the problems described, the power of analysis from these experiments is quite low. However these encouraging results would suggest further study of such mutants may prove fruitful.



# Chapter 4

## Discussion

## 4.1 Introduction

The exact function of hMLH1 in MMR is not fully understood, although it has been shown to be essential for *in vitro* MMR activity. Loss of hMLH1 expression is seen in a number of tumour cell lines resistant to cytotoxic drugs such as cisplatin, and restoration of MMR leads to restoration of drug sensitivity (Brown et al., 1997). This has led to the hypothesis that MMR is involved in coupling DNA damage to an apoptotic response. Signalling to a number of downstream cellular response pathways (c-Abl, p53 and p73) in response to DNA damage has been shown to be lost in MMR deficient cells (Duckett et al., 1999; Gong et al., 1999; Nehme et al., 1997). Exactly how MMR couples to these apoptotic pathways is unclear. The aim of this study was to identify proteins, using the yeast two-hybrid system, that interact with hMLH1, to attempt to further elucidate its role in MMR and the engagement of downstream damage response pathways.

The yeast two-hybrid system is an *in vivo* system used to identify protein-protein interactions (Fields and Song, 1989). The protein of interest is expressed as a hybrid protein and can be tested directly for an interaction with other candidate hybrid proteins. Alternatively the hybrid protein can be used to screen a high complexity yeast two-hybrid cDNA library, to identify a gene encoding a novel protein that interacts with it. Using a library screen large numbers of interacting proteins can be identified. The advantages of this system include the fact that the identification of interacting proteins is relatively simple as plasmid DNA can be isolated from positive interacting clones and the inserts sequenced. Therefore purified proteins or antibodies to the protein of interest are not required. The interacting domains of the individual proteins can also be identified within this system through site directed mutagenesis or deletion analysis.



## 4.2 The Yeast Two-Hybrid System: hMLH1-hPMS2 Interaction

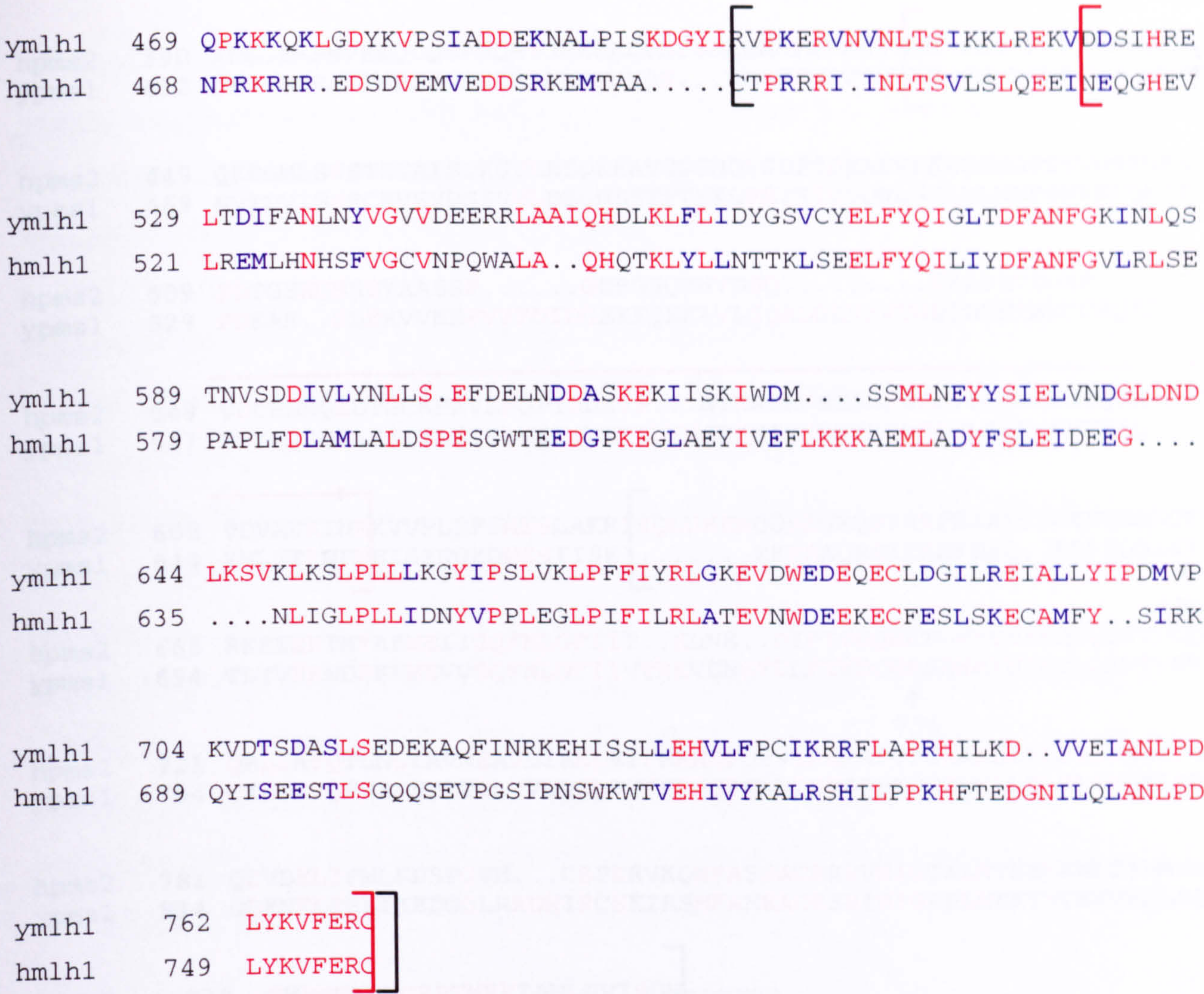
The aim of this thesis was to identify novel proteins capable of interacting with the human MMR protein, MLH1. Therefore making a yeast two-hybrid construct expressing a functional hMLH1 hybrid protein was required. The yeast two hybrid system is an *in vivo* system for detecting protein-protein interactions. Proteins of interest are expressed as hybrid proteins with the DNA binding domain and activation domain of a transcription factor, in this study GAL4, and tested for an interaction within yeast. Interactions between two-hybrid proteins results in reconstitution of the transcription factor which activates the expression of a reporter gene (*LACZ* in this study) which is under the transcriptional control of the particular transcription factor being utilised by the system.

hMLH1 protein has been shown to co-purify with hPMS2 as a heterodimer (Umar et al., 1996). The yeast two-hybrid system was also used to identify an interaction between yMlh1p and yPms1p (hPMS2 homologue) (Prolla et al., 1994). Therefore it was believed that a yeast two-hybrid interaction between hMLH1 and hPMS2 would be detectable, providing a positive control for using the hMLH1 hybrid protein for further studies. Constructs expressing full length and overlapping fragments of hMLH1 and hPMS2 hybrid proteins were made for use in this system. This study would also allow mapping of the domains involved in the hMLH1-hPMS2 interaction.

hMLH1 and hPMS2 clearly interacted in the yeast two-hybrid system. In particular, hMLH1 full length (amino acids 1-756) and fragment 4 (amino acids 514-756) hybrid proteins both interacted with hPMS2 fragment 3 (F3, amino acids 430-647) and 4 (F4, amino acids 630-862) hybrid proteins in the yeast two-hybrid system. These observations are in agreement with earlier studies with the yeast homologues of these proteins which implicate the C-termini of the proteins in this interaction (Pang et al., 1997). The C-termini of the yeast proteins are highly homologous to the human proteins (see Figure 19 and 20). yMlh1p amino acids 501-769 and full length hybrid protein were shown in the yeast two-hybrid system to interact with C-terminal fragments of yPms1p.

A second study was reported during the present study, examining the interactions of hMLH1 and hPMS2, using GST-fusion protein binding assays (Guerrette et al., 1999). In the yeast two-hybrid studies described in this thesis hMLH1-F4 (F4, amino acids 514-756) and full length hMLH1 could interact with hPMS2-F3 (F3, amino acids 430-647) and

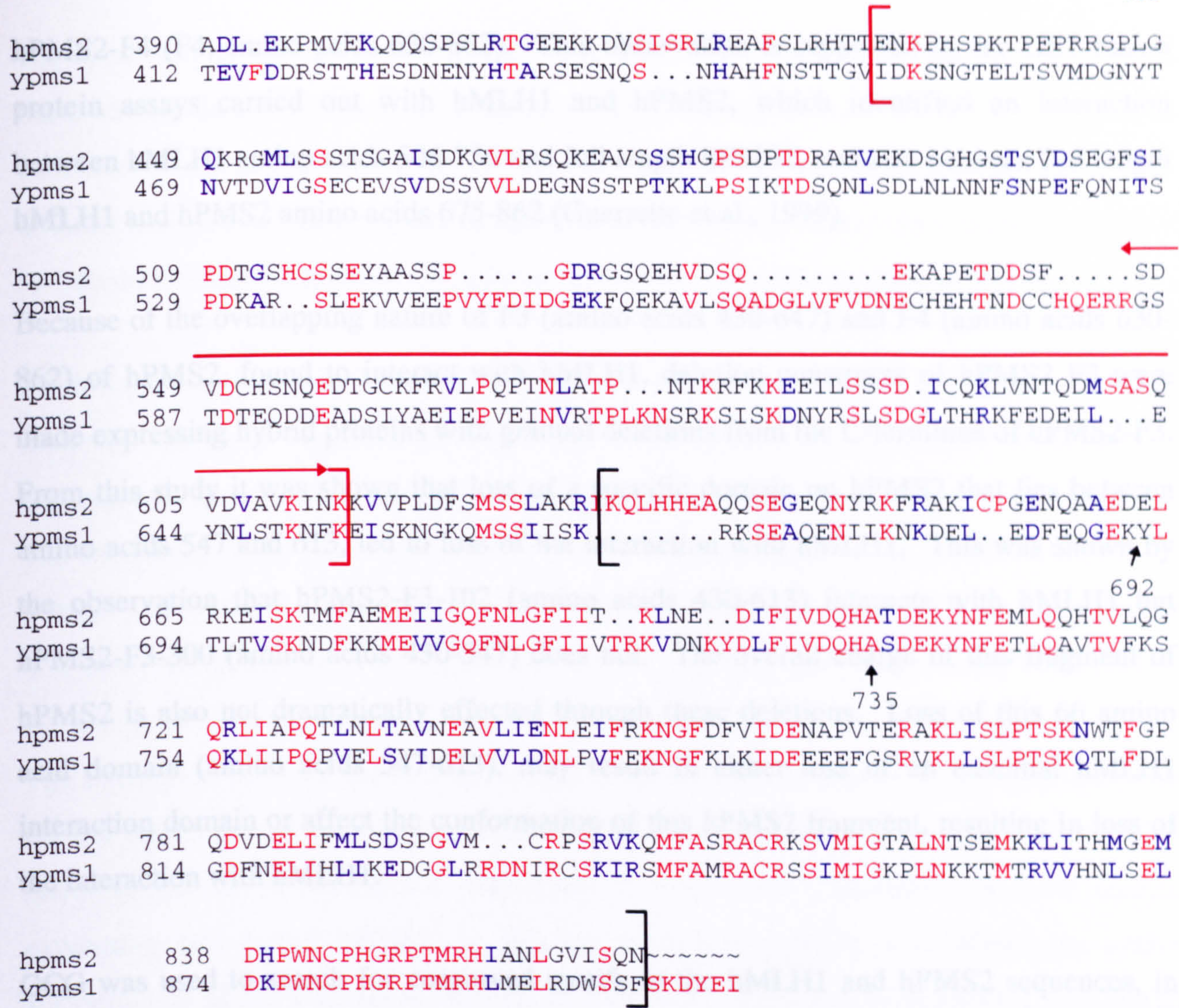





**Figure 19: Alignment of the C-terminal End of yMlh1p (ymlh1) and hMLH1 (hmlh1) Proteins.**

The C-termini of both proteins are highly homologous. The C-terminal fragment of yMlh1p which interacts with yPms1p is enclosed by [..]. The C-terminal fragment of hMLH1 which interacts with hMLH1 is enclosed by [..]. The C-terminal fragments of both proteins identified in this protein-protein interaction include the same regions in both proteins which are highly homologous to each other.





**Figure 20: Alignment of the C-terminal End of yPms1p (ypms1) and hPMS2 (hpms2) Proteins.**

Again the C-termini of both proteins are highly homologous. The two regions of hPMS2 identified from the yeast two-hybrid interaction studies with hMLH1 are amino acids 430-613 [..] and hPMS2-F4, amino acids 630-862 [..]. The C-terminal half of the first fragment, amino acids 430-613 and hPMS2-F4 are highly homologous to the yeast protein, yPms1p.  marks the region of hPMS2 between amino acids 547 and 613. Also marked on the alignment is amino acids 692 and 735 which mark a region of yPms1p which has been shown to be required for the interaction with yMlh1p.



hPMS2-F4 (F4, amino acids 630-862). This observation is supported by the GST-fusion protein assays carried out with hMLH1 and hPMS2, which identified an interaction between hMLH1 amino acids 506-756 and full length hPMS2, and also between full length hMLH1 and hPMS2 amino acids 675-862 (Guerrette et al., 1999).

