The Role of DNA Mismatch Repair in Cellular Responses to DNA damage and Drug Resistance

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ABSTRACT

DNA mismatch repair (MMR) is a system of evolutionary conserved DNA surveillance proteins that maintain the integrity of the genome by correcting errors of DNA replication and by regulating DNA excision and recombinational repair processes. Loss of human MMR is associated with microsatellite instability (MI), a mutator phenotype, and hereditary as well as sporadic cancers. Cells deficient in MMR display resistance to the cytotoxic effects of an expanding list of DNA-damaging agents such as DNA methylating, platinating and antimetabolite drugs, many of which are routinely used in the treatment of cancer. MMR deficiency has also been linked to defects in G_2 cell cycle arrest in response to DNA damage.

To explore the role that MMR has in cellular responses to DNA damage in isogenic strains of *S. cerevisiae*, individual disruptions in the MMR genes *MSH2*, *MSH3*, *MSH6*, *MLH1*, *MLH2* and *PMS1* were tested for cytotoxic and cell cycle responses to various DNA damaging agents. A significant 2.6 - 5.6-fold increase ($P<0.05$) in clonogenic resistance to a 24 hour exposure of 1mM cisplatin was seen in all mutants except for *pms1*, compared to wild type. All mutants, but not *mlh2*, displayed a 10-30-fold increased forward mutation frequency, indicative of loss of MMR function. These results indicate that specific components of MMR, but not necessarily MMR *per se*, are involved in conferring sensitivity to cisplatin. Re-introducing *ScMLH1* back into the *mlh1* mutant using a high copy yeast expression vector restored cisplatin sensitivity to levels greater than wild type.

Strains null for the recombinational repair gene *RAD52* and the recombination/nucleotide excision repair gene *RAD1* were hypersensitive to cisplatin. *mlh1*, *msh2* and *mlh2* strains in *rad52* and *rad1* backgrounds were as sensitive as single *rad52* and *rad1* strains indicating that the presence of these recombination/excision repair proteins is required for the MMR-deficient-induced resistance phenotype. Wild

type cells grown to confluence and hence under growth inhibitory conditions were more resistant to killing by cisplatin than logarithmically growing cells, whereas no difference was seen in the *msh2* mutants. This suggests a requirement for DNA replication for the MMR-dependent cytotoxic response. A model in which MMR acts to inhibit recombinational bypass of cisplatin-induced DNA adducts is described.

Cell cycle analysis of these strains indicated that *mlh1* and *msh2* disruption lead to an early release from G_2-M arrest in response to a 1 hour, 4mM exposure to cisplatin. MMR mutants exposed to 100gy ionising irradiation, after a transiently quicker entry into G_2-M, did not display any significant difference in arrest pattern or cytotoxicity compared with wild type. These results suggest that MMR in budding yeast functions in a cisplatin-specific DNA damage response pathway that leads to a prolonged G_2 cell cycle arrest response. A model is described in which MMR induces *S. cerevisiae* cell cycle checkpoint arrest in response to persistent cisplatin-induced DNA lesions and/or replication inhibition.

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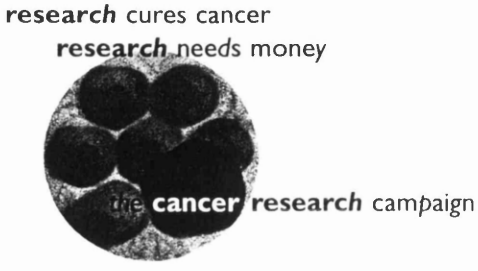

DECLARATION

I, **Stephen Thomas Durant**, declare that I am the author of this thesis, that all the references have been consulted by myself in the preparation of the manuscript, and that all the work described herein was performed by myself unless otherwise stated. This work has not been previously accepted for a higher degree.

Stephen T. Durant

October, 1999

ABBREVIATIONS

AGT	Alkylguanine alkyltransferase
ATM	Ataxia telangiecstasia mutant
BER	Base excision repair
CDDP	cis-diamminedichloroplatinum(II), cisplatin
CDS	Chromatin Denaturation Solution
DSB	Double strand break
FACS	Fluorescence activated cell sorting
5-FU	5-Fluorouracil
GSE	Genetic Supressor Element
HNPCC	Human non-polyposis colorectal carcinoma
L.D. 90	Lethal Dose at 90% cell death
IR	Ionising radiation
Kb	Kilobases
MGMT	Methylguanine methyltransferase
MI	Microsatellite Instability
MLH	MutL Homologue
MMR	Mismatch repair
MNNG	N-methyl-N'-nitro-N-nitrosoguanidine
MSH	MutS Homologue
NER	Nucleotide excision repair
MNU	N-methylnitrosourea
NHEJ	Non-homologous end-joining
PBS	Phosphate Buffered Saline
PCNA	Proliferating cell nuclear antigen
PCR	Polymerase Chain Reaction
PI	Propidium Iodide
PMS	Post Meiotic Segregation
RAD	Radiation sensitive
R.F	Resistance Factor

S. cerevisiae	*Saccharomyces cerevisiae*
SC	Synthetic complete media
S. pombe	*Schizosaccharomyces pombe*
SF	Surviving Fraction
SSA	Single strand annealing
SSB	Single strand break
UV 254	Ultraviolet radiation at 254nm
WT	Wild type
YPD	Yeast extract Peptone Dextrose

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INTRODUCTION

1. INTRODUCTION

The maintenance of genetic stability is fundamental for cell and organism. Biosynthetic errors of DNA replication, physically- or chemically-induced damage to DNA and unrepaired intermediates of DNA metabolism continually threaten the integrity of the genome and are the major causes of mutagenesis and cancer (Loeb, 1991; Freidberg *et al,* 1995). Maintaining genetic fidelity depends on the ability to monitor, repair and signal appropriate responses to these perturbations.

DNA mismatch repair (MMR) is a complex evolutionary-conserved DNA surveillance system that not only rectifies errors of DNA replication (Modrich, 1991), but also has activities in recombinational (Rayssigiuer *et al,* 1989; Datta *et al,* 1995; Saparbaev *et al,* 1996) and nucleotide excision repair pathways (Mellon and Champe, 1996; Kirkpatrick and Petes, 1997).

Loss of MMR function has been associated with microsatellite instability and a mutator phenotype (Shibata *et al,* 1994; Umar *et al,* 1994; Bhattacharya *et al,* 1994). Germline mutations in MMR genes have been linked to hereditary non-polyposis colorectal cancer or Lynch syndrome (Leach *et al,* 1993; Fischel *et al,* 1993; Bronner *et al,* 1994; Nicolaides *et al,* 1994; Papadopoulos *et al,* 1994; Tannergard *et al,* 1995; Fischel and Kolodner, 1995; Lui *et al,* 1995) and loss of MMR is also a common finding in many types of sporadic human cancers (Parsons *et al,* 1993; Umar *et al,* 1994; Risinger *et al,* 1995; Hatta *et al,* 1998).

In addition, MMR deficiency has been associated with tolerance to DNA methylating agents (Goldmacher *et al,* 1986; Griffin *et al,* 1994; Karran and Bignami 1994), resistance to an expanding list of DNA damaging agents (Anthoney *et al,* 1996;

Drummond *et al*, 1996; Brown *et al*, 1997) and defects in DNA damage-induced cell cycle arrest (Koi *et al*, 1994; Hawn *et al*, 1995; Carethers *et al*, 1996; Davis *et al*, 1998). This thesis explores the current literature and presents data using isogenic strains *of S. cerevisiae* to investigate the role of MMR in cellular responses to DNA damage.

1.1. The Maintenance of Genomic Stability

1.1.1. The *E.coli* MutHLS mismatch repair pathway

The first to be identified and best understood function of MMR is its role in the correction of DNA biosynthetic errors. By relying on normal Watson-Crick base pairing, the MMR system can distinguish mismatched bases (with efficiencies: G·T > G·G ≅ A·C > C·C) (Holmes *et al,* 1990) and other abnormal structures such as small looped sequences that distort the DNA helix (Modrich, 1997). These are estimated to arise approximately one in every 10^5 - 10^6 bases synthesised during proof-read DNA polymerase I and III-activated DNA replication (Darnell *et al,* 1990). The fully characterised MutHLS MMR system in *Escherichia coli* has revealed the mechanisms of bacterial strand discrimination, strand incision and repair.

DNA in *E.coli* is postreplicationally methylated by the Dam methylase. This modification asymmetry is used to direct MMR towards the transiently unmethylated adenine of newly synthesised palindromic d(GATC) sequences. It is in these sequences that the newly synthesised, transiently unmethylated daughter strand is discriminated. Incision of the unmodified strand at a hemimethylated d(GATC) site results in a nick and this is what directs repair (Kolodner and Alani, 1994).

All components of the MutHLS system of *E.coli* have been characterised (Lahue *et al,* 1989; Modrich 1991; Grilley *et al,* 1993; Fischel and Kolodner 1995). After the MutS protein binds to mismatched DNA, MutL then forms a heterodimer which then recruits the MutH endonuclease to cleave at the d(GATC) site (Hall and Matson, 1999). After this, DNA unwinding occurs at the nick by UvrD (helicase II) and proceeds to a point beyond the error. The action of one of several exonucleases - ExoI, Exo VII and RecJ - excises the strand containing the mismatch and this can occur on either side of the mismatch, reflecting the bidirectionality of the system. ExoVII and RecJ being 5' → 3'

and ExoI being 3' → 5' (Cooper *et al,* 1993) (See Fig. 1). In the presence of DNA polymerase III holoenzyme and single-stranded DNA-binding protein (SSB), repair synthesis occurs to fill the gap and DNA ligase seals the final nick (Modrich and Lahue, 1996).

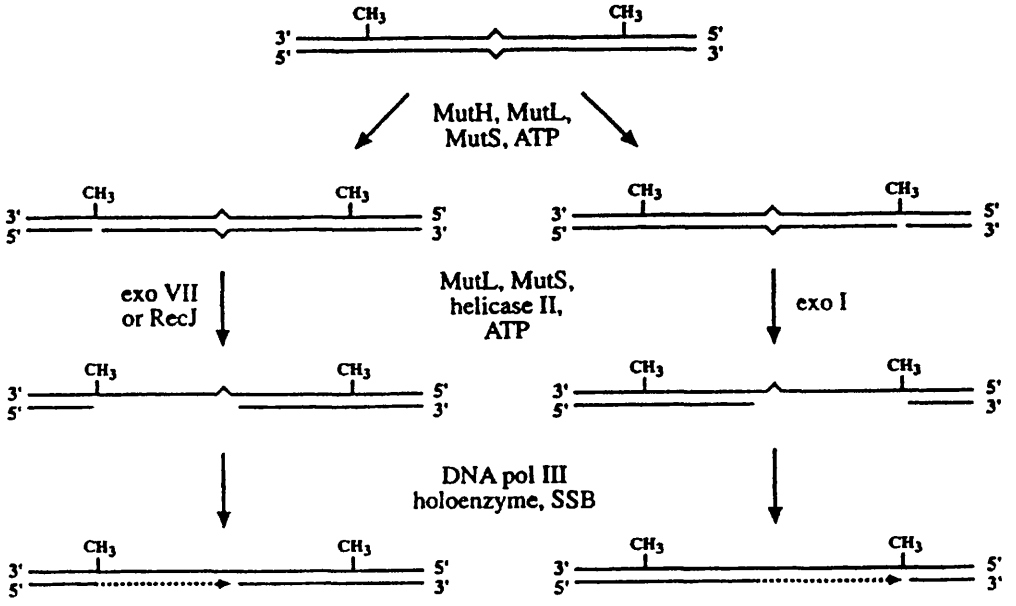


Figure 1 The E. coli methyl-directed MutHLS mismatch repair system

(taken from Grilley *et al,* 1993)

The requirement for MutH and the use of Dam methylation to distinguish new from template DNA is unique to certain bacteria and certainly not a common mechanism for daughter strand discrimination in any eukaryotic cell studied. The *Streptococcus pneumoniae* Hex MMR system is not known to utilise a MutH homologue or methylation asymmetry (Modrich, 1991; Fischel and Kolodner 1995; Modrich and Lahue 1996). Since DNA methylation does not seem to occur in *S. cerevisiae* (Proffitt *et al,* 1984), *Drosophila* or *Caenorhabditis elegans* (Modrich, 1991; Fischel and

Kolodner 1995; Freidberg *et al,* 1995; Modrich and Lahue 1996), the way in which the newly synthesised strand is recognised in higher systems is not known.

1.1.2. Eukaryotic MMR

When Williamson and colleagues (1985) used a unique disomic haploid strain of the budding yeast *Saccharomyces cerevisiae* containing two copies of chromosome III to identify meiotic hyperrecombination mutants, they found that a series of *pms*1-6 mutants displayed mitotic mutator phenotypes, making them candidates for MMR-deficiency. *PMS1* proved to be the first eukaryotic MMR gene identified.

Since then, homologues of bacterial MMR proteins have been found in yeast, fly, nematode, mouse and human cells and a remarkable degree of homology exists between them (Modrich and Lahue, 1996). The larger numbers of gene products identified in eukaryotes indicates the increased complexity and functional diversity evolved in these systems. At least six MutS homologues (MSH) have now been found in *S. cerevisiae* (Williamson *et al,* 1985; Bishop *et al,* 1987; Kramer *et al,* 1989; Reenan and Kolodner 1992; Kolodner 1995). *scMSH1-6* show high degrees of amino acid similarity to bacterial MutS and homologues of these have been identified in murine and human cell lines (Bhattacharyya *et al,* 1994; Shibita *et al,* 1994; Boyer *et al,* 1995; Liu *et al,* 1995; Papadopoulos *et al,* 1995). Three of these proteins, Msh2, Msh3 and Msh6 function in a eukaryotic MutHLS-like manner (Strand *et al,* 1995; Marsischky *et al,* 1996).

Purified Msh2 from both *S. cerevisiae* and human cells recognises with high affinity single base mispairs and multiple base insertion and deletion loops (Fischel *et al,* 1994; Alani *et al,* 1995). Msh3 and Msh6 (GTBP/p160) both copurify separately with Msh2 (Drummond *et al,* 1995; Palombo *et al,* 1995; Marsischky *et al,* 1996) and it is now known, through rates of single-base substitution verses frameshift mutation studies, that the Msh2-Msh6 heterodimer binds specifically to single mismatches and Msh2-Msh3 binds preferentially to looped insertion/deletions (Strand *et al,* 1995; Marsischky *et al,* 1996).

Homologues of the MutL protein have been identified in humans. Two proteins, *Sc*Mlh1 and *Sc*Pms1 (Pms2 in humans) are required for mismatch repair (Prolla *et al*, 1994; Modrich and Lahue, 1996). These proteins exist as a heterodimer and interact with Msh2 bound to a mispaired base. *S. cerevisiae mlh1* and *pms1* mutants show similar strong mutator phenotypes equivalent to that of *msh2* mutants (Strand *et al*, 1993). Human Pms1 is a third MutL protein identified by virtue of a germ-line mutation in *PMS1* in a single patient with a family history of colon cancer (Nicolaides *et al*, 1994). It was not known if this mutation caused Microsatellite instability.

Other components involved in the mismatch repair pathway have been implicated. *S. cerevisiae* strains lacking *RAD27/RTH1* (human *FEN1/MF-1*, *EXOIV*), a 5'-3' endo/exonuclease, have a mutator phenotype (Johnson *et al*, 1995) as strong as *msh2*, *mlh1* or *pms1* strains. However, double mutant combinations of *rad27* and either *msh2*, *mlh1*, or *pms1* show a three-five fold synergistic effect which suggests that *RAD27* exists as part of a separate pathway from *MSH2*, *MLH1* and *PMS1*. Rad27 is a member of the Rad6 epistasis group and is involved in repair of other types of DNA damage. *rad27* mutants also display cell cycle arrest defects (Reagan *et al*, 1995).

Human Exo1 has been identified in a GST-fusion screen as a protein that interacts with human Msh2 (Schmutte *et al*, 1998). *Exo1 S. pombe* mutants show a weak mutator phenotype (Szankasi and Smith 1995) and has been proposed to function in an MSH2-dependent manner (see Fig. 1). Both DNA polymerases II (epsilon) and III (delta) have been shown to participate in correcting mismatches in *S. cerevisiae* and these are the only DNA polymerases known to possess an intrinsic 3' to 5' exonuclease in *S. cerevisiae* (Morrison and Sugino, 1994). Human Replication protein A (RPA), which is also part of the replication fork machinery, is known to be required for mismatch repair (Lin *et al*, 1998). Proliferating cell nuclear antigen (PCNA) is known to bind to human Mlh1 in a yeast two-hybrid screen (Umar *et al*, 1996) and *S. cerevisiae* null for PCNA display a mutator phenotype (Johnson *et al*, 1996).

Recently, Med1, a novel human methyl-CpG-binding endonuclease, has been shown to bind to human Mlh1 and transfecting a *med1* mutant lacking the methyl-CpG-binding domain resulted in microsatellite instability (Bellacosa *et al*, 1999). This may

represent the putative homologue of bacterial MutH since MED1 has homology to bacterial DNA repair enzymes, displays endonuclease activity, binds with varying affinity to and hence discriminates between methylated and hemimethylated CpG sequences and stabilises Microsatellite sequences. This observation may also suggest that cytosine methylation may play a role in human mismatch repair. However, the lack of DNA methylation in *Drosophila*, *Caenorhabditis elegans* and *S. cerevisiae* (Modrich and Lahue, 1996) would represent a substantial evolutionary skip in the mechanism of strand discrimination.

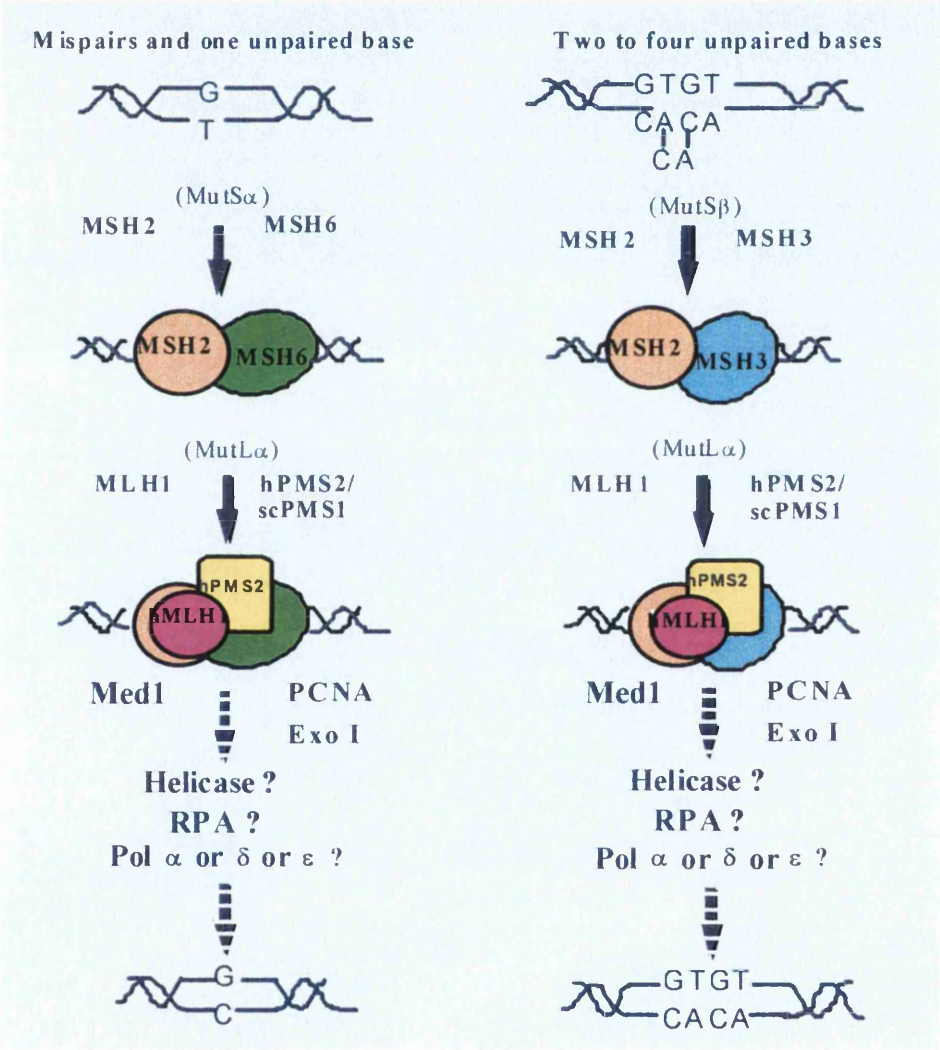


Figure 2 Schematic diagram of eukaryotic MMR

There is recent evidence to show that bacterial MutS and MutL, as well as eukaryotic Msh2 and Msh6 proteins act as molecular switches dependent on ATP hydrolysis. It has long been known that ATP is required for MutHLS mismatch repair (Welsh *et al,* 1987). Haber and Walker (1991) then showed that MutS bound and hydrolysed ATP and that a mutant MutS containing a mutation in the ATP phosphate binding loop consensus displayed reduced ATPase activity. It was then shown that point mutations in the ATP binding helix-turn-helix domain of *S. cerevisiae* Msh2 did not affect mismatch binding of the Msh2-msh6p complex, but did cause biochemical defects in mismatch repair downstream (Alani *et al,* 1997; Studamire *et al,* 1998). Additionally, crystal structure analysis of MutL has shown that it belongs to an emerging ATPase superfamily that includes DNA topoisomerase II and Hsp90 (Ban and Yang, 1998). A model has been proposed which suggests that a molecular switch is in operation so that when Msh2-Msh6 is in the ADP-bound form it is ON and binds to mismatched nucleotides, and OFF in the ATP-bound form and released (Gradia *et al,* 1997; Fischel 1998; Ban *et al,* 1999). In this way, it is proposed that the timing of ATP-ADP hydrolysis controls the sequential steps of downstream components of MMR.

1.1.3. The role of MMR in homologous recombination

Homologous recombination is a mechanism used for mitotic and meiotic strand exchange and DNA-double strand break repair. Various components of bacterial, yeast and mammalian MMR have evolved to function in both of these systems.

1.1.3.1. Mitotic and meiotic recombination

Homologous recombination is usually thought of as occurring between sequences at identical positions on homologous chromosomes (allelic recombination), but it can also occur between similar (homeologous) sequences at nonallelic (ectopic) locations. These similar sequences are found in large numbers of repeated regions found dispersed throughout eukaryotic genomes.

Such homeologous events can generate novel gene conversions and are likely to be important mechanisms for the evolution of multigene families. It is also implied that these events represent a source of diversity at immunoglobulin loci (Maizels 1989). However, crossing-over between more dispersed repeats can lead to genome rearrangements such as deletions, insertions and translocations, events commonly found in tumour cells. MMR has been shown to regulate these recombinant events, thus adding weight to its role as a genome stabiliser.

It has been known for some time that prokaryotic MMR acts as a potent barrier to recombination between divergent (homeologous) sequences (Rayssigiuer *et al,* 1989). More recently, it was demonstrated that the *S. cerevisiae* MMR genes *MSH2*, *MSH3* and *PMS1* regulated mitotic cross-overs between diverged sequences and that this effect was dependent on the degree of sequence divergence (Datta *et al,* 1995). In contrast, it was shown that Msh2 and Msh3 proteins are actually components of the Rad1/10

pathway of mitotic recombination (Saparbaev *et al,* 1996), obscuring the role MMR has in regulating recombination events.

A study which looked at the involvement of the *S. cerevisiae* MMR protein Mlh1 during meiosis revealed that this protein was unique among all the other components of MMR in its ability to promote meiotic crossing-over (Hunter and Borts, 1997). This function was originally assigned to the MMR-independent, exclusively meiotic proteins Msh4 and Msh5 (Ross-Macdonald and Roeder, 1994; Hollingsworth *et al,* 1995). The fact that these proteins are not required for mitotic recombination indicates that they act in mutually exclusive pathways for mitotic and meiotic recombination.

1.1.3.2. DNA double-strand break repair

Double strand breaks in DNA result from normal enzymatic activities of endonucleases and from the genotoxic effects of agents such as ionising radiation. They pose a considerable threat to Genomic integrity and cell survival. If left unrepaired, a single double-strand break is sufficient to cause cell death and, if inefficiently or inappropriately repaired, can generate potentially oncogenic chromosomal aberrations (Hiom, 1999).

Homologous recombination and non-homologous end-joining (NHER) are two mechanisms used to repair double-strand breaks in eukaryotes. NHER requires the Ku70 and Ku80 subunits of DNA protein kinase (DNA-PK) to bind to and initiate the re-joining of double-stranded DNA ends. This repair pathway is error-prone and the preferred pathway for double-strand break repair in mammalian cells. Homologous recombination utilises stretches of homologous donor sequences to repair the double strand break. This pathway is less error-prone, is the preferred mechanism in yeast and requires specific components of MMR.

Two major pathways of homologous recombination exist in *S. cerevisiae* – gene conversion and single-strand annealing (SSA). Both have the same first step of

processing the DNA ends which entails the 5' to 3' degradation of DNA strands that yields single-stranded 3' ends that act as donor templates for repair synthesis. Any nonhomologous bases contained at the 3' tail of this invading strand must be removed and this has been shown to be dependent on the nucleotide excision repair endonuclease Rad1/Rad10 and the MMR proteins Msh2 and Msh3 (Sugawara *et al,* 1997). Pms1 and Mlh1 proteins are not required (Sugawara *et al,* 1997). Only when 3' tails are 30 nucleotides long or longer are Rad1, Msh2 and Msh3 proteins necessary as shorter tails can be removed at least in part by the 3' to 5' proof-reading activity of DNA polymerase δ (Paques and Haber, 1997). After this initial process is complete the key process of Rad51-Rad52-dependent pairing and strand-exchange between homologous DNA molecules then proceeds (Shinohara and Ogawa, 1998).

1.1.4. Nucleotide excision repair and MMR

The present literature concerning the functional and mechanistic overlap between MMR and excision repair is contentious. Mismatch repair proteins predominantly repair base mismatches and small looped sequences whereas NER primarily removes pyrimidine dimers (induced by UV radiation) and other helix distorting adducts. It has been shown in *S. cerevisiae* however that repair of 26-base loops involves both the NER protein Rad1 (human ERCC4) and Msh2 (Kirkpatrick and Petes, 1997). By using elevated levels of post meiotic segregation (PMS) events as opposed to gene conversions it was demonstrated that homozygous deletions in either RAD1 or MSH2 caused a three-fold increase in PMS. However, double *rad1/msh2* mutants had the same PMS frequency indicating that these proteins function in the same pathway. Rad1 acts in conjunction with Rad10 (human ERCC1) to cleave 5' and Rad2 cuts 3' to a pyrimidine dimer during NER. Rad14 acts as the DNA damage recognition protein. Both *rad2* and *rad14* mutants did not increase PMS frequency indicating that complete NER is not required (Kirkpatrick and Petes, 1997).

A mutation of mei-9, the *Drosophila melanogaster* homologue of *sc*Rad1 (Sekelsky *et al,* 1995) increases PMS, and thus may be defective in meiotic mismatch repair (Carpenter 1982). A surprising observation came from Yang *et al,* (1996) who showed that a mutant *scRad3* conferred a mutator phenotype and actually enhanced the efficiency of heteroduplex repair. In two studies looking at base mismatch correction or short patch MMR (not dependent on MMR proteins), the human NER complex was found to act on G/G and G/A mismatched substrates (Huang *et al,* 1994), and more recently, it was shown that *S. pombe* NER proteins Rhp14p (*sc*Rad14), Swi10p (*sc*Rad10) and Rad16p (*sc*Rad1), which are components of a short-patch MSH2- and PMS1- independent mismatch repair pathway, corrects mainly C/C mismatches (the error least recognised by long patch MMR) (Fleck *et al,* 1999).

Another link between MMR and NER has been made specifically with respect to transcription-coupled NER. The removal of many types of DNA damage occurs more rapidly in transcriptionally active regions of DNA. This rapid repair has been shown to be due to faster repair of damage in the transcribed strand than non-transcribed strand of active genes. The process is a highly conserved mechanism of excision repair occurring in bacteria (Mellon and Hanawalt, 1989), yeast (Sweder and Hanawalt, 1992) and humans (Mellon *et al,* 1987; Leadon and Lawrence, 1991).

It has been demonstrated that mutant *E.coli* strains (*uvrA, uvrB, uvrC* and *uvrD*) defective in nucleotide excision repair could not repair cyclobutane pyrimidine dimers from expressed or non-expressed strands of the lactose operon. However, in this study, strains lacking either MutS or MutL selectively abolished repair in the transcribed strand and rendered the cells moderately sensitive to UV irradiation (Mellon and Champe, 1996). Similarly, several MMR-deficient tumour cell lines and HNPCC-derived lymphoblastoid cell lines deficient in either Msh2, Mlh1, or Pms2 were also found to be deficient in transcription-coupled repair (TCR) of UV damage, thus extending the connection between prokaryotic TCR and MMR to humans (Mellon *et al,* 1996). Another study reported that the hMSH2 gene product, but not hMlh1 was required for the removal of ionising radiation (IR)-induced oxidative damage, including thymine glycols, from the transcribed strand of an active gene (Leadon and Avrutskaya, 1997). In contrast however, the sensitivity to IR or UV was unaltered.

Several studies dispute the overlapping role of these two repair pathways. No defects in TCR of UV damage have been found in yeast mutants for *msh2*, *mlh1*, *pms1* or *msh3* (Sweder *et al,* 1996), which may indicate some functional difference between yeast and human MMR proteins in TCR. More studies on yeast exposed to other DNA damaging agents are needed before it can be ruled out that MMR plays any role in yeast TCR. In an *in vitro* study that analysed the activities of human MMR and excision repair upon a compound DNA lesion, i.e. one containing a mismatch as well as a UV-induced pyrimidine dimer adduct, no difference in repair activity was seen in MMR defective cell extracts indicating that MMR has no activity in UV-damage repair (Mu *et al,* 1997). Similarly, a recent complementary study showed that a human cell line defective in both MMR and NER was no different in cytotoxic or sister chromatid

exchange responses to UV compared to the derived NER-defective cell line (O'Driscoll *et al,* 1999). Therefore, since UV damage is a common genetic lesion, the authors dispute the idea that MMR acts as a general sensor of DNA damage.

1.1.5. Cancers associated with Microsatellite instability (MI) and MMR-deficiency

1.1.5.1. HNPCC and related disorders

It is widely considered that genetic instability is an integral component of human neoplasia (Loeb, 1991). An unstable, mutator phenotype would provide a continuing pool of mutants upon which selection could act to promote a tumour. It was of great interest therefore, when a series of seminal studies showed a link between heterozygous germ-line mutations in MMR genes, instability in tandem repeat sequences (microsatellites) and hereditary nonpolyposis colorectal cancer (HNPCC or Lynch Syndrome) (Leach *et al,* 1993; Fischel *et al,* 1993; Bronner *et al,* 1994; Nicolaides *et al,* 1994; Papadopoulos *et al,* 1994; Tannergard *et al,* 1995).

HNPCC is a common autosomal dominant disorder characterised by an inherited predisposition to early onset of colorectal cancer (mean age, approximately 40-45) and accounts for 1-6% of all colorectal cancers (Lynch *et al,* 1993). It is also associated with an increased incidence of carcinomas of the endometrium, ovary, small intestine, stomach, ureter and renal pelvis, termed Lynch Syndrome (LS) (Lynch *et al,* 1996; Kinzler and Vogelstein, 1996).

Mutations in *MLH1* and *MSH2* are seen in >90% of HNPCC kindreds, whereas mutations in *PMS2*, *PMS1* and *MSH6* are considerably less frequent. These are heterozygous mutations, so individuals retain a functional allele and are apparently competent in MMR. Subsequent somatic mutation in the wild-type allele leads to the mutator cancer phenotype (Parsons *et al,* 1993). In individuals with homozygous deletions of *MLH1*, haematological malignancies (leukaemias and/or lymphomas) and neurofibromatosis Type 1 also present at a very early age (Ricciardone *et al,* 1999;

Wang *et al,* 1999). HNPCC has also been associated with a mutant allele for *MLH1* in breast cancer patients (Risinger *et al,* 1996).

A closely related disorder is Muir-Torre syndrome (MTS). MTS is a rare autosomal-dominant condition characterised by the occurrence of sebaceous skin lesions and visceral tumours. It has been demonstrated that microsatellite instability (MI) and mutations in MMR genes occurs in at least a subset of cases and that unrelated cases of MTS have been associated with germline mutations in *MSH2* (Kruse *et al,* 1996).

1.1.5.2. Sporadic cancers

Outside of HNPCC/LS/MTS, the properties of many established colorectal adenocarcinoma cell lines are consistent with MMR defects. MI (Shibata *et al,* 1994), defects in MMR function in cell extracts (Umar *et al,* 1994) and mutator phenotypes (Bhattacharya *et al,* 1994) have been observed. Specific loss of MMR genes have also been documented. The MMR-defective LoVo cell line is homozygous for a partial deletion of the *MSH2* gene (Umar *et al,* 1994) and the MI^{+}, mutator cell line HCT116 has a mutation in the *MLH1* gene (Parsons *et al,* 1993).

Other sporadic and familial cancers have been shown to contain unstable microsatellites. MI has been associated with 67% of familial gastric cancers sampled, although only 1/6 showed a somatic mutation in *MSH2* (Akiyama *et al,* 1996). MI has been seen in 20% of melanoma cases (Peris *et al,* 1995), 41% of acute/lymphomatous adult T-cell leukaemia cases (ATL) (Hatta *et al,* 1998), all six tumours from a Turcot syndrome patient (a disorder associated with brain and colon cancers in early adult life) with a germline *PMS2* mutant allele (Miyaki *et al,* 1997) and 25% of uterine sarcomas, with a subset showing mutations in *MSH2* (Umar *et al,* 1994; Risinger *et al,* 1995). Furthermore, MI has also been strongly linked to the susceptibility of previously operated cancer patients to develop multiple primary cancers (Horii *et al,* 1994).

Finally, it has been seen that MI is highly frequent (86%) in hepatitis B positive livers of cirrhotic patients compared to HBV-negative cirrhotic samples (35%) and that this is reflected in the propensity for HBV to induce hepatocellular carcinoma (Salvucci *et al,* 1996). Similarly, MI is found to be more prevalent in Kaposi's sarcoma and aggressive lymphomas obtained from HIV-infected patients compared to tumours of the same histological type from HIV-negative patients (Bedi *et al,* 1995). Although MMR status was not studied in the context of viral carcinogenesis, it seems more likely that similarities in replication cycles of HBV and retroviruses (Tiollais *et al,* 1985) may play a role in inducing the high instabilities observed (Salvucci *et al,* 1996).

1.1.6. Mechanisms of oncogenesis induced by loss of MMR

Before the multistage cascade of tumour progression in a MMR-defective pathway initiates, mechanisms other than inherited defects or spontaneous mutations that compromise MMR can take place. Epigenetic silencing of MMR by DNA methylation is one such mechanism. Organisms like vertebrates and others that methylate their genomes for essential processes such as embryonic development, contain regions of CpG dinuleotides which are the predominant sites of methylation. Regions where they are more common, termed CpG islands, often reside in gene promoter sequences (Jones, 1996). CpG islands remain free of methylation but when methylated, gene expression becomes inactivated. Several studies provide evidence that aberrant hypermethylation of the human *MLH1* promoter correlates with lack of expression of Mlh1 in MMR-defective sporadic colon tumours and other MMR-defective tumour cell lines (Kane *et al,* 1997; Herman *et al,* 1998; Strathdee *et al,* 1999).

Colorectal cancers with MI have also been shown to contain hypermethylation of tumour suppresser genes themselves. The cell cycle regulator p16, the angiogenesis inhibitor thrombospondin-1 (TSP-1) and insulin growth factor II (IGF2) genes were all found to be hypermethylated in MI^+ compared to MI^- colorectal cancers (Ahuja *et al,* 1997).

It has been suggested that two separate pathways leading to tumourigenesis can operate, one by p53 malfunction and one associated with MI, although this is disputed. It has been shown that MI^+ cancers often show lower frequencies of mutations in p53 and in some activating oncogenes than do MI^- cancers (Aaltonen *et al,* 1993; Ionov *et al,* 1993, Kim *et al,* 1994, Cottu *et al,* 1996). However, in contrast, a positive correlation between p53 mutations and MI was seen in therapy-related paediatric secondary malignant

neoplasms (Gafanovich *et al,* 1999) and no correlation was seen at all between p53 mutation and MI in sporadic colorectal cancers (Ilyas *et al,* 1996).

More stringent and facilitated controls of genetic background and manipulation make animal models attractive to the study of MMR-deficient-induced neoplasia. Mutant strains of mice deficient for the murine homologues of the human genes *MLH1*, *PMS1*, *PMS2* (Prolla *et al,* 1998) and *MSH2* (Reitmair *et al,* 1996; de Wind *et al,* 1998) have been generated in order to investigate the tumourigenic phenotypes induced by each mutant. By comparing the tumour distribution of mice with humans, surprisingly different tumours arose. By one year of age, homozygous *mlh1*$^{-/-}$ and *msh2*$^{-/-}$ mice usually succumbed to T-cell lymphomas and autopsies revealed the development of intestinal adenomas and adenocarcinomas, and to a lesser extent, skin tumours and sarcomas (Prolla *et al,* 1998; Reitmair *et al,* 1996). However, *msh2* hemizygous (*Msh2*$^{+/-}$) disruptions did not affect survival and did not induce lymphomas.

Because of the difference in tumour distribution and spectra, it was proposed that dietary intake might effect the development of cancers particularly in the gut of HNPCC patients. A study which treated mice with ethylnitrosourea (ENU) synergistically enhanced lymphomagenesis in completely *msh2*-deficient mice. However, by crossing the *msh2* mutant strain with an immunocompromised *tap1*$^{-/-}$ mutant (a gene that controls antigen presentation) lymphomas were not induced by ethylnitrosourea, with mice generally succumbing to HNPCC-like tumours. This suggests the HNPCC tumour spectrum is determined by exposure of MMR-deficient cells to exogenous mutagens, rather than by tissue specific loss of the wild-type MMR allele or by immune surveillance (de Wind *et al,* 1998).

However, evasion from immune surveillance has been suggested to occur in MMR-defective colorectal carcinoma cells. β 2-microglobulin, responsible for presenting antigen to cytotoxic T-lymphocytes (CTL's), has been shown to be mutated to the point of loss of expression in mutator cells (Bicknell *et al,* 1996).

Additionally, intestinal tumours of *MLH1*-null mice express little or no adenomatous polyposis coli (Apc) tumour suppresser protein, similar to that seen in HNPCC

(Edelmann *et al*, 1999) and the frequency of intestinal tumours rose by 40-100 fold when an Apc gene mutation was bred into the *mlh1* mutant mice (Edelmann *et al*, 1999). Interestingly, when one allele of Apc was disrupted, it was seen that loss of the wild type *MSH2* allele occurred in a significant fraction of $Apc^{+/-}/msh2^{+/-}$ mice (de Wind *et al*, 1998). A complementary loss of Msh2 and Apc function may be a mechanism for the development of the HNPCC tumour spectrum.

It was discussed earlier that MI^+ tumours often displayed less frequent mutations in tumour Supressor genes such as p53 than MI-negative tumours. However, other oncogenic transformations have been linked to MI and MMR-deficiency. Inactivation of transforming growth factor-β type II receptor (Type II TGF-β R) by a single-nucleotide frameshift in a polyadenosine tract of exonic sequence correlated highly with MI colon cancer cell lines compared to MI-negative cell lines (Markowitz *et al*, 1995). TGF-β is a potent inhibitor of multiple epithelial cell types and loss of this negative regulation is thought to contribute to neoplasia (Markowitz *et al*, 1995).

Similar somatic frameshift mutations have been reported in the pro-apoptotic BAX (Rampino *et al*, 1997) and caspase-5 (Schwartz *et al*, 1999) genes and in insulin-like growth factor II receptor gene (Souza *et al*, 1996) of MI^+ colon and endometrial cancer cell lines. Ikeda and colleagues (1998) have also found a close correlation between repeat CAG mutations of the transcriptional activator E2F4 gene (a member of the E2F family that is regulated by p107 and p130, close relatives of retinoblastoma suppresser) and MI^+ colorectal cancer cells with frameshift mutations in *MSH3*.

Finally, increased activity of the anti-senescence enzyme telomerase is a common phenotype in the majority of tumour specimens from most cancer types (Kim *et al*, 1994); and it has been seen that normal and carcinoma samples from HNPCC patients show higher levels of activity of telomerase compared to normal and polyps tissue from non-HNPCC individuals (Cheng *et al*, 1998). It has been suggested that MMR-deficiency may indirectly activate the telomerase enzyme by loss of regulation (Cheng *et al*, 1998).

1.2. Cellular Responses to DNA Damage and Mechanisms of Drug Resistance

All organisms are exposed constantly and unavoidably to foreign chemicals (xenobiotics), toxic metabolites, free radicals and radiation, all of which can react with DNA. The response a cell makes to these nucleophiles determines its sensitivity or resistance to them. A cell may manifest resistance to genotoxic agents such as chemotherapeutic drugs, which may be primary (present when the drug is first given) or acquired (developing during treatment with the drug). Acquired resistance may be due to adaptation or to mutation, with the emergence of cells which are less/unaffected by the agent, thus conferring a selective advantage over sensitive cells. A cell may respond in several distinct ways to the presence of the agent itself or to the effects it has upon DNA. The effect of a DNA-targeting genotoxic drug is determined by responses such as:

1) Reducing the physical interaction with DNA. This involves upregulating processes that a) decrease that amount of drug taken up by the cell; b) increase the physical efflux of an agent out of the cell; c) bind toxicants to intracellular proteins and d) detoxify agents by enzymatic biotransformation.
2) Increasing the activity of DNA repair pathways
3) Allowing SOS translesion bypass of a DNA adduct
4) Arresting the cell cycle
5) Activating programmed cell death

1.2.1. Reducing import into the cell

Down-regulation of specialised membrane receptors and transport processes are adaptive mechanisms for reducing the delivery of drugs to target molecules. Endocytosis of some toxicant-protein complexes, such as Cd-metallothionein or lipoprotein receptor-mediated endocytosis of lipoprotein-bound toxicants can be regulated as well as the internalisation of cationic aminoglycosides and heavy metals associated with anionic phospholipids (Laurent *et al,* 1990; Casarett and Doull, 1996).

1.2.2. Increasing export from the cell

Intracellular toxicants may be transported out of cells containing the ATP-dependent membrane transporter known as the multidrug-resistance (MDR) protein, or P-glycoprotein. This protein extrudes many types of chemicals e.g. the neurotoxic pesticide ivermectin, that are substrates for this efflux pump and the MDR gene may up-regulated to increase this activity (Elbling *et al,* 1993; Schinkel *et al,* 1994). Studies that have analysed relationships between sensitivity of cancer cell lines to chemotherapeutic drugs and P-glycoprotein expression have shown that drugs that induce MDR activity include doxorubicin, mitoxantrone and vincristine; where as non-MDR drugs include cisplatin, ifosfamide and bleomycin (Hoffmann *et al,* 1999).

However, enhanced expression of the cMOAT protein, another efflux transporter pump, has been shown to increase the extrusion of the cisplatin-GSH conjugate (see Section 1.2.4) from AH66 tumour cells (Minamino *et al,* 1999).

1.2.3. Association with intracellular binding proteins

An increase in the pool of nontarget intracellular binding proteins can also reduce the concentration of toxicants at the target site, at least temporarily. As mentioned above, Metalothionein, a cysteine-rich cytoplasmic protein sequesters cadmium during acute cadmium intoxication (Goering *et al,* 1995). The heat shock response is another

mechanism that can protect a cell from physical injury and the toxic effects of many chemicals. Heat shock proteins (Hsp) have been classified into different families based on molecular mass, are highly conserved in eukaryotes, and are induced by a number of pathophysiological states e.g. heat shock, anoxia, oxidative stress, toxicants, heavy metal exposure and tissue trauma (Jaatela and Wissing, 1992; Hightower, 1991). These proteins play an important housekeeping role in the maintenance of normal protein structure and the degradation of damaged proteins. Several studies looking at DNA damaging agents have shown that cisplatin toxicity correlates with Hsp expression. Continuous cisplatin incubation increased HSP27 levels and induced HSP27 phosphorylation in GLC4 (human small-cell lung carcinoma) cells. However, no correlation was seen in the cisplatin resistant subline (Hettinga *et al,* 1996). Testis tumour cells in vitro retain their sensitivity to chemotherapeutic drugs, radiation and heat shock and having shown that this was associated with low constitutive levels of heat shock protein (HSP) 27, overexpressing cells were shown to be more resistant to heat shock, cisplatin, and doxorubicin (Richards *et al,* 1996).

1.2.4. Detoxication

Upregulating biotransformation enzymes in the cytoplasm is an important detoxication mechanism. These proteins can convert a lypophyllic absorbent agent to a hydrophyllic form which is more easily eliminated from the body by excretion (Casarett and Doull, 1996). However, this process can also be responsible for the biotransformation of a chemical into an active metabolite (e.g. the formation of glutathione conjugates with nitrosoguanides). Phase I enzymes (e.g. cytochrome P450 and carboxylesterases) are involved in initial hydrolysis, reduction and oxidation reactions which expose or introduce an electrophyllic group (-OH, $-NH_2$, $-SH_2$, or -COOH). They usually result in only a small increase in the hydrophylicity of an agent whereas Phase II reactions (i.e. glutathione and amino acid conjugation, glucuronidation, acetylation, methylation and sulfonation) occur at the functional groups present or introduced/exposed by phase I enzymes (with the exception of methylation/acetylation) and result in a large increase in

hydrophilicity. Therefore, they greatly promote the excretion of xenobiotics (Casarett and Doull, 1996).

The conjugation of xenobiotics with the phase II detoxicant glutathione is catalysed by a family of glutathione S-transferases (GST) and is a mechanism of resistance acquired during cisplatin therapy. This is because substrates for GST are hydrophobic and contain an electrophilic atom. Good substrates are those which contain leaving groups such as a nitro group and the displacement reaction which leads to conjugation is increased by the presence of other electron-withdrawing groups (e.g. -Cl). Both of these groups exist on agents such as 1,2-Dichloro-4-nitrobenzene and on cisplatin (cis-diaminedichloroplatinum) (see Fig.4).

Resistance to toxic compounds is often associated with an over-expression of GST (e.g. resistance of insects to DDT or corn to atrazine). Recent studies have shown that cisplatin stimulates GST activity in blood platelets after incubation with the drug. Levels of glutathione were shown to be decreased because the complex of cisplatin with glutathione was formed (Olas and Zbikowska, 1999). Glutathione has been used effectively in cisplatin treatment regimens for the management of the toxic side-effects prevalent during cisplatin chemotherapy of advanced ovarian cancer (Bohm *et al,* 1999). However, it is noteworthy that in this study, and in another which studied neurotoxicity during ovarian cancer treatment, glutathione cotreatment did not impair cisplatin antineoplastic effectiveness (Bogliun *et al,* 1996).

1.2.5. DNA Repair

Mechanisms involved in the repair of genetic lesions caused by DNA damaging agents may be upregulated in response to the presence of DNA damage. The removal of alkyl groups from bases is carried out by the alkyltransferases which are regulated by the presence of alkylating agent-induced DNA damage. This is a major mechanism of resistance to DNA alkylating drugs e.g. MGMT expression during MNNG treatment (See Section 1.3.1). Base excision repair (BER) involves the complete removal of a damaged base followed by insertion of a new complementary base. Oxidative damage

can cause this kind of lesion. BER involves the actions of N-glycosylase, apurinic/apyrimidinic endonuclease, exonulease, polymerase and ligase enzymes.

Nucleotide excision repair (NER) excises the damaged base, sugar and phosphate and is triggered in response to UV-induced damage and other agents that cause bulky lesions. Recombinational repair acts upon single or double strand breaks and responds to agents such as ionising radiation and chemicals that induce DNA strand breaks e.g. bleomycin (See Section 1.1.3).

In addition to efficient pre-replicative excision repair mechanisms, cells have also evolved SOS-like damage tolerance pathways enabling them to replicate lesion-containing DNA molecules either by directly replicating through the damaged base (translesion synthesis, TLS) or by employing the locally undamaged complementary strand thus avoiding the lesion (damage avoidance pathways, DA). During the SOS response the error-prone TLS pathway is strongly stimulated (approximately 20-fold) at the expense of the error-free DA pathways (Becheral and Fuchs, 1999).

The enzymes involved in all of these repair pathways can be regulated with varying degrees depending on cell/tissue type. In these cases, upregulation leads to resistance. However, as will be seen in the next Section, MMR defects or lack of MMR protein expression leads to resistance to a wide variety of DNA damaging agents.

1.2.6. Cell cycle arrest

A cell can respond to DNA damage by arresting the cell cycle. This limits the chances of DNA replication occurring passed potentially mutagenic lesions and presumably allows time for correctable damage to be repaired. Any variance in this capability (mutations/polymorphisms in genes controlling this response) will affect the sensitivity a cell has to DNA damaging agents. The cell cycle has specific checkpoints at which arrest can occur, either before DNA synthesis (G_1 arrest) or after DNA synthesis (G_2 arrest). In mammalian cells, the products of the p53 and p21 genes have a key role in

response (See Section 1.3.5). Cisplatin, an agent that crosslinks DNA, is known to induce predominantly a G_2 arrest in mammalian cells (Eastman, 1990) although the drug also induces p53 and p21 expression (Anthoney *et al,* 1996).

1.2.7. Cell death

The chemotherapeutic agent cisplatin exerts its cytotoxic effects by forming covalent adducts on DNA, blocking polymerases, and preventing replication, transcription and cell division (Bruhn *et al,* 1990). Consequently, mammalian cells characteristically die by apoptosis, or programmed cell death, in which nuclear condensation, blebbing and cellular shrinkage results. In unicellular organisms such as yeast, this process is called autolysis and can also be induced by genotoxic agents (Megan *et al,* 1996).

As well as being crucial during the development of multicellular organisms, this response may function to ensure the elimination of cells (unicellular or somatic) that are too severely damaged for normal functioning. Any cell with malfunctioning cell death pathways or those with uncoupled signal transduction pathways which connect damage detection systems to the cell death mechanism will become tolerant to the damage sustained (See Section 1.3.6). Chemotherapeutic agents like cisplatin are known to trigger cell death in mammalian cells (Eastman, 1990) and in yeast (Megan *et al,* 1996).

In mammalian cells, the Bcl-2 family of genes are important regulators of apoptosis and among these, Bcl-2 and Bax control cell death, thus contributing to both tumour growth and drug sensitivity. After treatment with cisplatin, paclitaxel and other chemotherapeutic agents, Bax expression was shown to change from negative to positive in TC901 cells. Bcl-2/Bax status was correlated with drug sensitivity and treatment with chemotherapeutic agents induced apoptosis in these cancer cells (Kawakami *et al,* 1999).

1.3. The Role of MMR Deficiency in Drug Resistance

Bacterial and yeast strains as well as the more recently isolated mammalian cell lines that harbour defects in MMR demonstrate chemoresistance to a wide variety of DNA damaging agents. Various theories have been constructed to explain how this phenomenon exists and the following sites the literature used to support these hypotheses.

1.3.1. MMR-deficiency and resistance to alkylating agents

The involvement of MMR in the processing of O-6-methylguanine DNA residues in *E. coli* has been known for quite some time (Karran and Marinus, 1982). After *E. coli* MMR mutants (Eadie *et al,* 1984) and human lymphoblastoid B-cell lines were shown to be tolerant to the DNA methylating agent N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) (Goldmacher *et al,* 1986), Kat and colleagues (1993) reported that derivatives of these human cell lines were defective in strand-specific mismatch repair. Extracts from these cell lines also failed to form complexes with substrates containing methylated bases (Griffin *et al,* 1994; Karran and Bignami 1994) and from here on, human MMR became heavily implied in the recognition and sensitisation of cells to methylation damage.

Subsequently, the human colorectal adenocarcinoma cell line HCT116 known to be deficient in *MLH1* due to a homozygous mutation in *MLH1* resulting in a truncated non-functional product, displayed a 200-fold increase in colony forming ability after 10 days exposure to MNNG compared to an HCT116+Ch.3 transformant (chromosome 3 containing MLH1) (Koi *et al,* 1994). Moreover, the homozygous *MSH2* deficient

human endometrial adenocarcinoma cell line HEC59 was shown to be resistant to MNNG compared to a chromosome 2 transformant (HEC59) which re-introduced *MSH2* (Umar *et al,* 1997). Similarly, the *PMS2*-deficient endometrial adenocarcinoma cell line HEC-1-A was shown to be more resistant to MNNG when compared to MMR-proficient KLE cells (Risinger *et al,* 1995).

The dominance of the MMR-defective alkylation tolerant phenotype over the hypersensitive phenotype resulting from O^6-alkylguanine-DNA alkyltransferase (AGT)-deficiency was revealed by Branch *et al,* (1995). MMR proficient cells that lack the DNA repair enzyme AGT (or O^6-methylguanine-DNA methyltransferase, MGMT), (termed mex$^-$ cells for MGMT expression) cannot remove the alkyl group from the O^6 position of guanine and become sensitive to the cytotoxic lesion (Branch *et al,* 1995; Liu *et al,* 1996). Even though the human colorectal adenocarcinoma cell line SW48 was not shown to express detectable levels of the enzyme MGMT, it was seen to be highly resistant to N-methyl-N-nitrosourea and also slightly to methyl methanosulphonate (Branch *et al,* 1995). This cell line displayed a spontaneous mutator phenotype, MI and lack of *hMLH1* mRNA (Liu *et al,* 1995), indicating that MMR-deficiency overrides the sensitivity conferred by loss of methylation repair.

A study using yeast as an isogenic model for alkylation tolerance then demonstrated that deletion of any of the MMR genes, *MLH1*, *MSH2*, *MSH3*, *MSH6* and *PMS1* did not rescue *mgt*Δ O^6 MeG DNA repair methyltransferase-deficient cells from killing by MNNG (Xiao *et al,* 1995; Bawa and Ziao, 1997). However, a strain (XS-14) carrying a mutated form of the *MSH5* gene, but not an *msh5*Δ null mutant, was responsible for cellular tolerance to MNNG (Bawa and Ziao, 1997). These cells did not display a mutator phenotype but did have a meiotic defect (spore inviability) consistent with the role *scMSH5* has in meiosis (Hollingsworth *et al,* 1995). It was suggested that some mutated forms of *MSH5* and possibly of MutS homologues in mammalian cells may be involved in alkylation tolerance.

Another methylating agent, busulphan, has been reported to be, at least in part, dependent on MMR for cytotoxicity. A MMR-proficient human glioblastoma

multiforme xenograft was more sensitive to busulphan than a procarbazine-selected MMR-deficient derivative (Freidman *et al,* 1997). However, the bifunctional methylator, 1,3-bis(2-chloroethyl)-1-nitrosourea that forms O^6-chloroethylguanine DNA adducts, and the nitrogen mustards, melphalan and perfosfamide (an active form of cyclophosphamide) were not shown to be differentially cytotoxic to MMR-proficient or deficient cell lines (Rode *et al,* 1996).

1.3.1.1. The abortive MMR hypothesis

During these studies, theories attempting to resolve the precise mechanism of MMR deficient-mediated resistance have been constructed. A mechanism of MMR-dependent cytotoxicity exerted by methylation damage has been proposed. The methylating agents MNU, MNNG, and the clinically relevant monofunctional methylating drugs procarbazine and its activated form, temozolamide form a variety of DNA adducts of which O^6-methylguanine is the most cytotoxic (Fink *et al,* 1998). In the case of O^6-methylguanine adducts, MMR only seems to recognise O^6-methylguanine opposite a thymine mispair (Griffin *et al,* 1994; Duckett *et al,* 1996). Such mispaired errors occur after DNA replication and one hypothesis is that MMR, after recognising the mismatch, will continue to replace the thymine with another thymine opposite the unrepaired distorting lesion after each replication cycle. Such futile attempts at repair is envisioned to increase the chances of a double-strand break being encountered at the time of the next S-phase – an event that triggers apoptosis (Karran *et al,* 1994).

However, the abortive MMR theory is not conclusive for several reasons. Support for the theory comes from the fact that MGMT-deficient (mex^-) cells require MMR for sensitivity to MNNG which takes away the dominance of direct genotoxicity of unrepaired O^6 MeG. However, 1) the mammalian cell lines used were not mutant for MGMT. Some were transcriptionally down regulated (Ostrowiski *et al,* 1991) and others actually interconvertible (Cairns-Smith and Karran, 1992); 2) alkylation tolerance was only achieved after several rounds of selection under mutagenic

conditions (Goldmacher *et al,* 1986) so the phenotype could be the manifestation of several mutations, and 3) these cell lines were derived from tumours that possibly harboured other mutations that could effect alkylation tolerance.

Furthermore, the study using isogenic yeast MMR mutants does not provide strong concomitant evidence to support what is seen in human tumour cell lines (Bawa and Xiao, 1997). Finally, 6-thioguanine is an agent that can be incorporated into DNA, subsequently causing mispairing with thymine (see Section 1.2.3). Because correct pairing with 6-thioguanine adducts can occur, studies suggest that the miscoding frequency of this lesion is not high enough for the level of toxicity observed (Rappaport, 1993). However, despite these criticisms, the abortive theory has yet to be disproved.

1.3.2. MMR-deficiency and resistance to DNA platinating agents

Cis-Diaminedichloroplatinum(II), or cisplatin, is widely used in the treatment of ovarian, testicular and head and neck carcinomas (Loehrer and Einhorn, 1984). The popularity of this agent as an effective treatment for these tumours is marred by the emergence of drug resistance, representing a major factor in treatment failure (Kaye, 1996). A substantial body of evidence indicates that cisplatin exerts its cytotoxic effect by forming DNA platinum adducts, primarily in the form of intrastrand crosslinks at the N-7 positions between adjacent purines (mostly guanines) (Sherman and Lippard, 1987). Of these, 1,2 intrastrand crosslinks comprise about 90% and 1,3 crosslinks account for a further 5% (Fichtinger-Schepman *et al,* 1985). The 1,3 crosslink is efficiently removed by nucleotide excision repair (Moggs *et al,* 1996) but the predominant 1,2 crosslink remains refractory to removal by this pathway (Szymkowski *et al,* 1992) (See Fig. 4).

Carboplatin is a structurally related molecule to cisplatin, the only difference being the addition of a 1,1-cyclobutanedicarboxylato-leaving group (Fig. 3). The types of DNA adducts caused by cisplatin and Carboplatin are the same. Carboplatin has a slower

reaction time with DNA, a shorter pharmacokinetic half-life and is generally less toxic than cisplatin (Cavalli *et al*, 1997). Oxaliplatin has recently been shown to be effective in colorectal cancer treatment (Links M, *personal communication*); the spectrum of DNA adducts being the same despite its larger molecular size. The inactive *trans* isomer, transplatin does not cause intrastrand crosslinks (citing evidence for the importance of this lesion for cytotoxicity) (Calvert *et al*, 1993) and is not used clinically.

Figure 3 Structures of platinum drugs.

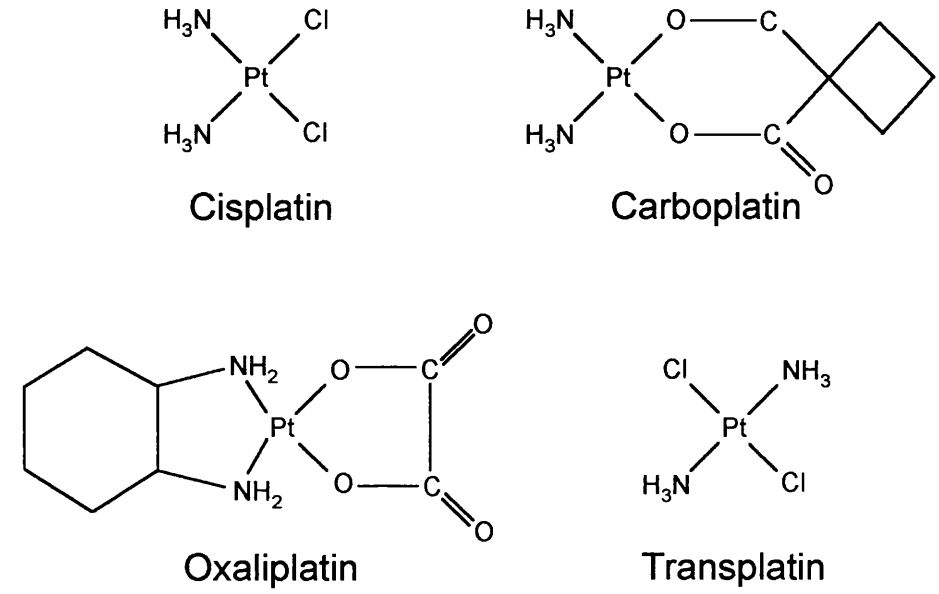
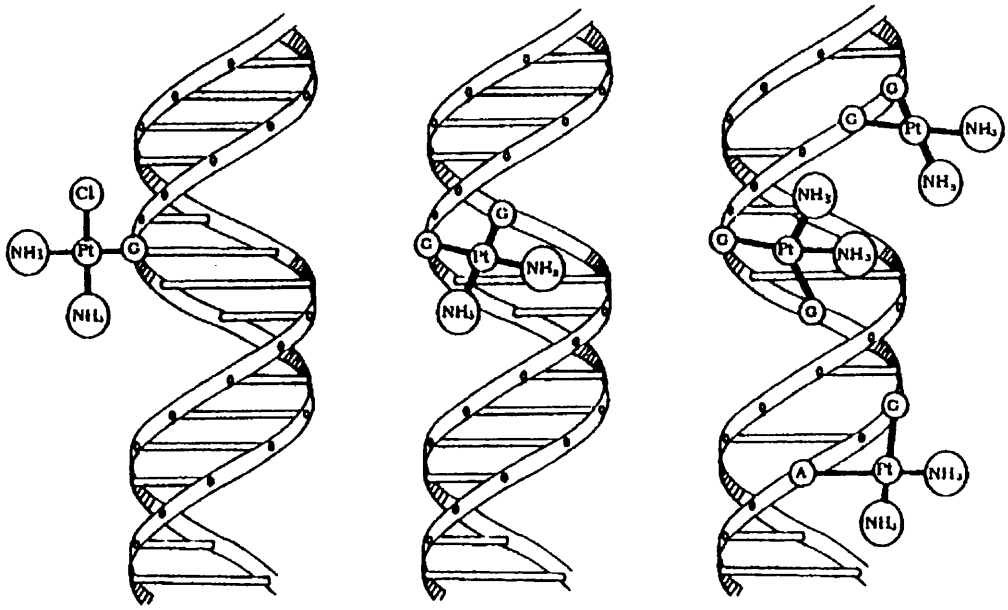


Figure 4 Illustration of DNA adducts formed by cisplatin

(courtesy of Brown and Bissett, 1993)



The first indication of an involvement of MMR in cellular sensitivity to platinum compounds came from Fram *et al,* (1985) who showed that cisplatin hypersensitive *dam E.coli* mutants became resistant by introduction of mutant *MutS* or *MutL.* It was not until studies using tumour cell lines with known defects in MMR that the same was shown to be true in mammalian cells. Anthoney *et al,* (1996) demonstrated that cisplatin resistant ovarian cell lines acquired MI and that this was associated with defects in strand-specific mismatch repair (Drummond *et al,* 1996). It was also seen in cisplatin-resistant human colon and endometrial cancer cell lines with deficiencies in hMLH1 or hMSH2 function that sensitivity to cisplatin could be complemented by chromosome transfer (Aebi *et al,* 1996).

It was then reported that pure hMsh2 bound to platinated DNA in mobility shift assays (Mello *et al,* 1996). The hMutSα heterodimer of MSH2 and MSH6 was then shown to

bind to the 1,2 intrastrand crosslink (Duckett *et al,* 1996) and the highest affinity of binding to this lesion was when a thymine was mispaired with a 3' guanine (Yamada *et al,* 1997).

Subsequently, it has been shown that 9/10 independent subclones of the A2780 ovarian carcinoma cell line, selected for resistance to cisplatin (and subsequently shown to be cross-resistant to MNU, 6-thioguanine and doxorubicin), lost protein expression of Mlh1 and Pms2 as measured by immunoassay (Brown *et al,* 1997). mRNA levels for *MLH1*, but not *PMS2*, were markedly reduced, possibly indicating that Pms2 was unstable without its MutLα partner. Both the parental and resistant subclones retained both copies of the *MLH1* gene. It was also reported from a small sample population that 10% of ovarian tumours from patients that had not undergone chemotherapy were negative for MLH1 expression as measured by immunoblot, compared to 36% of tumours samples taken at second look laparotomy from patients after cisplatin and cyclophosphamide chemotherapy (Brown *et al,* 1997). Although sample size was small (n=50), these data may indicate that tumour cells *in vivo* that lose expression of Mlh1 and become resistant to cisplatin, survive treatment.

Further support for this comes from a study which showed that a variety of drugs could enrich for MMR-deficient cells. A 50:50 mixed population of MMR-proficient and -deficient cells were exposed to cisplatin, Carboplatin, doxorubicin, etoposide or 6-thioguanine. Each drug enriched for MMR deficient cells and these cells became clonogenically resistant to the drug used (Fink *et al,* 1998).

1.3.2.1. The replication stalling model

An alternative model to the abortive, futile cycles of repair hypothesis (Karran and Bignami, 1994), to explain MMR-deficient-induced resistance has been proposed by Brown *et al,* (1997). Here, it is proposed that cisplatin adducts cause replication stalling in MMR proficient cells which leads to cell death. Absence of MMR, or at least components of MMR, allows replication bypass of the lesion and cell survival.