Because of the overlapping nature of F3 (amino acids 430-647) and F4 (amino acids 630-862) of hPMS2, found to interact with hMLH1, deletion constructs of hPMS2 F3 were made expressing hybrid proteins with gradual deletions from the C-terminus of hPMS2-F3. From this study it was shown that loss of a specific domain on hPMS2 that lies between amino acids 547 and 613, led to loss of the interaction with hMLH1. This was shown by the observation that hPMS2-F3-102 (amino acids 430-613) interacts with hMLH1 but hPMS2-F3-300 (amino acids 430-547) does not. The overall charge of this fragment of hPMS2 is also not dramatically effected through these deletions. Loss of this 66 amino acid domain (amino acids 547-613), may result in either loss of an essential hMLH1 interaction domain or affect the conformation of this hPMS2 fragment, resulting in loss of the interaction with hMLH1.

GCG was used to search for conserved motifs in the hMLH1 and hPMS2 sequences, in particular the motifs command was used, which looks for sequence motifs by searching through the protein sequence for patterns defined in the PROSITE dictionary of protein sites and patterns. A number of websites which allow searches of protein sequences for known motifs such as, <http://www.bioweb.pastuer.fr/seqanal/interfaces/pscan.html>, were also utilised to examine the sequences of hMLH1 and hPMS2 for conserved motifs. In particular the interaction domains on these two proteins were examined. However no conserved motifs were identified through these searches, other than the conserved N-terminal GFREAL, ATP-binding domain.

GST-fusion protein studies with GST-hMLH1 and GST-hPMS2, full length and fragments, identified two hPMS2 regions capable of interacting with hMLH1, amino acids 602-862 and amino acids 675-862 (Guerrette et al., 1999). These observations correlate with the yeast two-hybrid results demonstrating an interaction between hMLH1 and hPMS2-F3 (F3, amino acids 430-647) and hPMS2-F4 (F4, amino acids 630-862). Also observations of interactions between yMlh1p and yPms1p using the yeast two-hybrid system identified an interaction between yMlh1p and yPms1p, amino acids 524-904, and amino acids 692-904 but not with yPms1p amino acids 735-904 (Pang et al., 1997). The loss of a highly conserved region (between yPms1p and hPMS2) of yPms1p, between amino acids 692 and



735 apparently effects the ability of the two proteins to interact (see Figure 20). However, these studies do not rule out the possibility that a second domain on hPMS2 exists that facilitates an interaction with hMLH1. Therefore the observation of an interaction between hMLH1 and two separate regions of hPMS2, amino acids 430-613 and amino acids 630-862 could outline two separate domains involved in this protein-protein interaction.

No interaction was seen between hMLH1 full length or F4 with full length hPMS2. Although the entire sequence of the hPMS2 construct was not sequenced the protein is expressed in frame and restriction enzyme digests would suggest the sequence is correct. The expression of the full length hPMS2 protein as a hybrid protein may effect its ability to interact with hMLH1. A low level of interaction between full length yMlh1p and yPms1p was observed as measured by  $\beta$ -galactosidase activity in a quantitative assay compared to the strong interaction of yMlh1p with a C-terminal fragment of yPms1p (Pang et al., 1997). The interaction between the two full length proteins in these studies, is undetectable with the full length human proteins, or in the case of the yeast study which used a quantitative measurement of transcriptional activation, is very low. Therefore in both studies expressing full length hPMS2 or yPms1p as hybrid proteins may effect the native conformation of these proteins resulting in loss of an interaction with hMLH1 or yMlh1p respectively.

Overall this yeast two-hybrid study clearly identified an interaction between hMLH1 and hPMS2. It was shown that hMLH1-F4 (F4, amino acids 514-756) and full length hMLH1 could interact with hPMS2-F3 (F3, amino acids 430-647) and hPMS2-F4 (F4, amino acids 630-862). Deletion analysis demonstrated two separate regions of hPMS2, amino acids 430-613 and amino acids 630-862 which can interact with both full length hMLH1 and hMLH1-F4 (F4, amino acids 514-756). Loss of a specific domain on hPMS2 that lies between amino acids 547 and 613, led to loss of the interaction with hMLH1. This was shown by the observation that hPMS2-F3-102 (amino acids 430-613) interacts with hMLH1 but hPMS2-F3-300 (amino acids 430-547) does not.



### 4.3 Yeast Two-Hybrid cDNA Library Screening with a hMLH1 Hybrid Protein.

The pGBT9-hMLH1 construct had been shown to produce a hybrid hMLH1 protein with the GAL4 binding domain that did not activate *LACZ* transcription autonomously or when expressed with other unrelated hybrid proteins (AD hybrid proteins). hMLH1 protein was also detected by western blot in extracts made from yeast cells transformed with this construct. The interaction studies with hPMS2 hybrid proteins demonstrates that this full length hMLH1 hybrid protein can interact with a known binding partner, hPMS2, in this system. Therefore this hMLH1 hybrid protein is clearly functional for binding activity. The pGBT9-hMLH1 construct used in the interaction studies with hPMS2 hybrid proteins was therefore deemed suitable for use in further yeast two-hybrid studies.

This functional hMLH1 hybrid protein was used to screen a MATCHMAKER™ human normal mammary gland yeast two hybrid cDNA library (Clontech, UK).  $1 \times 10^5$  colonies were screened in this screen and a total of 15 novel clones were identified as interacting with hMLH1. Five of the fifteen clones identified are unknown, i.e. encode sequences that have not yet been assigned a function. From this screen the interaction of hMLH1 with c-MYC was particularly interesting. Identification of proteins interacting with hMLH1 that have known roles in signal transduction pathways was the main aim of this study. c-MYC is known to be involved in a number of pathways including signalling to apoptotic cell death pathways. This interaction will be discussed later. However a number of other proteins shown to interact with hMLH1 in the yeast two-hybrid system are also potentially important (see Table 16).

This screen was relatively small and would not be fully representative of the range of possible interactions that hMLH1 may be involved in. Therefore a second construct was made, pGBKT7-hMLH1. This construct expressed a hMLH1 hybrid protein which could also interact with hPMS2-F3 and hPMS2-F4 and the c-MYC construct pulled out from the first cDNA library screen. This hybrid protein did not autonomously activate *LACZ* expression in the yeast two-hybrid system. It was therefore also deemed suitable for use in a second yeast two-hybrid cDNA library screen, this time using a pre-transformed MATCHMAKER™ normal ovary cDNA library. This second system from Clontech, MATCHMAKER™ 3, is a more advanced system, with much fewer false positives coming through the screen and also easier isolation of the library constructs and hence identification of the interacting proteins. From this screen an estimated  $5.3 \times 10^6$  clones



**Table 16: Summary of Proteins Identified as Interacting with hMLH1 with the Yeast Two-Hybrid Screens.**

<b>hMLH1 Interacting Protein</b>	<b>Function</b>	<b>Comment</b>
PMS1, MLH3, MED1	PMS1 and MLH3 are human MutL homologues which may play a role in MMR.  MED1 is a mismatch specific T/U DNA glycosylase.	Previously identified interacting proteins.
c-Myc	c-MYC protein has a number of functions including regulation of cellular proliferation and apoptosis	Potential role in signalling pathways involving hMLH1.
Polypeptide chain elongation factor 1- alpha	Elongation factor with a role in protein synthesis, also plays a role in cytoskeletal organisation	Predicted to have a role in apoptosis, may also be involved in signalling pathways involving hMLH1.
Ribosomal protein L26	Ribosomal protein.	Possible false positive.
U4/U6 small nuclear ribonucleoprotein (hPrp3)	snRNP, splicing factor.	Possible false positive.
Protein phosphatase methyltransferase 1 (PME1)	A methyltransferase, demethylates protein phosphatase 2A.	Possible false positive, as identified in another screen with a completely unrelated protein.
Endothelial PAS domain protein 1 (EPAS1)	Homologue of HIF-1 $\alpha$ , both hypoxia inducible. Also can activate expression of VEGF.	Interaction with hMLH1 may block transcriptional activation properties of this protein under some conditions.
Alpha-L-iduronidase gene	A lysosomal hydrolase, a deficiency in which results in the lysosomal storage disorder mucopolysaccharidosis type I	-



DC3	Function not known.	-
RIGUI putative mammalian ortholog of PER	Function not known, possible role in circadian rhythm regulation.	-
Uncoupling protein 2 (UCP2)	Function unknown, may act in similar way to its murine homologue which acts as an uncoupler of oxidative phosphorylation.	-
FHL2	A LIM domain protein preferentially expressed in human heart.	-
ELPIN	Cytoskeletal protein preferentially expressed in human epithelial cells. Expression of this protein has been shown to be lost in a number of cancer cell lines (Maul and Chang, 1999).	-
KIAA09134, KIAA0167, mRNA from chromosome 5q 21-22, mRNA full length insert cDNA clone, cDNA DKFZp434E2321	Unknown functions.	-



were screened. Ten different clones were identified from this screen as interacting with hMLH1 which included three known binding partners of hMLH1. These were hMLH3, hPMS1 and MED1.

Although the c-MYC-hMLH1 interaction is the only interaction that has been examined further, a number of other protein-protein interactions were discovered from these screens (see Table 16). These protein-hMLH1 interactions would have to be verified with additional assays such as co-immunoprecipitation assays, as shown for the c-MYC-hMLH1 interaction, before concluding that they are real. However the initial observations from these yeast two-hybrid cDNA library screens are indicative of the capacity of these proteins to interact with hMLH1. Some of the proteins pulled out from these screens such as the ribosomal protein L26 and the ribonucleoprotein hPrp3 could be false positives even though they do not fill the criteria for false positives in this system. These types of proteins have been reported as common false positives in this system. This web page from the Golemis lab, which has utilised the yeast two-hybrid screening system on numerous occasions, lists a range of proteins commonly found as false positives in the yeast two-hybrid system: <http://www.fccc.edu/research/labs/golemis/Table1.html>.

A few of the proteins described have been relatively well characterised functionally. These include elongation factor 1 alpha and EPAS1. Elongation factor 1 alpha (EF-1 alpha) was shown to interact with hMLH1 in both yeast two-hybrid library screens. EF-1 alpha is an enzyme that catalyses the GTP-dependent binding of aminoacyl-tRNA to acceptor sites of ribosomes during protein synthesis. This protein has also more recently been discovered to play a role in cytoskeletal organisation. In particular EF-1 alpha functions in microtubule severing (Shiina et al., 1994). Translation of the protein is also selectively activated upon mitogenic stimulation demonstrating regulation of protein expression (Jefferies and Thomas, 1994). The observation that EF-1 alpha plays a key role in both protein synthesis and cytoskeletal organisation, led to further studies as to whether EF-1 alpha expression levels could affect apoptosis levels. Apoptosis was induced in cells by serum deprivation. Addition of antisense EF-1 alpha to these cells led to a significant protection from apoptotic cell death (Duttaroy et al., 1998). In addition overexpression of EF-1 alpha increased the rate of cell death. These observations suggest that EF-1 alpha levels could be a global regulator modulating the rate of apoptosis. Increased expression of EF-1 alpha leads to an increased protein synthesis rate, which would include specific pro-apoptotic proteins and also could play a role in the cytoskeletal changes associated with apoptosis. Support for this model came from a study which demonstrated up-regulation of EF-1 alpha



by p53 in murine cells (Kato, 1999). p53 is known to play a role in a number of apoptotic pathways. These studies suggesting a role for EF-1 alpha in regulating apoptosis are particularly interesting in light of the hMLH1-EF-1 alpha interaction seen in the yeast two-hybrid system. hMLH1 has been implicated in mediating sensitivity to a range of anti-cancer agents such as cisplatin (Aebi et al., 1996; Anthony et al., 1996). Loss of hMLH1 in cells is associated with the emergence of resistance to treatment with such drugs, suggesting hMLH1 somehow signals apoptosis in response to treatment. This interaction therefore could possibly represent one mechanism through which hMLH1 exerts this effect.

hMLH1 was also demonstrated to interact with EPAS1 and RIGUI in the yeast two-hybrid system. Endothelial PAS-1 (EPAS1) protein is a basic helix-loop-helix and PAS domain transcription factor. This protein shares 48% sequence identity with hypoxia inducible factor (HIF-1 $\alpha$ ) (Tian et al., 1997). EPAS1 can also, like HIF-1 $\alpha$ , activate transcription of VEGF through the HIF-1 $\alpha$  binding site (Maemura et al., 1999). Activation of both proteins is stimulated by hypoxic conditions. The acronym PAS derives from the first three members of the family: the period gene (*PER*), the arylhydrocarbon receptor nuclear translocator (*ARNT*) gene and the *single minded* gene. RIGUI is a putative mammalian orthologue of the *Drosophila PER* gene (Sun et al., 1997). This protein also has a basic helix-loop-helix and a PAS domain. However, in both cases, the C-terminal region of the two proteins involved in the interaction with hMLH1 does not include these domains. EPAS1 has a C-terminal transactivation domain, which does not seem to effect its use in the yeast two-hybrid system as the construct expressing this protein did not autonomously activate *LACZ* expression alone or when co-transformed into yeast with a lamin expressing construct. Within this domain a number of di-leucine repeats have been identified, and such repeats are important for the interaction of co-activators such as p300 with transcription factors (O'Rourke et al., 1999). p300 has been shown to bind this domain in HIF-1. The identification of the hMLH1 interaction with the C-terminal leucine zipper domain of c-MYC would suggest that perhaps hMLH1 could also interact with EPAS1 through this leucine rich region and perhaps block binding of p300 and effect the transactivation function of this protein.

The observed interactions between hMLH1 and three known binding partners hPMS1, hMLH3 and MED1 act as excellent internal controls for the binding function of the hybrid hMLH1 protein (Bellacosa et al., 1999; Leung et al., 2000; Lipkin et al., 2000; Raschle et al., 1999). The constructs isolated expressing hMLH3 and hPMS1 contain the C-terminal regions of these proteins previously shown to be required for this interaction with hMLH1.



Overall a range of proteins have been identified as interacting with hMLH1 in the yeast two-hybrid system. These include known binding partners as well as novel interacting proteins. The identification of proteins that could play a role in signalling from DNA damage, via hMLH1, to downstream cellular response pathways was the main aim of this project. Therefore the identification of an interaction with c-MYC, a protein known to have a number of functions including signalling apoptosis, was intriguing. However some of the other interactions identified were also particularly interesting. One protein, EF-1 alpha was identified in both screens. In the second yeast two-hybrid screen numerous clones were also identified as interacting with hMLH1. Therefore this EF-1 alpha-hMLH1 interaction represents another potentially interesting study. The recent identification of a link between EF-1 alpha expression and apoptosis also signals the possible role of this interaction in damage response pathways.



#### 4.4 c-MYC Interacts with hMLH1

From the first yeast two-hybrid cDNA library screen an interaction between hMLH1 and c-MYC was clearly demonstrated. c-MYC is a protein with diverse roles, a putative transcription factor which plays a role in cell growth control, regulation of the cell cycle, differentiation, and apoptosis. The aim of the screen was to identify possible pathways through which MMR via hMLH1, signals downstream cellular responses after DNA damage occurs. The identification of a hMLH1-c-MYC interaction could represent a specific route through which hMLH1 signals from DNA damage to various response pathways, such as cell death, in response to treatment with DNA-damaging agents. The hMLH1-c-MYC interaction observed was an exciting observation but needed to be confirmed through additional independent methods.

The pACT2 (GAL4 AD plasmid) library construct expressing c-MYC encodes the C-terminal half of the protein. c-MYC consists of 439 amino acids, amino acids 245-stop codon are expressed from this pACT2 library plasmid. This C-terminal half of the protein encodes the basic helix-loop-helix (bHLH) leucine zipper domains. These domains are known to be required for the biological activity of c-MYC. The leucine zipper domain of the protein facilitates protein-protein interactions and is a domain seen in a range of other proteins such as FOS and JUN. The bHLH domain is required for binding DNA. An essential function of c-MYC is to form a complex with MAX, which binds a conserved DNA recognition sequence and is proposed to activate the transcription of certain genes. Confirmation of the specificity of the hMLH1-c-MYC interaction within this system was facilitated through using c-MYC mutants and testing whether they could still interact with hMLH1.

Mutants of avian c-MYC were available from Dr. D. Crouch (University of Dundee) and were used in the yeast two-hybrid system. The region of c-MYC involved in the interaction with hMLH1 is highly conserved from chicken to human cells. Yeast two-hybrid pACT-2 constructs were made expressing the same C-terminal region of avian c-MYC, v-MYC and also mutants of avian c-MYC. These constructs were all tested for autonomous activation of the yeast two-hybrid reporter gene and none were positive. Therefore the constructs were tested for an interaction with hMLH1.

Both avian c-MYC and v-MYC interacted with hMLH1 in the yeast two-hybrid system. Mutations in both the basic region and the two helices of the HLH domain of avian c-MYC



had no effect on the interaction with hMLH1. However deletion of the C-terminal 10 and 28 amino acids of avian c-MYC led to the loss of an interaction between avian c-MYC and hMLH1. This region of c-MYC encodes the leucine zipper domain of the protein, a domain previously identified as being crucial for protein-protein interactions. The original human c-MYC construct from the library screen was shown to specifically interact with hMLH1 F4, the same domain of hMLH1 required for an interaction with hPMS2. The avian c-MYC and v-MYC constructs were also tested for an interaction with hMLH1 F4 and showed the same pattern of interactions as with full length hMLH1.

From these experiments the critical region of c-MYC for the interaction with hMLH1 is shown to be the very C-terminal sequence encompassing the leucine zipper domain. The use of mutants of c-MYC in the yeast two-hybrid system demonstrating this specific region of the protein required for the interaction with hMLH1 lends further support for this being a functionally relevant interaction. Use of specific mutants of c-MYC in these assays eliminates the possibility of this hMLH1-c-MYC being a non-specific interaction due to charge. However alternative assays should be used to demonstrate this interaction independently. Therefore co-immunoprecipitation assays were utilised to confirm this interaction.

These co-immunoprecipitation assays utilise cell extracts and specific polyclonal antibodies against the proteins of interest. Saturating amounts of the antibodies were incubated with cell extract and the immunoprecipitates collected on sepharose beads. From these assays hMLH1 was clearly shown to co-immunoprecipitate with c-MYC polyclonal antibody. hMLH1 did not co-immunoprecipitate with the non-specific rabbit pre-immune serum. Unfortunately the monoclonal c-MYC antibodies available are not very effective and the reciprocal detection of c-MYC co-immunoprecipitating with the hMLH1 polyclonal antibody proved more difficult. Avian cell extracts (from MC29 cells) were prepared, which have elevated c-MYC expression, and used in these assays. In these experiments hMLH1 polyclonal antibody was shown to be able to co-immunoprecipitate both avian MLH1 and avian c-MYC. Again these associations are specific, as these proteins did not co-immunoprecipitate with pre-immune serum. This demonstration of co-immunoprecipitation of the two proteins provides additional evidence for an interaction between c-MYC and hMLH1.

Finally, further confirmation for an interaction between hMLH1 and c-MYC came from the GST pull down assays done in collaboration with Dr. E. Homer. In these assays hMLH1



full length and F1-F4 (corresponding to the fragments of hMLH1 used in the yeast two-hybrid system) were expressed as fusion proteins with GST. MAX, a known binding partner of c-MYC, was also expressed as a GST fusion protein. In a similar manner to the co-immunoprecipitation protocol the GST fusion proteins were incubated with cell extract and the proteins bound to these GST fusion proteins examined by western blot. From these assays, as in the co-immunoprecipitation assays, hMLH1 was clearly demonstrated to bind c-MYC. c-MYC is shown in these assays to bind GST-MAX, GST-hMLH1 and hMLH1-F4 but not GST alone. The demonstration of endogenous c-MYC being pulled down with F4 of hMLH1 as well as full length hMLH1 supports the observations from the yeast two-hybrid system.

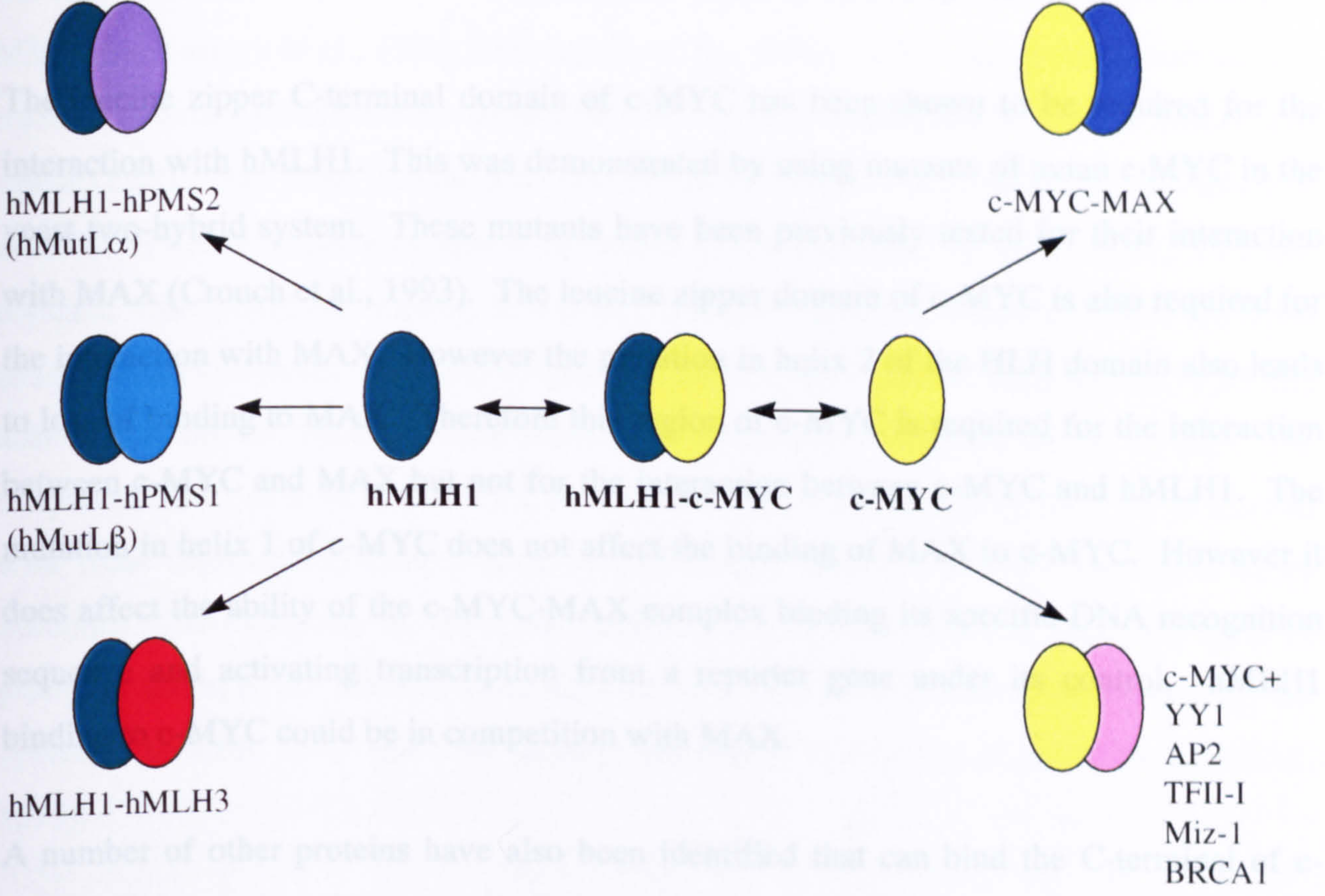
In summary, the observed interaction between hMLH1 and c-MYC in the yeast two hybrid system has been confirmed by demonstrating this interaction in a number of other independent systems. The specific domains on the two proteins required for this interaction have been elucidated using the yeast two-hybrid system. Co-immunoprecipitation assays and GST pull down assays have both demonstrated a specific interaction between hMLH1 and c-MYC under the experimental conditions used in these assays.

Preliminary studies have also demonstrated an interaction between hMSH2 and MAX (pers. comm. Dr. E. Homer). This interaction between hMSH2 and MAX was shown using GST pull down assays and further verified using co-immunoprecipitation assays. These observations further substantiate the potential importance of the c-MYC-hMLH1 interaction, as this known c-MYC binding partner MAX binds a second MMR protein hMSH2.

Within the yeast two hybrid system and also from the GST pull down assays the interaction region on hMLH1 for binding c-MYC has been localised to hMLH1 F4, the C-terminal fragment of hMLH1 (amino acids 514-756). This C-terminal fragment of hMLH1 also contains the binding domain for hPMS2. Therefore this interaction between hMLH1 and c-MYC could be competing with this binding of hMLH1 to hPMS2. Interactions between hMLH1 and other MutL homologues, hMLH3 and hPMS1 recently discovered, are also mediated through the C-terminal of hMLH1 (Leung et al., 2000; Lipkin et al., 2000; Raschle et al., 1999). c-MYC could be competing with these other proteins for binding to hMLH1 (see Figure 21). It will therefore be of interest to define more specifically the



interaction domain on hMLH1. The c-MYC clone pulled out of the yeast two hybrid screen that interacts with hMLH1 does not interact with hPMS2 P3 or P4. These results suggest that although the C-terminal of the MutL homologues are relatively homologous, the interaction of the C-terminal half of c-MYC with hMLH1 is specific.



**Figure 21: hMLH1 and c-MYC Protein-Protein Interactions.**

hMLH1 binds to three other MutL homologues, hPMS2, hPMS1 and hMLH3. Complex formation is through the C-terminal half of the protein, the same region involved in the interaction of hMLH1 with c-MYC. c-MYC interacts with its major binding partner MAX through its C-terminal half. Interaction between c-MYC and a range of other proteins (listed) have also been identified which are also mediated through the C-terminal of the protein.

This has led to the proposal that c-MYC somehow activates the repressing function of this POZ domain and inhibits the normal transactivation function of Miz-1. The interaction of Miz-1 with the HLH domain of c-MYC would support this model, as it has been shown that it is through this domain that c-MYC as part of a heterodimer (c-MYC-MAX) binds DNA. BRCA1 when overexpressed can inhibit the transactivation function of c-MYC and also c-MYC/MAX co-transformation, again demonstrating the importance of the HLH domain of c-MYC and the interaction with MAX for its different roles (Wang et al., 1998). However the full implications of this interaction with BRCA1 are yet to be elucidated.



interaction domain on hMLH1. The c-MYC clone pulled out of the yeast two-hybrid screen that interacts with hMLH1 does not interact with hPMS2 F3 or F4. This would suggest that although the C-termini of the MutL homologues are relatively homologous, the interaction of the C-terminal half of c-MYC with hMLH1 is specific.