Support comes from the fact that cellular proliferation and hence presumably DNA replication is required for induction of apoptosis by cisplatin (Evans *et al,* 1994). Indeed, it has been shown that cisplatin-resistant *hMLH1* and *hMSH6* defective cell lines displayed 2.5-6 fold increases in replicative bypass of cisplatin adducts (Vaisman *et al,* 1998). However, the authors interpret this as MMR-deficiency contributing to increased replicative bypass and therefore to drug resistance by preventing the lethality exerted by futile rounds of translesion synthesis (abortive theory).

Studies have shown that DNA polymerase δ and ε are able to bypass 1,2 intrastrand crosslinks induced by cisplatin in structures that resemble replication forks (Hofmann *et al,* 1996). The antibiotic, aphidicolin, inhibits DNA polymerases α, δ and ε by binding to polymerase nucleotide binding sites (Wood and Shivji, 1997). Recently, it was shown that aphidicolin increases the sensitivity of MMR-deficient ovarian carcinoma cell lines to cisplatin and MNU to a greater extent than their MMR-proficient counterparts (Moreland *et al,* 1999). It is proposed that by inhibiting the elevated levels of lesion bypass in MMR-deficient cells, replication stalling at the lesion reduces drug tolerance and leads to cell death.

1.3.3. MMR deficiency and resistance to other drugs

6-Thioguanine is not an alkylating agent in itself but after incorporation into DNA, it can be methylated by S-adenosylmethionine to form S^6-methylthioguanine. This is prone to mispairing with thymine which is recognised by MutSα (Swann *et al,* 1996). Even its natural pairing with cytosine is recognised by MutSα (Griffin *et al,* 1994; Waters and Swann, 1997) and MMR-deficient cells have been shown to be 5- to 10-fold more resistant to 6-thioguanine (Aebi *et al,* 1997; Hawn *et al,* 1995). Adducts produced by 6-thioguanine are not good substrates for MGMT (Swann *et al,* 1996) and so would be expected to persist in DNA until encountered by MMR.

Loss of mismatch repair has also been associated with resistance to the antibiotic, doxorubicin (adriamycin) (Drummond *et al,* 1996) and the topoisomerase II inhibitor, etoposide (Aebi *et al,* 1997). Doxorubicin forms a variety of adducts in DNA and so the substrates recognised by MMR induced by this drug are less clearly defined. Even less well understood is the mechanism of resistance conferred by MMR deficiency to etoposide. This agent is not known to interact directly with DNA so mechanisms of MMR-induced cytotoxicity are speculative. It could be that MMR recognises and signals the presence of certain damaged structures caused by the inhibition of DNA unwinding normally carried out by topoisomerase II.

1.3.3.1. The mutator theory

The development of cross-resistance to a variety of drugs has led to the theory that MMR deficiency, which is associated with an elevated mutator phenotype, leads to global genomic mutations. These may include mutations in cytoplasmic resistance genes that encode for proteins responsible for actions such as detoxification and drug transport. A long established axiom has described tumourigenesis as a form of somatic evolution; a concept that places the selection of advantageous mutations as the underlying mechanism of the multistep process towards neoplasia (*for review, see* Tomlinson *et al,* 1996). This has been extended to explain the development of cross-resistance to drugs in cells that have lost MMR. It has been suggested using mathematical models that MMR-defective cells that have an elevated mutation rate will, over repeated rounds of division, accumulate mutations in resistance genes (Tomlinson *et al,* 1996).

Recently, it was shown that exposing MMR-proficient and deficient cell lines to cisplatin led to cross resistance to topotecan, gemcitabine and possibly paclitaxel – drugs commonly combined with cisplatin in cancer treatment regimens (Lin and Howell, 1999). Indeed the folds of resistance observed with these drugs were greater than those obtained for cisplatin.

The mutator theory predicts that re-introducing MMR back into resistant cells will have no effect on drug resistance because the cell would have undergone the fixation of mutations involved in drug resistance. However, there are a number of complementation studies examining the involvement of MMR in engaging cell death that dispute this. Sensitivity to MNNG is restored in HCT116-36 chromosome 3 transformants where the *MLH1* gene was re-introduced (Koi *et al,* 1994) and the homozygous *MSH2* deficient human endometrial adenocarcinoma cell line HEC59 was shown to be more resistant to MNNG compared to an *MSH2*-restored HEC59 Chromosome 2 derivative (Umar *et al,* 1997). Sensitivity to cisplatin in human colon

and endometrial cancer cell lines with deficiencies in Mlh1 or Msh2 function could be complemented by chromosome transfer (Aebi *et al,* 1996). However, the mechanism(s) of resistance conferred by loss of MMR to drugs such as etoposide and doxorubicin remain elusive.

1.3.4. The response of MMR-defective cell lines to ionising radiation

The evidence surrounding the involvement of MMR in cytotoxic responses to ionising radiation is inconsistent. *MLH1*-defective mouse cells were shown to be slightly more resistant to γ-irradiation than MMR competent controls (Fritzell *et al*, 1997) but *hMLH1*-deficient HCT116 colorectal cancer cells were shown to be slightly γ-irradiation sensitive and showed defects in G_2 cell cycle arrest (*see* Section 1.2.5) (Davis *et al*, 1998). No difference was seen between MSH2-proficient and deficient cells defective in NER in response to IR (O'Driscoll *et al*, 1999).

The role of MMR in IR-induced DNA damage processing is unclear. IR is known to cause single and double-strand breaks in DNA (Hiom, 1999). It has not been shown that MMR proteins bind to double-stranded breaks, although single-stranded DNA is targeted by MMR in nick-directed repair and is a substrate for homologous recombination - a pathway known to be regulated by MMR (see Section 1.1.3).

1.3.5. MMR and cell cycle arrest

There is substantial evidence supporting an active involvement of proteins of mismatch repair in the G_2 cell cycle arrest response to DNA damage. Although it has been shown that certain components of MMR are themselves cell cycle regulated (Kramer *et al,* 1996; Harris *et al,* 1997), evidence for a functional role of MMR proteins in the induction of cell cycle arrest comes from several studies using human cell lines. MMR-deficient human colon carcinoma (HCT116) cells which lacked Mlh1 expression were more resistant to 6-thioguanine and lacked a G_2-M arrest response compared to the 6-thioguanine-treated MMR proficient (HCT116 3-6) derivative (Koi *et al,* 1994; Hawn *et al,* 1995). Incidentally, a study that re-introduced wild type *hMLH1* in HCT116 cells showed that cellular proliferation and rate of DNA synthesis was markedly reduced (Shin *et al,* 1998).

In a study that used seven MMR-deficient and proficient human cancer cell lines (with and without confirmed mutations in *MLH1* or *MSH2*), G_2 growth arrest in response to MNNG treatment was seen in the proficient cell lines. The MMR-deficient cells, however, escaped an initial G_2 delay and resumed a normal growth pattern (Carethers *et al,* 1996). Radio-sensitive, MMR-deficient HCT116 cells also showed a reduced delay of re-entry into the cell cycle after G_2 arrest in response to ionising radiation (Davis *et al,* 1998). Similar IR responses were also noted between murine *mlh1* knockout compared to wild-type primary embryonic fibroblasts (Davis *et al,* 1998).

Furthermore, in addition to its role in G_1 arrest induction, p53 is known to be required for sustained G_2-M arrest after DNA damage (Bunz *et al,* 1998; Innocente *et al,* 1999) and it has been shown that the MMR-deficient ovarian carcinoma (A2780-cp70) cell line shows loss of p53 function as measured by reduced $p21^{CIP1}$ mRNA compared to the MMR-proficient (A2780) derivative in response to cisplatin (Anthoney *et al,* 1996).

To explain the apparent involvement of MMR in G_2-M arrest as opposed to G_1 arrest, the following studies suggest that DNA replication is required for MMR-dependent DNA damage processing. The MutS complex is known to bind to the DNA 1,2 intrastrand crosslink with much greater affinity if opposite a mismatched base, suggesting that DNA synthesis past the lesion is required for recognition (Duckett *et al,* 1996; Brown *et al,* 1997). Also, MMR deficient ovarian carcinoma (A2780-cp70) cells that show resistance to cisplatin compared to proficient (A2780) cells have a significantly reduced MMR-dependent resistance phenotype when treated with aphidicolin - a DNA polymerase inhibitor (Moreland *et al,* 1999).

1.3.6. MMR and the signal transduction pathway for cell death

Eukaryotic cells respond to the presence of cisplatin adducts in DNA by activating signal transduction pathways that result in cell cycle arrest, an increase in the activity of certain types of DNA repair, and apoptosis (Eastman, 1990). Although the signalling pathways activated as part of the cisplatin-induced injury response are as yet poorly characterised, studies have demonstrated that the response involves activation of the stress-activated protein kinase JNK and the nuclear c-Abl protein kinase (Kharbanda *et al,* 1995).

JNK phosphorylates the transcription factors c-Jun, activator of transcription factor 2, and Elk-1 and stimulates their transcriptional activities (Hibi *et al,* 1993; Gupta *et al,* 1995; Cavigelli *et al,* 1995). c-Abl associates with the retinoblastoma (Rb) protein thus indicating a role in cell cycle control (Welch and Wang, 1993). c-Abl has also been shown to phosphorylate and stimulate the transcription of RNA polymerase II (Welch and Wang, 1993).

Nehme and colleagues (1997) investigated the effect of loss of MMR function on the ability of cisplatin to activate the stress-activated protein kinase JNK and the protein tyrosine kinase c-Abl, as these were reported to be activated in response to a variety of DNA damaging agents (Kharbanda *et al,* 1995). They showed that cisplatin activated JNK 3.8-fold more efficiently in MMR-proficient that MMR-deficient cells and that c-Abl activation was completely absent in MMR-deficient cells (Nehme *et al,* 1997). This suggests that JNK and c-Abl may be targets for the detector proteins of MMR in coupling cisplatin-induced DNA damage to apoptosis.

In addition, it has recently been demonstrated that cells proficient in MMR increased the level of the p53-related protein, p73 in response to cisplatin (by increasing its half-life) (Gong *et al,* 1999). p73 can activate p53- responsive promoters and induce

apoptosis when overexpressed in certain p53-deficient tumor cells. p73 encodes several isoforms including p73 alpha and p73 beta, each of which can induce permanent growth arrest with markers of replicative senescence when overexpressed in a tetracycline-regulatable manner in human cancer cells lacking functional p53 (Jost *et al,* 1997; Fang *et al,* 1999).

Gong *et al,* (1999) recently showed that the p73 half-life in MMR-deficient HCT116 cells lacking the *MLH1* gene was not increased in response to cisplatin compared to levels in MMR proficient cells. Furthermore, c-Abl enhanced the function of p73 in cisplatin-treated MMR proficient cells by stabilising the protein. These proteins have indeed been shown to associate with each other (Agami *et al,* 1999; Yuan *et al,* 1999). c-Abl was found to be inactivated in MMR-deficient cells in response to cisplatin (Gong *et al,* 1999). It was also demonstrated that cisplatin could induce two parallel death-response pathways, one dependent on p53 and the other on p73. p53 was induced by cisplatin in Mlh1- and c-Abl-deficient murine embryonic fibroblasts indicating the lack of requirement for these proteins in the p53 response. p73 was induced in p53-deficient cells in response to cisplatin but not in the absence of Mlh1 or c-Abl (Gong *et al,* 1999). These results indicate that c-Abl and p73 are components of a MMR-dependent apoptosis pathway which contributes to the cytotoxic effect of cisplatin.

1.4. Objectives of Thesis

From the existing evidence, it seems that the mechanisms underlying MMR-dependent cytotoxicity are multifactorial. The generation of the lethal signal is still under debate and the spectrum of different types of DNA lesions caused by varying agents is still unclear. In addition, a large amount of eukaryotic MMR homologues have been identified, many of which have no known function. To this end, this thesis aims to investigate what genes and mechanisms are involved in eukaryotic MMR-dependent responses to DNA damage.

1.4.1. Cisplatin as a model DNA damaging agent

The following observations explain why the cytotoxic DNA damaging agent cisplatin was chosen for the study of MMR-dependent cellular responses.

a) the recognition proteins, MutS homologues, of MMR bind with strong affinity to the 1,2 intrastrand crosslink,

b) this adduct is poorly repaired by NER. Transplatin, which does not induce this adduct, is not active which suggests that this is a cytotoxic lesion.

c) previous studies using human cell lines have reported MMR deficiency in response to cisplatin exposure and that this selection process lead to resistance to other DNA damaging drugs.

1.4.2. *S. cerevisiae* as a model organism

Particularly in the field of DNA repair, the use of microbiological systems to study human mechanisms has proven invaluable because of the homologous systems that have persisted through evolution. The yeast *S. cerevisiae* has gained popularity as a model for pre-screening new drugs, elucidating their mode of action and understanding

the mechanisms that regulate their effects on biological systems (Hannan 1988; Nittis and Wang 1988; Delitheos *et al,* 1995; Hartwell *et al,* 1997). This has recently become important due to the completion of the *S. cerevisiae* genome sequence database. One major advantage of using *S. cerevisiae* over human cell lines is the guarantee of isogenicity when comparing phenotypes of strains with known gene manipulations. To this end, this thesis takes advantage of the *S. cerevisiae* model in the study of eukaryotic MMR.

1.4.3. Aims

1) To find out if *S. cerevisiae* strains defective in certain MMR genes have altered cytotoxic responses to cisplatin. This will be carried out by setting up a standard clonogenic assay for specific isogenic strains of *S. cerevisiae* defective in individual genes involved in MMR.

2) To explore specifically which MMR genes in *S. cerevisiae* are involved in cisplatin cytotoxicity

3) To set up a complementary gene re-introduction system to investigate if restoring MMR function in MMR-defective *S. cerevisiae* strains also directly restores responses to cisplatin

4) To identify if other DNA repair genes i.e. *RAD52* and *RAD1* involved in recombination and excision repair, are involved in MMR-dependent responses to cisplatin

5) To examine if *S. cerevisiae* MMR genes are involved in cell cycle arrest in response to a variety of DNA damaging agents, i.e. cisplatin, transplatin (control), ultraviolet radiation and ionising radiation.

EQUIPMENT

2. EQUIPMENT

Hybaid Touchdown PCR Thermal Cycler

Hybaid Omnigene PCR Thermal Cycler

Innova 4300 Incubator shaker - New Brunswick Scientific

Innova 4000 Incubator shaker - New Brunswick Scientific

Omega Prestige Medical autoclave

Flow laboratories CO_2 incubator 220

Swan U170-80H -70°C refrigerator

Stratagene UV Stratalinker 2400

Voss eppendorf Rotator

MixiMatic shaker - Jencons Scientific Ltd

MSE Soniprep 150 sonicator

Hetovac speedivac DNA prep dryer

IEC Centra-7R refrigerated centrifuge

Sorvall Instruments - Dupont RC3C refrigerated centrifuge

Sorvall Instruments - Dupont RC5B refrigerated Superspeed centrifuge

IEC Micromax centrifuge

Luckham 4RT rocking table

Corning pH meter 240

Pharmacia Biotech Electrophoresis power pack supply EPS 600

ABI 373A DNA sequencer

MPH Multipurpose gel electrophoresis chamber

Bio-Rad gel doc 1000 UV box

Zeiss Axioskop fluorescent microscope connected with JVC ½ INCH CCD digital videocamera

Applied Biosystems 392 DNA/RNA oligonucleotide synthesiser

Beckman DU 650 Spectrophotometer connected to IBM Personal system 2 colour display and Epson LX-800 dot matrix printer

Techne Dri-Block DB2A and DB3D heating blocks

Mettler AE100 and PM300 weighing balances

Bigneat Ltd Captair fume hood

Becton Dickinson Facsan linked to Apple Macintosh Quadra 650 with CellQuest software

Oce 3107c digital colour printer

Kodak ColourEase printer

Pdi 420oe 1-D gel scanner

REAGENTS

3. REAGENTS

Buffers, solutions and media

Yeast Peptone Dextrose Media

10g/500ml Difco Peptone
5g/500ml Yeast extract

Add 475ml H_20, adjust pH to 5.8 and autoclave in pressure cooker.
Cool to ~50°C and add dextrose (glucose) to 2% by adding 25ml of a 40% stock solution (filter sterilise) to each 500ml of medium.

Synthetic dropout media

Amino acids	**mg**
adenine	800
arginine	800
aspartic acid	4000
histidine	800
leucine	800
lysine	1200
methionine	800
phenylalanine	2000
threonine	8000
tryptophan	800
tyrosine	1200
uracil	800
isoleucine	1200 (optional)

valine 600 (optional)

Grind powders up together and store at RT. Leave out the amino acid that you are making dropout. Everything added for synthetic complete.

870 mg/L

6.7g yeast nitrogen base w/o amino acids

20g glucose

1L dH_20 then pH to 5.5 -6.0 using 1M NaOH. Autoclave.

9g/500ml Agar (if needed for plates)

DNA loading dye

0.25% Bromophenol blue

0.25% Xylene cyanol

30% Glycerol

L-broth

1% Tryptone

0.5% Yeast extract

1% NaCl

Phosphate buffered saline (PBS)

0.8% NaCl

0.115% Na_2HP04

0.02% KCl

0.02% KH_2PO4

PLATE solution

90ml sterile 45% PEG 4000 (Sigma)

10ml 1M lithium acetate

1ml 1M Tris-HCl, pH 7.5

0.2ml 0.5M EDTA

vortex mix.

TBE (1x)

89mM Tris borate

89mM Boric acid

2.5mM EDTA

***Qiagen®* Plasmid Purification buffers:**

Buffer	**composition**	**storage**
Buffer P1 (Resuspension Buffer)	50mM Tris·Cl, pH 8.0; 10mM EDTA; 100µg/ml RNase A	4°C
Buffer P2 (Lysis Buffer)	200mM Na OH, 1% SDS	room temperature
Buffer P3 (Neutralisation Buffer)	3.0M potassium acetate, pH 5.0	room temperature or 4°C
Buffer QBT (Equilibration Buffer)	750mM NaCl; 50mM MOPS, pH 7.0 15% isopropanol; 0.15% Triton® X-100	room temperature
Buffer QC (Wash Buffer)	1.0M NaCl; 50mM MOPS, pH 7.0 15% isopropanol	room temperature
Buffer QF (Elution Buffer)	1.25M NaCl; 50mM Tris, Tris·Cl, pH 8.5 15% isopropanol	room temperature

Buffer QN (Elution Buffer)	1.6M NaCl; 50mM MOPS, pH 7.0; 15% isopropanol	room temperature
STE	100mM NaCl; 10mM Tris·Cl, pH 8.0 1mM EDTA	room temperature
TE	10mM Tris·Cl, pH 8.0 1mM EDTA	room temperature

CDS (Chromatin denaturation solution):

Dissolve 2.5ml 4 *N* HCl, 0.5g Triton X-100 (Sigma), 1.75g NaCl in 97.5ml dH_2O. Heat to dissolve Triton X-100 and cool on ice before using to treat fixed cells.

Formaldehyde solution (0.25% v/v):

Mix 0.156ml formaldehyde solution (16% ultrapure formaldehyde, EM Grade, Polyscience, Warrington, PA) in 10ml PBS.

Zymolyase solution (40U/ml):

Dissolve 20mg 20T zymolyase (20U/mg; ICN ImmunoBiochemicals, Lislie, IL) in 10ml PBS; prepare solution right before use and store on ice.

FACS buffer:

211mM Tris, 78mM NaCl, 75mM MgCl pH 7.8

METHODS

4. METHODS

4.1. *Saccharomyces cerevisiae* mutant strains

Haploid isogenic *S. cerevisiae* strains were obtained in collaboration with Dr. Rhona Borts of the Yeast Genetic department, Institute of Molecular Medicine, John Radcliffe Hospital, Oxford, UK. All strains are derivatives of RHB2096-1b (*ura3-1 lys2-d met13-4 cyh2 leu2-R MATα*). (Hunter and Borts, 1997). Strains were generated by complete gene deletion using PCR-mediated recombinational insertion of the *LEU2* or *KANMX* cassette (Wach *et al,* 1994; Hunter and Borts, 1997).

Description	Strain
Wild type	2096-1B
Δ*msh2*	RHB 2348
Δ*msh3*	RHB 2347
Δ*msh6*	NHT 173
Δ*mlh1*	RBT 311
Δ*mlh2*	RBT 324
Δ*pms1*	RBT 289
Δ*rad52*	RHB 2692
Δ*rad52*/Δ*msh2*	RHB 2700
Δ*rad52*/Δ*mlh1*	RHB 2698
Δ*rad52*/Δ*mlh2*	RHB 2699
Δ*rad1*	RBT 302
Δ*rad1*/Δ*msh2*	RHB 2694
Δ*rad1*/Δ*mlh1*	RHB 2693
Δ*rad1*/Δ*mlh2*	RHB 2695

Upon arrival, strains are inoculated onto YPD agar plates, grown for 2-3 days in 30°C and each lawn is scraped off and inoculated into 15% glycerol stocks and stored at 70°C.

4.2. Drug treatment of *S. cerevisiae* strains

1. Scrape off cells from frozen stock and grow to saturation in 10ml YPD medium by incubating universal for ~2 days in a 30°C shaking incubator.
2. Dilute cells to $2x10^7$ cells/ml, with a range of DMSO dissolved cisplatin concentrations e.g. 0, 0.5, 1, 1.5, 2mM cisplatin.
3. Incubate cells in a universal tube in 30°C shaking incubator for 24hrs.
4. (Take an aliquot for fixing with 70% ethanol for FACS analysis). Dilute and plate out cells on solid YPD at 400 cells per plate.
5. Incubate four plates per concentration of cisplatin for ~2 days in a 30°C incubator.
6. Count colonies and calculate percent survival relative to controls.

4.3. Irradiation of *S. cerevisiae*

1. Scrape off cells from frozen (expanded) stock and grow to saturation in 10ml YPD medium by incubating universal for ~2 days in a 30°C shaking incubator.
2. Dilute cells to $2x10^7$ cells/ml. Place universals under a source of γ-radiation emitter (Cobalt-60) and expose to 100gy (dose equivalent at the time was: 58mins of 1.43gy/min)
3. Immediately after irradiation take an aliquot for fixing with 70% ethanol for FACS analysis/Fluorescent microscopy - see section 5.8 & 5.9. Dilute and plate out cells on solid YPD at 400 cells per plate.
4. Incubate plates for ~2 days in a 30°C incubator. Count colonies and calculate percent survival relative to controls.

4.4. *S. cerevisiae MLH1* gene re-introduction

4.4.1. PCR of *S. cerevisiae MLH1*

cDNA from *Saccharomyces cerevisiae* (Sigma) was used for amplifying *ScMLH1*. Polymerase chain reaction of *ScMLH1* was carried out in-frame including the 5' ATG start site and ending at 3' TAA stop codon. The following cycle was performed:

94°C	5 mins	
94°C	1 min	x 30 cycles
Annealing temp.	1 min	
72°C	1 min	
72°C	5 min	

Hold at 15°C

4.4.2. *S. cerevisiae MLH1* oligonucleotide primers

F-BamHI:

5'- TCGCCG GGATCC ATG TCT CTC AGA ATA AAA GCA -3'

BamHI start ANNEALING TEMP: 48.2°C

R-Mlu:

5'- AGGAT ACGCGT TTA ACA CCT CTC AAA AAC TTT G -3'

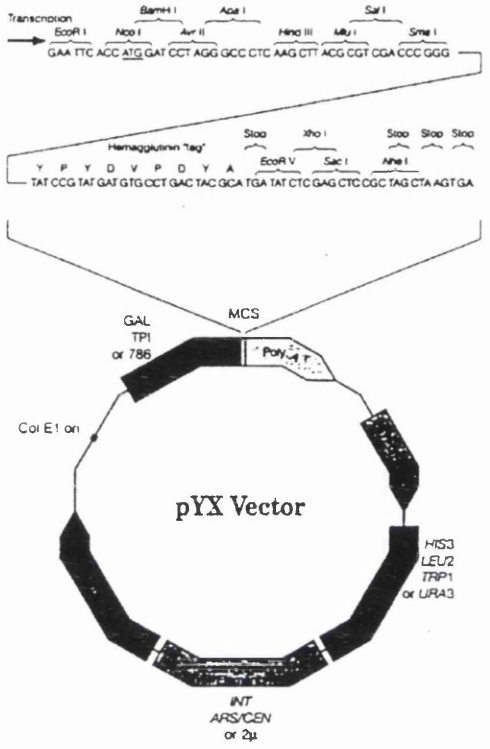
MluI stop ANNEALING TEMP: 48.6°C

Enzyme restriction sites *BamHI* and *MluI* were inserted into the primers as non-annealing sequences surrounded by 5' random sequences (to enable digestion). These ends were then used to clone into pYX212 after digestion.

4.4.3. Coding sequence for S. cerevisiae *MLH1* gene

See Appendix

4.4.4. Cloning *ScMLH1* into yeast expression vector



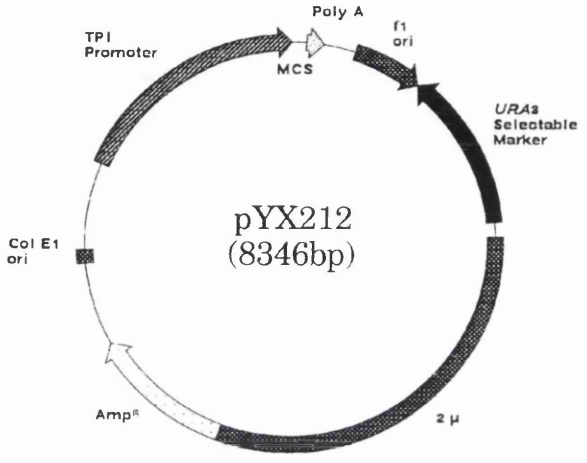


Figure 5 pYX212 yeast expression vector

(*R&D Systems*). 2μ /URA / TPI promoter - This replicates autonomously in yeast and is usually stable being maintained at 25-100 copies per cell. Selective pressure recommended for maintenance.

1. Digest the plasmid and PCR products (flanked with restriction sites) using BamHI and MluI. Always use 10x concentration of enzyme to DNA (i.e. equal volumes with 10U/μl enzyme. In small eppendorf, add in order:

   7μl dH_2O

   1μl appropriate buffer (10x)

   1μl DNA: ~1μg/μl of plasmid and ~1μg/μl insert

   1μl enzyme (10x) - put first enzyme in with lower salt buffer. Incubate at 37°C on heating block for 2-4hrs. Put on ice. Adjust salt concentration and add

second enzyme for sequential digest. Incubate at 37°C on heating block for 2-4hrs. Put on ice.

Make sure that the glycerol in enzyme solution is diluted by at least 1/10th final volume.

2. For ethanol precipitation of restriction enzyme-cut products:
   -add 3M NaAc (pH8) to give final conc. of 0.3M. i.e. 1/10th volume.
   - add 1µl glycogen (optional)
   - add 2 volumes ice cold ethanol and mix well. Place at -20°C or on dry ice for 0.5-1hr to allow DNA to precipitate.
   - spin at 490g (13,000rpm on Sorvall RC3C) for 15min at 4°C.
   - remove supernatent. Add 1ml 70% ethanol and put on dry ice (or -20°C) for 0.5hr. Respin at 490g for 15min at 4°C.
   - remove supernatant, allow to air-dry and resuspend in TE/water (pH 7.6) (20µl).

   Use 0.6 volumes isopropanol instead of ethanol to precipitate larger fragment (leaving unwanted small R.E product in supernatant).

4. Set up the following ligation reactions maintaining a final volume of not more than than 10µl:
   - transfer 50ng plasmid DNA to each tube (50ng/µl)
   - add 20x (1µg), 10x (500ng) and 5x (250ng) concentration of insert DNA
   - add 4µl 5x T4 DNA ligase buffer
   - add 2 units T4 DNA ligase (4units/ml)
   - add 2µl Bovine serum albumen (acetylated, from 10mg/ml stock)
   Adjust to 20µl with dH_20. Incubate at 16°C over night.

5. Purify DNA using *Wizard™PCR Preps DNA Purification System.* See Section 4.5

6. To detect successful ligation reaction in samples, set up electrophoresis gel by making up 100ml gel. Heat dissolve:

agarose	0.8g

	0.5x TBE	100ml
-While hot, add:	0.5μg/ml ethidium bromide	100μl of 0.5mg/ml

7. Set gel in chamber with comb and allow to cool for 20mins.
8. Emerse in tank filled with running buffer:

	0.5x TBE	400ml
(optional)	0.5μg/ml ethidium bromide	400μl of 0.5mg/ml

9. Load samples to each well containing:

2μl DNA

2μl 6x loading dye

8μl TE pH.8.0

10. Run at 80V, 100mAmps
11. Image gel under transilluminator.

4.4.5. *E.coli* DH5α transformation

Preparation of agar plates with Ampicillin for selection

1. Add 7.5g bactoAgar to 500ml LB.
2. Autoclaved LB bottles.
3. Add 100μg/ml Amp (final conc.) i.e. 1ml of 50mg/ml stock by filter sterilisation.
4. Poor into bacteria-grade petri-dishes up to line and leave to set.
5. Wrap in Parafilm "M" and store at 4°C.

E.coli (DH5α - competent strain) transformation.

1. Transfer 20μl thawed cells into chilled eppendorf tubes.
2. Add 1μl of plasmid DNA to separate tubes (1-10ng/μl), shake gently.
3. Put on ice for 30 mins.
4. Heat shock for 40 secs. in 42°C heat block, then place on ice.
5. Add 80μl LB medium and shake tubes at 225rpm for 1hr at 37°C.
6. Pipette all cells on to ampicillin-containing agar plate and spread evenly, incubate overnight.

4.4.6. Isolating DH5α colonies containing correctly sized vector inserts.

Vector Primers:
pYX112 F1 (187) 5' GTC TAG CTC CAG CTT TTG 3'
pYX112 R1 (6454) 5' CCT TTG CAT TAG CAT GCG 3'

pYX212 F1 (8154): 5' TAT ACC TTT GGC TCG GCT 3'
pYX212 R1 (187): 5' CAA AAG CTG GAG CTA GAC 3'

1. Prepare appropriate numbers of universals containing 5ml LB and thin-walled eppendorfs containing master-mix for PCR.
2. After growing bacterial colonies, use pipette tips or tooth picks to scrape off individual colonies and emerse in a) warmed up 10ml LB containing 100μg/ml Ampicillin (20μl of 50mg/ml stock) (go to step 3) and b) mastermix tube (go to step 7)
3. Shake in incubator at 37°C for 2-3hrs.
4. Pour 1 litre of LB into each flask, add 100μg/ml Amp (2ml of 50mg/ml) and warm in incubator.
5. Add 10ml of cells into flasks and shake at 37°C overnight.
6. Freeze down stock by placing 150μl autoclaved glycerol into plastic tubes, add 850μl cells and store at -70°C
7. PCR the bacterial plasmid template using vector primers and screen for correct insert. Using 0.5ml thin-walled tubes.

   Make up the following master mix for 50μl reaction:

10x PCR buffer	5μl
25mM MgCl	3μl
10mM dNTPs	1μl
Expand High Fidelity Taq polymerase	

(2-5 units/μl)	0.5μl
autoclaved dH_2O	36.5μl

to appropriate reaction:

primer 1 (10μM)	1μl
primer 2 (10μM)	1μl

8. Mix gently and layer 2 drops (~100μl) of mineral oil over the reaction.
9. PCR cycle (see 4.4.1)
10. Analyse 10 to 20μl of amplified sample using agarose gel electrophoresis.
11. Discard unsuccessful cultures and remaining liquid cultures.
12. Make glycerol preps of successful cultures (150μl pure glycerol to 850μl LB)

4.4.7. Isolation of vectors

Extracting Plasmid DNA from E.coli

(Quaigen Maxi Prep Plasmid System - for buffer constituents see Reagents)

1. Pre-chill buffer P3 (Neutralisation buffer)
2. Spin down cultures in large centrifuge beaker at 3000rpm for 5 mins.
3. Resuspend pellet in 10ml buffer P1 (Resuspension buffer) containing RNaseA.
4. Add 10ml buffer P2 (Lysis buffer), mix gently and incubate at room temp for 5 mins.
5. Add 10ml chilled buffer P3 (Neutralisation buffer), mix immediately but gently and incubate on ice for 20 mins.
6. Make sure tubes are balanced (very viscous precipitate).
7. Spin down at 290g (10,000rpm on Sorvall RC3C) at 4°C for 30 mins and remove supernatant promptly.
8. Repeat Step 7.
9. Equilibrate a Qiagen-tip 500 by running through 10ml buffer QBT (Equilibration buffer) and allow column to empty by gravity flow. Collect in 50ml Falcon tubes.
10. Run supernatant through, then wash through with 60ml buffer QC (Wash buffer) (10ml at a time).
11. Elute the DNA with 15ml buffer QF (Elution buffer) and collect.
12. Precipitate DNA by adding 0.7 volumes of isopropanol (10.5ml) and mix at room temperature.
13. Mark outside of tube where pellet will appear and spin down immediately at 290g at 4°C for 30 mins. Carefully remove supernatant.
14. Wash DNA with 15ml 70% ethanol, spin down again, air-dry pellet for 5 mins and redissolve in TE buffer / water (200µl approx.)

4.5. Purification of PCR products

(Wizard™ PCR Preps DNA Purification System)

1. Transfer the aqueous (lower) phase to a clean eppendorf. The presence of too much mineral oil in the sample can lead to a decreased yield in the PCR product purification.
2. Aliquot 100µl of Direct Purification Buffer into a 1.5ml eppendorf. Add the PCR product (30-300µl) and vortex briefly to mix.
3. Add 1ml of Resin and vortex briefly 3x over a one minute period.
4. Make up 80% isopropanol. For each PCR product, prepare one Wizard Minicolumn. Set aside the plunger from a 2.5ml syringe. Attach the syringe barrel provided in kit to the Minicolumn.
5. Pipette the resin/DNA mix into the syringe barrel, insert and push the plunger slowly through. Discard the elute.
6. Detach the syringe barrel from the Minicolumn and remove the plunger. Reattach the syringe barrel and pipette in 2ml of 80% isopropanol. Insert the plunger and gently push through to wash the column. Discard the elute.
7. Remove the syringe and transfer the Minicolumn to a 1.5ml eppendorf. Centrifuge for 2min at 9300g (10,000rpm in microfuge) to dry the resin.
8. Pre-heat an eppendorf full of TE to 60°C. Transfer the Minicolumn to a new eppendorf. Apply 50µl of 60°C TE and wait 1min. Centrifuge the Minicolumn for 20sec at 9300g to elute the bound DNA. Remove and discard the Minicolumn and store DNA at 4°C or -20°C according to use.

4.5.1. Measuring DNA concentration by spectrophotometry

1. Dilute DNA 1:500 i.e. 2µl into 998µl TE.
2. Using Spectrophotometer, blank with TE and measure absorbance of nucleic acid samples (OD, optical density) at 260nm.

3. Calculate concentration: abs at 260nm x dilution factor x 50µg/ml
   = µg/ml of DNA

4.6. Li-acetate yeast transformation

1. Take 0.5ml of culture and spin 10 seconds in a microcentrifuge. Decant supernatant by
   inverting the tube and shaking it once
2. Add 10µl of carrier DNA (100µg salmon sperm DNA - boiled for 10mins) and 1µg transforming DNA, subject to vortex mixing.
3. Add 0.5ml PLATE solution (PEG/Li-acetate/TE, see Reagents), subject to vortex mixing.
4. Incubate overnight at room temperature on bench top
5. Spread mixture directly on selective plate. (Cells tend to settle overnight - plate 0.2ml from bottom as PEG solution slows growth)

4.7. Sequencing of *ScMLH1* insert

Taq Terminator Sequencing (on ABI 373A)

Fragments of approximately 200bp are suitable for sequencing. Six 5' primers and six 3' primers were designed along ScMLH1:

seqMLH1 - F1: 5'- ATG TCT CTC AGA ATA AAA GCA -3'
seqMLH1 - R1: 5'- AAA AAG GTC TTC AAC TAG GAT -3'
seqMLH1 - F2: 5'- AAG TTA AAG AAG ACA GAT GTG -3'
seqMLH1 - R2: 5'- TTG GCA GAT AAT TGG AGT AAA -3'
seqMLH1 - F3: 5'- ATA ATA GAC TAG TGA CAT GTG -3'
seqMLH1 - R3: 5'- AAC TGT TGA CTT GAG GAT AAA -3'
seqMLH1 - F4: 5'- TAA ACT AGT CAG AAT AGA TGC -3'
seqMLH1 - R4: 5'- AGC TTT AAG TCA TGC TGA ATA -3'
seqMLH1 - F5: 5'- AAA TTT GAA TTA CGT TGG GGT -3'
seqMLH1 - R5: 5'- AGA TGG AAT GTA GCC TTT TAA -3'
seqMLH1 - F6: 5'- AAG CTA AAA TCT CTA CCA CTA -3'
seqMLH1 - R6: 5'- GTT AAC ACC TCT CAA AAA CTT -3'

1. Mix 0.5 - 1μg DNA with 3.2pmoles primer and make volume to 6μl with ddH_2O.

Oligo size	**ng equivalent of 3.2pmoles**
16mer	16.90
18mer	19.00
20mer	21.12
22mer	23.23
24mer	25.34

2. Add 4μl reaction mix
3. PCR with following conditions:

with Perkin Elmer 9600: with Perkin Elmer 480:

preheat to 96°C	96°C x 30sec
96°C x 15sec	50°C x 15sec
50°C x 1min	60°C x 4mins
60°C x 4mins	
	25 cycles
25 cycles	4°C
4°C	

4. Add 2µl of 3M Sodium Acetate pH4.5 and 50µl Ethanol to the final aqueous phase (Use 0.5ml tubes for this and future steps)
5. Mix and stand at -70°C for 15mins
6. Spin for 15mins at full speed in Eppendorf microfuge
7. Carefully take off supernatant
8. Wash pellet with 250µl 70% Ethanol
9. Spin 5mins
10. Again remove supernatant carefully
11. Speedivac for 5mins to dry off pellet.

Samples prepared to stage 11 were then given to In-house Sequencer service.

4.8. Mutator phenotype testing

4.8.1. Forward mutation frequency

Since wild type CAN1 confers sensitivity to L-canavanine, any mutation results in recessive resistance:

1. Make up agar plates containing synthetic complete media with arginine dropped out (as arginine represses L-canavanine uptake) and 0.006% L-canavanine (3ml of 2% solution per litre media).
2. Plate out 10^6 cells on YPD-arginine$^-$ + agar + 0.006% L-canavanine and 400 cells on YPD + agar control. Count colonies surviving.
3. Calculate forward mutation frequency:

$$\frac{\text{No. Can colonies x 400}}{\text{No. ctrl colonies x } 10^6}$$

4.8.2. Mutation Rate - fluctuation analysis

Work carried out on mutation rate determination was carried out by Dr Gill Hirst. Fluctuation analysis was determined by plating out strains to obtain individual colonies.

1) Make up SC-Arg media plates.
2) Pick 12 single colonies >3mm diameter and resuspend in sterile 0.5ml H_20.
3) Dilute cells 1/10 and 1/100 and seed onto Sc-Arg to obtain numbers of viable cells and Sc-Arg/L-canavanine plates to determine mutant colony number.
4) Grow for 2-3 days at 30° C and count.
5) The method of Lea and Coulson (1949) is then used to analyse data.

4.9. Yeast FACS Analysis

1. From an exponentially growing culture remove 5mls and sonicate for 10sec at a frequency of 2500 using an MSE Soniprep 150 sonicator. Spin down 5min 100g (1000rpm bench top centrifuge).
2. Resuspend in 2ml 70% EtOH
3. Leave for 1hr room temp.
4. Spin cells for 5 minutes, 100g (1000rpm in microfuge) and resuspend in 0.5ml ice-cold CDS and incubate 10mins on ice.
5. Spin down cells for 1 minute, 350g (2000rpm in microfuge) and resuspend in 0,5ml formaldehyde solution for 30min room temp.
6. Spin down cells for 1 minute, 350g and wash once with 0.5ml PBS.
7. Resuspend in 800µl 50mM Tris and add 200ul 5mg/ml pre-boiled RNAse A (*Kramel Biotech*) in 50mM Tris, pH7.8 (i.e. using stock of 2mg/ml RNAse A). Incubate for 1hr in 37C room.
8. Spin down cells for 1 minute, 350g and resuspend pellet in 0.5ml zymolyase solution (or, 0.5ml of 5mg/ml pepsin in 55mM HCl. for 30 minutes at 37C) and incubate at 30C for 10min.
9. Spin down cells for 1 minute, 350g and resuspend in 1ml Tris/NaCl/MgCl - 200mM Tris Ph7.5, 211mM NaCl, 78mM MgCl
10. Spin down for 1 minute, 350g and resuspend in 0.5ml Tris/NaCl/Mg/Cl. Add 55µl 500µg/ml propidium iodide in water for final conc. of 50µg/ml PI and soon after, read on FACS. Transfer 50ul cells to FACS tube and add 2mls 50mM Tris, pH 7.8

4.10. Yeast fluorescent microscopy

1. Harvest 1ml of cells. Sonicate for 10 secs. at a frequency of 5000 (MSE Soniprep 150 sonicator), transfer to plastic tube and spin down cells (10 secs at 18300g or 14000 rpm in microfuge). Wash pellet in 50mM Tris 7.8. Resuspend in 1ml 70% ethanol and 50mM Tris 7.8 (50µl of 1M Tris 7.8). Incubate O/N. (The cells can be stored for months at 4°C at this stage)
2. Spin cells down for 1 minute, 350g and wash pellet 2x with 50mM Tris 7.8. Resuspend in 1ml 50 mM tris 7.8 and add 20µl 10mg/ml RNase A (DNase free - boil for 5mins). Incubate O/N at RT on a rotator.
3. Spin cells down for 1 minute, 350g and wash pellet 1x with FACS buffer (200mM Tris 7.5, 211 mM NaCl, 78mM $MgCl_2$). Resuspend in 0.5ml FACS buffer and add 55µl of 0.5mg/ml propidium iodide. The cells can be stored frozen for at least 2 weeks at this stage.
4. Sonicate for 10 seconds at a frequency of 5000 (MSE Soniprep 150 sonicator) and place under a fluorescent microscope. Place one drop of oil on a cover-slip and observe under the microscope.

RESULTS

5. RESULTS

5.1. Isogenic MMR mutant *S. cerevisiae* strains

Previous studies have tested the relative difference between parental and MMR-defective tumour cell lines to the cytotoxic effects of cisplatin and other DNA damaging agents (Anthoney *et al,* 1996; Brown *et al,* 1997). Studies using yeast as an isogenic model have only tested for alkylation tolerance (Xiao *et al,* 1995; Bawa and Ziao, 1997) and not for cisplatin resistance. They demonstrated that deletion of any of the MMR genes, *MLH1*, *MSH2*, *MSH3*, *MSH6* and *PMS1* did not rescue *mgt*Δ O^6 MeG DNA repair methyltransferase-deficient cells from killing by MNNG (Xiao *et al,* 1995; Bawa and Ziao, 1997). However, a strain (XS-14) carrying a mutated form of the *MSH5* gene, but not an *msh5*Δ null mutant, was responsible for cellular tolerance to MNNG (Bawa and Ziao, 1997) and it was suggested that some mutated forms of *MSH5* and possibly of MutS homologues in mammalian cells may be involved in alkylation tolerance.

This thesis has taken advantage of isogenic strains of *S. cerevisiae* with specific disruptions in known MMR genes and tested the relative cytotoxicity of each mutant to cisplatin treatment. Certain fundamental differences between yeast and mammalian cells have to be taken into account when screening for cytotoxic phenotypes. First, the plasma membrane of yeast is made up of the polysaccharide chitin and β-1,3 glucan making up a densely interwoven fibrillar mesh (Pringle *et al,* 1997). This requires much higher concentrations of drug to achieve cytotoxicity and previous studies that have analysed the cytotoxicity of cisplatin in yeast have used concentrations ranging up to 1.2mM in the medium (Megan *et al,* 1996). This is approximately one thousand times the concentration of cisplatin used for tumour cell line studies (Anthoney *et al,* 1996; Brown *et al,* 1997).

An advantage of using yeast is the doubling time. Budding yeast doubling times are much quicker, typically 90-120 minutes, compared to mammalian cells in culture (Pringle *et al*, 1997). Therefore, assays can be carried out over a 2-3 day basis compared to those that span a week for mammalian studies.

Isogenic *S. cerevisiae* strains were generated in collaboration with Dr. Rhona Borts, IMM, John Radcliffe Hospital, Oxford, UK. Individual MMR genes were disrupted by recombination plasmids containing *KANMX* and *LEU2* selection markers (Wach *et al*, 1994). Before the strains were tested for cisplatin cytotoxicity, each strain was screened for the correct selectable marker (See Table 1) as determined by resistance to the aminoglycoside antibiotic, kanamycin, growth on leucine drop-out media and for *ADE* selection as determined by red colony formation. Approximately 400 cells were seeded per plate. Fig. 6 shows an example of *KANMX* resistant and sensitive strains and Fig. 7 shows strains able to grow without the presence of leucine in the media.

Description	Strain	Phenotypes
Wild type	2096-1B	Matα, *leu2, trp1,ade1*
msh2	RHB 2348-9b	Matα, *leu2, trp1,ADE1*
msh3	RHB 2347-4b	Matα, *LEU2, trp1,ADE1*
msh6	NHT 173	Matα, *KANMX, leu2, trp1,ade1*
mlh1	RBT 311	Matα, *LEU2, trp1,ade1*
mlh2	RBT 324	Matα, *KANMX, leu2, TRP,ADE1*
pms1	RBT 289	Matα, *LEU2, TRP,ade1*
rad52	RHB 2692-1c	Matα, *LEU2, trp1,ADE1*
rad52/msh2	RHB 2700-1b	Matα, *LEU2, trp1,ADE1*
rad52/mlh1	RHB 2698-5c	Matα, *LEU2, trp1,ADE1*
rad52/mlh2	RHB 2699-4c	Matα, *LEU2, KANMX, trp1,ADE1*
rad1	RBT 302	Matα, *LEU2, trp1,ADE1*
rad1/msh2	RHB 2694-9c	Matα, *LEU2, trp1,ADE1*
rad1/mlh1	RHB 2693-8a	Matα, *LEU2, trp1,ADE1*
rad1/mlh2	RHB 2695-2b	Matα, *LEU2, KANMX, TRP,ADE1*

Table 1 Phenotypes of *S. cerevisiae* MMR mutant strains

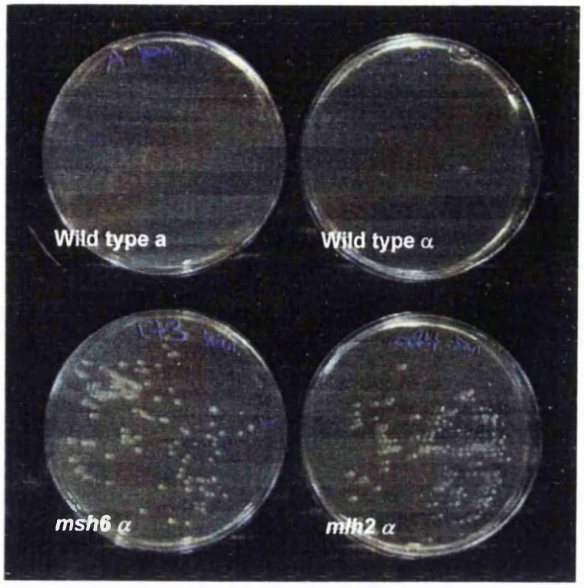


Figure 6 *KANMX* resistance - phenotype test

The strains with *KANMX* resistance gene inserted grow on *KAN* selective media. This confirms the presence of the *KANMX* cassette plasmid used for MMR gene disruption in the appropriate strains (See Table 1 for phenotypes).

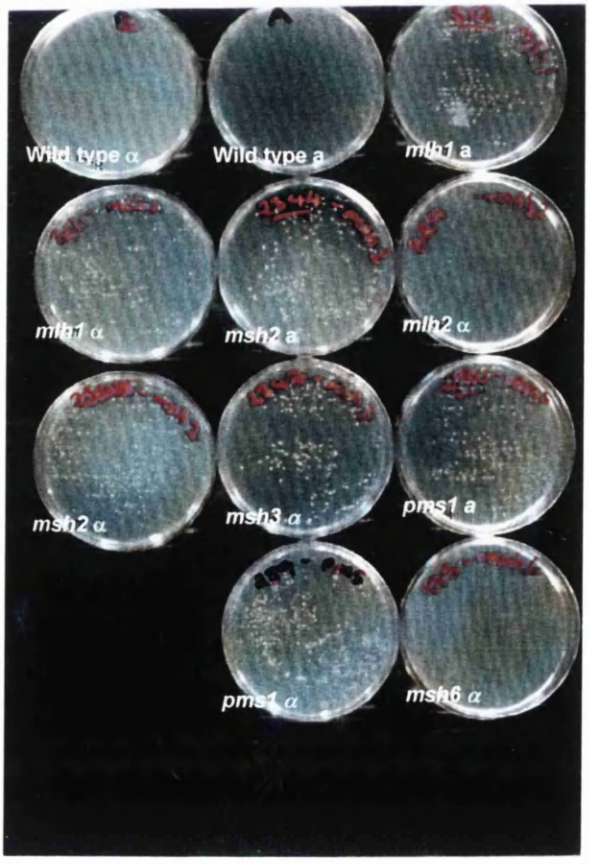


Figure 7 *LEU2* gene insertion - phenotype test

The strains with the *LEU2* gene inserted grow on Leu drop-out selection media. This confirms the presence of *LEU2* plasmids used for MMR gene disruption in the appropriate strains (See Table 1 for phenotypes).

5.2. Clonogenic responses of MMR mutants to cisplatin

Each strain was seeded at $2x10^7$ cells in 1ml of liquid YPD media and exposed to 0-1mM cisplatin (dissolved in 10µl of DMSO) as previously described (Megan *et al*, 1996). Cells were initially exposed to cisplatin for 1 hour, washed and plated out on YPD agar at 400 cells per plate. No cytotoxic response was seen in wild type or mutant strains as measured by colony forming ability after 2-3 days incubation. Therefore, the concentration range was increased from 0 to 2mM cisplatin and time of exposure was increased to 24 hours in separate experiments. Under these conditions, a standard kill curve was achieved for wild type cells exposed for 24 hours and to the higher concentration of cisplatin. This enabled a comparison of cisplatin sensitivity of the isogenic MMR mutants. Fig. 8 shows photographs of plates with colonies formed from wild type and *mlh1* mutants in response to a 24 hour exposure of 0, 0.5, 1, 1.5 and 2mM cisplatin. Fig. 9 shows the clonogenic responses averaged from at least four separate, repeated experiments using wild type, *mlh1*, *msh2*, *msh3*, *msh6*, *mlh1*, *pms1* and *mlh2* strains.

Table 2 shows the inhibitory constant at 90% inhibition of colony formation for each mutant tested. The L.D. 90 value for the wild type strain under these conditions was 1mM, whereas the L.D. 90 values for *msh2*, *msh3*, *msh6*, *mlh1* and *mlh2* were significantly different ($P<0.05$), as tested by the student's unpaired, two-tailed t-test at 1.7, 1.8, 1.7, 1.5 and 2mM respectively. The L.D. 90 value of 1.1mM for *pms1* was not significantly different from wild type ($P>0.05$). The surviving fractions (SF) at 1mM cisplatin treatment for each strain were: wild type (0.14), *mlh1* (0.39), *msh6* (0.55), *msh2* (0.61), *pms1* (0.29), *msh3* (0.69) and *mlh2* (0.82) (See Appendix).

These results demonstrate that all the MMR mutants tested, but not *pms1*, were 1.5 - 2 fold more resistant to 1mM cisplatin than wild type cells at the L.D. 90 value. These folds of resistance are slightly lower than those seen in mammalian cell lines deficient

MMR which have typically shown 2-5 folds of resistance (Anthoney *et al,*1996; Brown *et al,* 1997).

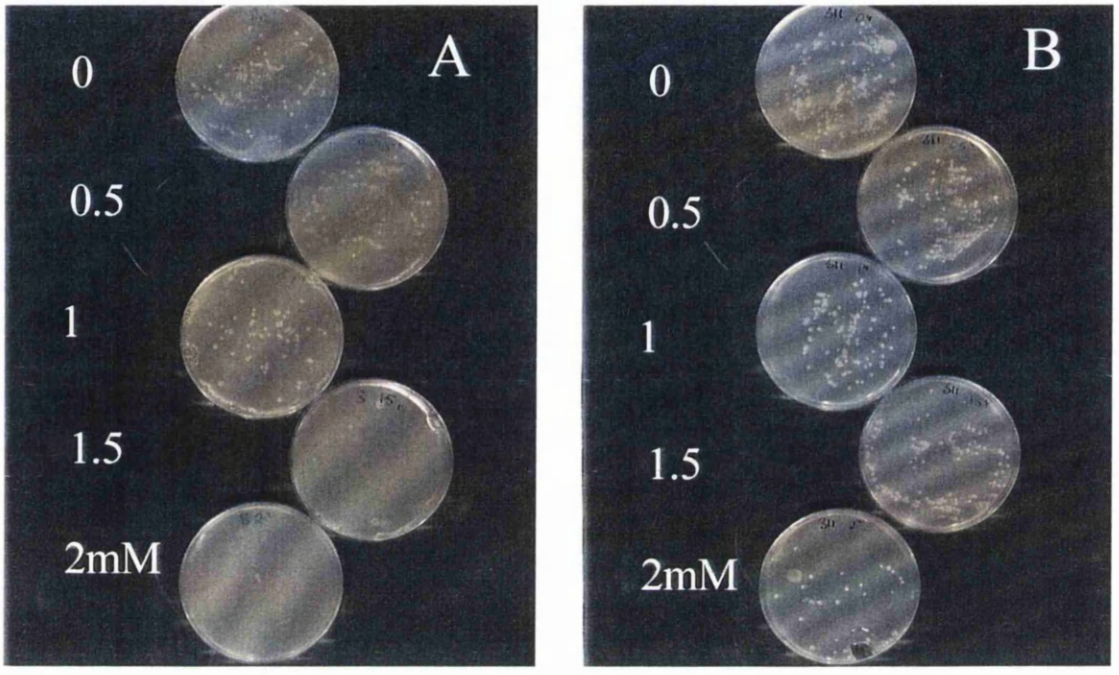


Figure 8 Photographs of colonies formed after cisplatin exposure.

A typical example of colonies grown on YPD agar. Approximately 400 cells were inoculated onto YPD agar plates after exposure to 0-2mM cisplatin for 24 hours in liquid culture. (A) shows wild type and (B) shows *mlh1* colonies grown after 2-3 days incubation at 30°C. *mlh1* colonies are seen to grow after 1.5 and 2mM whereas wild type cells do not form colonies at these concentrations.

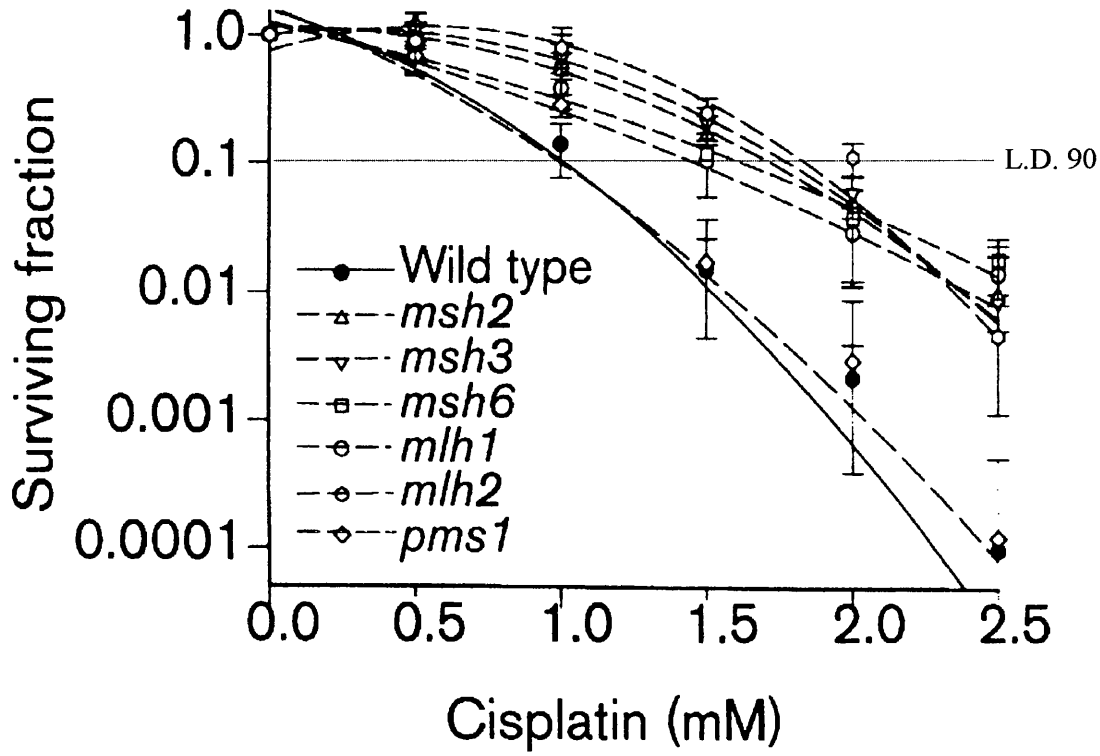


Figure 9 Clonogenic responses of wild type and MMR mutants to 24hr exposure of cisplatin.

All MMR mutants, but not *pms1*, show a 2.6 - 5.6-fold significant increase ($P<0.05$, as measured by student's unpaired two-tailed t-test) in resistance to 1mM cisplatin compared to wild type. At L.D. 90 values, resistance values range from 1.4 - 2-fold. (See appendix for statistical analysis). Mutants listed in descending order of resistance are *mlh2*, *msh2*, *msh3*, *msh6* and *mlh1*. Regression lines were drawn to 2 orders and error bars represent 99% confidence limits.

Description	Strain	L.D. 90 (cisplatin, mM)	Resistance Factor.
Wild type	2096-1B	1	1
msh2	RHB 2348-9b	1.7	1.7*
msh3	RHB 2347-4b	1.8	1.7*
msh6	NHT 173	1.7	1.6*
mlh1	RBT 311	1.5	1.4*
mlh2	RBT 324	2.0	2*
pms1	RBT 289	1.1	1.1

Table 2 Lethal Doses at 90% cell death and resistance factors of MMR mutants.

Clonogenic survival showed that all MMR mutant strains tested for cisplatin toxicity, but not *pms1*, showed significant resistance values ($P<0.05$, student's unpaired two-tailed t-test). Asterix indicates significant difference determined by student's unpaired two-tailed t-test.

5.3. Mutator phenotypes

5.3.1. Forward mutation frequency

Loss of MMR function is associated with a mutator phenotype. Therefore, to assess the MMR proficiency of these strains, forward mutation frequency was calculated. This is carried out by measuring the frequency of colonies developing mutations in the gene controlling sensitivity to L-canavanine. 0.006% L-canavanine in YPD agar was made up and plates were inoculated with a concentration of cells that produced a countable number of colonies after incubation. This was calibrated at 10^6 cells per plate. Forward mutation frequency was calculated as shown in Methods. Table 3 shows the mutation frequencies obtained from wild type, *mlh1*, *msh2*, *pms1* and *mlh2* mutants. The mutant frequencies tested place the mutator phenotype of each strain in descending order of mutant frequency: *pms1* > *mlh1* > *msh2* > *mlh2*. The *mlh2* strain did not display an elevated mutation frequency compared to wild type.

5.3.2. Mutation rate

A more accurate determination of mutagenicity is achieved by carrying out fluctuation analysis to measure mutation rate. Ten single colonies or more are isolated and cells from these colonies were assessed for L-canavanine resistance. Mutation rate was calculated using the formula shown in Methods and carried out by Dr. Gill Hirst.

Table 3 shows the comparison between frequencies and rates calculated. Again *mlh2* was shown not to have a significant difference from wild type and *pms1* showed an high mutation rate, with an equivalent difference observed in mutation frequency.

Table 3 Mutation frequencies and rates of MMR mutant strains

Strain	Forward mutation frequency	Mutation rate
wild type	1.6×10^{-6}	3.7×10^{-8}
msh2	2.5×10^{-5}	nd
mlh1	4.7×10^{-5}	nd
pms1	8.7×10^{-5}	8.7×10^{-6}
mlh2	9.6×10^{-7}	6.7×10^{-8}

These results show that *pms1* mutants have the highest mutation frequency and rate but do not confer a significant increase in resistance to cisplatin. Whereas *mlh2* mutants do not show a significantly elevated ($P>0.05$) mutation frequency or rate but do confer the highest significant increase in resistance to cisplatin compared to the other mutants and wild type strain. This provides evidence to suggest that separate components of MMR have separate roles in conferring drug resistance and elevated mutation frequencies/rates. Specific components of MMR but not MMR activity *per se* are required for sensitivity to cisplatin.

5.4. *ScMLH1* gene re-introduction

It has previously been shown that the majority of human tumour cell lines that were selected for resistance to cisplatin did not express *hMLH1* as measured by mRNA levels. *PMS1* and *MSH2* mRNA levels in cells derived from the human ovarian cell line A2780 could be detected. Consequently, these cell lines showed cross resistance to MNU and doxorubicin (Brown *et al*, 1997). Of the MMR-specific genes identified, the MutL complex (Mlh1/Pms2 heterodimer in humans and the Mlh1/Pms1 heterodimer in yeast) is the most down stream known to date.

For these reasons, it was considered important to examine if there was something particular about *ScMLH1* that was important for sensitising cells to cisplatin. Consequently, *ScMLH1* was cloned and re-introduced back into the *mlh1* mutant. This could then be used as a complementary system to ascertain if restoring wild type *ScMLH1* function could restore cisplatin sensitivity in yeast. This was carried out to exclude the possibility that the mutator hypothesis has a mechanistic role in the development of cisplatin resistance.

Furthermore, re-introducing the *hMLH1* gene alone by plasmid transfection into human cell lines has proven difficult in the past. Instead, previous studies using human cell lines have carried out chromosome 3 transfer, the chromosome containing h*MLH1*, to restore wild type protein in *MLH1*-defective cell lines. Therefore, transfecting the sc*MLH1* gene into yeast using a yeast expression vector was performed in order to test for cisplatin toxicity without introducing any other possibly confounding chromosomal sequences.

Fig. 10 shows a screen for DH5-α *E. coli* transformants selected for growth on Ampicillin plates with the yeast expression vectors containing the Amp resistance gene. Bands were generated using the *ScMLH1* primers shown in Methods and annealing positions shown in Appendix. PCR fragments generated show the putative *ScMLH1*

size (2.2kb) as measured by the 100kb ladder. A larger ladder would have been more appropriate for size determination, however due to availability and convenience, the 100kb ladder was used. Yeast expression vectors used were the low copy plasmid pYX112 and the high copy plasmid pYX212. Only from the high copy vectors were correctly-sized fragments generated. The reason for the lack of detection in the pYX112 vector has been identified. An error of design of pYX112 primers was made in that the 5' forward primer and 3' reverse primer were reversed. This therefore generated PCR products across the entire sequence of the vector and not across the cloning site. Indeed it can be seen that low yields of very large fragments exist towards the top of the gel. Therefore the selection for colonies containing the correct clones could not be made for this vector.

pYX212-transformed colonies containing the appropriately sized fragments were then grown up to isolate vector-plus-inserts ready for sequencing and transforming into yeast. Wild type and *mlh1* mutant strains were transformed using the Li-acetate method (see Section 7.1). Cells were incubated with vector + insert and vector alone using salmon sperm DNA as a carrier. Cells were then grown on SC-leucine and SC-tryptophan drop-out media depending on vector used for transformation. The few colonies that grew (plating efficiency: <10 from a 1.5ml inoculation of 10^7 cells) were then isolated.

Fig. 11 shows the result of a PCR reaction using *ScMLH1* primers and pYX112 / pYX212 vector primers shown in Methods to detect the correctly sized inserts within wild type and *mlh1* mutant strains of *S. cerevisiae*. Yeast cells were placed directly in the PCR reaction so that high temperature lysis of cells allowed for direct template DNA for the reaction. Fig. 11 shows the presence of *ScMLH1*-sized fragments in the *mlh1* mutant. Both vector and *ScMLH1* primers detected the 2.2kb-sized insert, the vector primers generating a slightly larger band due to the extra sequences flanking the insert region.