The leucine zipper C-terminal domain of c-MYC has been shown to be required for the interaction with hMLH1. This was demonstrated by using mutants of avian c-MYC in the yeast two-hybrid system. These mutants have been previously tested for their interaction with MAX (Crouch et al., 1993). The leucine zipper domain of c-MYC is also required for the interaction with MAX. However the mutation in helix 2 of the HLH domain also leads to loss of binding to MAX. Therefore this region of c-MYC is required for the interaction between c-MYC and MAX but not for the interaction between c-MYC and hMLH1. The mutation in helix 1 of c-MYC does not affect the binding of MAX to c-MYC. However it does affect the ability of the c-MYC-MAX complex binding its specific DNA recognition sequence and activating transcription from a reporter gene under its control. hMLH1 binding to c-MYC could be in competition with MAX.

A number of other proteins have also been identified that can bind the C-terminal of c-MYC. These include YY1, TFII-I, BRCA1, MIZ-1 and AP2. Both MIZ-1 and BRCA1 specifically bind the HLH domain of c-MYC and do not require the leucine zipper domain of this protein for binding (Peukert et al., 1997; Wang et al., 1998). MIZ-1 is known to bind the Inr element of a number of genes including cyclin D1, activating transcription of these genes (Peukert et al., 1997). Overexpression of the MIZ-1 protein leads to growth arrest. However co-expression of c-MYC leads to inhibition of this arrest and is dependent on formation of the MIZ-1-c-MYC heterodimer and also on a functional POZ domain in MIZ-1. This has led to the proposal that c-MYC somehow activates the repression function of this POZ domain and inhibits the normal transactivation function of MIZ-1. The interaction of MIZ1 with the HLH domain of c-MYC would support this model, as it has been shown that it is through this domain that c-MYC as part of a heterodimer (c-MYC-MAX) binds DNA. BRCA1 when overexpressed can inhibit the transactivation function of c-MYC and also c-MYC/RAS co-transformation, again demonstrating the importance of the HLH domain of c-MYC and the interaction with MAX for its different roles (Wang et al., 1998). However the full implications of this interaction with BRCA1 are yet to be elucidated.



The region of c-MYC required for interaction with both YY1 and TFII-I has also been examined (Roy et al., 1993; Shrivastava et al., 1993). Although these studies were not conclusive these interactions are unlikely to require the leucine zipper domain but instead the HLH domain of c-MYC. YY1 can bind c-MYC to the exclusion of MAX binding c-MYC (Shrivastava et al., 1993; Shrivastava et al., 1996). YY1 acts to both activate and repress transcription and also is found to be involved in transcription initiation. The *in vivo* interaction between these two proteins occurs mostly in cells that express elevated levels of c-MYC. These two proteins appear to have a reciprocal effect on each other. YY1 can compete with MAX for binding to c-MYC, but has also been found to affect the TAD (transactivation domain) of c-MYC although not through a direct interaction. YY1 can also inhibit the co-transformation activity of c-MYC/RAS (Austen et al., 1998). The reciprocal effect of c-MYC on YY1, acts to interfere with the ability of YY1 to interact with basal transcription factors such as TBP and/or TFII-B. c-MYC can again block initiation of transcription by the transcription factor TFII-I which can bind two types of promoter element, an initiator (Inr) element and also an E-box site, by preventing co-operation between TFII-I and TBP through binding to TFII-I (Roy et al., 1993; Roy et al., 1993).

AP2 also interacts with the C-terminal of c-MYC but the specific domains of c-MYC involved in this interaction have not been elucidated (Gaubatz et al., 1995). It was demonstrated that binding of c-MYC by AP2 does not preclude binding of MAX to c-MYC. However AP2 binding of c-MYC does impair DNA binding of this complex to DNA.

hMLH1 is therefore also competing with these proteins for binding to c-MYC. Is it possible that like some of these other proteins, hMLH1 through its interaction with c-MYC can effect this proteins functions, such as its ability to activate transcription of certain genes or its co-transformation activity with activated RAS. c-MYC through its interaction with hMLH1 could also exert an effect on the functions of this protein in MMR or its role in signalling downstream cellular responses after treatment with a range of DNA-damaging agents. These two proteins clearly interact, the next challenge therefore is elucidating the functional role of this interaction.



## 4.5 Elucidating the Functional Significance of the hMLH1-c-MYC Interaction

The interaction between hMLH1 and c-MYC has been demonstrated utilising a number of independent assay systems. hMLH1 requires the leucine zipper domain of c-MYC for the formation of a hMLH1-c-MYC complex. This was demonstrated using mutants of avian c-MYC in the yeast two-hybrid system. The leucine zipper domain has been shown to be required for the transforming ability of c-MYC, specifically through its interaction with MAX. This region of c-MYC was also previously shown to be required for the c-MYC-MAX interaction, but unlike the hMLH1 interaction, MAX also requires helix 2 of the HLH domain of c-MYC for complex formation (Crouch et al., 1993).

Binding to MAX is required for both the oncogenic and transactivation functions of the c-MYC protein (Amati et al., 1993; Amati et al., 1992). As described earlier c-MYC can co-operate with activated RAS leading to cellular transformation. The identification of an N-terminal transactivation domain and also a C-terminal bHLH-Zip domain (which is commonly found in a number of transcription factors) in the c-MYC protein also suggested a role for this protein in transcriptional control. c-MYC through an interaction with MAX can bind a consensus DNA recognition binding sequence and activate transcription of a reporter gene in a number of *in vivo* reporter gene expression systems (Amati et al., 1992; Kretzner et al., 1992). The interaction of c-MYC-MAX with TRRAP and the subsequent recruitment of the histone acetyltransferase GCN5 provided a direct link between c-MYC and modes of transcription activation (McMahon et al., 1998; McMahon et al., 2000). c-MYC has also been shown to repress transcription of certain genes through its interactions with proteins such as YY1 and TFII-I.

MAX is a protein that is central to two opposing networks of protein-protein interactions. MAX can bind other proteins such as MAD and MXI1 (Ayer et al., 1993; Zervos et al., 1993). These complexes can compete with the c-MYC-MAX heterodimer for binding to the E-box recognition sequences and can inhibit both the transactivation and oncogenic potential of c-MYC (Ayer et al., 1993). Could hMLH1 be in competition with MAX for binding to c-MYC? If this were the case, loss of hMLH1 would lead to loss of this competition and therefore increased formation of c-MYC-MAX heterodimer through which c-MYC exerts many of its functions. For example if in the absence of hMLH1, such an increase in c-MYC-MAX complex formation did result, this could have an effect on the balance of the proposed dual control that c-MYC has on the cell, i.e. proliferation versus



cell death. Loss of hMLH1 is associated with loss of sensitivity in response to DNA damaging agents. Therefore one possible model for the function of this hMLH1-c-MYC interaction is that it acts to mediate the response from DNA damage recognition to downstream effector pathways.

To address whether the association of hMLH1 with c-MYC can preclude binding of c-MYC to MAX, electrophoretic mobility shift DNA binding assays (EMSA) were utilised. c-MYC and MAX proteins dimerise and bind DNA through their basic-helix-loop-helix-leucine zipper motifs (b-HLH-LZ) (Blackwood and Eisenman, 1991; Blackwood et al., 1992). c-MYC-MAX heterodimer binds with highest affinity to the consensus sequence CACG/ATG, the E-box sequence (Prendergast and Ziff, 1991). c-MYC-MAX and MAX-MAX complexes can form and bind to this E-box DNA core sequence. However c-MYC alone does not bind this sequence. Electrophoretic mobility shift DNA binding assays were carried out with purified GST-MYC, HIS-MAX and GST-MLH1. From these assays using a wide range of GST-hMLH1 concentrations, hMLH1 did not have an effect on c-MYC-MAX binding to its recognition E-box sequence. Furthermore, no ternary complex formation was observed, supporting the observed overlapping nature of the regions of interaction on c-MYC for these two proteins.

hMLH1 in these bandshift assays could not inhibit the formation of c-MYC-MAX complexes or the binding of these complexes to their recognition DNA binding sequence, the E-box recognition sequence. Under these experimental conditions therefore it is concluded that the affinity of hMLH1 for c-MYC is not high enough to disrupt a c-MYC-MAX complex. Using purified proteins not expressed as fusions could prove more useful in this type of experiment. Interpretation of the results would not be complicated by the possible distortions in protein structure introduced through the fusion protein. This would require expression and purification of functional full length c-MYC. Alternatively, to determine whether hMLH1 precludes binding of c-MYC to MAX, GST-hMLH1 could be incubated with purified c-MYC, c-MYC and MAX and MAX alone and subsequently test which proteins co-immunoprecipitated with hMLH1.

Although a negative result in this assay is somewhat difficult to interpret it is unlikely that hMLH1 can actively compete with MAX for binding to c-MYC in a similar way to, for example to the competition for binding to MAX by c-MYC and the other MAX binding partners such as MAD. It has been previously reported that much of the c-MYC expressed in the cell is in complex with MAX (Blackwood et al., 1992). hMLH1 also forms



complexes with a number of proteins specifically involved in MMR, the MutL homologues, hPMS2, hPMS1 and hMLH3 (Leung et al., 2000; Li and Modrich, 1995; Lipkin et al., 2000; Raschle et al., 1999). Another interaction between hMLH1 and a novel mismatch specific T/U DNA glycosylase MED1 has also been described (Bellacosa et al., 1999).

It is possible therefore that the interaction between hMLH1 and c-MYC acts at another specific junction in cellular activity. For example hMLH1 is specifically involved in MMR. However following exposure of cells to DNA-damaging agents this protein is clearly involved in signalling from the DNA damage site to downstream cellular responses. Is it possible that this pathway through hMLH1 co-operates with a second signal transduction pathway acting via c-MYC which together, through the hMLH1-c-MYC interaction, signal a single response element such as cell cycle arrest or apoptosis.

#### **4.5.1 Could Elevated c-MYC Expression Ablate hMLH1 Function?**

The proto-oncogene *c-MYC* was first discovered through its homology with the transforming gene, *v-MYC*, of avian myelocytomatosis virus, MC29 (Vennstrom et al., 1982). The *v-MYC* retroviral gene causes the development of a variety of tumours in infected chickens, supporting the idea that *c-MYC* would have oncogenic activity in human cells (Bishop, 1982). Subsequently the *c-MYC* oncogene has been found to be activated in many animal and human tumours (Nesbit et al., 1999). The *c-Myc*, *L-Myc* and *N-Myc* family members have each been implicated in the onset of human neoplasia.

Activation of human *c-MYC* genes occurs through several mechanisms leading to the onset of cancer. An early observation of a link between *c-MYC* and cancer was the observed translocation of *c-MYC* on chromosome 8 so that it is juxtaposed with the regulatory elements of one of three immunoglobulin genes on chromosome 2, 14 or 22 in Burkitt's lymphoma (BL) (Cole, 1986; Nesbit et al., 1999). This leads to the activation of *c-MYC* and subsequent onset of the BL lymphoid malignancy. Elevated expression of the *c-MYC* gene has been associated with a number of cancers, including breast, lung, prostate, gastrointestinal, some melanomas and myeloid leukaemia. How this amplification of c-MYC expression arises and its links to disease progression are yet to be clearly elucidated.

The coincidence of elevated c-MYC expression and oncogenesis is clearly a common occurrence. Is it possible that part of the oncogenic effect of this elevated c-MYC



expression acts through inhibition of functional MMR, perhaps through sequestration of hMLH1? Under normal cellular conditions this interaction between hMLH1 and c-MYC may have a specific role in mediating cellular responses to DNA damage which are ablated in transformed cells. To address the possibility that elevated c-MYC expression can act to impair functional MMR, the Rat-1 c-MYCER<sup>TM</sup> system was utilised. As described earlier this system allows inducible expression of c-MYC. Therefore the effect of normal expression versus elevated c-MYC expression on MMR can be examined.

The first experiments carried out to address the possible effect of elevated c-MYC on MMR utilising this system were bandshift experiments with a mismatched oligonucleotide. Mismatch repair protein complexes can specifically bind heteroduplex DNA for example in the form of a GT mismatch within a short oligonucleotide sequence (Branch et al., 1993; Jiricny et al., 1988). Recognition of the mismatch and initial binding is mediated through the MutS proteins (Fishel et al., 1994; Miret et al., 1993; Modrich, 1989; Su and Modrich, 1986). MutL proteins have also been shown to be part of this complex (Grilley et al., 1989; Matton et al., 2000; Prolla et al., 1994). Formation of a mismatch specific retardation complex was shown to be reduced in the hMLH1 deficient cell line A2780/cp70 compared to the mismatch repair, hMLH1 proficient A2780 parental cell line (Anthony et al., 1996). Therefore in the presence of elevated c-MYC expression, if c-MYC can sequester hMLH1 and effect its function in MMR, a reduction in binding of a mismatch specific protein complex may be observed.