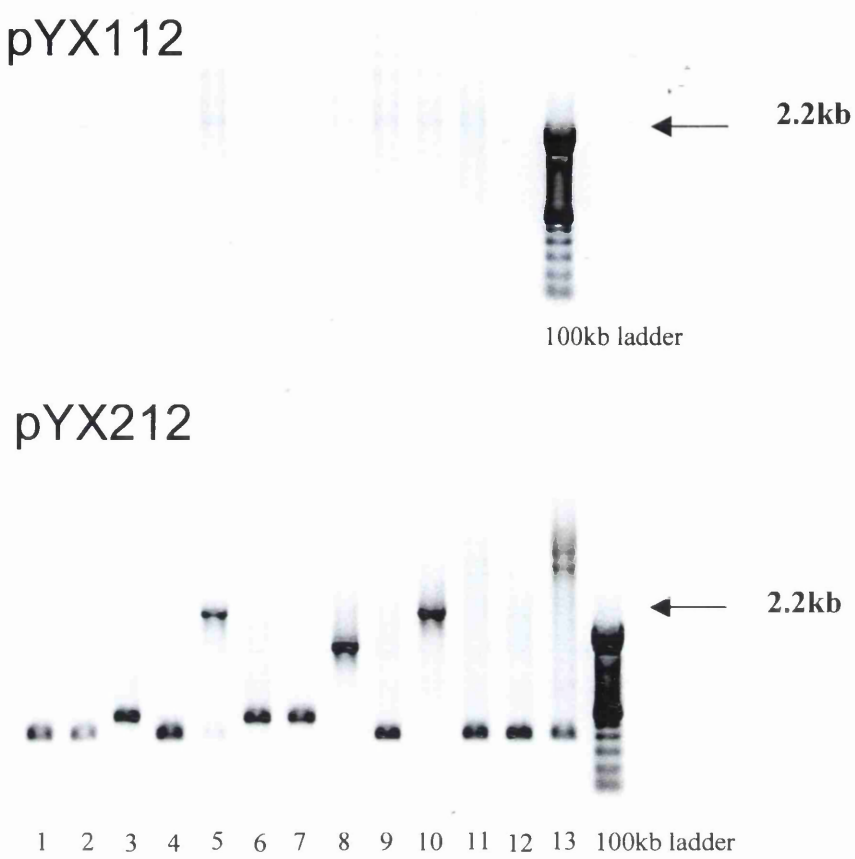


Figure 10 A screen of 13 *E.coli* colonies for each plasmid transfection.

Correctly sized (2.2kb) *ScMLH1* PCR fragments using vector primers are indicated. Vector pYX112 does not show clear bands at the putative size for the *MLH1* gene. However, vector pYX212 primers yield two potential colonies that contain the correctly-sized fragments. Colony from pYX212 Lane 10 was selected due to size and yield.

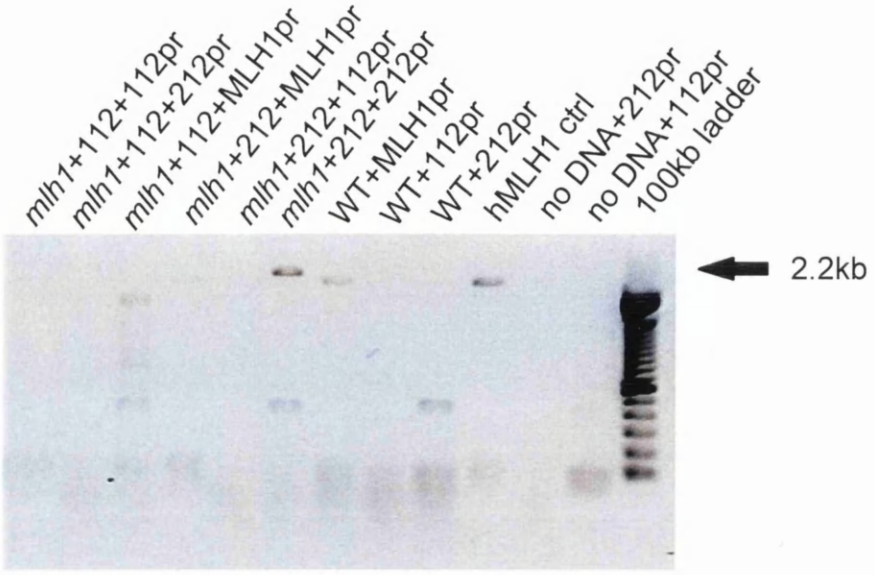


Figure 11 Agar gel electrophoresis for the detection of *scMLH1* in yeast strains.

The *ScMLH1* primers (*MLH1*pr) and vector primers (pYX112pr or pYX212pr) were used to detect the presence of *ScMLH1* in the yeast transformants. Cells were placed directly into the PCR reaction. Lanes 4 and 6 show the presence of correctly sized fragment using both *MLH1*pr and pYX212pr vectors (note the pYX212pr yields slightly larger fragment due to the inclusion of vector sequence).

5.5. *ScMLH1* Sequencing

In order to confirm the presence of the correct sequence of the insert, sequencing of the *ScMLH1* insert in pYX212 was carried out on two separate occasions using the same cloned sequence as template. Forward and reverse primers were designed to generate PCR sequences that stretched across the entire scMLH1 sequence. In the appendix, sequences generated by each primer are shown. Both FASTA and BESTFIT analysis using GCG software were carried out to align the PCR sequences generated with the known scMLH1 gene sequence in the database (Saccharomyces Genome Database - Accession number: U07187).

The results show that 5 point mutations were detected.

Cys208Trp
Ile263Val
Ala712Thr
Ser717Asp
Leu770Pro
(Numbers indicate codon)

Sequencing was repeated using the same template sample. Therefore it cannot be ruled out that the template itself was correct in sequence but of a quality insufficient for Taq terminator sequencing. The same primers were also used which may have influenced the fidelity of the PCR products especially at the ends of long sequences (see Appendix).

The *Sc*Mlh1 protein has 781 amino acids with the most highly conserved sequence residing in the amino-terminus. Here, the GFRGEAL MutL box is located, a region of high sequence homology between the MutL proteins in all eukaryotes studied. The majority of point mutations detected in this sequence lie nearer the carboxy-terminus

where the greatest degree of sequence variation exists (Peltomaki *et al*, 1997; Papadopoulos *et al*, 1994; Shimodaira *et al*, 1998). Most mutations occurring in human *MLH1* in HNPCC patients are frameshift non-sense mutations. (Peltomaki *et al*, 1997; Papadopoulos *et al*, 1994).

5.6. Clonogenic response of *ScMLH1*-re-introduced transformant to cisplatin

Fig. 12 shows the response obtained by treating isolated clones of *mlh1* mutants transformed with pYX212 vector alone (legend: mlh1 vec), pYX112 (unknown insert, legend: mlh1 112), and pYX212 containing the sequenced *ScMLH1* insert (legend: mlh1 212). The figure shows the hypersensitive response obtained by the scMLH1 transformant compared to wild type and mlh1-vector alone controls. The L.D. 90 value for this transformant was 0.5mM, as shown in Table 2.

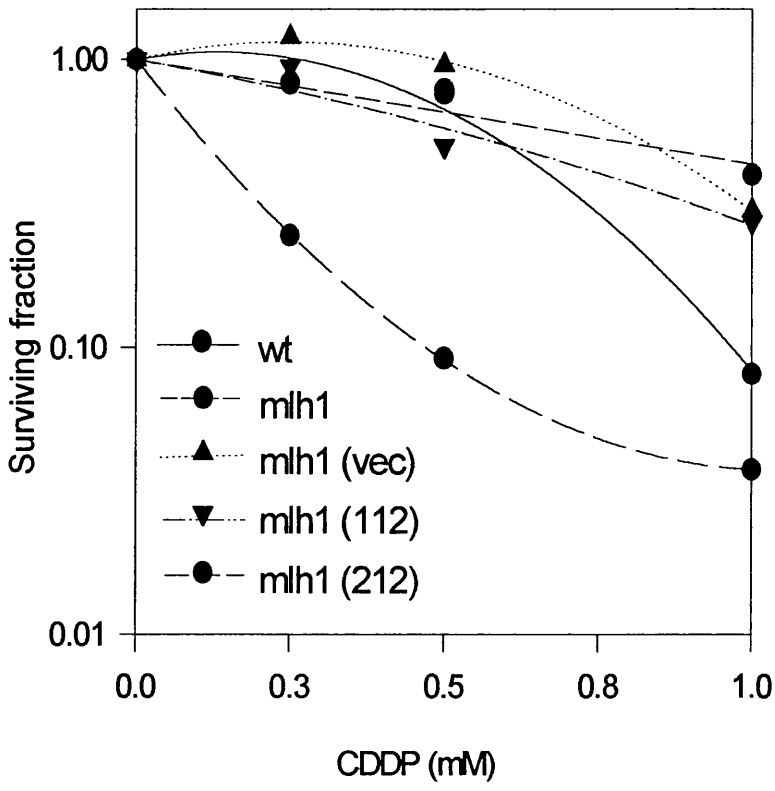


Figure 12 Survival curves for *ScMLH1* re-introduced strains.

The transformed *ScMLH1* mutant with the *ScMLH1*-inserted expression vector (pYX212) restores sensitivity to levels higher than wild type.

5.7. Clonogenic response of MMR mutants in a Rad52-deficient background

The involvement of MMR in recombinational repair is well documented and discussed in Section 1.1.3. However, previous studies have not addressed the issue of the involvement of MMR in recombinational repair with respect to DNA damage responses. Homologous recombination is used to repair double strand breaks in DNA by using homologous chromosomes as templates for repair. Mutations of the genes in the Rad52-epistasis group (*RAD50-RAD57*) confer sensitivity to X-rays and to chemicals that cause strand breaks in DNA (Game *et al*, 1974; Friedberg 1988). Rad52 is an essential component of this repair pathway (Shinohara and Ogawa, 1998). Previous studies have indicated that Rad52-dependent recombinational repair is modulated by MMR (Paques and Haber, 1997; Sugawara *et al*, 1997). By studying any difference that might exist in response to cisplatin-induced DNA damage between *S. cerevisiae* MMR mutants and MMR mutants in a recombinational repair-deficient background, any dependence on the presence of Rad52 could be assessed.

Isogenic *rad52* mutants were generated by *LEU2* gene insertion and *rad52*/MMR double mutants were generated by isolating segregants of crosses between the *rad52* with each of the MMR mutants: *mlh1*, *msh2* and *mlh2*.

Fig. 13 shows the clonogenic responses of the *rad52* mutant compared to wild type. The hypersensitivity observed (L.D. 90 value of 0.7mM) is significant ($P<0.05$) as determined by the student's unpaired, two-tailed t-test. Fig. 13 also shows the relative sensitivities of MMR/*rad52* double mutants. These are all as sensitive as the single *rad52* mutant (for L.D. 90 values and resistance factor values see Table 3).

5.8. Clonogenic response of MMR mutants in a Rad1-deficient background

The involvement of MMR in NER is discussed in Section 1.1.4. It has been shown in *S. cerevisiae* that repair of 26-base loops involves both the NER protein Rad1 (human ERCC4) and Msh2 (Kirkpatrick and Petes, 1997). A mutation of mei-9, the *Drosophila melanogaster* homologue of *sc*Rad1 (Sekelsky *et al*, 1995) increases PMS, and thus may be defective in meiotic mismatch repair (Carpenter 1982). Cisplatin-induced DNA interstrand crosslinks are known to be repaired by the nucleotide excision repair (NER) system. It was of interest, therefore, to investigate the effects of disrupting individual components of MMR in an NER-compromised background. Used as a control, the availability of a *rad1* mutant was taken advantage of and used to assess the effect of knocking out the genes from a separate repair system (MMR). However, Rad1, as discussed in Section 1.1.3, has also been shown to be involved in recombinational repair. Therefore the present data suggest an involvement of Rad1 in MMR and recombinational repair. To elucidate the role this protein has in MMR-dependent cisplatin cytotoxicity, isogenic rad1 mutants were generated, crossed with the MMR mutants and tested for clonogenic sensitivity to cisplatin.

Fig. 14 shows the response of the *rad1* strain to cisplatin treatment. As shown in Table 4, the hypersensitive *rad1* strain displayed an L.D. 90 value of 0.6mM, a value significantly different from wild type ($P<0.05$). The kill curves obtained for the MMR/rad1 double mutants were indistinguishable from the single *rad1* strain and displayed similar responses to those seen in the *rad52* background. These results suggest that MMR-dependent cytotoxicity is also dependent on the presence of Rad1 protein.

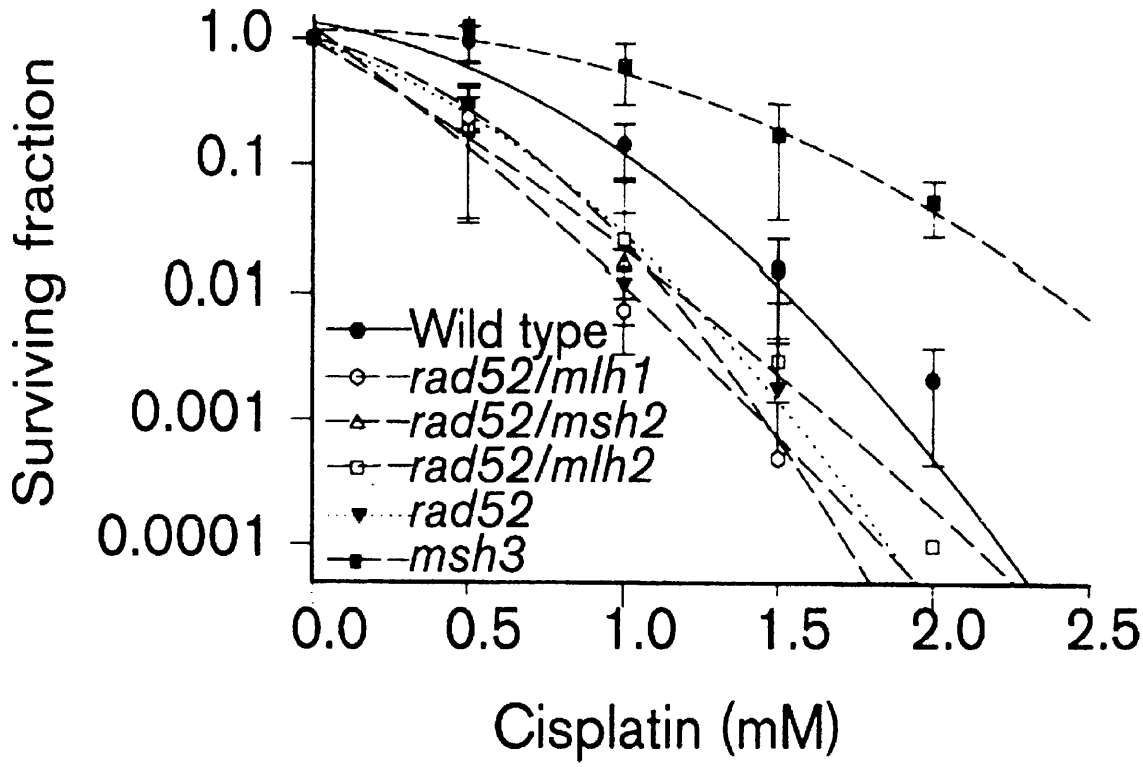


Figure 13 Clonogenic responses of MMR mutants in a *rad52* background.

Mlh1, *msh2* and *mlh2* mutants show the same resistant phenotype as *rad52* single mutant (not statistically different as determined by student's unpaired two-tailed t-test), therefore losing the single MMR mutant resistant phenotype. Regression lines were drawn to 2 orders and error bars represent 99% confidence limits.

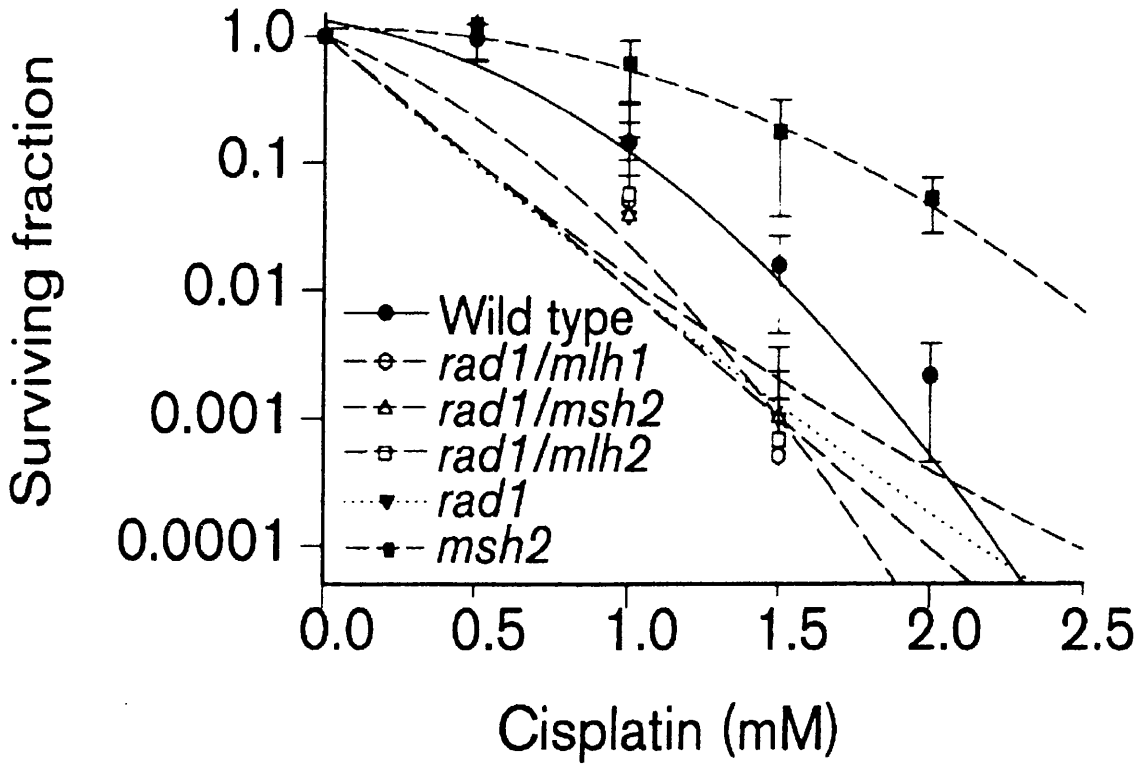


Figure 14 Clonogenic response of MMR mutants in a *rad1* background.

mlh1, *msh2* and *mlh2* mutants show the same resistant phenotype as *rad1* single mutant (not statistically different as determined by student's unpaired two-tailed t-test), therefore losing the resistant phenotype when *RAD1* was present. Regression lines were drawn to 2 orders and error bars represent 99% confidence limits.

Description	Strain	L.D. 90 (cisplatin, mM)	Resistance Factor.	Mutant Frequency
Wild type	2096-1B	1	1	$1.6x10^{-6}$
msh2	RHB 2348-9b	1.7	1.7*	$2.5x10^{-5}$
msh3	RHB 2347-4b	1.8	1.7*	n.d.
msh6	NHT 173	1.7	1.6*	n.d.
mlh1	RBT 311	1.5	1.4*	$4.7x10^{-5}$
mlh2	RBT 324	2.0	2*	$9.6x10^{-7}$
pms1	RBT 289	1.1	1.1	$8.7x10^{-5}$
mlh1+vector	RBT311:v	1.6	1.6*	n.d.
mlh1+pMLH1	RBT311:MLH1	0.5	0.5	n.d.
rad52	RHB 2692-1c	0.7	0.7*	n.d.
rad52/msh2	RHB 2700-1b	0.7	0.7	n.d.
rad52/mlh1	RHB 2698-5c	0.5	0.5	n.d.
rad52/mlh2	RHB 2699-4c	0.6	0.6	n.d.
rad1	RBT 302	0.6	0.6*	n.d.
rad1/msh2	RHB 2694-9c	0.6	0.6	n.d.
rad1/mlh1	RHB 2693-8a	0.7	0.7	n.d.
rad1/mlh2	RHB 2695-2b	0.6	0.6	n.d.

Table 4 Sensitivities to cisplatin and mutator phenotypes of MMR mutants.

The table shows lethal doses taken at 90% reduction in colony forming ability (L.D. 90) compared to untreated controls. Resistance factors (asterixed) indicate significant increase ($P<0.01$) as determined by student's unpaired, two-tailed t-test. Forward mutation frequency measured by number of resistant colonies arising on 0.006% L-Canavanine agar plates per 10^6 colonies grown without L-canavanine on agar.

5.9. Effect of growth inhibition on sensitivity of MMR mutants to cisplatin

There are a number of studies which suggest that DNA replication is required for MMR-dependent sensitivity to cisplatin. It has been shown that the human MutS complex binds to the DNA 1,2 intrastrand crosslink with much greater affinity if opposite a mismatched base, suggesting that base misincorporation and DNA synthesis past the lesion is required for recognition (Duckett *et al,* 1996; Brown *et al,* 1997). Also, it has been shown that MMR deficient ovarian carcinoma (A2780-cp70) cells which show resistance to cisplatin compared to proficient (A2780) cells have a significantly reduced MMR-dependent resistance phenotype when treated with aphidicolin - a DNA polymerase inhibitor (Moreland *et al,* 1999).

Therefore to address whether or not DNA replication is required in yeast for MMR-dependent cisplatin cytotoxicity, growth inhibitory conditions were put on the strains. Strains were grown to confluence and reached a stationary phase at a concentration of < 1.5×10^8 cells/ml (see Fig. 15).

After this was determined, wild type and *msh2* strains (chosen because they showed greater resistance to cisplatin than *mlh1* cells) were grown logarithmically and seeded at concentrations that reached confluence. Cells were then exposed to cisplatin. Fig. 16 and 17 show the cytotoxic responses of each strain. Fig. 16 shows that for wild type cells, the effect of growth inhibition significantly increased the resistance to cisplatin at 2mM. However, Fig. 17 shows that growth inhibition had no significant effect on the sensitivity of *msh2* mutants to cisplatin.

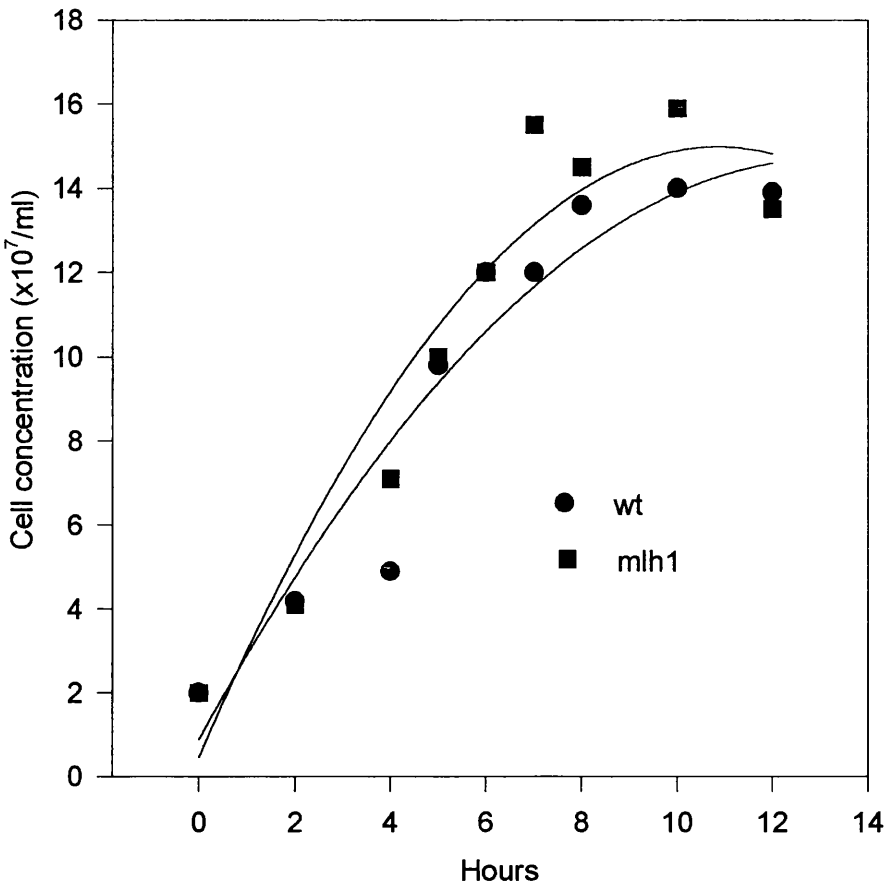


Figure 15 Growth curves for wild type and *mlh1* mutant strains.

The curves show that both strains of 1ml of 2 x 10^7 cells grown in closed-capped universals in shaking incubator reached a plateau in growth concentration of 1.4 x 10^8 /ml as measured by haemocytometer after 12 hours incubation.

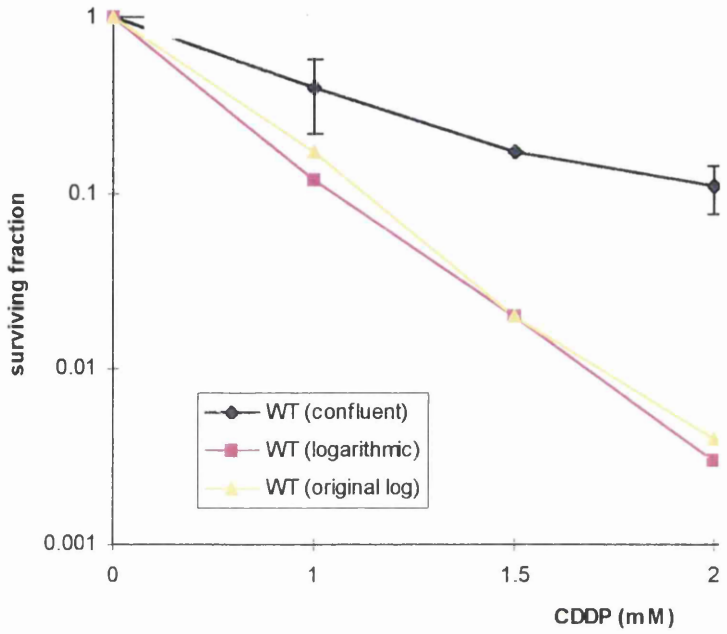


Figure 16 Clonogenic response for growth suppressed and exponentially growing wild type cells exposed to cisplatin.

Cells grown to confluence (>$1.6x10^8$/ml) show significantly increased resistance ($P<0.05$) as determined by student's unpaired two-tailed t-test, to 2mM cisplatin compared to logarithmically growing cells (seeded at $2x10^7$/ml). Original log curves are included from the previous toxicity data and show that the results are repeatable in this assay. Error bars represent standard deviation from the mean.

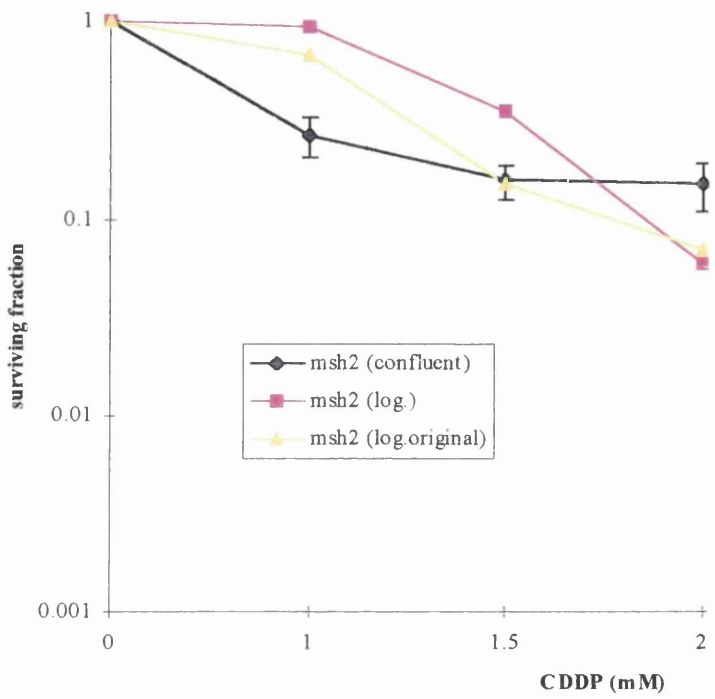


Figure 17 Clonogenic response for growth suppressed and exponentially growing *msh2* mutants exposed to cisplatin

Cells grown to confluence do not show a significant difference (as determined by student's unpaired two-tailed t-test), in sensitivity to 2mM cisplatin ($P>0.05$) compared to logarithmically growing cells. Error bars represent standard deviation from the mean.

5.10. Cell cycle analysis

Cell cycle analysis was carried out for the following reasons: i) The DNA replication inhibition experiments here show that MMR-dependent cytotoxicity is, at least in part, dependent on DNA replication and thus progression through S-phase of the cell cycle; ii) previous studies have shown that *MLH1* transfection induced growth suppression in an *MLH1*-defective cell line (Shin *et al,* 1998) and MMR-proficient cells were able to enter G_2 arrest while MMR-defective cells continued cycling in response to certain types of DNA damage (Koi *et al,* 1994; Hawn *et al,* 1995; Carethers *et al,* 1996), and iii) the cell cycle of *S. cerevisiae* MMR mutants has not previously been analysed in response to cisplatin-induced DNA damage.

5.10.1. FACS analysis

After cells were treated with cisplatin, stained with PI and analysed by FACS, G_2 peaks were observed as shown in Fig. 18. To span the 24 hour period of drug treatment cells were synchronised to allow accurate peak histogram study of cell cycle responses. Mating Type a (Mata) cells were studied due to the availability of the cell cycle arrest pheromone, α-Factor. Mating Type α (Matα) cells, which were the strains used in the cytotoxicity assays, could not be used due to the commercial unavailability of the highly lipophilic A-Factor. Cells were treated with 15μg/ml α-Factor (Peira *et al,* 1998) and samples were taken every hour during synchronisation. Cells were sonicated, enzymatically treated to partially digest the cell membrane, fixed in 70% ethanol, stained with propidium iodide and analysed by FACS. Fig.18 shows that in row 1 cells show G1 and G2 peaks for at least 6 hours of pheromone treatment. After 24 hours, cells became asynchronous. Yeast begin to ignore α-Factor after approximately 4 hours due to down regulation of α-Factor receptor (E. Scheibel, personal communication). Therefore cell synchronisation was carried out for 2 hours and FACS analysis was

carried out after cells started cycling again. Row 2 shows the G1 and G2 peaks during the initial pheromone treatment and row 3 shows the response of cells after being washed from pheromone. Surprisingly a large G2 peak is seen initially, followed by cells becoming asynchronous after approximately 3 hours. Cycling yeast cells become asynchronous after one cell cycle because mother and daughter cells are of different sizes (Pringle *et al*, 1997). This has made the synchronisation of yeast difficult for longer periods of time and FACS analysis difficult to interpret.

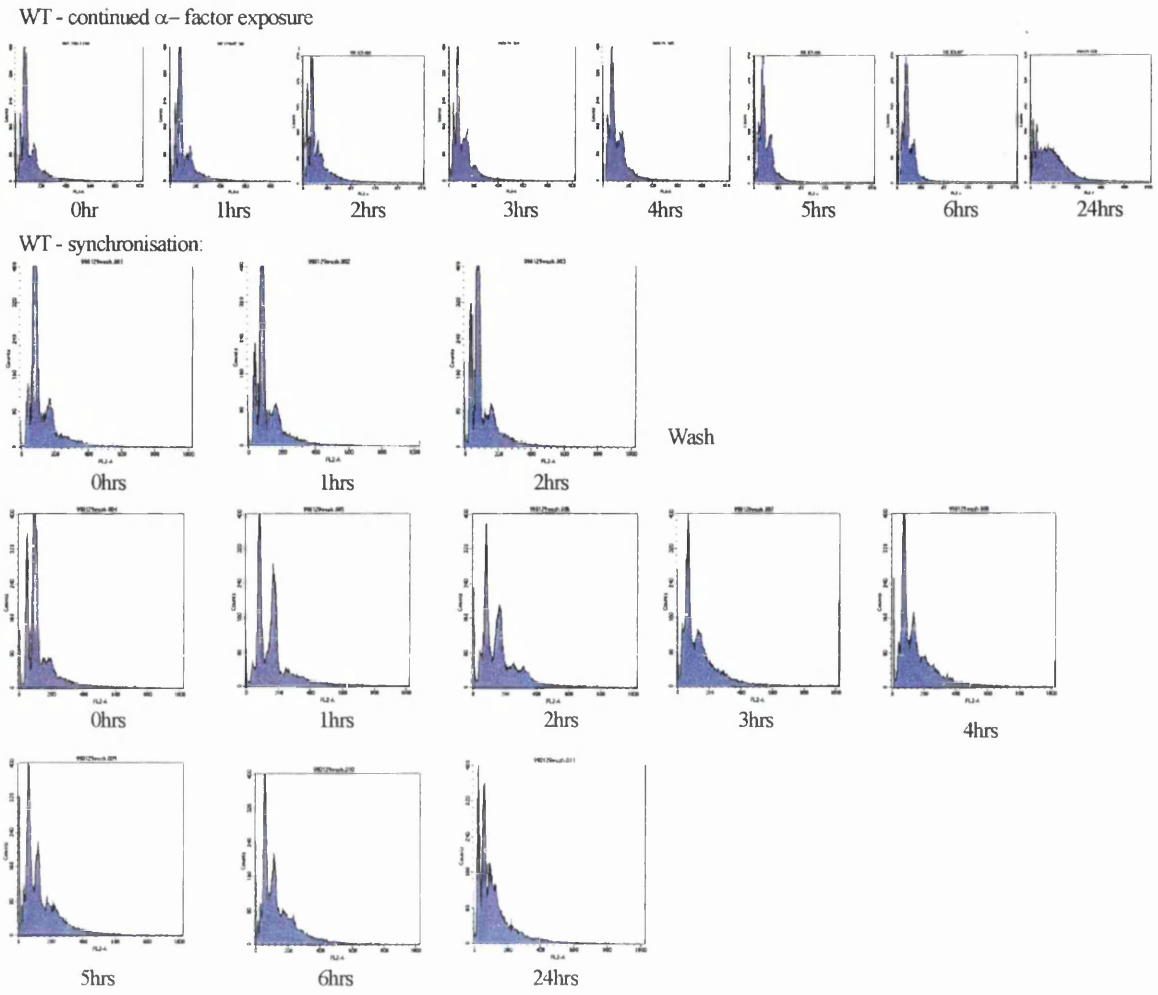


Figure 18 FACS analysis of wild type cells in response to α-Factor.

To synchronise cells for FACS analysis, cells were exposed to 15μg/ml α-Factor and analysed. G_1 and G_2 peaks are evident during the first 6hrs exposure. After 24hrs, cells come out of arrest and become asynchronous. When cells were exposed for 2hrs and washed off the pheromone cells displayed a large G_2 peak followed by cells becoming out of phase after three hours. The reason for this G_2 peak is unclear. It may be artefactual as this was not repeated.

5.10.2. FACS analysis after cisplatin treatment

Wild type, *mlh1* and *msh2* cells were then treated with 1mM cisplatin to investigate cell cycle responses after synchronisation. Fig. 19 shows that exposure to 1mM cisplatin after 4 hours with 4 hours treatment with pheromone resulted in wild type cells entering predominantly a G_1 arrest. Pheromone alone caused a G_1 and G_2 peak. Again after 24 hours, cells became asynchronous and difficult to interpret. Cisplatin however did show signs of sustaining a G_1 arrest in cells that otherwise became asynchronous to a 24 hour exposure to α-Factor. This cell cycle response was also seen in the MMR mutants although no G_2 peak was seen in response to α-Factor alone. This may indicate a defect in MMR-defective yeast cells in inducing G_2 arrest. The figure shows the difficulty in distinguishing between G_1, S, G_2 and M phases. Background noise, which was reduced by slowing the rate of flow cytometry, remains high, making analysis difficult to interpret.

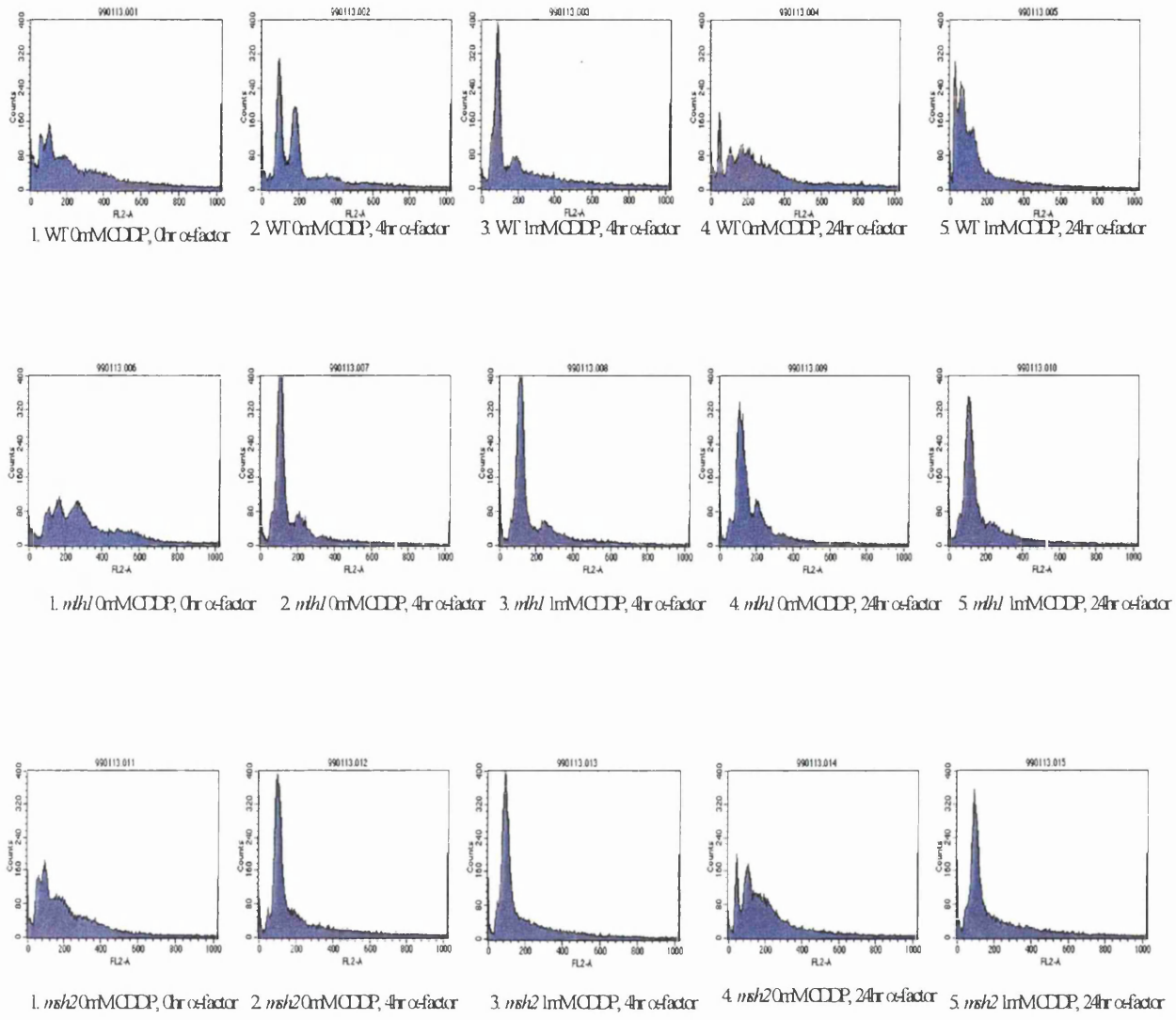


Figure 19 FACS analysis of wild type (WT), *mlh1* and *msh2* mutants exposed to α-Factor and cisplatin.

Graphs show G_1 peak and G_2 peaks in wild type cells in response to α-Factor and predominantly a G_1 peak in response to α-Factor with 1mM cisplatin. MMR mutant strains displayed this response also but possibly showed a defect in G_2 arrest in response to pheromone alone.

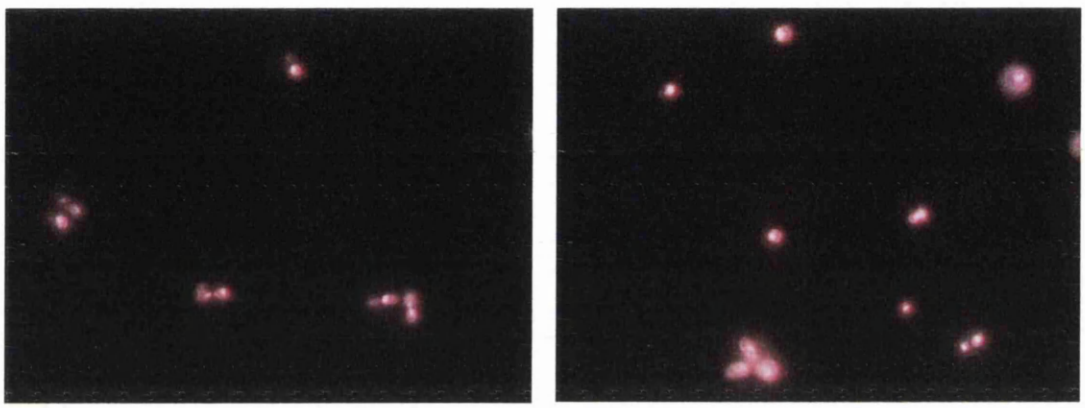
5.11. Fluorescent Microscopy

Due to the problems encountered by FACS analysis of yeast, a more rapid method for measuring cell cycle arrest in budding yeast which does not require the synchronisation of cells for analysis was therefore adopted. Budding yeast arrest in G_2-M by forming enlarged, equally sized buds with a 2N nucleus at the bridge between the unseparated buds (resembling a dumb-bell structure) (Lowndes N, *personal communication*; Pringle *et al,* 1997).

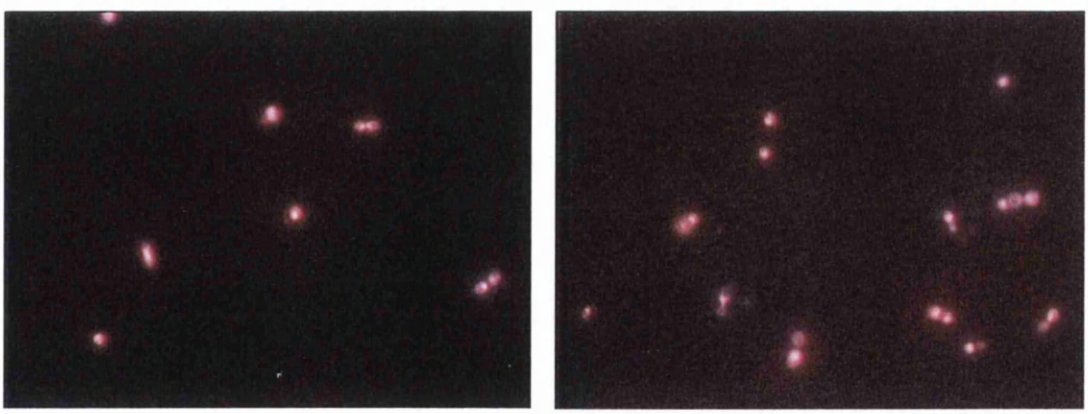
At the time of carrying out these experiments, it was considered important that cells should not be exposed to cisplatin for such a long period as cell cycle responses may be affected by prolonged exposure. Cisplatin exposure was therefore reduced to a 1 hour treatment but at a higher concentration of 4mM instead of 1mM. The PI stained samples were scored under a fluorescent microscope and Fig. 20 shows an example of the micrographs used to score for G_2 buds. Wild type and *ScMLH1* transformant cells did not visually start exiting from G_2 arrest until 12-16 hours after the initial exposure to cisplatin, therefore, samples were taken over a 24 hour period until background levels were reached (See Figs. 20). Fig. 21 shows a magnified example of a G_2 budded structure and also an example of cell exposed to cisplatin indicating signs of nuclear fragmentation typical of mammalian programmed cell death. Fig. 22 shows the G_2 arrest response pattern of wild type, *mlh1*, *msh2* and the *mlh1* transformants after a 1 hour exposure to 4mM cisplatin.

Figure 20 Micrographs of WT and *mlh1* responses to a 1 hour exposure of 4mM cisplatin.

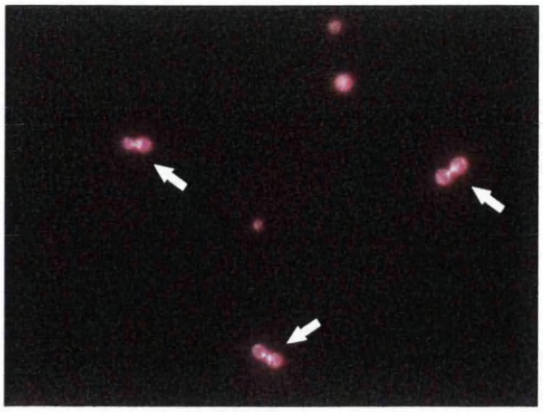
Random field-of-view pictures were taken of cells fixed, RNAse digested and propidium iodide stained at various time points after initial exposure to cisplatin. Pictures show on left wild type (WT) cells prolonged in G_2-associated enlarged, equal-sized budded structures compared to *mlh1* mutants on the right. White arrows indicate G_2-buds, blue arrows indicate cells progressed through mitosis with separated nuclei.



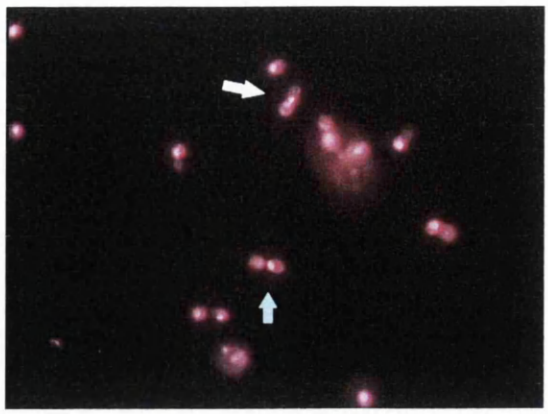
WT - 0hr **mlh1 - 0hr**



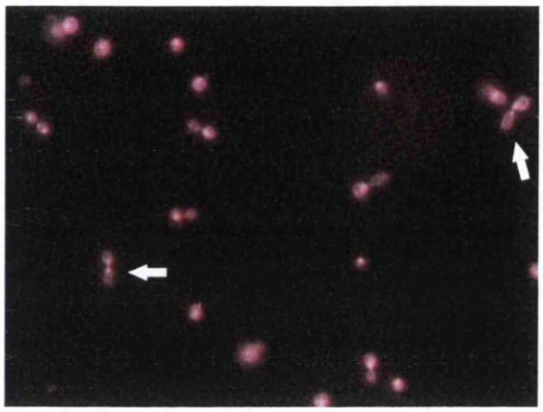
WT - 2hrs ***mlh1* - 2hrs**



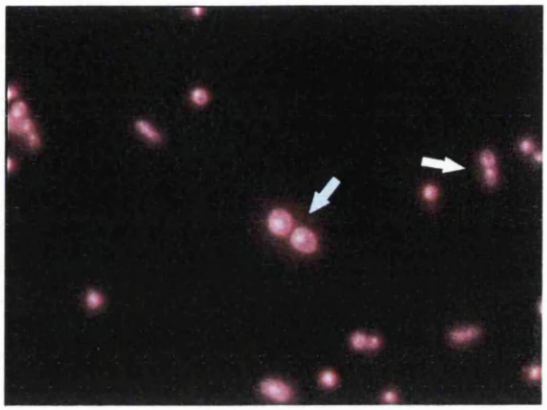
WT - 4hrs



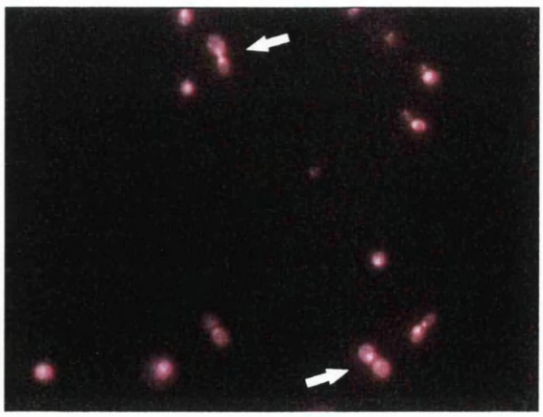
***mlh1* - 4hrs**



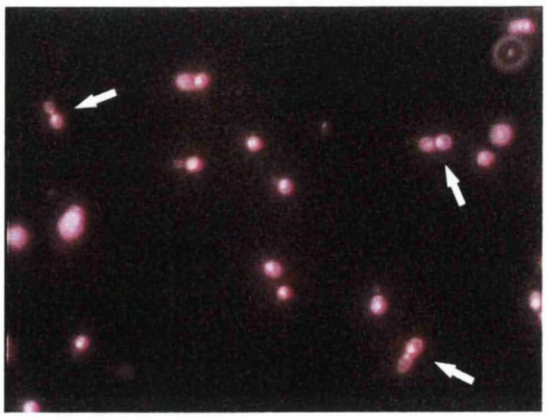
WT - 6hrs



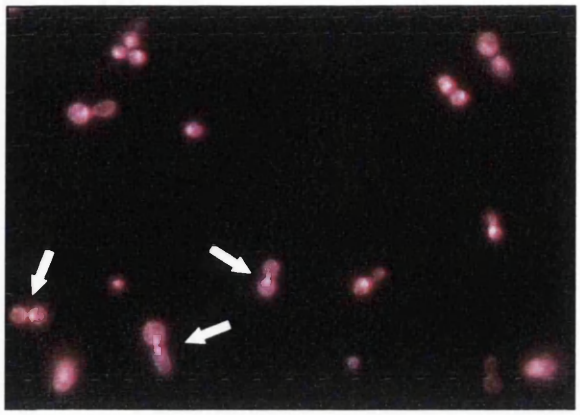
***mlh1* - 6hrs**



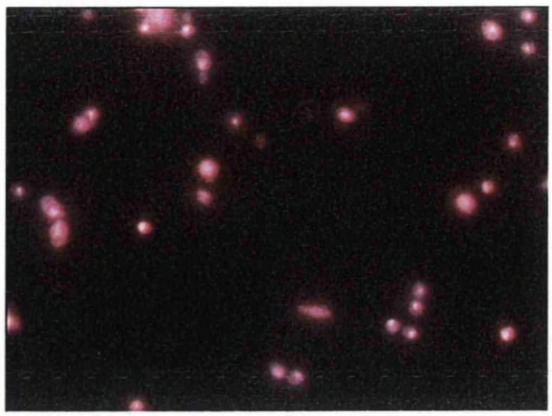
WT - 8hrs



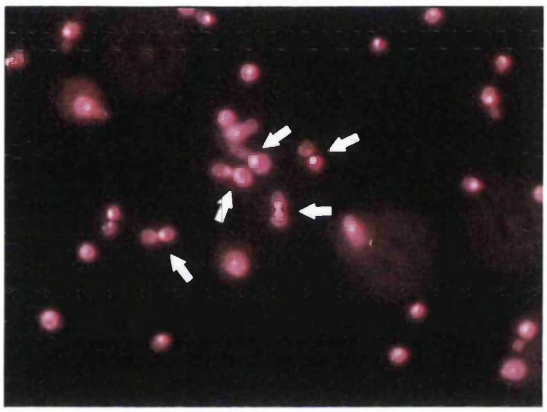
***mlh1* - 8hrs**



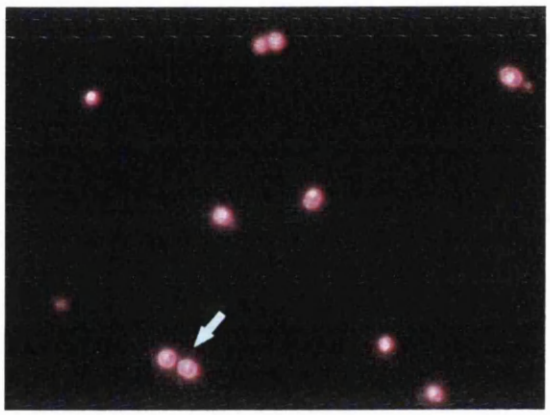
WT- 10hrs



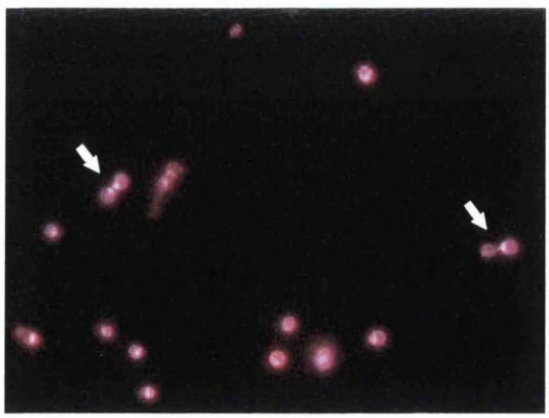
mlh1- 10hrs



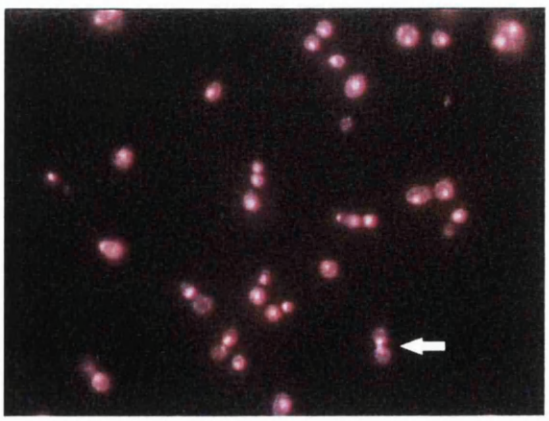
WT - 12hrs



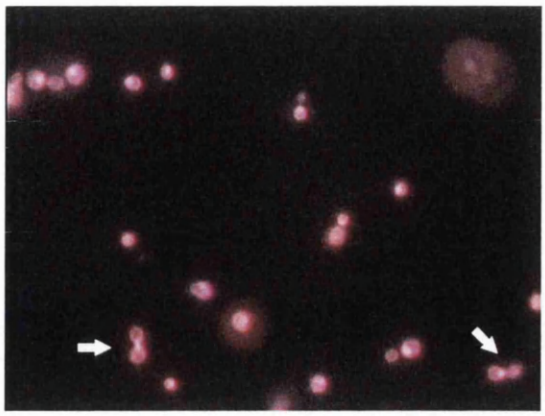
mlh1 - 12hrs



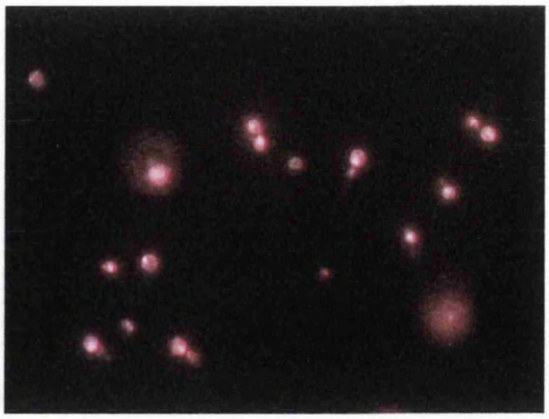
WT - 14hrs



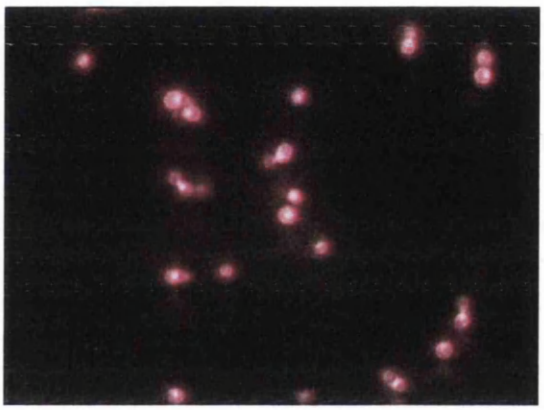
mlh1 - 14hrs



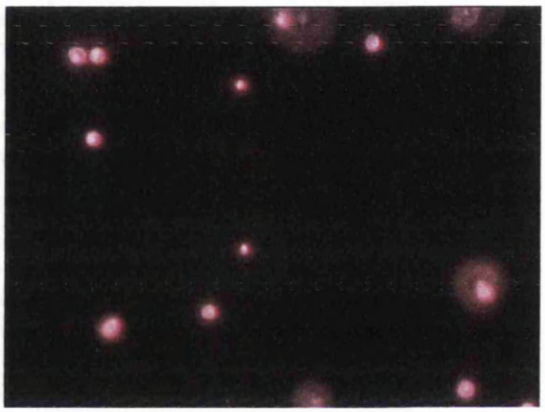
WT - 16hrs



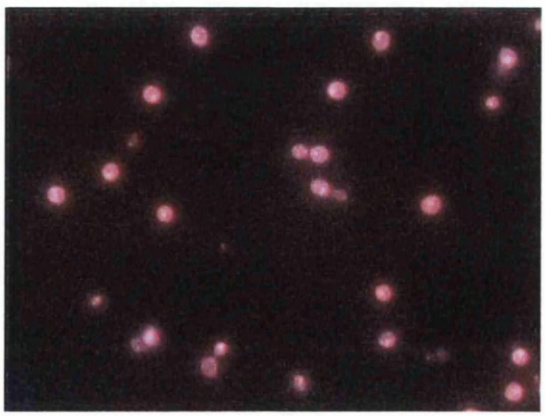
***mlh1* - 16hrs**



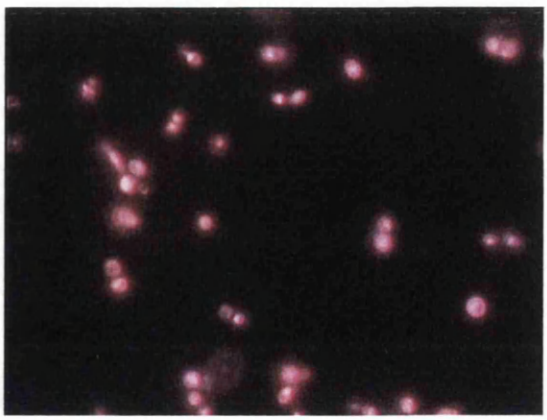
WT - 18hrs



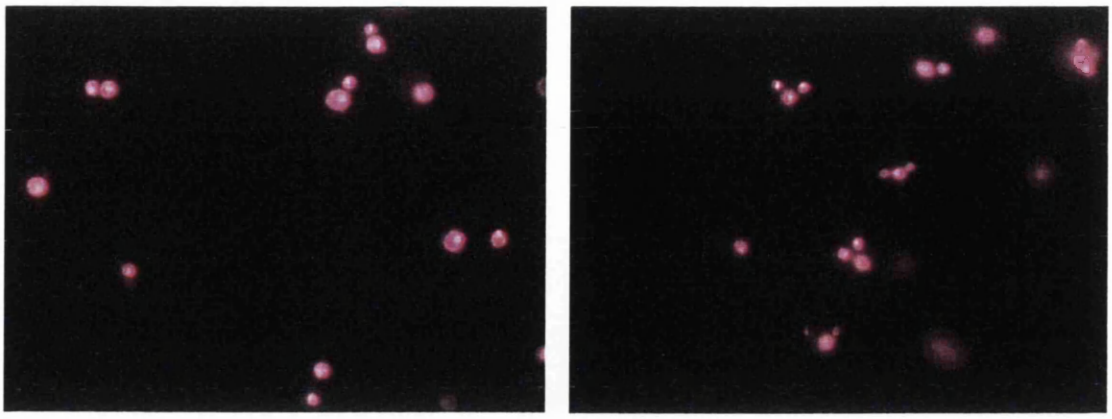
***mlh1* - 18hrs**



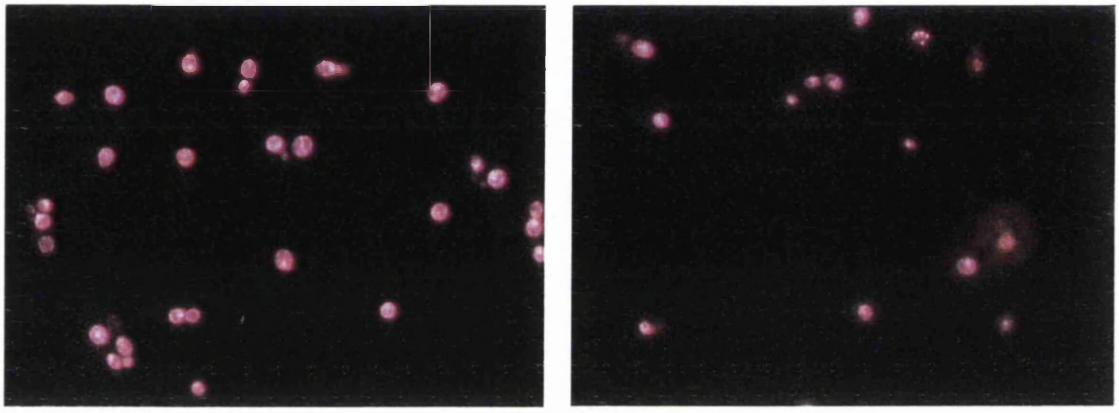
WT - 20hrs



***mlh1* - 20hrs**



WT - 22hrs ***mlh1*** **- 22hrs**



WT - 24hrs **mlh1 24hrs**

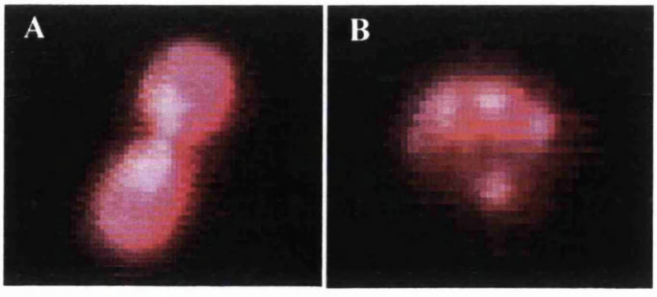


Figure 21 Magnified pictures of PI-stained *S. cerevisiae* cells.

PI stain shows (A) a wild type cell exposed to 12 hours with cisplatin showing equal-sized buds typical of cells in G_2 and (B) a wild type cell exposed to cisplatin for 24 hours showing signs of DNA fragmentation.

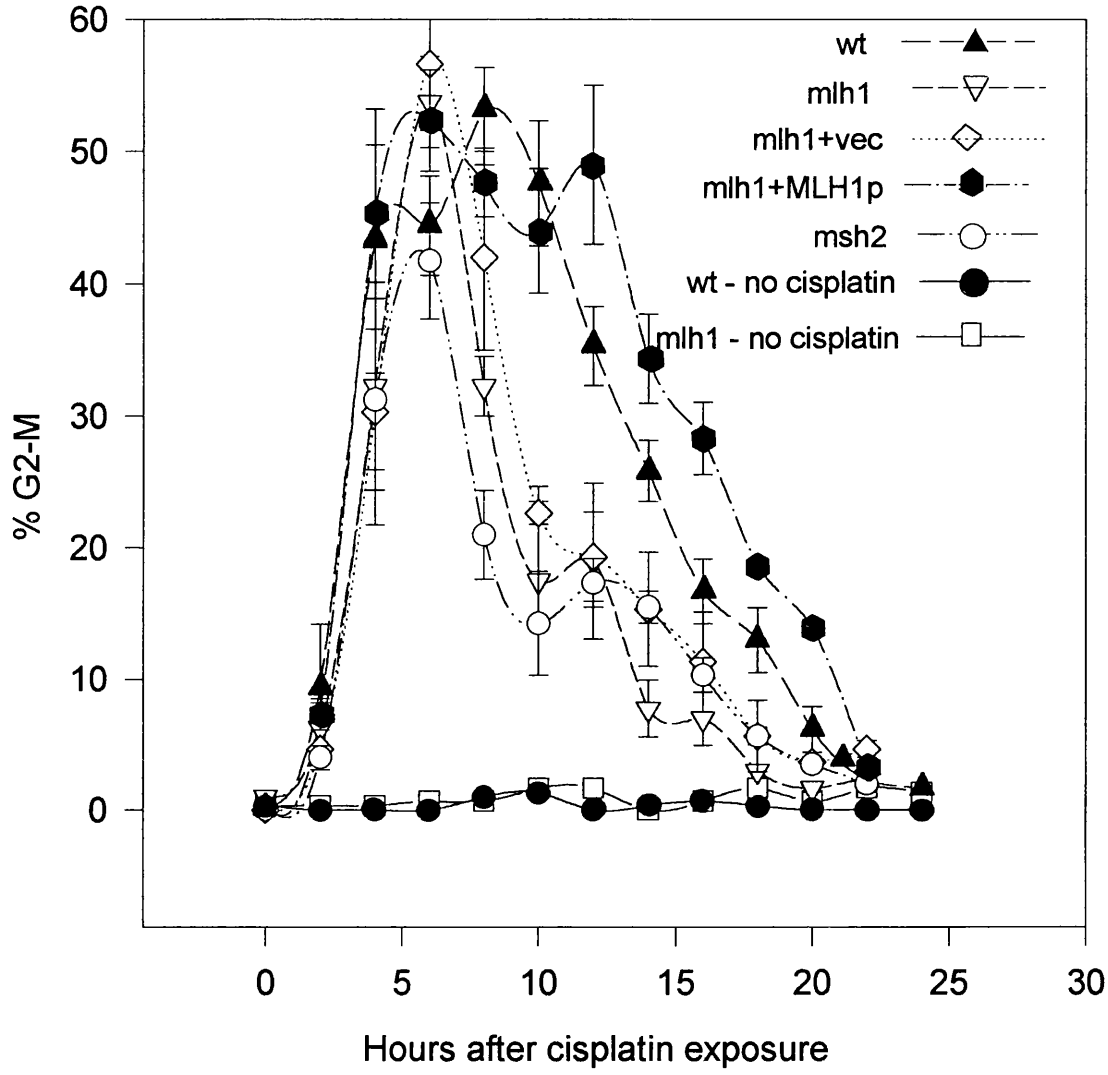


Figure 22 Percentage of cells of each strain in G_2-M arrest in response to cisplatin.

Cells in logarithmic growth phase were exposed to 4mM cisplatin in liquid YPD media for 1 hour. After exposure, cells were resuspended in YPD, placed in shaking incubator and taken off every 2 hours for sampling. G_2 buds were scored out of 200 cells counted under a fluorescent microscope. Experiments were repeated three times. *mlh1* and *msh2* mutants show an early release from G_2 arrest after 6 hours compared to a sustained 10 hour peak for WT. Re-introducing *ScMLH1* back into the mlh1 mutant restores the prolongation of G_2-M arrest (error bars represent standard error of the mean).