Experiments were carried out with nuclear extracts from Rat-1 c-MYCER<sup>TM</sup> cells without elevated c-MYC expression and at time points ranging between 2 and 24 hours after treatment with 4-hydroxy tamoxifen (4-OHT). These extracts were incubated with a <sup>32</sup>P end labelled 34 bp oligonucleotide with a GC base pair or a GT mismatch at position 16. In extracts from both A2780 and Rat-1 c-MYCER<sup>TM</sup> (without activation of c-MYC expression by 4-OHT) a mismatch specific protein-DNA complex was observed in the gel shift assays. In this study the elevated c-MYC expression in extracts made from Rat-1 c-MYCER<sup>TM</sup> cells at 2, 4, 8 and 24 hours after treatment with 4-OHT did not effect the formation of this mismatch specific complex. Any differences in intensity of the mismatch specific bands were found to be due to extract quality, as shown by performing bandshift experiments with these extracts using an oligonucleotide with an SP1 binding site.

Therefore in this system under these experimental conditions the increased expression of c-MYC does not effect the formation of a mismatch specific protein complex on the



heteroduplex DNA. c-MYC may require other co-factors to exert any effect on the formation of this complex. However the possible effect exerted by c-MYC on MMR through binding to hMLH1 may not act at this step of the repair process. The MutL protein in bacteria is known to be involved in generating a signal between mismatch recognition and other components of the repair pathway in particular recruitment of the MutH endonuclease (Hall et al., 1998; Hall and Matson, 1999). hMLH1 has also been demonstrated to play a crucial role in signalling downstream cellular responses in response to DNA damaging agents for example cisplatin leading to the activation of c-ABL, JNK and p73 (Duckett et al., 1999; Gong et al., 1999; Nehme et al., 1997). The effect of overexpression of c-MYC on hMLH1 and MMR may therefore materialise at a later point in this process, when perhaps hMLH1 is available to bind and is no longer in complex with the MutS proteins. The actual effect of this proposed sequestration of hMLH1 by c-MYC may be more easily measured using alternative systems.

Utilising the MYCER system to induce expression of c-Myc in cells, it has been shown that elevated c-MYC levels can result in genomic instability (Felsher and Bishop, 1999; Mai et al., 1996). Transient induction of elevated *c-MYC* gene expression led to a significant increase in the tumorigenicity of Rat1a cells as measured in xenograft experiments (Felsher and Bishop, 1999). This effect was seen even in cells 30 days after the return of normal c-MYC levels. Genomic instability, including karyotypic anomalies and gene amplification, was seen upon elevated c-MYC expression in this system. This instability may be the result of c-MYC elevated expression leading to an accelerated passage of the cells through G<sub>1</sub> and only arresting at G<sub>2</sub>. This study has led to the proposal that elevated c-MYC expression may contribute to a neoplastic phenotype through a dominant mutator effect (Felsher and Bishop, 1999).

It was decided to test whether this effect of overexpressing c-MYC in the Rat-1 c-MYCER<sup>TM</sup> cell system could induce a mutator phenotype. More specifically, testing whether overexpression of c-MYC could induce a mutator phenotype which can be directly linked to MMR inhibition. Mutation rates at a selectable gene, *HPRT*, were measured in response to treatment with 6-thioguanine. These cells were grown up from single colonies in the presence or absence of 4-hydroxy tamoxifen i.e. the presence or absence of elevated c-MYC expression. Cells were subsequently plated out at a high density in the presence of the anti-metabolite 6-thioguanine. Fluctuation analysis was carried out by the Luria-Delbruck method and the mutation rates calculated for the untreated cells and the cells treated with 4-OHT and hence with elevated expression of c-MYC (Delbruck, 1943). Cells



with elevated c-MYC expression were clearly shown to possess a significantly higher mutation rate compared to those with normal c-MYC expression. Therefore elevated c-MYC expression in this system also induces a mutator phenotype.

Resistance to the chemotherapeutic agent 6-thioguanine (6-TG) arises due to mutations in the hypoxanthine-guanine phosphoribosyl transferase (*HPRT*) gene. Loss of mismatch repair is known to lead to an increased mutation rate at *HPRT* and consequently increased resistance to 6-TG (Bhattacharyya et al., 1994; Eshleman et al., 1995; Glaab et al., 1998). In an MMR deficient background acquired resistance to 6-thioguanine has been found to be associated with hotspot mutations in the *HPRT* gene (Bhattacharyya et al., 1994; Kat et al., 1993). In particular a specific frameshift hotspot at a run of six guanine residues in exon 3 of the *HPRT* gene, accounted for a high proportion of *HPRT* mutations in an MMR deficient cell line background.

To examine whether the mutator phenotype induced by overexpression of c-MYC can be specifically linked to inhibition of MMR, mutant colonies which grew on plates from cells grown both in the presence and the absence of elevated c-MYC expression (+/- 4-OHT) were picked and expanded for further examination. Genomic DNA was extracted from these mutant clones and tested for the presence of mutations at a specific run of six guanine (G) bases in exon 3 of the *HPRT* gene. Comparison of mutant clones from cells treated or not with 4-OHT and hence with or without elevated c-MYC expression showed differences with respect to mutations at this run of G residues. All 22 mutant clones from cells grown in the absence of 4-OHT had no frameshift mutations. However 4 out of the 25 mutant clones from cells treated with 4-OHT, i.e. with elevated c-MYC expression, had frameshift mutations at this run of six G bases. Although due to the small numbers involved, this number of mutants in the cells grown in the presence of 4-OHT is not significant, as measured by Fisher's Exact Test,  $p\text{-value}=0.071$  ( $p\text{-value}$  of less than 0.05 is taken as significant). However, these results do suggest the possibility that there may be a link between the mutator phenotype, induced through overexpression of c-MYC, and loss of MMR. Larger numbers of mutant clones would have to be examined to strengthen the power of the analysis.

An elevated mutation rate has previously been identified at this run of G residues to be associated with loss of MMR in particular loss of hMLH1 expression. The fact that elevated c-MYC expression leading to an increased mutation rate at this selectable gene, *HPRT*, also results in mutations in a number of mutant clones at this frameshift mutation



hotspot seen in hMLH1 deficient cells, suggests the possibility that the mutator phenotype seen in this assay could be due to inhibition of MMR. The relatively small number of mutants examined due to the fact that mutant numbers were small and also the difficulties encountered growing up mutant clones means that only 22 mutant colonies from cells not treated with 4-OHT (i.e. no elevated c-MYC expression) were examined and only 25 mutants from 4-OHT treated cells. Therefore the possibility remains that if a larger number of mutants had been available for examination this outcome may have differed. A significant number of the mutant clones from cells treated with 4-OHT, i.e. elevated c-MYC expression, with a frameshift mutation at this mutation hotspot similar to that seen in the hMLH1 deficient cell lines described earlier, may have been observed.

Overall this set of experiments testing the possibility that elevated c-MYC expression could lead to inhibition of MMR are somewhat inconclusive. In the case of the mismatch specific gel shift experiments, the contents of the mismatch specific protein-DNA retarded complex has not been fully defined. It is known that MutL and its eukaryotic homologues are capable of binding MutS (and homologues) bound to the mismatch and in fact can enhance the binding capacity of the MutS proteins to the mismatched DNA (Grilley et al., 1989; Habraken et al., 1997; Prolla et al., 1994). However these observations come from experiments utilising purified proteins, demonstrating recognition and binding of the mismatch by the MutS proteins followed by ternary complex formation with the MutL proteins. One inherent problem with these experiments is that they do not account for the possible additional factors involved in MMR that could also bind at this point of the repair pathway. Therefore although a mismatch specific retardation complex is clearly visible upon addition of nuclear cell extracts the actual components of this complex have not been defined. A recent study involving immunoprecipitation of proteins crosslinked specifically to heteroduplex DNA revealed the presence of both human MutS and MutL proteins bound in this complex (Matton et al., 2000).

Acknowledging the caveats of using purified proteins for such mismatch specific gel shift experiments, it is possible that a more definitive answer as to the effect of c-MYC on mismatch specific complex binding may be more forthcoming through experiments utilising purified proteins. However such experiments would be a considerable undertaking as the production of the various proteins especially c-MYC is not trivial. If such experiments were to be considered, the inclusion of MAX may also be useful as it is known that many functions of c-MYC are facilitated through the c-MYC-MAX complex. The recent discovery of an interaction between MAX and hMSH2 may also suggest that



the inclusion of hMSH2 in these experiments could also prove worthwhile. It has also not been shown that the binding of c-MYC to hMLH1 and MAX is mutually exclusive. Interestingly many recently defined protein-protein interactions have been found to be part of a bigger multi-protein complex, one example being the discovery of the BASC complex (Wang et al., 2000). BASC is a super complex of proteins associated with BRCA1, which is believed to be involved in the recognition and repair of DNA adducts. Interestingly both hMLH1 and human MutS proteins have been identified as components of this multi-protein complex. It is possible that the interaction between MMR proteins and c-MYC is representative of such a multi-protein complex.

The anti-cancer agent cisplatin (CDDP) is used to treat a range of cancers. The development of tumour resistance to treatment with platinum drug therapies is a primary reason why treatment fails. Aberrant oncogene expression, e.g. *c-MYC*, differentiates between malignant and normal cells. Since a number of studies have shown a correlation between de-regulated *c-MYC* gene expression and poor prognosis, it was thought possible that high levels of *c-MYC* expression could also play a role in the emergence of platinum drug resistance.

*In vitro* reporter gene assays where the reporter gene is under the control of the *c-MYC* promoter were used to demonstrate that in a number of cell lines treated with CDDP, *c-MYC* gene expression was stimulated (Eliopoulos et al., 1995). Using a solid transplantable tumour model in rats, *c-MYC* expression was examined after tumours were treated intravenously with cisplatin (Walker et al., 1996). The tumour cells that survived treatment demonstrated elevated *c-MYC* expression. Additional studies with both xenograft and cell line models from cancers with associated *c-MYC* oncogene amplification were carried out. From these it was shown that the treatment of cells with antisense *c-MYC* in combination with CDDP, was able to restore CDDP sensitivity to these CDDP resistant models (Citro et al., 1998; Leonetti et al., 1999; Mizutani et al., 1994; Van Waardenburg et al., 1997). This restoration of sensitivity through the synergistic effect of CDDP and antisense *c-MYC* specifically led to an increased apoptotic response (Leonetti et al., 1999; Van Waardenburg et al., 1997). One such study examined the effect of *c-MYC* expression levels on sensitivity to both CDDP and ionising radiation (IR). Elevated *c-MYC* expression led to increased CDDP resistance but had no effect on the sensitivity of cells to IR treatment. It has been proposed from this study that c-MYC acts either in a direct or indirect manner on a DNA repair pathway involved in recognising DNA damage induced by treatment with CDDP.



These observations suggest that it may be worthwhile extending the mismatch specific bandshift studies to similar studies utilising oligonucleotides which instead of a mismatched base pair carry the major CDDP induced adduct a 1,2-intrastrand d(GpG) crosslink. The human MutS $\alpha$  complex has been shown to recognise and bind such adducts as well as base pairs between O<sup>6</sup>-methylguanine and thymine or cytosine (Duckett et al., 1996). Loss of functional MMR leads to resistance to a range of DNA-damaging agents including CDDP (Aebi et al., 1996; Anthoney et al., 1996). Both hMSH2 and hMLH1 have been implicated in the signalling of downstream response pathways in response to treatment with CDDP (Gong et al., 1999; Nehme et al., 1997). One model would suggest that recognition and binding of CDDP induced adducts by the human MutS proteins and binding of these complexes by the human MutL proteins is vitally important for signalling a cellular response to the DNA damage induced by CDDP. The most likely candidate for the specific signalling function being the hMLH1 protein. Therefore the observed increased resistance to CDDP treatment in cells with elevated c-MYC presents the intriguing possibility that in these cells resistance could possibly be induced through the possible effect of elevated c-MYC expression on the MMR pathway.

#### **4.5.2 Model for the Role of a hMLH1-c-MYC Complex**

c-MYC is required for signalling both proliferation and apoptosis, two opposing pathways. A 'dual signal model' has been proposed to explain these paradoxical roles for the protein (Harrington et al., 1994; Prendergast, 1999). This model proposes that c-MYC acts to regulate both cell division and cell death. However these functions of c-MYC cannot be separated as distinct entities. Therefore this proposal has been re-modelled to suggest that c-MYC can co-ordinately prime both cell death and drive proliferation (Prendergast, 1999). Priming of cell death for example could involve the transcriptional regulation of c-MYC target genes. Triggering of cell death is proposed to subsequently act through an additional pathway.

An interaction between c-MYC and the BIN1 protein was recently identified (Elliott et al., 1999; Sakamuro et al., 1996). BIN1 is able to inhibit the transactivation properties of c-MYC-MAX on a number of artificial promoters examined *in vivo* and also on c-MYC endogenously activated promoters such as ODC (Elliott et al., 1999). These studies also demonstrated the ability of BIN1 to suppress c-MYC/RAS co-transformation. BIN1 is found on chromosome 2q14 which localises to a region often found deleted in metastatic prostate cancer (Sakamuro and Prendergast, 1999). Re-introduction of BIN1 into tumour



cell lines lacking the endogenous protein results in inhibition of cell growth which is mediated by an apoptotic cell death. Bin1 has been proposed to act as a 'cell fate adaptor protein' which acts to co-ordinate various pathways upon exit from the cell cycle (Prendergast, 1999; Sakamuro and Prendergast, 1999). An example of this role is the fact that Bin1 seems to play a role in mediating a c-MYC dependent apoptotic response (Prendergast, 1999). The interaction between c-MYC and BIN1 is required to trigger apoptosis but is not necessary to drive proliferation or transformation. The transition from 'priming' cell death to actually 'triggering' cell death by c-MYC could therefore involve for example, interactions between BIN1 and the c-MYC target gene products (Prendergast, 1999).

This 'dual control' exerted by c-MYC is intriguing with respect to the interaction of this protein with hMLH1. In some ways hMLH1 could be considered as a specific cell fate adaptor protein in response to certain types of DNA damage. DNA damage is recognised by the MMR system and where possible repaired. However upon treatment with DNA-damaging agents which cause major DNA adducts, the MMR system can recognise and bind such adducts but cannot repair them (Duckett et al., 1996). Therefore the MMR system through proteins such as hMLH1 are proposed to signal from the DNA damage site to downstream effector molecules which are involved in signal transduction pathways that lead to cell death through apoptosis (Duckett et al., 1999; Gong et al., 1999; Nehme et al., 1997). The interaction with c-MYC could be therefore potentially critical in the differentiation between successful adduct repair and hence continued cell cycle progression and proliferation compared to recognition of an adduct which cannot be repaired by MMR followed by signalling for an apoptotic response in response to the DNA-damaging agent in question.

The MMR system is able to recognise and bind the adducts that result from treatment with a number of DNA damaging agents (Duckett et al., 1996). A number of models have been proposed to suggest how MMR mediates sensitivity to treatment with these agents. One early model proposed to explain the methylation tolerance phenotype seen in the absence of functional MMR is the 'futile repair' model. This proposes that MMR recognises the DNA adduct produced, and triggers double strand breaks through futile attempts at repair. This model suggests that these double strand breaks are recognised by other damage sensors which signal the downstream response pathways. The observations that MMR is in fact involved in mediating sensitivity to a broad range of DNA-damaging agents led to the proposal that the MMR system may be directly involved in the signalling of downstream



pathways. Support for this model came from the observations that the up-regulation of expression of some downstream effectors, for example c-ABL, in response to treatment with cisplatin is MMR dependent. Some suggestions have been made that the mutation avoidance role of MMR and the role in signalling downstream pathways in response to DNA damaging agents may be separable. These have been proposed because of the observation that some alkylation tolerant cell lines have mild or undetectable mutator phenotypes (Hampson et al., 1997).

The MMR system has been clearly implicated in signalling downstream events in response to treatment with DNA-damaging agents such as the methylating agents MNU and MNNG or platinum drugs such as cisplatin. Treatment of MMR proficient and deficient cell lines with cisplatin have shown an MMR dependent activation of c-ABL and c-ABL induced stimulation of p73 in response to cisplatin (Nehme et al., 1997). Cisplatin also stimulates expression of p53 but this is independent of MMR status. Similar experiments have been carried out with MMR deficient and proficient cell lines treated with the methylating agents MNU and MNNG (Duckett et al., 1999). In these experiments phosphorylation of p53 at serine 15 and 392 in response to treatment with these drugs was shown to be dependent on functional MMR. Triggering of MMR dependent cell death pathways in response to treatment with such methylating agents has also been shown independent of functional p53. Cell death is observed in p53 deficient HeLa cells and also methylation tolerant cell lines defective in MMR have been isolated in this genetic background (Aquilina et al., 1997; Aquilina et al., 1995; Duckett et al., 1999). This p53 independent cell death pathway in response to treatment with methylating agents may also occur through c-ABL and p73, however this remains undetermined.

MMR through hMLH1 therefore signals more than one cell death signalling pathway, in response to DNA damage, after treatment with anti-cancer drugs. Drug resistance is a major problem in the treatment of cancer. Resistance to the drug cisplatin has been linked to both loss of functional MMR but also to elevated levels of c-MYC expression. c-MYC is an enigmatic protein with many cellular roles not all of which are fully understood. This protein was once described as a 'citadel of incomprehensibility', but over the past decade with further study the multiple roles which intricately cross different and apparently opposing pathways are beginning to be understood (Luscher and Eisenman, 1990). The observed interaction of hMLH1 with c-MYC in the studies described in this thesis pose the intriguing possibility that hMLH1 as part of the MMR system may act through this



interaction with c-MYC to trigger cellular responses after treatment with agents, like cisplatin, that are known to cause DNA damage.

A number of possibilities of how such a relationship could be mediated exist. This interaction may act as a decisive step for c-MYC function i.e. maintenance of cellular proliferation or apoptotic cell death. The 'dual control' model proposed to explain the opposing functions of c-MYC suggests that c-MYC can co-ordinately prime both cell death and drive proliferation. In response to suitable signals c-MYC is proposed to trigger apoptosis through downstream pathways. Therefore the interaction with hMLH1 may actively link the priming of cell death to the triggering of cell death. This would suggest that the interaction of these two proteins occurs specifically in response to treatment with DNA-damaging agents. Because c-MYC expression is known to be upregulated in response to treatment with cisplatin, this interaction may only therefore occur, or at least preferably occur when c-MYC expression is high. This model is especially plausible when one considers the relatively low levels of c-MYC expression in normal cells. hMLH1 may interact with c-MYC and directly trigger an apoptotic response through proteins such as BIN1. Alternatively this interaction may form part of the MMR dependent activation of the p53 or p73 dependent apoptotic pathways. The observation that in a number of human tumours c-MYC expression is elevated, and this is associated with resistance to cisplatin, would also have to be accounted for by such a model. A fine balance obviously exists between the various functions of the c-MYC protein. Upon treatment of cells with cisplatin, the increased level of c-MYC expression proposed here to act in concert with MMR to trigger cell death is different to the c-MYC activation seen in some cancer cells. This represents a transient effect versus a more permanent effect mediated through for example APC mutations in colon cancer cells which lead to loss of c-MYC regulation. Further studies will be needed to address the functional significance of this interaction and more specifically address the possible scenarios proposed above.



# Appendix



Appendix 1

Human MLH1 Details of the Sequence from the  
National Centre for Biotechnology Information