5.12. Clonogenic response to 1 hour exposure of 4mM cisplatin

In order to ascertain the cytotoxicity of this cisplatin exposure, clonogenic assays were performed on samples before fixation and staining for cell cycle analysis. 200 cells were seeded out onto YPD agar plates and incubated at 30°C for 2-3 days. Colonies were counted and surviving fractions calculated from no treatment controls (Table 5).

Number of colonies		Wild type	*mlh1*	*msh2*
10μl DMSO control	1.	277	152	180
	2.	210	194	116
	3.	220	143	195
mean		233.91	161.56	159.68
S.F		1	1	1
4mM cisplatin dissolved in 10μl DMSO, 1hr	1.	270	168	145
	2.	128	125	158
	3.	219	159	121
mean		196.34	149.46	140.48
S.F		0.84	0.93	0.88
students' t test compared to no treatment, t=		0.57	0.58	0.47

Table 5 Clonogenic response to a 1 hour exposure of 4mM cisplatin

Figures show that a 1 hour exposure to 4mM cisplatin was not significantly cytotoxic to any of the strains tested as measured by colony forming ability on YPD agar.

5.13. Cell cycle responses to ionising radiation

The literature concerning cell cycle responses made by tumour cell lines defective in MMR to ionising radiation (IR) is inconsistent. Some studies have observed an attenuated G_2 arrest and others have observed no difference at all (See Section 1.2.4). Therefore, isogenic yeast strains defective in specific components of MMR was carried out to investigate the responses made in *S. cerevisiae*. The experiment was also carried out as a control, since it has not been shown that MMR proteins bind to DNA damaged by ionising radiation.

Exponentially growing yeast were exposed in liquid culture in universal tubes to 100gy γ-irradiation (Weinert and Hartwell, 1988) to induce cell cycle arrest. Cells were then sampled every two hours as before, fixed, PI stained and scored for G_2 buds under fluorescent microscopy. Fig. 23 shows the responses made by wild type, *mlh1*, *msh2*, *rad1* and *rad52* strains.

rad1 and *rad52* mutants show a significantly elevated G_2-M arrest response ($P<0.05$) compared to wild type at 0 and 2 hours after IR exposure. The *mlh1* and *msh2* strains show similar responses to that of wild type. However, a significant difference ($P<0.05$) is observed between *mlh1* and wild type at 2 and 4 hours after IR exposure and between *msh2* and wild type at 2 hours after IR exposure. No difference was seen after this initial response.

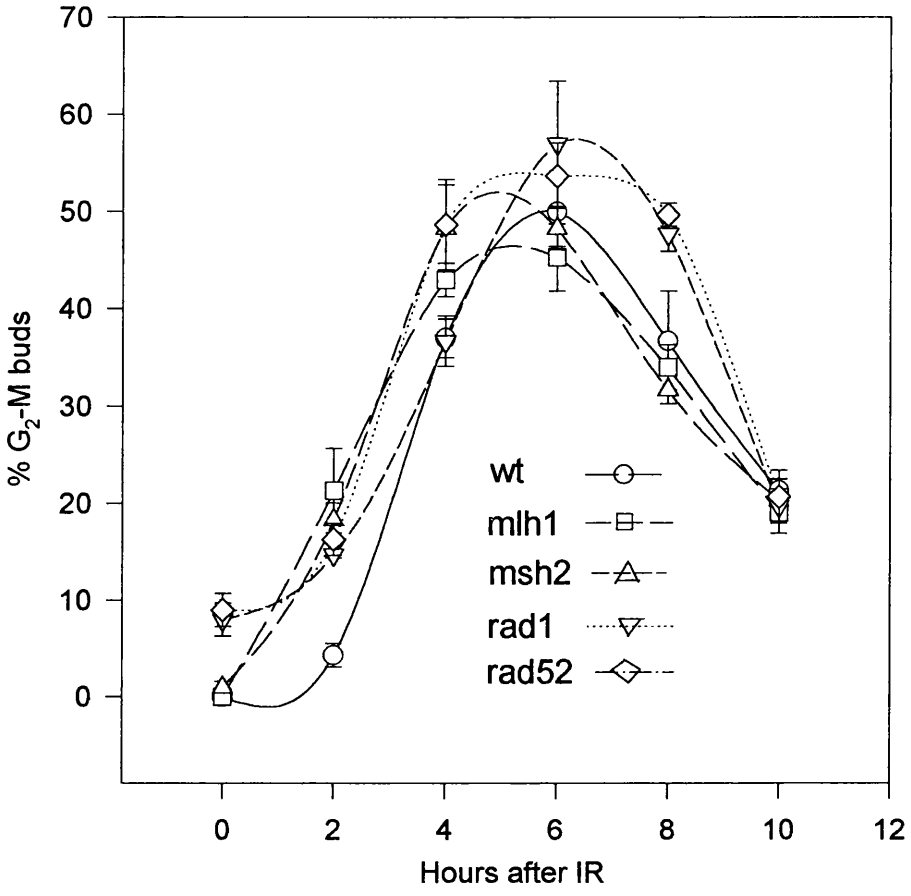


Figure 23 Percentage of cells of each *S. cerevisiae* strain in G_2-M arrest in response to IR.

Exponentially growing strains were exposed to 100gy γ-IR and samples were taken at 2 hourly intervals after exposure. G_2-M - associated equally sized buds were scored by propidium iodide-staining followed by fluorescent microscopy. Error bars indicate standard deviation from the mean.

5.14. Clonogenic response to ionising radiation

The clonogenic responses of these strains were also tested to ascertain if 100gy IR was cytotoxic to the cells. This dose was chosen to induce G2 arrest and not to kill the cells outright (Weinert and Hartwell, 1988). If the dose used was cytotoxic to the cells, then cell cycle analysis would be confounded by the presence of severe DNA damage and the activation of cell death signals.

Table 6 shows the surviving fraction obtained from cells taken after IR exposure before the fixation and staining procedures for cell cycle analysis. 200 hundred cells were plated out on YPD agar and colonies counted after 2-3 days incubation. The table shows that colony forming ability was reduced, especially for the *rad1* mutants. *rad52* mutants showed no difference in cytotoxicity compared to wild type. This is surprising due to the fact that Rad52 is involved in double strand break repair by recombination. *rad* mutants should be IR-sensitive. This is seen in *rad1* mutants but not *rad52*. The reason for this is unclear. Statistical analysis was not possible due to the single experiment carried out for the no treatment control.

	WT	***mlh1***	***msh2***	***rad1***	***rad52***
0gy	238	113	290	309	175
100gy	69	44	99	56	92
	64	49	118	76	44
	102		185	45	55
S.F	0.33	0.41	0.46	0.19	0.36

Table 6 Number of colonies formed after ionising radiation exposure
The surviving fraction (S.F) values show that colony forming ability was reduced in response γ-ray IR exposure. The T-test could not be calculated due to the single assay carried for the no treatment control.

These results suggest that the dose of IR used was partially cytotoxic to all the strains exposed. Caution must therefore be taken in the analysis of cell cycle phenotypes when considering the amount of DNA damage induced. The relative number of replication

forks encountering IR-induced DNA lesions must be taken into account when assessing the differences between strains in ability to undergo cell cycle arrest. However, it is difficult not to induce cell death in a proportion of cells exposed to cell cycle arresting doses of IR and the majority of stained cells scored for G_2 arrest in the cell cycle analysis would probably have been viable considering that cells subjected to lethal doses of IR would not cycle at all.

DISCUSSION

6. DISCUSSION

6.1. Cytotoxic response of MMR mutants to cisplatin

Strains of *S. cerevisiae* with specific MMR gene disruptions were used to test the relative clonogenic responses to cisplatin exposure compared to wild type. Each strain, exposed for 24 hours to 0-2mM cisplatin in liquid culture, was assessed for colony forming ability on agar. Fig. 9 shows the relative sensitivities of wild type, *msh2, msh3, msh6, mlh1, pms1* and *mlh3* mutants. The graph shows that all of the mutants, except for *pms1*, displayed a 1.4- to 2-fold significant ($P < 0.05$) increase in resistance to 1mM cisplatin. Table 2 shows the L.D. 90 (mM) and the resistance factor (RF) for each strain. The RF's for each strain are in the descending order of resistance: *mlh2* > *msh2* ≡ *msh3* > *msh6* > *mlh1*> *pms1*.

Since not all of the genes knocked out for MMR produced the resistance phenotype (i.e. *pms1*), these results suggest that MMR activity *per se*, or at least MMR involving *PMS1*, need not be required for cisplatin-induced cytotoxicity. In support of this, it has been noted in patients with HNPCC a surprisingly low frequency of germ-line *PMS2* (*PMS1* in yeast) mutations exists compared to *MLH1* mutations especially given that mutations in both of these genes cause the same type of MMR defect (Boyer *et al*, 1995). Additionally, Mlh1 in *S. cerevisiae* has been shown to be unique among MMR proteins in its involvement in recombinational processes (Hunter and Borts, 1997). In this context, it has been suggested that eukaryotic Mlh1 and Pms1/2 do not play

equivalent roles in the cell even though they are subunits of the same complex, or that like Msh3 and Msh6, there is a function that is redundant with *Sc*Pms1/hPms2 (Kolodner, 1996).

Disrupting *ScMLH2* lead to the strongest resistant phenotype of all the MMR mutants. No known function for *scMLH2* has yet been ascribed although it was suggested that the gene might be involved in homeologous recombination (Prolla *et al,* 1994a). Interestingly, *scMLH2* is more closely related in sequence to the human *PMS* genes than to *hMLH1* (Kolodner, 1996) which is surprising considering the differences observed here in cytotoxic responses of the two mutants. However, to date no human *MLH2* gene has been identified and the percentage sequence similarity observed between *scMLH2* and *hPMS2* was only 39.87% (McIlwrath *et al,* 1998).

These strains were also exposed to carboplatin, doxorubicin and UV irradiation (Durant *et al,* 1999). Resistance to carboplatin was also seen and each strain displayed similar orders of resistance (*mlh2* > *mlh1* > *msh2* ≡ *msh6* > *msh2* > *pms1*). This is consistent with the fact that Carboplatin is a structurally related compound to cisplatin known to induce the same spectrum of DNA adducts. Even higher folds of resistance to doxorubicin was observed, but no difference was seen between wild type and mutants exposed to UV irradiation. Doxorubicin induces a range of adducts in DNA including double strand breaks and redox cycling-induced DNA crosslinks (Sladanowski and Konopa, 1994). Therefore it may be the case that more than one doxorubicin-induced substrate is recognised by MMR, increasing the chance of a lethal signal being generated.

Mismatched bases damaged by UV irradiation (inducing cyclobutane pyrimidine dimers) have also been reported to be substrates for bacterial MutS (Feng *et al,* 1991; Wang *et al,* 1997) and human MutSα (Wang *et al,* 1999). However, the major cytotoxic lesion induced by UV is removed efficiently by NER and is therefore not expected to persist (Sweder *et al,* 1996).

The cytotoxic responses to these DNA damaging agents and the low level of resistance obtained are in concordance with what is seen in cisplatin-resistant tumour cell lines (Fink *et al,* 1996), lending credence to the extrapolation of the yeast model to human cells. The low level of resistance (≤ 2-fold) observed *in vitro* is regarded as clinically relevant to the acquisition or enrichment of chemotherapeutically resistant tumour cells during treatment and is therefore sufficient to account for treatment failure (Andrews *et al,* 1990).

6.2. *ScMLH1* gene re-introduction

Previously, it has proven difficult to re-introduce *hMLH1* into *MLH1*-deficient tumour cell lines. Instead, chromosome 3 transfer which carries the *MLH1* gene has been carried out (Koi *et al*, 1994; Aebi *et al*, 1996; de la Alas, 1997). The obvious disadvantage of this is that many other genes are re-introduced which might confound the phenotypic effect under study. To test if *ScMLH1* had a direct role in conferring sensitivity to cisplatin, *ScMLH1* alone was re-introduced back into the *mlh1* mutant using a yeast expression vector. The cisplatin toxicity assay was then carried out on the yeast transformants with the wild type *ScMLH1* gene detected and sequenced. As there is no commercially available antibody for scMlh1 protein, PCR and sequencing were the principle methods used for the detection of the cloned *ScMLH1* gene in yeast.

6.2.1. Detection of *ScMLH1* in pYX vectors

Fig. 10 shows the absence of an *ScMLH1*-sized band in *E. coli* colonies transformed with the cloned pYX112 (low copy yeast expression vector). However, a correctly sized band is shown in colonies transformed with the cloned pYX212 (high copy yeast expression vector). During the preparation of this thesis, the reason for the lack of detection in the pYX112 vector was identified. During the design of the vector primers that flank the cloning sites of the two vectors, an error of design of pYX112 primers was made in that the 5' forward primer and 3' reverse primer were reversed. This therefore generated PCR products across the entire sequence of the vector and not across the cloning site. Therefore the selection for colonies containing the correct clones could not be made for this vector.

6.2.2. *ScMLH1* cloned into yeast

Fig. 11 shows the PCR products produced using *ScMLH1* primers and vector primers from individual yeast colonies isolated on selective media. This confirms the re-introduction of an *ScMLH1*-sized product with the pYX212 transformed yeast clone isolated. The slightly larger fragment seen using the vector primers is consistent with the extra vector sequences flanking the insert region.

6.2.3. *ScMLH1* sequencing

Following two separate sequencing tests (using the same forward and reverse primers in each) on the same *ScMLH1* insert cloned into yeast, the same five point mutations were detected (See Appendix). The following lists the missense mutations detected.

Cys208Trp
Ile263Val
Ala712Thr
Ser717Asp
Leu770Pro

scMlh1: 781 amino acids (see Appendix)

Note that none of these are nonsense frameshift mutations. According to the ICG-HNPCC database, 90% of *hMSH2* mutations and 69% of *hMLH1* mutations are nonsense, frameshift and splice site mutations (Peltomaki *et al,* 1997; Papadopoulos *et al,* 1994; Shimodaira *et al,* 1998), indicating that pathological mutations, for the majority, represent gene disruptions that completely knock out their function. Furthermore, the majority of the point mutations detected here lie in the C-Terminus of scMlh1. The region of high sequence homology between the MutL proteins lies within

the N-Terminus, where the so called MutL box: GFRGEAL resides. Results presented here show that the cytotoxic function of *ScMLH1* was not affected sufficiently by these mutations/polymorphisms. It would have been interesting to see if these point mutations had any effect on MMR function as measured by mutation frequency/rate. This was not carried out due to time constraints.

6.3. Mutator phenotypes

6.3.1. Mutation frequency & rate

After testing the forward mutation frequencies and rates of the MMR mutants as determined by L-canavanine resistance, Table 3 shows the values established for each strain. Both methods revealed that *pms1* mutants were the highest mutators, followed by *mlh1* and *msh2* mutants. *mlh2* mutants did not show a significantly different mutator phenotype as measured by frequency or rate, compared to wild type. These data agree with previous reports that showed that *MLH2*, originally identified and cloned as a homologue of the yeast MutL genes, is not a mutator when knocked out (Prolla *et al*, 1994a; Borts R, *personal communication*).

These mutator phenotypes provide additional evidence to support a separation of roles of MMR proteins involved in the cytotoxic response to DNA damage. The *mlh2* mutant did not display a mutator phenotype but conferred the highest level of resistance to cisplatin and the *pms1* mutant displayed the highest mutator phenotype but did not confer resistance to cisplatin. This indicates again that not all components of MMR may be involved in the cytotoxic response and that mismatch repair *per se*, or at least MMR requiring Pms1, need not be required.

6.4. Cytotoxic response of MMR/*rad52*/*rad1* mutants to cisplatin

Fig. 13 & 14 show the clonogenic responses of the *rad52* and *rad1* mutants compared to wild type. The hypersensitivity observed in both mutants was significant ($P<0.05$) indicating that *RAD52* and *RAD1* are involved in the repair of cisplatin-induced DNA damage. The figures also show responses made by MMR/*rad52*- and MMR/*rad1*-double mutants. Without the presence of either Rad52 or Rad1, the resistance phenotype originally conferred by the absence of *msh2*, *mlh1* or *mlh2*, is lost (no significant difference seen between *rad52*- or *rad1*-MMR double mutants and the hypersensitive *rad1* or *rad52* mutants alone, $P>0.05$). This indicates that the presence of Rad52 and Rad1 are required for MMR-dependent cytotoxicity in response to cisplatin and implicates a recombinational-dependent process in damage tolerance (Durant *et al*, 1999).

Rad52 is an essential component of homologous recombination. It has been proposed that bypass of lesions during DNA replication is carried out by recombinational repair (Zou and Rothstein, 1997). Here, *rad52* mutation in mitotically growing yeast were shown to greatly reduce the level of Holliday junctions, S-phase specific structures of recombination stimulated to repair replication-related lesions.

Rad1 is a component of NER but has also been implicated in recombination repair. *RAD1* was shown to be required for removal of nonhomologous DNA from the 3' ends of recombining DNA, a process analogous to the excision of photodimers during repair of ultraviolet-damaged DNA (Fishman-Lobell and Haber, 1992). In a similar role to Rad52, Rad1 has been suggested to act to resolve Holliday junctions (Habraken *et al*, 1994) explaining the recombination defect observed in *rad1* mutants. It has also been shown that purified *Sc*Rad1 and *Sc*Rad10 interacted with a synthetic bubble structure and incised the DNA at the 5'-side of the centrally unpaired region, resembling the dual

incision step in nucleotide excision repair in vivo (Fishman-Lobell and Haber, 1992; Davies et al, 1995).

6.5. Recombination bypass and DNA replication

A model to explain the dependence on the presence of Rad52/Rad1 proteins for MMR-dependent cytotoxicity is shown in Fig. 24.

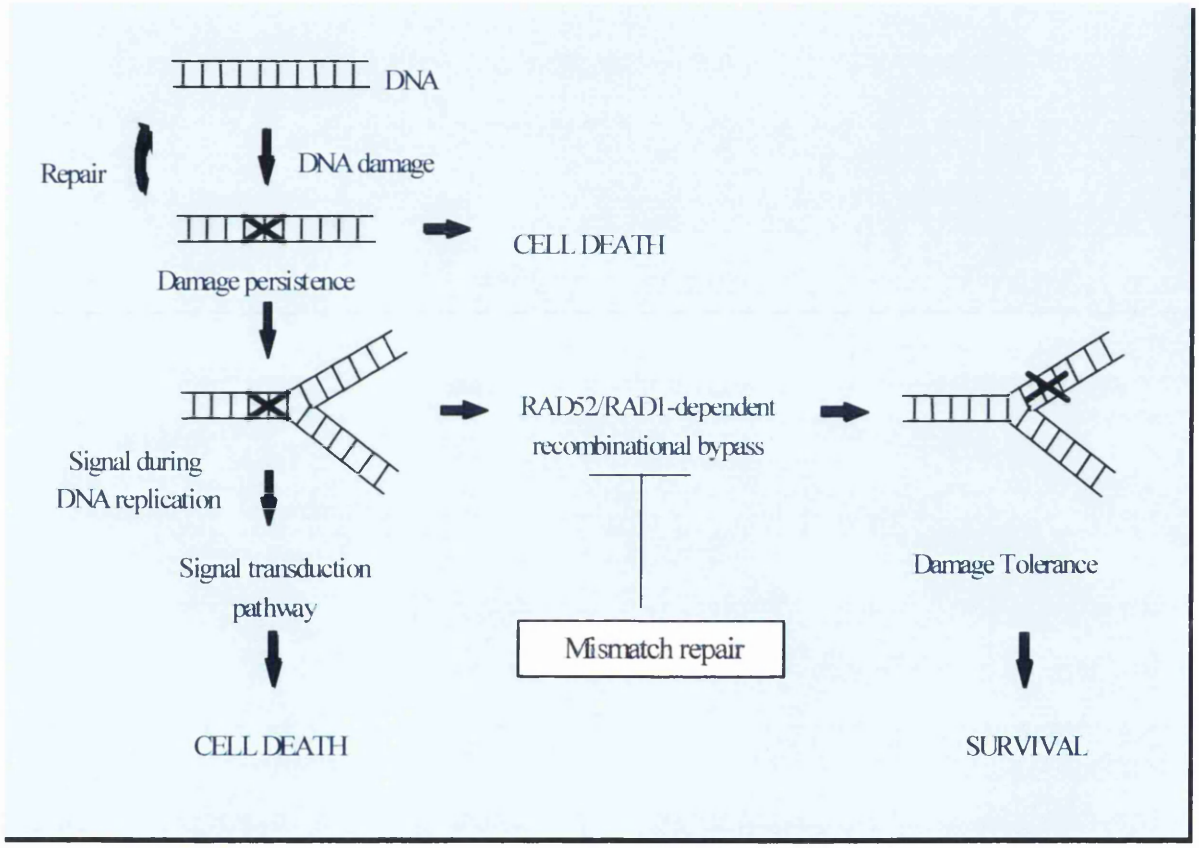


Figure 24 Model of recombination-dependent bypass

Mismatch repair proteins inhibit a *RAD52/1*-dependent cisplatin adduct bypass mechanism either by blocking the bypass apparatus at the adduct or by direct functional inhibition.

The bypass model predicts that loss of MMR proteins will reduce the probability of lethal signals being generated during replication by allowing adduct bypass to proceed. Studies which support such a model have shown that MMR-defective ovarian carcinoma cell lines display enhanced replicative bypass of platinum adducts compared to MMR-proficient derivatives (Mamenta *et al,* 1994; Vaisman *et al,* 1998). Indeed it has been shown, without inducing any DNA damage, that re-introducing human *MLH1* into HCT116 *MLH1*-deficient cells decreased cellular proliferation and rate of DNA synthesis (Shin *et al,* 1998). This suggests that MMR, or at least Mlh1, has a function in inhibiting DNA replication and possibly in inhibiting recombination-dependent DNA replication passed specific lesions.

To test if DNA replication was indeed necessary for MMR-dependent drug sensitivity, the cytotoxic responses of the wild type and *msh2* mutant strain to cisplatin under growth inhibitory (and therefore reduced DNA replication) conditions were analysed. Cells were grown exponentially and at stationary phase by seeding at growth-promoting ($2x10^7$/ml) and confluent (>$1.5x10^8$) concentrations, respectively. Fig. 16 shows a significantly increased resistance of wild type cells exposed to cisplatin during stationary phase of growth (L.D. 90 of 1.8mM) compared with logarithmically growing cells (L.D. 90 of 1.0mM). However, Fig. 17 shows that the cisplatin-sensitivity of the *msh2* strain is not affected by growth phase. These results suggest that DNA replication is required for MMR-dependent cytotoxicity.

One must lend caution to this observation. *RAD52/RAD1*-dependent recombinational bypass of genetic lesions occurs during DNA replication as a system to restore collapsed replication forks (Zou and Rothstein, 1997). It has been shown that the recombination intermediate, the Holliday Junction, reaches its highest level during S phase (Zou and Rothstein, 1997). Inhibiting DNA replication may therefore also inhibit the bypass mechanism and thus have no net effect on drug sensitivity. One interpretation of the results is that recombination is not as dependent on S-phase as the lethal signal generated by MMR. Another is simply that more DNA damage is generated and/or are channelled into the recombinational repair pathway during replication, with recombination not being directly dependent on replication.

6.6. Cell cycle analysis

Cell cycle analysis was carried out for the following reasons. i) The DNA replication inhibition experiments here show that MMR-dependent cytotoxicity is, at least in part, dependent on DNA replication and thus progression through S-phase of the cell cycle; ii) previous studies have shown that *MLH1* transfection induced growth suppression in an *MLH1*-defective cell line (Shin *et al,* 1998) and DNA damage sensitive MMR-proficient cells were able to enter G_2 arrest while DNA damage resistant MMR-defective cells continued cycling (Koi *et al,* 1994; Hawn *et al,* 1995; Carethers *et al,* 1996), and iii) the cell cycle of *S. cerevisiae* MMR mutants has not previously been analysed in response to cisplatin-induced DNA damage.

6.6.1. FACS analysis

After cells were treated with cisplatin, stained with PI and ran through the FACS machine, G_2 peaks were analysed as shown in Fig. 18. Cells were synchronised to allow accurate peak histogram study of cell cycle responses. Mating Type A (MatA) cells were studied due to the availability of the cell cycle arrest pheromone, α-Factor. Mating Type α (Matα) cells, which were the strains used in the cytotoxicity assays, could not be used due to the commercial unavailability of the highly lipophilic A-Factor. The analysis showed that MatA wild type cells became synchronised by treatment with the yeast pheromone α-Factor. This induced a G_1 and a G_2 arrest in preparation for meiosis. However, this response lasted only for some time after 6 hours, after which cells began cycling out of phase (due to the initial difference between mother and daughter cell size and physiology). Yeast begin to ignore external pheromonal influences after approximately 3-4 hours by down regulating receptor levels (Schiebal E., *personal communication*), therefore synchronisation can only be maintained for this period of time.

Cells were therefore washed off the pheromone after a 2 hour treatment (Pereira *et al,* 1998) and the analysis shows that cells became asynchronous after 2-3 hours. Fig. 19 shows the analysis of wild type, *mlh1* and *msh2* cells treated with α-Factor and 1mM cisplatin. The peaks show that wild type cells entered a G_1 and G_2 arrest after 4hrs exposure to drug and α-factor. After this period, the distinction between cell cycle stages became difficult to interpret. The *mlh1* and *msh2* mutants responded by displaying a G_1 peak with little or no G_2 peak. This may indicate a defect in G2 arrest induction in response to α-Factor. FACS analysis in response to cisplatin was confounded by a lack of clear distinction between peaks and background noise.

6.6.2. Fluorescent microscopy

A simpler and quicker method for measuring cell cycle arrest in budding yeast exists which does not require synchronisation of cells for analysis. Using a fluorescent microscope, budding yeast can be scored by counting G_2-M structures that form enlarged, equally sized buds with a 2N nucleus at the bridge between the unseparated buds. This resembles a dum-bell-like structure (Goldstone *et al*, 1997; Lowndes N, *personal communication*).

At this juncture, it was taken into consideration that cells exposed to cisplatin for such a prolonged period of time (24 hours) might affect cell cycle responses. Other factors such as repair activities and prolonged replication stalling times might confound the arrest response. Cisplatin exposure was therefore reduced to a 1 hour treatment but at a higher concentration of 4mM, compared to 1mM. The PI stained samples were scored under a fluorescent microscope and Fig. 20 shows an example of the micrographs used to score for G_2 buds for wild type and *mlh1* strains and Fig. 21 shows more clearly an example of a G_2 bud structure and a cell undergoing DNA fragmentation.

Fig. 22 shows the data for G_2 arrest responses for wild type, *mlh1*, *msh2*, *mlh1* vector alone and *ScMLH1* transformants, from three separate experiments. Although FACS analysis could have been used under these conditions, scoring for G_2 buds under the microscope proved to be effective. Fig. 22 shows the percentage of cells in G_2-M arrest in response to a 1 hour exposure to 4mM cisplatin. The *mlh1* and *msh2* strains show an early (approximately 4 hour) release from G_2-M arrest compared to wild type. In fact, *msh2* cells only reached a 40% peak G_2-M arrest before release compared to a near 60% G_2-M peak observed for wild type. *mlh1* G_2-M arrest responses are significantly different ($P<0.05$) compared to wild type at 10, 14, 16 and 18 hours after cisplatin treatment. *msh2* G_2-M arrest responses are significantly different ($P<0.05$) at 8, 10, 12, 14 and 18 hours after cisplatin treatment. The *mlh1*+vector alone transformant control showed no difference compared to *mlh1* mutant and the *mlh1+MLH1* transformant

showed a prolonged G_2-M arrest possibly even greater than wild type (significantly greater than wild type at 16 and 20 hours, $P<0.05$ 1-tailed T-test).

The cytotoxicity of a 1 hour exposure of 4mM cisplatin was assessed by plating approximately 400 cells before the fixing and staining procedures. Table 5 shows that this exposure was not significantly cytotoxic to any of the strains tested. Surviving fractions reached 0.84, 0.93 and 0.88 for wild type, *mlh1* and *msh2* strains respectively. Therefore, this indicates cells maintained viability after release from G_2 arrest.

These results strongly suggest that the *S. cerevisiae* MMR genes *MLH1* and *MSH2* are involved in prolonging G_2-M cell cycle arrest in response to cisplatin. This may have implications for a role of MMR proteins in the inhibition of cells entering mitosis in response to cisplatin-induced DNA damage (See Section 6.6.3).

6.6.3. Cisplatin-induced cell cycle arrest pathways involving MMR

Studies using *S. cerevisiae* and *S. pombe* have shed some light on the possible mechanisms at fault in human disease. The $Mec1^{sc}/Rad3^{sp}$ (human Atr) protein pathway is central to the cell cycle checkpoint response in yeast, with the closely related protein, Atm, being mutated in individuals with the rare disease ataxia telangiecstasia (Sanchez *et al*, 1996). Studies exploiting the yeast *S. pombe* and *S. cerevisiae* have defined many proteins involved in checkpoint control (Carr and Hoekstra, 1995). $Mec1^{sc}$ is a protein essential for viability and is required for all known DNA structure checkpoints by inhibiting G_2-M progression via $Pds1^{sc}/Chk1^{sp}$ kinase (Carr 1997). $Pds1^{sc}/Chk1^{sp}$ in turn phosphorylates and inhibits $Wee1^{sp}$, a $p34^{cdc2}$ tyrosine kinase. Two checkpoint signals during S-phase exist in yeast - one generated by DNA replication inhibition (in which DNA-protein complexes e.g. Rad proteins, formed during different stages of S-phase, repair and recombination could be structures monitored by this checkpoint pathway) (Carr 1997) and the other by DNA damage.

Both require activation of a protein kinase cascade dependent on Mec1. In the DNA damage checkpoint, Mec1 is required for phosphorylation of Rad53, a protein activated by Rad9 binding which is in turn phosphorylated in response to DNA damage (Sun *et al,* 1998). Rad53 disruption in *S. cerevisiae* and inhibition of Rad9-Rad53 binding abolishes G2/M cell cycle arrest in response to DNA but does not affect replication inhibition-induced arrest (Sun *et al,* 1998).

The results presented here may provide evidence to suggest that MMR proteins act up-stream in either of these cell cycle arrest pathways in response to cisplatin-induced DNA damage. A model to explain the early release from G_2 arrest in MMR mutants in response to cisplatin damage could be that MMR proteins, upon binding to poorly repaired cisplatin intrastrand crosslinks, either prevent DNA replication/lesion bypass or signal the presence of a damaged site (see Fig. 25). This then provides an additional persistent signal that prolongs the phosphorylation events and leads to a sustained cell cycle arrest.

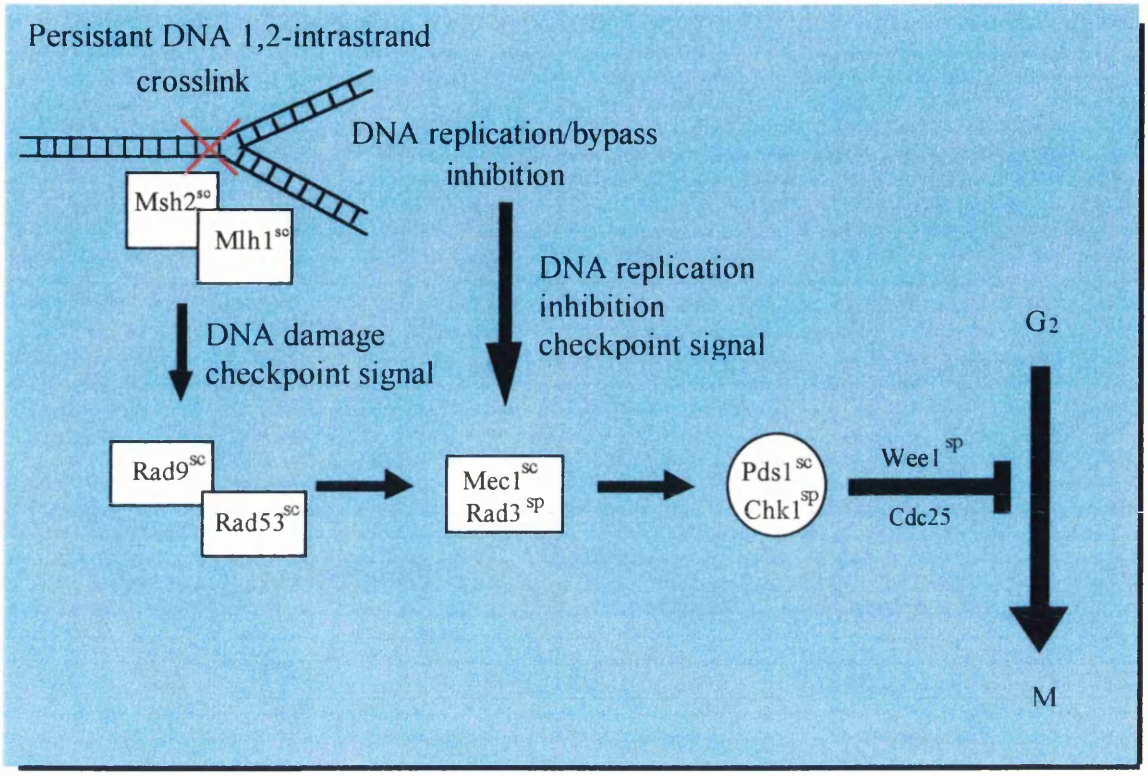


Figure 25 Model of yeast cell cycle arrest involving MMR

MMR proteins bound to unrepaired cisplatin-induced DNA adducts (i.e. 1,2 intrastrand crosslinks) cause replication stalling and bypass inhibition - which then activates the Mec1/Rad3-dependent pathway of cell cycle arrest. Or, MMR proteins signal a DNA damage response pathway dependent on Rad9 and Rad53 which also leads to cell cycle arrest. Either way, the persistent lesion which is not bypassed, leads to a prolonged cell cycle arrest.