LOCUS	HSHMLHI	2503 bp	mRNA	PRI	31-MAR-1994
DEFINITION	Human DNA mismatch repair (hmlh1) mRNA, complete cds.				
ACCESSION	U07418				
VERSION	U07418.1 GI:466461				
KEYWORDS	.				
SOURCE	human.				
ORGANISM	Homo sapiens				
	Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi; Mammalia; Eutheria; Primates; Catarrhini; Hominidae; Homo.				
REFERENCE	1 (bases 1 to 2503)				
AUTHORS	Papadopoulos,N., Nicolaides,N.C., Wei,Y.F., Ruben,S.M., Carter,K.C., Rosen,C.A., Haseltine,W.H., Fleischmann,R.D., Fraser,C.M., Adams,M.D., Venter,J.C., Hamilton,S.R., Petersen,G.M., Watson,P., Lynch,H.T., Peltomaki,P., Mecklin,J.P., Chapelle,A.D., Kinzler,K.W. and Vogelstein,B.				
TITLE	Mutation of a mutL Homolog is Associated with Hereditary Colon Cancer				
JOURNAL	Science 263, 1625-1629 (1994)				
MEDLINE	94174309				
REFERENCE	2 (bases 1 to 2503)				
AUTHORS	Wei,Y.F.				
TITLE	Direct Submission				
JOURNAL	Submitted (04-MAR-1994) Ying-Fei Wei, Molecular Biology, Human Genome Sciences, Inc., 9620 Medical Center Drive, Rockville, MD 20850, USA				
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2281 ctgatctata caaagtcttt gagaggtgtt aaatatggtt atttatgcac tgtgggatgt
2341 gttcttcttt ctctgtattc cgatacaaag tgttgtatca aagtgtgata tacaagtgt
2401 accaacataa gtgttggtag cacttaagac ttatacttgc cttctgatag tattccttta
2461 tacacagtgg attgattata aataaataga tgtgtcttaa cat

```







R2		
721	AACTGATAGAAATTGGATGTGAGGATAAAACCCTAGCCTTCAAAATGAATGGTTACATAT -----+-----+-----+-----+-----+-----+-----+ L I E I G C E D K T L A F K M N G Y I S	780
781	CCAATGCAAACACTACTCAGTGAAGAAGTGCATCTTCTTACTCTTCATCAACCATCGTCTGG -----+-----+-----+-----+-----+-----+-----+ N A N Y S V K K C I F L L F I N H R L V	840
841	TAGAATCAACTTCCTTGAGAAAAGCCATAGAAACAGTGTATGCAGCCTATTTGCCCAAAA -----+-----+-----+-----+-----+-----+-----+ E S T S L R K A I E T V Y A A Y L P K N	900
901	ACACACACCCATTTCCTGTACCTCAGTTTAGAAATCAGTCCCCAGAATGTGGATGTTAATG -----+-----+-----+-----+-----+-----+-----+ T H P F L Y L S L E I S P Q N V D V N V	960
F3		
961	TGCACCCACAAAGCATGAAGTTCACCTCCTGCACGAGGAGAGCATCCTGGAGCGGGTGC -----+-----+-----+-----+-----+-----+-----+ H P T K H E V H F L H E E S I L E R V Q	1020
R2		
1021	AGCAGCACATCGAGAGCAAGCTCCTGGGCTCCAATTCCTCCAGGATGTACTTCACCCAGA -----+-----+-----+-----+-----+-----+-----+ Q H I E S K L L G S N S S R M Y F T Q T	1080
1081	CTTTGCTACCAGGACTTGCTGGCCCCCTCTGGGGAGATGGTTAAATCCACAACAAGTCTGA -----+-----+-----+-----+-----+-----+-----+ L L P G L A G P S G E M V K S T T S L T	1140
1141	CCTCGTCTTCTACTTCTGGAAGTAGTGATAAGGTCTATGCCCACCAGATGGTTCGTACAG -----+-----+-----+-----+-----+-----+-----+ S S S T S G S S D K V Y A H Q M V R T D	1200
1201	ATTCCCGGGAACAGAAGCTTGATGCATTTCTGCAGCCTCTGAGCAAACCCCTGTCCAGTC -----+-----+-----+-----+-----+-----+-----+ S R E Q K L D A F L Q P L S K P L S S Q	1260
1261	AGCCCCAGGCCATTGTCACAGAGGATAAGACAGATATTTCTAGTGGCAGGGCTAGGCAGC -----+-----+-----+-----+-----+-----+-----+ P Q A I V T E D K T D I S S G R A R Q Q	1320
1321	AAGATGAGGAGATGCTTGAACCTCCAGCCCCTGCTGAAGTGGCTGCCAAAAATCAGAGCT -----+-----+-----+-----+-----+-----+-----+ D E E M L E L P A P A E V A A K N Q S L	1380
1381	TGGAGGGGGATACAACAAAGGGGACTTCAGAAATGTCAGAGAAGAGAGGACCTACTTCCA -----+-----+-----+-----+-----+-----+-----+ E G D T T K G T S E M S E K R G P T S S	1440
1441	GCAACCCAGAAAGAGACATCGGGAAGATTCTGATGTGGAATGGTGGGAAGATGATTCCC -----+-----+-----+-----+-----+-----+-----+ N P R K R H R E D S D V E M V E D D S R	1500
1501	GAAAGGAAATGACTGCAGCTTGTACCCCCCGGAGAAGGATCATTAACCTCACTAGTGTTT -----+-----+-----+-----+-----+-----+-----+ K E M T A A C T P R R R I I N L T S V L	1560
F4		
1561	TGAGTCTCCAGGAAGAAATTAATGAGCAGGGACATGAGGTTCTCCGGGAGATGTTGCATA -----+-----+-----+-----+-----+-----+-----+ S L Q E E I N E Q G H E V L R E M L H N	1620



1621 ACCACTCCTTCGTGGGCTGTGTGAATCCTCAGTGGGCCTTGGCACAGCATCAAACCAAGT 1680  
-----+-----+-----+-----+-----+  
H S F V G C V N P Q W A L A Q H Q T K L

1681 TATACCTTCTCAACACCACCAAGCTTAGTGAAGAACTGTTCTACCAGATACTCATTTATG 1740  
-----+-----+-----+-----+-----+  
Y L L N T T K L S E E L F Y Q I L I Y D

1741 ATTTTGCCAATTTTGGTGTTCCTCAGGTTATCGGAGCCAGCACCGCTCTTTGACCTTGCCA 1800  
-----+-----+-----+-----+-----+  
F A N F G V L R L S E P A P L F D L A M

1801 TGCTTGCCTTAGATAGTCCAGAGAGTGGCTGGACAGAGGAAGATGGTCCCAAAGAAGGAC 1860  
-----+-----+-----+-----+-----+  
L A L D S P E S G W T E E D G P K E G L

1861 TTGCTGAATACATTGTTGAGTTTCTGAAGAAGAAGGCTGAGATGCTTGCAGACTATTTCT 1920  
-----+-----+-----+-----+-----+  
A E Y I V E F L K K K A E M L A D Y F S

1921 CTTTGGAATTGATGAGGAAGGGAACCTGATTGGATTACCCCTTCTGATTGACAACCTATG 1980  
-----+-----+-----+-----+-----+  
L E I D E E G N L I G L P L L I D N Y V

1981 TGCCCCCTTTGGAGGGACTGCCTATCTTCATTCTTCGACTAGCCACTGAGGTGAATTGGG 2040  
-----+-----+-----+-----+-----+  
P P L E G L P I F I L R L A T E V N W D

2041 ACGAAGAAAAGGAATGTTTTGAAAGCCTCAGTAAAGAATGCGCTATGTTCTATTCCATCC 2100  
-----+-----+-----+-----+-----+  
E E K E C F E S L S K E C A M F Y S I R

2101 GGAAGCAGTACATATCTGAGGAGTCGACCCTCTCAGGCCAGCAGAGTGAAGTGCCTGGCT 2160  
-----+-----+-----+-----+-----+  
K Q Y I S E E S T L S G Q Q S E V P G S

2161 CCATTCCAAACTCCTGGAAGTGGACTGTGGAACACATTGTCTATAAAGCCTTGCGCTCAC 2220  
-----+-----+-----+-----+-----+  
I P N S W K W T V E H I V Y K A L R S H

2221 ACATTCTGCCTCCTAAACATTTACAGAAGATGGAAATATCCTGCAGCTTGCTAACCTGC 2280  
-----+-----+-----+-----+-----+  
I L P P K H F T E D G N I L Q L A N L P

2281 CTGATCTATACAAAGTCTTTGAGAGGTGTAA  
-----+-----+-----+-----+  
D L Y K V F E R C \*

← R3

← R4



Appendix 3

Human PMS2, Details of the Sequence from the  
National Centre for Biotechnology Information

LOCUS	HSU14658	2697 bp	mRNA	PRI	12-OCT-1994
DEFINITION	Human DNA mismatch repair gene homologue (hPMS2) mRNA, complete cds.				
ACCESSION	U14658				
VERSION	U14658.1 GI:557469				
KEYWORDS	.				
SOURCE	human.				
ORGANISM	Homo sapiens Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi; Mammalia; Eutheria; Primates; Catarrhini; Hominidae; Homo.				
REFERENCE	1 (bases 1 to 2697)				
AUTHORS	Bronner,C.E., Baker,S.M., Morrison,P.T., Warren,G., Smith,L.G., Lescoe,M.K., Kane,M., Earibino,C., Lipford,J., Linblom,A., Tannergard,P., Bollag,R.J., Godwin,A.R., Ward,D.C., Nordenskjold,M., Fishel,R., Kolodner,R.D. and Liskay,R.M.				
TITLE	Mutation in the DNA mismatch repair gene homologue hPMS2 is associated with hereditary nonpolyposis colon cancer				
JOURNAL	Unpublished				
REFERENCE	2 (bases 1 to 2697)				
AUTHORS	Morrison,P.T.				
TITLE	Direct Submission				
JOURNAL	Submitted (14-SEP-1994) Paul T. Morrison, Molecular Biology Core Facility, Dana-Farber Cancer Institute, 44 Binney Street, Boston, MA 002115, USA				
FEATURES	Location/Qualifiers				
source	1..2697 /organism="Homo sapiens" /db_xref="taxon:9606"				
gene	3..2591 /gene="hPMS2"				
CDS	3..2591 /gene="hPMS2" /function="DNA mismatch repair" /note="similar to S. cerevisiae PMS1 Swiss-Prot Accession Number P14242, S. cerevisiae MLH1 GenBank Accession Number U07187, E. coli MUTL Swiss-Prot Accession Number P23367, Salmonella typhimurium MUTL Swiss-Prot Accession Number P14161, Streptococcus pneumoniae Swiss-Prot Accession Number P14160" /codon_start=1 /protein_id="AAA50390.1" /db_xref="GI:557470" /translation="MERAESSSTEPAKAIKPIDRKSVHQICSGQVVLSTAVKELVE NSLDAGATNIDLKLDYGVLDLIEVSDNGCGVEEENFEGLTLKHHTSKIQEFADLTQVE TFGFRGEALSSLCALSDVTISTCHASAKVGTRLMFDHNGKIIQKTPYPRPRGTTVSVQ QLFSTLPVRHKEFQRNIKKEYAKMVQVLHAYCIIISAGIRVSCTNQLGQGKRQPVVCTG GSPSIKENIGSVFGQKQLQSLIPFVQLPPSDSVCEEYGLSCSDALHNLFYISGFISQC THGVGRSSTDQRQFFFINRRPCDPAKVCRLVNEVYHMYNRHQYPFVVLNISVDSECVDI NVTDPDKRQILLQEEKLLLAVLKTSLIGMFDSDVNKLNVSQQPLLDVEGNLIKMHAAADL EKPMVEKQDQSPSLRTGEEKKDVSIISRLREAFSLRHTTENKPHSPKTPPERRSPLGQK RGMSSSTSGAISDKGVLRSQKEAVSSSHGPSDPTDRAEVEKDSGHGSTSVDSSEGFSI PDTGSHCSSEYAASSPGDRGSQEHVDSQEKAPETDDSFSDVDCHSNQEDTGCKFRVLP QPTNLATPNTKRFKKEEILSSSDICQKLVNTQDMSASQVDVAVKINKKVPLDFSMSS LAKRIKQLHHEAQQSEGEQNYRKFRAKICPGENQAAEDELNRKEISKTMFAEMEIIIGQF NLGFIITKLNEDIFIVDQHATDEKYNFEMLOQHTVLOGQRLIAPQTLNLTAVNEAVLI ENLEIFRKNGFDFVIDENAPVTERAKLISLPTSKNWTFGPQDVDELIFMLS DSPGVMC RPSRVKQMFASRACRKSVMIGTALNTSEMKKLITHMGEMDHPWNC PHGRPTMRHIANL GVISQN"				
BASE COUNT	797 a	588 c	653 g	659 t	
ORIGIN	1 ccatggagcg agctgagagc tcgagtacag aacctgctaa ggccatcaaa cctattgata 61 ggaagtcagt ccatcagatt tgctctgggc aggtggtact gagtctaagc actgcggtaa 121 aggagttagt agaaaacagt ctggatgctg gtgccactaa tattgatcta aagcttaagg 181 actatggagt ggatcttatt gaagtttcag acaatggatg tggggtagaa gaagaaaact 241 tcgaaggctt aactctgaaa catcacacat ctaagattca agagtttgcc gacctaac 301 aggttgaaac ttttggcttt cggggggaag ctctgagctc actttgtgca ctgagcgatg 361 tcaccatttc tacctgccac gcatcggcga aggttggaac tcgactgatg tttgatcaca 421 atgggaaaat tatccagaaa accccctacc cccgcccag agggaccaca gtcagcgtgc 481 agcagttatt ttccacacta cctgtgcgcc ataaggaatt tcaaaggaat attaagaagg 541 agtatgccaa aatggtccag gtcttacatg catactgtat catttcagca ggcattccgtg				



601 taagttgcac caatcagctt ggacaaggaa aacgacagcc tgtggtatgc acaggtggaa  
 661 gccccagcat aaaggaaaat atcggctctg tgtttgggca gaagcagttg caaagcctca  
 721 ttccttttgt tcagctgccc cctagtact cctgtgtga agagtacggt ttgagctggt  
 781 cggatgctct gcataatctt ttttacatct caggtttcat ttcacaatgc acgcatggag  
 841 ttggaaggag ttcaacagac agacagtttt tctttatcaa ccggcggcct tgtgacctag  
 901 caaaggtctg cagactcgtg aatgaggtct accacatgta taatcgacac cagtatccat  
 961 ttgttggtct taacatttct gttgattcag aatgcgttga tatcaatggt actccagata  
 1021 aaaggcaaataa ttgctacaa gaggaaaagc ttttggtggc agtttttaaag acctctttga  
 1081 taggaatggt tgatagtgat gtcaacaagc taaatgtcag tcagcagcca ctgctggatg  
 1141 ttgaaggtaa cttaataaaa atgcatgcag cggattttgga aaagcccatg gtagaaaagc  
 1201 aggatcaatc cccttcatta aggactggag aagaaaaaaa agacgtgtcc atttccagac  
 1261 tgcgagaggc cttttctctt cgtcacacaa cagagaacaa gcctcacagc ccaaagactc  
 1321 cagaaccaag aaggagccct ctaggacaga aaaggggtat gctgtcttct agcacttcag  
 1381 gtgccatctc tgacaaaggc gtcctgagat ctcagaaaga ggcagtgagt tccagtcacg  
 1441 gaccagtgga ccctacggac agagcggagg tggagaagga ctcggggcac ggcagcactt  
 1501 ccgtggattc tgaggggttc agcatcccag acacgggcag tctactgcagc agcgagtatg  
 1561 cggccagctc cccaggggac aggggctcgc aggaacatgt ggactctcag gagaaagcgc  
 1621 ctgaaactga cgactctttt tcagatgtgg actgccattc aaaccaggaa gataccggat  
 1681 gttaaatttc agttttgcct cagccaacta atctcgcaac cccaaacaca aagcgtttta  
 1741 aaaaagaaga aattctttcc agttctgaca tttgtcaaaa gttagtataat actcaggaca  
 1801 tgtcagcctc tcagggtgat gtagctgtga aaattaataa gaaagttgtg cccctggact  
 1861 tttctatgag ttcttttagct aaacgaataa agcagttaca tcatgaagca cagcaaagtg  
 1921 aaggggaaca gaattacagg aagtttaggg caaagatttg tcctggagaa aatcaagcag  
 1981 ccgaagatga actaagaaaa gagataagta aaacgatgtt tgcagaaatg gaaatcattg  
 2041 gtcagtttaa cctgggattt ataataacca aactgaatga ggatatcttc atagtggacc  
 2101 agcatgccac ggacgagaag tataacttcg agatgctgca gcagcacacc gtgctccagg  
 2161 ggcagaggct catagcacct cagactctca acttaactgc tgtaaatgaa gctgttctga  
 2221 tagaaaatct ggaaatattt agaaagaatg gctttgattt tgttatcgat gaaaatgctc  
 2281 cagtcactga aagggtctaa ctgatttcct tgccaactag taaaaactgg accttcggac  
 2341 cccaggacgt cgatgaactg atcttcatgc tgagcgacag ccctggggtc atgtgccggc  
 2401 cttcccagat caagcagatg tttgcctcca gagcctgccg gaagtcggtg atgattggga  
 2461 ctgctctcaa cacaagcgag atgaagaaac tgatcaccca catgggggag atggaccacc  
 2521 cctggaactg tccccatgga aggccaaacca tgagacacat cgccaacctg ggtgtcattt  
 2581 ctcagaactg accgtagtca ctgtatggaa taattggttt tatcgcatat ttttatgttt  
 2641 tgaaagacag agtcttcact aacctttttt gttttaaaat gaaacctgcg gccttcg



Appendix 4. Human PMS2 Nucleotide Sequence and Peptide Sequence with Primers used to make the yeast Two-Hybrid Constructs.

F1 →		
1	CCATGGAGCGAGCTGAGAGCTCGAGTACAGAACCTGCTAAGGCCATCAAACCTATTGATC -----+-----+-----+-----+-----+-----+-----+ M E R A E S S S T E P A K A I K P I D R	60
61	GGAAGTCAGTCCATCAGATTTGCTCTGGGCAGGTGGTACTGAGTCTAAGCACTGCCGTAA -----+-----+-----+-----+-----+-----+-----+ K S V H Q I C S G Q V V L S L S T A V K	120
121	AGGAGTTAGTAGAAAACAGTCTGGATGCTGGTGCCACTAATATTGATCTAAAGCTTAAGG -----+-----+-----+-----+-----+-----+-----+ E L V E N S L D A G A T N I D L K L K D	180
181	ACTATGGAGTGGATCTTATTGAAGTTTCAGACAATGGATGTGGGGTAGAAGAAGAAACT -----+-----+-----+-----+-----+-----+-----+ Y G V D L I E V S D N G C G V E E E N F	240
241	TCGAAGGCTTAACCTCTGAAACATCACACATCTAAGATTCAAGAGTTTGCCGACCTAACTC -----+-----+-----+-----+-----+-----+-----+ E G L T L K H H T S K I Q E F A D L T Q	300
301	AGGTTGAACTTTTGGCTTTCGGGGGAAGCTCTGAGCTCACTTTGTGCACTGAGCGATG -----+-----+-----+-----+-----+-----+-----+ V E T F G F R G E A L S S L C A L S D V	360
361	TCACCATTTCTACCTGCCACGCATCGGCCGAAGGTTGGAACCTCGACTGATGTTTGATCACA -----+-----+-----+-----+-----+-----+-----+ T I S T C H A S A K V G T R L M F D H N	420
421	ATGGGAAAATTATCCAGAAAACCCCTACCCCGCCCCAGAGGGACCACAGTCAGCGTGC -----+-----+-----+-----+-----+-----+-----+ G K I I Q K T P Y P R P R G T T V S V Q	480
481	AGCAGTTATTTTCCACACTACCTGTGCGCCATAAGGAATTTCAAAGGAATATTAAGAAGG -----+-----+-----+-----+-----+-----+-----+ Q L F S T L P V R H K E F Q R N I K K E	540
541	AGTATGCCAAAATGGTCCAGGTCTTACATGCATACTGTATCATTTTCAGCAGGCATCCGTG -----+-----+-----+-----+-----+-----+-----+ Y A K M V Q V L H A Y C I I S A G I R V	600
601	TAAGTTGCACCAATCAGCTTGGACAAGGAAAACGACAGCCTGTGGTATGCACAGGTGGAA -----+-----+-----+-----+-----+-----+-----+ S C T N Q L G Q G K R Q P V V C T G G S	660
661	GCCCCAGCATAAAGGAAAATATCGGCTCTGTGTTTGGGCAGAAGCAGTTGCAAAGCCTCA -----+-----+-----+-----+-----+-----+-----+ P S I K E N I G S V F G Q K Q L Q S L I	720
F2 → ←		
721	TTCCTTTTGTTTCAGCTGCCCCCTAGTGACTCCGTGTGTGAAGAGTACGGTTTGAGCTGTT -----+-----+-----+-----+-----+-----+-----+ P F V Q L P P S D S V C E E Y G L S C S	780



R1

781	CGGATGCTCTGCATAATCTTTTTTACATCTCAGGTTTCATTTACACAATGCACGCATGGAG	840
	D A L H N L F Y I S G F I S Q C T H G V	
841	TTGGAAGGAGTTCAACAGACAGACAGTTTTTCTTTATCAACCGGCGGCCTTGTGACCCAG	900
	G R S S T D R Q F F F I N R R P C D P A	
901	CAAAGGTCTGCAGACTCGTGAATGAGGTCTACCACATGTATAATCGACACCAGTATCCAT	960
	K V C R L V N E V Y H M Y N R H Q Y P F	
961	TTGTTGTTCTTAACATTTCTGTTGATTGAGAATGCGTTGATATCAATGTTACTCCAGATA	1020
	V V L N I S V D S E C V D I N V T P D K	
1021	AAAGGCAAATTTTGCTACAAGAGGAAAAGCTTTTGTGTCAGTTTAAAGACCTCTTTGA	1080
	R Q I L L Q E E K L L L A V L K T S L I	
1081	TAGGAATGTTTGATAGTGATGTCAACAAGCTAAATGTCAGTCAGCAGCCACTGCTGGATG	1140
	G M F D S D V N K L N V S Q Q P L L D V	
1141	TTGAAGGTAACCTTAATAAAAAATGCATGCAGCGGATTTGGAAAAGCCCATGGTAGAAAAGC	1200
	E G N L I K M H A A D L E K P M V E K Q	
1201	AGGATCAATCCCCTTCATTAAGGACTGGAGAAGAAAAAAGACGTGTCCATTTCCAGAC	1260
	D Q S P S L R T G E E K K D V S I S R L	

F3

1261	TGCGAGAGGCCTTTTCTCTTCGTCACACAACAGAGAACAAGCCTCACAGCCCAAAGACTC	1320
	R E A F S L R H T T E N K P H S P K T P	

R2

1321	CAGAACCAAGAAGGAGCCCTCTAGGACAGAAAAGGGGTATGCTGTCTTCTAGCACTTCAG	1380
	E P R R S P L G Q K R G M L S S S T S G	
1381	GTGCCATCTCTGACAAAGGCGTCCTGAGATCTCAGAAAGAGGCAGTGAGTTCCAGTCACG	1440
	A I S D K G V L R S Q K E A V S S S H G	
1441	GACCCAGTGACCCTACGGACAGAGCGGAGGTGGAGAAGGACTCGGGGCACGGCAGCACTT	1500
	P S D P T D R A E V E K D S G H G S T S	
1501	CCGTGGATTCTGAGGGGTTGAGCATCCCAGACACGGGCAGTCACTGCAGCAGCGAGTATG	1560
	V D S E G F S I P D T G S H C S S E Y A	
1561	CGGCCAGCTCCCCAGGGGACAGGGGCTCGCAGGAACATGTGGACTCTCAGGAGAAAGCGC	1620
	A S S P G D R G S Q E H V D S Q E K A P	
1621	CTGAAACTGACGACTCTTTTTTCAGATGTGGACTGCCATTCAAACCAGGAAGATACCGGAT	1680
	E T D D S F S D V D C H S N Q E D T G C	



1681 GTAAATTTTCGAGTTTTGCCTCAGCCAACTAATCTCGCAACCCCAAACACAAAGCGTTTTA 1740  
-----+-----+-----+-----+-----+-----+  
K F R V L P Q P T N L A T P N T K R F K

1741 AAAAAGAAGAAATTCTTTCCAGTTCTGACATTTGTCAAAAGTTAGTAAATACTCAGGACA 1800  
-----+-----+-----+-----+-----+-----+  
K E E I L S S S D I C Q K L V N T Q D M

1801 TGTCAGCCTCTCAGGTTGATGTAGCTGTGAAAATTAATAAGAAAGTTGTGCCCCCTGGACT 1860  
-----+-----+-----+-----+-----+-----+  
S A S Q V D V A V K I N K K V V P L D F

F4 →

1861 TTTCTATGAGTTCTTTAGCTAAACGAATAAAGCAGTTACATCATGAAGCACAGCAAAGTG 1920  
-----+-----+-----+-----+-----+-----+  
S M S S L A K R I K Q L H H E A Q Q S E

← R3

1921 AAGGGGAACAGAATTACAGGAAGTTTAGGGCAAAGATTTGTCCTGGAGAAAATCAAGCAG 1980  
-----+-----+-----+-----+-----+-----+  
G E Q N Y R K F R A K I C P G E N Q A A

1981 CCGAAGATGAACTAAGAAAAGAGATAAGTAAAACGATGTTTGCAGAAATGGAAATCATTG 2040  
-----+-----+-----+-----+-----+-----+  
E D E L R K E I S K T M F A E M E I I G

2041 GTCAGTTTAACCTGGGATTTATAATAACCAAACCTGAATGAGGATATCTTCATAGTGGACC 2100  
-----+-----+-----+-----+-----+-----+  
Q F N L G F I I T K L N E D I F I V D Q

2101 AGCATGCCACGGACGAGAAGTATAACTTCGAGATGCTGCAGCAGCACACCGTGCTCCAGG 2160  
-----+-----+-----+-----+-----+-----+  
H A T D E K Y N F E M L Q Q H T V L Q G

2161 GGCAGAGGCTCATAGCACCTCAGACTCTCAACTTAACTGCTGTTAATGAAGCTGTTCTGA 2220  
-----+-----+-----+-----+-----+-----+  
Q R L I A P Q T L N L T A V N E A V L I

2221 TAGAAAATCTGGAAATATTTAGAAAGAATGGCTTTGATTTTGTATCGATGAAAATGCTC 2280  
-----+-----+-----+-----+-----+-----+  
E N L E I F R K N G F D F V I D E N A P

2281 CAGTCACTGAAAGGGCTAAACTGATTTTCCTTGCCAACTAGTAAAAACTGGACCTTCGGAC 2340  
-----+-----+-----+-----+-----+-----+  
V T E R A K L I S L P T S K N W T F G P

2341 CCCAGGACGTCGATGAACTGATCTTCATGCTGAGCGACAGCCCTGGGGTCATGTGCCGGC 2400  
-----+-----+-----+-----+-----+-----+  
Q D V D E L I F M L S D S P G V M C R P

2401 CTTCCCGAGTCAAGCAGATGTTTGCCTCCAGAGCCTGCCGGAAGTCGGTGATGATTGGGA 2460  
-----+-----+-----+-----+-----+-----+  
S R V K Q M F A S R A C R K S V M I G T

2461 CTGCTCTCAACACAAGCGAGATGAAGAACTGATCACCCACATGGGGGAGATGGACCACC 2520  
-----+-----+-----+-----+-----+-----+  
A L N T S E M K K L I T H M G E M D H P

←

2521 CCTGGAAGTGTCCCCATGGAAGGCCAACCATGAGACACATCGCCAACCTGGGTGTCATTT 2580  
-----+-----+-----+-----+-----+-----+  
W N C P H G R P T M R H I A N L G V I S



————R4

2581 CTCAGAACTGA  
-----+--  
Q N \*



Appendix 5 Human c-MYC Details of the Sequence from the National Centre for Biotechnology Information

LOCUS	HSMYC1	2121 bp	mRNA	PRI	17-FEB-1997
DEFINITION	Human mRNA encoding the c-myc oncogene.				
ACCESSION	V00568				
VERSION	V00568.1 GI:34815				
KEYWORDS	complementary DNA; oncogene.				
SOURCE	human.				
ORGANISM	Homo sapiens				
	Eukaryota; Metazoa; Chordata; Vertebrata; Mammalia; Eutheria; Primates; Catarrhini; Hominidae; Homo.				
REFERENCE	1 (bases 1 to 2121)				
AUTHORS	Watt,R., Stanton,L.W., Marcu,K.B., Gallo,R.C., Croce,C.M. and Rovera,G.				
TITLE	Nucleotide sequence of cloned cDNA of human c-myc oncogene				
JOURNAL	Nature 303 (5919), 725-728 (1983)				
MEDLINE	83219310				
REFERENCE	2 (bases 833 to 834)				
AUTHORS	Watt,R.				
TITLE	Direct Submission				
JOURNAL	Submitted (18-JUL-1983) to the EMBL/GenBank/DDBJ databases				
COMMENT	Data kindly reviewed (18-JUL-1983) by Watt R.				
	The germ line c-myc oncogene given in <HSCMYC> differs in its 5' noncoding sequence from the sequence reported here, but the protein coding regions are the same.				
FEATURES	Location/Qualifiers				
source	1..2121				
	/organism="Homo sapiens"				
	/db_xref="taxon:9606"				
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# Appendix 6      Chicken (avian) c-MYC Details of the Sequence from the National Centre for Biotechnology Information

LOCUS            CHKMYC            4984 bp            DNA            VRT            28-APR-1993  
 DEFINITION      Chicken cellular myc proto-oncogene, complete cds.  
 ACCESSION       J00889  
 VERSION          J00889.1    GI:212354  
 KEYWORDS        c-myc proto-oncogene; integration site; myc oncogene;  
                   proto-oncogene.  
 SOURCE           Chicken (Rhode Island Red): genomic DNA from lambda library of  
                   R.Axel; and normal DNA.  
 ORGANISM        Gallus gallus  
                   Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi;  
                   Archosauria; Aves; Neognathae; Galliformes; Phasianidae;  
                   Phasianinae; Gallus.  
 REFERENCE       1 (bases 2205 to 4984)  
 AUTHORS          Watson,D.K., Reddy,P.E., Duesberg,P.H. and Papas,T.S.  
 TITLE            Nucleotide sequence analysis of the chicken c-myc gene reveals  
                   homologous and unique coding regions by comparison with the  
                   transforming gene of avian myelocytomatosis virus MC29,  
                   delta-gag-myc  
                   Proc. Natl. Acad. Sci. U.S.A. 80, 2146-2150 (1983)  
 JOURNAL          83169838  
 MEDLINE  
 REFERENCE       2 (bases 1 to 2399)  
 AUTHORS          Shih,C.-K., Linial,M.L., Goodenow,M.M. and Hayward,W.S.  
 TITLE            Nucleotide sequence 5' of the chicken c-myc coding region:  
                   Localization of a noncoding exon that is absent from myc  
                   transcripts in most avian leukosis virus-induced lymphomas  
                   Proc. Natl. Acad. Sci. U.S.A. 81, 4697-4701 (1984)  
 JOURNAL          84272700  
 MEDLINE  
 REFERENCE       3 (sites)  
 AUTHORS          Linial,M.L. and Groudine,M.  
 TITLE            Transcription of three c-myc exons is enhanced in chicken bursal  
                   lymphoma cell lines  
                   Proc. Natl. Acad. Sci. U.S.A. 82, 53-57 (1985)  
 JOURNAL          85113170  
 MEDLINE  
 COMMENT          [3] sites; exon 1 boundaries.  
                   The chicken cellular myc locus shows a great deal of homology to  
                   the onc locus of avian myelocytomatosis virus MC-29, differing in  
                   only 9 base pairs (resulting in seven amino acid substitutions)  
                   within the two coding exons.  
                   [2] found that in nearly all of the avian leukosis virus-induced  
                   chicken lymphomas that they examined, proviral sequences were  
                   located within the 1 to 1.5 kb region upstream of the coding exons  
                   of c-myc. Neel et al. (J. Virol. 44, 158-166 (1982)), sequenced the  
                   3' proviral integration boundaries in two chicken tumors (see  
                   separate entry).  
                   [3] found that the myc mRNA in cell lines derived from ALV-induced  
                   bursal lymphomas includes this non-coding exon. [3] also notes a  
                   potential TATA box at 1166-1171.  
                   Complete source information:  
                   Chicken (Rhode Island Red [2]): genomic DNA from lambda library of  
                   R.Axel [2]; and normal DNA [1],[3].  
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61

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121

tgcgggggtc ctcccgcccg gcgatccgtg ttcctccccg agcttctacg cttagaaatg

181

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241

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