6.6.4. *S. cerevisiae* responses to IR

MMR and *rad* mutant *S. cerevisiae* strains were also exposed to 100gy γ-irradiation under a ^{60}Co source. Fig. 23 shows the G_2-M arrest responses of each strain. *rad1* and *rad52* mutants showed a significantly elevated G_2-M arrest response ($P<0.05$) compared to wild type at 0 and 2 hours after IR exposure. This indicates that *rad1* and *rad52* cells enter G_2-M arrest quicker than wild type, possibly as a result of unrepaired IR-induced lesions triggering cell cycle arrest responses. The *mlh1* and *msh2* strains show very similar responses to that of wild type, however, a significant difference ($P<0.05$) was observed between *mlh1* and wild type at 2 and 4 hours after IR exposure and between *msh2* and wild type at 2 hours after IR exposure. This may suggest that *mlh1* and *msh2* mutants arrest in G_2-M earlier in response to IR. The reason for this is unclear. The lack of these DNA binding proteins may cause a transient reduction in IR shielding and increase the exposure of DNA to direct damage by IR. However, γ-IR mainly causes indirect chemical damage to DNA, such as the formation of hydroxyl free radicals from reactions with water, as opposed to the direct physical damage caused by α-emitters. No difference was seen after this initial phase.

In comparison with inconsistent studies using murine and human MMR-defective cell lines, these cell cycle results contradict with the majority finding. It was shown that radio-sensitive HCT116 cells show deficiency in G_2-M checkpoint arrest in response to ionising radiation (Davis *et al,* 1998) and similar IR responses were also noted between murine *MLH1* knockout compared to wild-type primary embryonic fibroblasts (Davis *et al,* 1998). However, O'Driscoll *et al,* (1999) showed that NER-defective cells were no different in sensitivity to γ-irradiation compared to an *MSH2*-/NER-defective derivative; cell cycle analysis was not carried out in this study. In the study using HCT116 cells, X-rays were used as a source of IR. Work presented here is based on γ-irradiation. X-rays have slightly less energy than γ-rays but the spectrum of adducts caused by both are generally considered to be the same (Hall, 1994). It is possible that MLH1-deficient HCT116 cells and embryonic fibroblasts have other mutations that might effect responses to IR. The results obtained here using isogenic MMR defective

yeast strains argues against the finding that MMR deficiency leads to an abrogation of G_2 arrest.

Clonogenic assessment on the IR-treated strains suggested that the exposure was partially cytotoxic to the strains (see Table 6) with surviving fractions of 0.33, 0.41, 0.46, 0.19 and 0.36 for wt, *mlh1*, *msh2*, *rad1* and *rad52* strains respectively. The degree of lethality must therefore be considered when studying cell cycle phenotypes. The proportion of replication forks encountering IR-induced DNA damage, the severity of the lesions themselves and the temporal persistence of unrepaired adducts will all play a role in determining the outcome of the cellular response. However, inviable cells receiving lethal doses of IR should not have cycled at all, would appear morphologically different and would represent a small proportion of the cells scored for G_2 arrest. Rad52, which is responsible for repairing double strand breaks, did not show sensitivity to this dose of IR, indicating either that double strand breaks were not the main adducts induced or the lesions induced were repaired by other pathways (non-homologous recombination). The former suggestion is consistent with the fact that α-particles and not γ-rays are responsible for causing DNA double strand breaks (Hall *et al,* 1994). The sensitivity observed in the *rad1* mutants suggests that IR resulted in chemical modifications to DNA that represent substrates for Rad1-dependent repair.

6.7. Future experiments

Due to time constraints several questions remain unaddressed. With regard to the *ScMLH1* re-introduced transformant strain generated, it is not known if re-introducing wild type *ScMLH1* restores MMR function as measured by mutation frequency or rate. These are experiments that could be repeated on the transformants isolated. Mutation frequency and rate was also not determined for the remaining MMR mutants.

Secondly, these transformants were not exposed to ionising radiation, since no significant difference in cell cycle response or cytotoxicity was seen between wild type and *mlh1* mutants. However, it would be interesting to see if the transformants, which under the pYX212 vector highly express *ScMLH1*, respond differently to IR, especially since the transformants demonstrated hypersensitivity to cisplatin. On the subject of IR, increasing the number of repeats for both the cytotoxicity and cell cycle experiments would statistically clarify/nullify the results obtained here.

Repeating the cloning of the *ScMLH1* gene into the low expression vector would be useful to explore any difference observed between low and high protein levels in cell cycle and cytotoxic responses. The point mutations detected in the *ScMLH1* gene insert, according to the phenotypes observed, may be gain-of-function mutations or artefactual mutations responsible for some other MMR-independent effect. Therefore, repeating the cloning in the high copy vector and sequencing of the insert in an attempt to reduce the number of point mutations obtained could be carried out.

Cloning of the human *MLH1* gene into the yeast vectors could be carried out, with the ultimate aim in cancer therapy to re-introduce sequences that restore cisplatin

sensitivity. Cell cycle and cytotoxic responses would then confirm or negate the use of these MMR mutant strains for higher eukaryotic extrapolation.

In testing for dependence on *RAD1* and *RAD52* for MMR-dependent cytotoxicity, it was shown that both defects abolished the MMR deficiency-induced resistant phenotype. Therefore, a suitable negative control is needed to confirm that only specific DNA proteins are necessary for MMR-dependent cytotoxicity. Therefore, strains with proteins from other rad epistasis groups would be useful to disrupt. Rad14 is a protein known to be exclusive to NER. A *rad14* background could then be used to test the MMR deficient strains.

Cell cycle analysis using FACS could not be carried out effectively. Yeast FACS analysis is notoriously difficult and a number of technical factors could be addressed to improve the method. Prolonged enzyme digestion of the cell wall (although partially carried out here), slowing the rate of flow cytometry, prolonged sonication of cells to reduce clumping and more sophisticated cell cycle synchronisation protocols could all be tested.

6.7.1. Longer term plans

The models constructed to explain the observations of *RAD1* and *RAD52* dependence on MMR-dependent cytotoxicity and G_2-cell cycle arrest attenuation in response to cisplatin treatment, both provide a platform for further work. At present there are no biochemical studies that link recombination repair pathways and G_2 checkpoint pathways to MMR. The yeast two-hybrid screen, immunoprecipitation and gel retard experiments could be used to pull out various proteins associated with these pathways.

In the follow up of the cell cycle story that has emerged, exploring the activation of specific cyclin-dependent kinases and/or checkpoint proteins with and without the presence of specific MMR genes in yeast would also provide information on the

pathways operating in cell cycle control. Phosphorylation, protein binding and local distribution of these proteins would be important studies to undertake.

The use of genetic suppresser elements to identify important functional sequences within MMR genes in yeast could be exploited. These studies may provide specific sequences that are possible to re-introduce into human cell lines with the aim of restoring drug sensitivity in MMR-defective resistant cells. More ambitious still, restoring MMR function in MMR-defective cells may protect against further mutagenesis and the multistep progression towards neoplasia.

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7.1. References to methods

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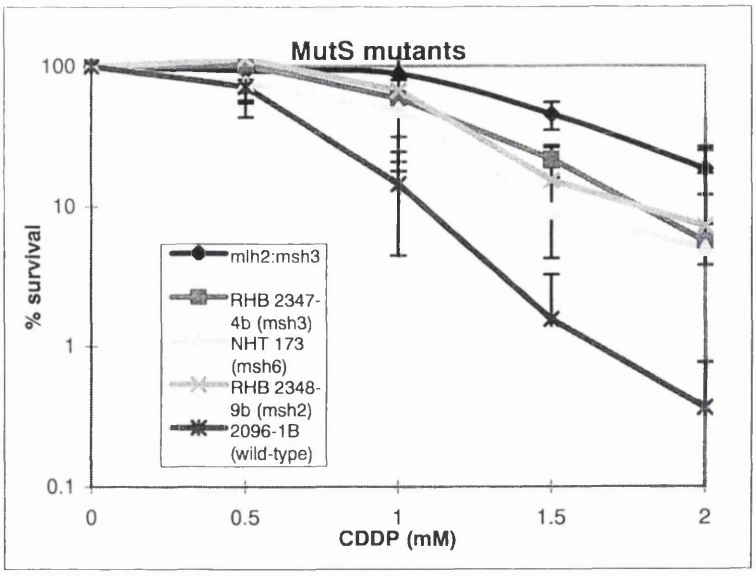
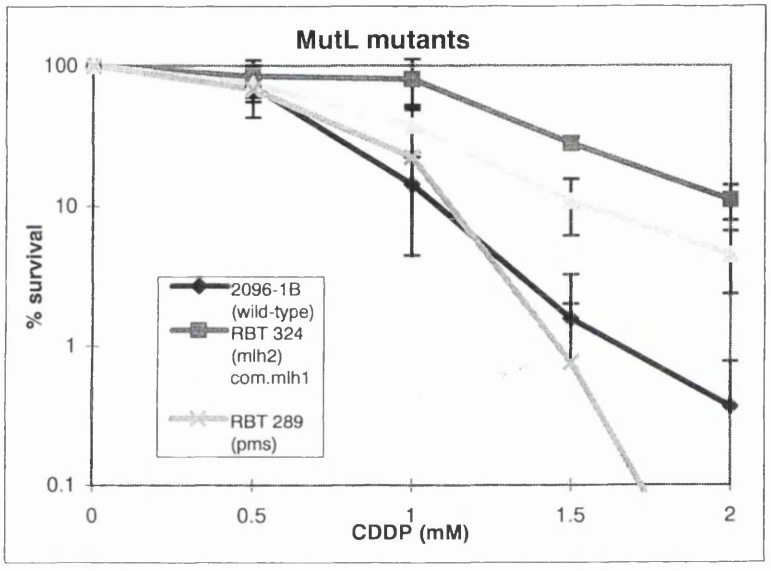
APPENDIX

9. APPENDIX

9.1. Statistics


MutS mutants
100
10
1
0.1
% survival
0
0.5
1
1.5
2
CDDP (mM)
mlh2:msh3
RHB 2347-4b (msh3)
NHT 173 (msh6)
RHB 2348-9b (msh2)
2096-1B (wild-type)


MutL mutants
100
10
1
0.1
% survival
0
0.5
1
1.5
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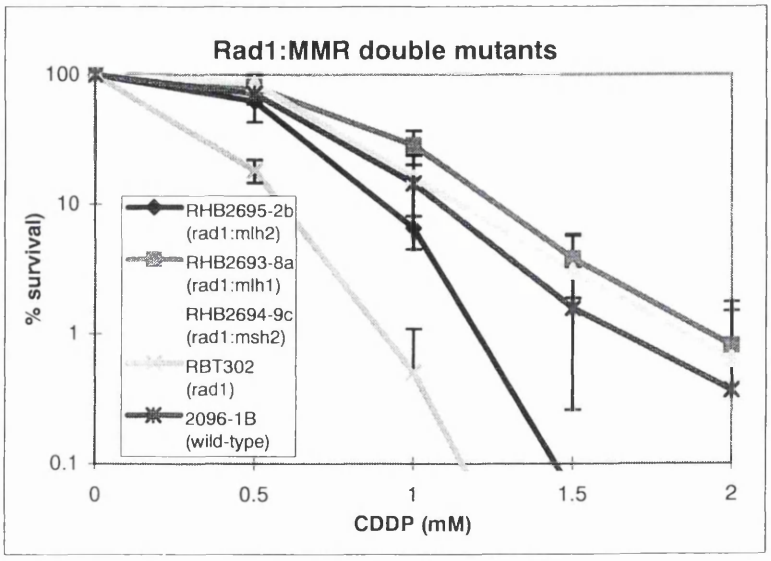
CDDP (mM)
2096-1B (wild-type)
RBT 324 (mlh2) com.mlh1
RBT 289 (pms)


Rad1:MMR double mutants
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% survival)
0
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1.5
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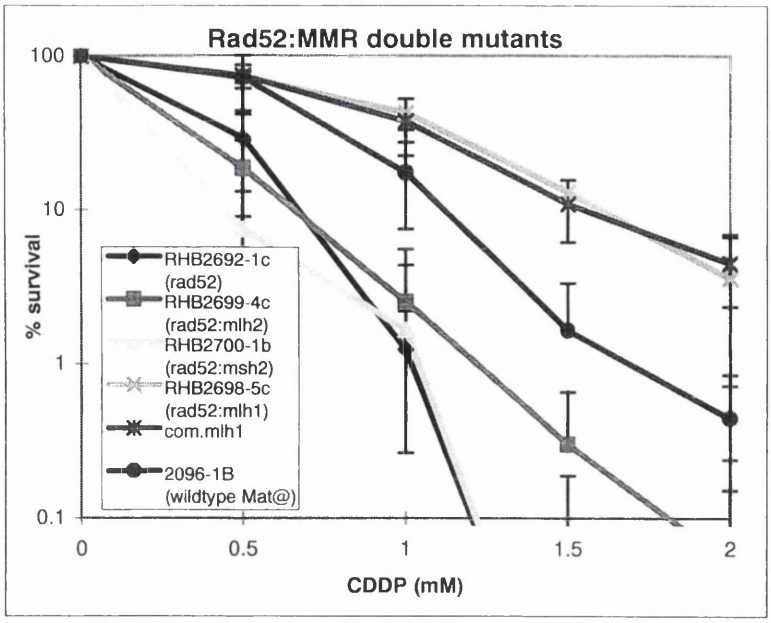
CDDP (mM)
RHB2695-2b (rad1:mlh2)
RHB2693-8a (rad1:mlh1)
RHB2694-9c (rad1:msh2)
RBT302 (rad1)
2096-1B (wild-type)


Rad52:MMR double mutants
100
10
1
0.1
% survival
0
0.5
1
1.5
2
CDDP (mM)
RHB2692-1c (rad52)
RHB2699-4c (rad52:mlh2)
RHB2700-1b (rad52:msh2)
RHB2698-5c (rad52:mlh1)
com.mlh1
2096-1B (wildtype Mat@)

survival of yeast mutants exposed for 24hr with cisplatin - ingle mutant strains:

splatin (mM)		0	0.5	1	1.5	2
96-1A	1	100	114	143		16.9
	2	100	89.2	116.6		16.8
	3	100	116.6	72.2		9.3
	4					
	5					
96-1B	1					
	2	100	64	38.2		1.1
	3	100	59.8	8		1
	4	100		21.6	6.3	0.003
	5	100	112.5	14	0.29	0.29
	6	100	73.4	9.2	0.43	0.0025
	7	100	83	12.7	0.72	0.97
	8	100	72.6	0.68	0.96	0.0025
	9	100	73.9	13.6	2.1	0.07
	10	100		27	2	0.007
	11	100	126	15.1	1.6	0.5
.7.97	12	100	38.8	4.8	0.5	0.3
	13	100	33.1	7.2	1.2	0.2
	14	100	48.4	14.7	1.1	0.3
BT 311	1	100	62.8	17.7		7.1
h1	2	100	50.4	47.8		1.1
	3	100		23.5	2.7	2
	4	100	67	30.2	8.2	0.03
	5	100	78.8	52	11	2.9
	6	100	83.1	33.1	11.8	4.3
	7	100	72.7	54.7	13.1	3.4
.7.97	8	100	87.1	56.2	16.6	2.8
IT 173	1	100	109	74.3		7.1
sh6	2	100		50.4	27.6	33.2
	3	100	55.2	18.9	4.1	1.7
	4	100	64.4	107.6	3.8	2.4
	5	100	75.3	36.8	13.2	4.6
	6	100	81.4	48.4	11.8	3.6
BT 289	1					
ns	2	100	63.9	13.2	2.5	0.004
	3	100	55.3	17.7	2.8	0.01
	4	100	71.8	15.1	1.6	0.008
	5	100	84.7	71.6	0.03	0.01
IB 2348-9b	1	100	159.8	160.1		52.2
sh2	2	100		32.2	13.6	3.7
	3	100	158.3	78.3	36.2	7.7
	4	100	100.8	36	10.2	3.7
	5	100	49.1	72.1	9.3	3.8
	6	100	147.7	85.2	17.7	6.6
IB 2347-4b	1					
sh3	2	100		40.2	23.9	4
	3	100	115.4	96	21.4	7.8
	4	100	98.2	76.8	29.3	7.3
	5	100	93.8	39.8	14.3	4.3
	6	100	141.8	92	19.1	7.1
BT 324	1	100	56.5	107		14.7

lh2	2					
	3	100	85.2	59.2	26.3	9.5
	4	100	90.7	55.8	26.7	8
	5	100	116.6	116.6	31.7	13.2
	6	100	104.3	69	18.8	13
BT341						
lh1	1	100	103.2	45	15.6	4.1
nd disruption	2	100	88.1	65.5	16.1	6.6
	3	100	88.4	70.5	38.8	6.3
	4	100	53.4	55.9	25.1	2.2
lh2:msh3	1	100	65.9	54.3	37.5	9
	2	100	86	95.4	39.3	19.7
	3	100	74.2		43.5	22.6
	4	100	145.6	114	59.7	22.5

EAN

splatin (mM)	0	0.5	1	1.5	2
lh2:msh3	100	92.925	87.9	45	18.45
HB 2347-4b (msh3)	100	102.0564	58.6051	21.51562	5.594181
HT 173 (msh6)	100	75.01259	48.83499	9.229677	5.015946
HB 2348-9b (msh2)	100	113.0827	66.852	15.25671	7.188673
996-1B (wild-type)	100	71.40909	14.36769	1.563636	0.365
BT 324 (mlh2)	100	84.46922	80.12343	28.13037	11.01986
m.mlh1	100	74.0975	37.4645	10.87917	4.494415
BT 289 (pms)	100	68.0861	22.41864	0.761351	0.007521

tandard deviation

splatin (mM)	0	0.5	1	1.5	2
996-1B	0	28.39822	9.922764	1.684472	0.406001
347-4B	0	21.76327	27.39029	5.571355	1.801388
BT324	0	24.6515	31.62462	3.008876	3.124633
BT311	0	12.73787	15.08869	4.733146	2.142221
HT173	0	20.49532	31.08567	9.675226	12.11853
BT289	0	12.4901	28.19373	1.244277	0.002828
348-9B	0	47.86536	46.22984	11.01612	19.30438
lh2:msh3	0	36.07209	30.54849	10.11731	6.441791

-TEST (student unpaired, two-tailed)

splatin (mM)	0	0.5	1	1.5	2
996-1B:2347-4b	0	0.073573	**0.010405**	**0.000857**	**0.001749**
996-1B:RBT324	0	0.79245	**0.005207**	**0.001873**	**0.000765**
996-1B:RBT311	0	0.25972	**0.002908**	**0.004731**	**0.01154**
996-1B:NHT173	0	0.634271	**0.023933**	0.07529	0.151425
996-1B:RBT289	0	0.202783	0.417084	0.943542	**0.027056**
996-1B:2348-9b	0	0.139563	**0.022048**	**0.033186**	0.172029
lh2:msh3-mlh2	0	0.917227	0.783208	0.023635	0.12335

ıd1:MMR double mutant strains -

32695-2b	100	62.5	6.3	0.0025	0.0025
1:mlh2	100	75	9.1	0.0025	0.0025
	100	67.2	7.5	0.0025	0.0025
	100	59.3	8.9	0.29	0.0025
	100	47	0.75	0.0025	0.0025
32693-8a	100	114.7	40	5.9	0.5
1:mlh1	100	53.8	17.7	2.6	2.3
	100	74.7	24.8	4.9	0.2
	100	79.6	31.3	1.7	0.2
32694-9c	100	101.3	25.7	3.8	1.9
1:msh2	100	93.6	18.4	6.6	0.3
	100	74.3	10.4	0.6	0.02
	100	72.7	8.8	1.1	0.3
T302	100	20.3	1.1	0.0025	0.0025
1	100	12.8	0.0025	0.0025	0.0025
	100	20.8	0.0025	0.0025	0.0025
	100	19	0.9	0.0025	0.0025

ːan

ɔlatin	0	0.5	1	1.5	2
B2695-2b (rad1:mlh2)	100	62.2	6.51	0.06	0.0025
B2693-8a (rad1:mlh1)	100	80.7	28.45	3.775	0.8
B2694-9c (rad1:msh2)	100	85.475	15.825	3.025	0.63
T302 (rad1)	100	18.225	0.50125	0.0025	0.0025
)6-1B (wild-type)	100	71.40909	14.36769	1.563636	0.365

ınndard dev.

ɔlatin	0	0.5	1	1.5	2
B2695-2b (rad1:mlh2)	0	10.34867	3.414015	0.128574	0
B2693-8a (rad1:mlh1)	0	21.5432	8.386517	1.902367	0.944458
B2694-9c (rad1:msh2)	0	14.19539	7.808702	2.766918	0.856894
T302 (rad1)	0	3.69538	0.581666	0	0
)6-1B	0	28.39822	9.922764	1.684472	0.406001

ɛst (between wt & mutants)

ɔlatin	0	0.5	1	1.5	2
B2695-2b (rad1:mlh2)	-	0.360386	**0.023846**	**0.014372**	0.007365
B2693-8a (rad1:mlh1)	-	0.565079	**0.048377**	0.10417	0.456984
B2694-9c (rad1:msh2)	-	0.231971	0.770088	0.379659	0.586988
T302 (rad1)	-	**8.46E-05**	**0.000284**	**0.011765**	**0.007365**

ɛst (between Rad-1 single and RAD-1 double mutants)

ɔlatin	0	0.5	1	1.5	2
HB2695-2b (rad1:mlh2)		**0.000254**	**0.015834**	0.373901	#DIV/0!
HB2693-8a (rad1:mlh1)		**0.014817**	**0.009627**	**0.030752**	0.212391
HB2694-9c (rad1:msh2)		**0.001621**	**0.029057**	0.116828	0.239257

RAD52:MMR double mutant strains -

cisplatin (mM)	0	0.5	1	1.5	2
RHB2692-1c	100	39.1	0.0025	0.0025	0.0025
	100	49.3	0.0025	0.0025	0.0025
	100	31.4	1.4	0.0025	0.0025
	100	27.5	2.7	0.0025	0.0025
	100	21.9	1.7	0.3	0.0025
	100	10.7	1.2	0.6	0.0025
	100	24.9	1.6	0.4	0.0025
RHB2699-4c	100	16.5	0.9	0.0025	0.2
	100	29.3	7	0.0025	0.0025
	100	22.1	1.7	0.7	0.0025
	100	6.6	0.5	0.5	0.0025
RHB2700-1b	100	16.8	6.3	0.0025	0.0025
	100	10.7	0.0025	0.0025	0.0025
	100	9.9	0.0025	0.0025	0.0025
	100	8.7	4.8	0.0025	0.0025
	100	1.4	0.0025	0.0025	0.0025
	100	2	0.5	0.0025	0.0025
	100	3.1	0.0025	0.0025	0.0025
RHB2698-5c	100	68	56.5	32.2	2.3
	100	81.7	34	4.1	1.1
	100	70.9	39.8	6.9	8.5
	100	70.4	40.3	8.9	2.4
RBT341	100	103.2	45	15.6	4.1
	100	88.1	65.1	16.1	6.6
	100	88.4	70.5	38.8	6.3
	100	53.4	55.9	25.1	2.2
	100	92.9	48.7	12.1	1
RBT340	100	58.1	15.1	0.7	0.0025
	100	26.8	4.9	1	0.2
	100	18.1	2.5	0.5	0.0025
	100	73.1	12	0.0025	0.0025
	100	22.5	8	1.2	0.0025

Mean

cisplatin (mM)	0	0.5	1	1.5	2
RHB2692-1c (rad52)	100	29.25714	1.229286	0.0025	0.0025
RHB2699-4c (rad52:mlh2)	100	18.625	2.525	0.30125	0.051875
RHB2700-1b (rad52:msh2)	100	7.514286	1.658571	2.50E-03	2.50E-03
RHB2698-5c (rad52:mlh1)	100	72.75	42.65	13.025	3.575
com.mlh1	100	74.0975	37.4645	10.87917	4.494415
2096-1B (wildtype Mat@)	100	72.2	17.42	1.65	0.445376

s.d

cisplatin (mM)	0	0.5	1	1.5	2
RHB2692-1c	0	12.40804	0.963867	0.18572	0.718131
RHB2699-4c	0	9.576838	3.024759	0.355897	0.099995
RHB2700-1b	0	5.638389	2.700527	5.35E-06	5.35E-06
RHB2698-5c	0	6.099454	9.66592		3.336041
RBT341	0	18.79614	10.74653	10.77697	2.464346

RBT340	0	25.89895	5.907834	0.420313	0.099995
2096-1B	0	22.49171	14.98318	2.302347	0.491082
				12.93403	

T-test

cisplatin (mM)	0	0.5	1	1.5	2
Diff between:					
Wildtype:Rad52		**0.000637**	**0.000443**	**0.022493**	0.025441
Wildtype:Rad52/mlh2		**0.000125**	**0.001868**	**0.036944**	**0.022465**
Wildtype:Rad52/msh2		**1.5E-05**	**0.000598**	**0.011765**	**0.007365**
Wildtype:Rad52/mlh1		0.885156	**0.003556**	0.174302	0.149796

T-test (diff between rad52 single mutant and doubles)

rad52:rad52/mlh2		0.355668	0.46007	0.594921	0.391002
rad52:rad52/msh2		**0.002627**	0.548209	0.094889	#DIV/0!
rad52:rad52/mlh1		**2.87E-05**	**0.003224**	0.141341	0.121651

Surviving Fraction (compared to wt)

cisplatin (mM)	0	0.5	1	1.5	2
2096-1B (wild-type)					
RBT 324 (mlh2)	1	1.182892	5.576639	17.99035	30.19141
com.mlh1	1	1.037648	2.607552	6.957607	12.31347
RBT 289 (pms)	1	0.953465	1.560351	0.48691	0.020606
mih2:msh3	1	1.301305	6.117893	28.77907	50.54795
RHB 2347-4b (msh3)	1	1.429179	4.07895	13.75999	15.32652
NHT 173 (msh6)	1	1.050463	3.398945	5.9027	13.74232
RHB 2348-9b (msh2)	1	1.583589	4.652939	9.757196	19.69499

Surviving Fraction (SF) (compared to rad1 mutant)

cisplatin (mM)	0	0.5	1	1.5	2
RHB2695-2b (rad1:mlh2)	1	3.412894	12.98753	24	1
RHB2693-8a (rad1:mlh1)	1	4.427984	56.7581	1510	320
RHB2694-9c (rad1:msh2)	1	4.689986	31.57107	1210	252
RBT302 (rad1)					

Surviving Fraction (compared to rad52 mutant)

cisplatin (mM)	0	0.5	1	1.5	2
RHB2692-1c (rad52)					
RHB2699-4c (rad52:mlh2)	1	0.636597	2.054038	120.5	20.75
RHB2700-1b (rad52:msh2)	1	0.256836	1.349216	1	1
RHB2698-5c (rad52:mlh1)	1	2.486572	34.69494	5210	1430
RBT341 (mlh1)	1	#REF!	#REF!	#REF!	#REF!
2096-1B (wildtype Mat@)	1	2.467773	14.17083	660	178.1506

N:\DATA\FIG1.SPW

	-1- Drug	-2- W.T.	-3- wt 0.5	-4- wt 1	-5- wt 1.5	-6- wt 2	-7- mlh1	-8- mlh1-0.5
1	0.0000	1.0000	1.6400	0.3800	0.0600	1.0000e-4	1.0000	0.6300
2	0.5000	1.0000	0.6000	0.0800	3.0000e-3	1.0000e-4	1.0000	0.5000
3	1.0000	1.0000	1.1200	0.2100	4.0000e-3	3.0000e-5	1.0000	0.6700
4	1.5000	1.0000	0.7300	0.1400	7.0000e-3	3.0000e-3	1.0000	0.7900
5	2.0000	1.0000	0.8300	0.0900	9.6000e-3	3.0000e-5	1.0000	0.8300
6			0.7200	0.1300	0.0200	9.7000e-3	1.0000	0.7300
7			0.7400	7.0000e-3	0.0210	2.5000e-5		0.8700
8			1.2600	0.1400	0.0160	7.0000e-4		
9				0.2700	5.0000e-3	7.0000e-5		
10				0.1500	0.0120	5.0000e-3		
11				0.0500	0.0110	3.0000e-3		
12				0.0700		2.0000e-3		
13						3.0000e-3		

N:\DATA\FIG1.SPW

	-9- mlh1 1	-10- mlh1 1.5	-11- mlh1 2	-12- msh6	-13- msh6 0.5	-14- msh6 1	-15- msh6 1.5	-16- msh6 2
1	0.1800	0.0270	0.0700	1.0000	1.0900	0.7400	0.2800	0.0700
2	0.4800	0.0820	0.0100	1.0000	0.5500	0.5000	0.0400	
3	0.2400	0.1100	0.0200	1.0000	0.6400	0.1900	0.0380	0.0170
4	0.3000	0.1200	3.0000e-4	1.0000	0.7500	1.0700	0.1320	0.0240
5	0.5200	0.1300	0.0300	1.0000	0.8100	0.3680	0.1180	0.0460
6	0.3300	0.1700	0.0430			0.4840		0.0360
7	0.5400		0.0340					
8	0.5600		0.0280					

N:\DATA\FIG1.SPW

	-17- msh2	-18- msh2 0.5	-19- msh2 1	-20- msh2 1.5	-21- msh2 2	-22- pms	-23- pms 0.5	-24- pms 1
1	1.0000	1.6000	0.3200	0.1360	0.0370	1.0000	0.6400	0.1320
2	1.0000	1.6000	0.7800	0.3620	0.0770	1.0000	0.5500	0.1770
3	1.0000	1.0000	0.3600	0.1020	0.0370	1.0000	0.7200	0.1500
4		0.4900	0.7200	0.0930	0.0380		0.8500	0.7160
5		1.4700	0.8500	0.1770	0.0660			

N:\DATA\FIG1.SPW

	-25- pms 1.5	-26- pms2	-27- msh3	-28- msh3-0.5	-29- msh3 1	-30- msh3 1.5	-31- msh3 2	-32- mlh2
1	0.0250	4.0000e-5	1.0000	0.9800	0.4000	0.2400	0.0400	1.0000
2	0.0280	1.0000e-4	1.0000	0.9400	0.9600	0.2100	0.0780	1.0000
3	0.0160	8.0000e-5	1.0000	1.4100	0.7700	0.2900	0.0730	1.0000
4	3.0000e-4	1.0000e-4	1.0000	1.1500	0.4000	0.1400	0.0430	1.0000
5					0.9200	0.1900	0.0710	

N:\DATA\FIG1.SPW

	-33- mlh2 0.5	-34- mlh2 1	-35- mlh2 1.5	-36- mlh2 2
1	0.5700	1.0700	0.2600	0.0950
2	0.8500	0.5900	0.2700	0.0800
3	0.9100	0.5600	0.3100	0.1320
4	1.1600	1.1700	0.1900	0.1300
5	1.0400	0.6900		0.1470

Column Statistics - N:\DATA\FIG1.SPW

	-1- Drug	-2- W.T.	-3- wt 0.5	-4- wt 1	-5- wt 1.5	-6- wt 2	-7- mlh1	-8- mlh1-0.5
Mean	1.0000	1.0000	0.9550	0.1431	0.0153	2.0581e-3	1.0000	0.7171
Std.Dev	0.7906	0.0000	0.3552	0.1028	0.0160	2.8217e-3	0.0000	0.1282
Std.Err	0.3536	0.0000	0.1256	0.0297	4.8326e-3	7.8260e-4	0.0000	0.0484
95% Conf	0.9816	0.0000	0.2970	0.0653	0.0108	1.7052e-3	0.0000	0.1185
99% Conf	1.6274	0.0000	0.4395	0.0922	0.0153	2.3907e-3	0.0000	0.1796
Size	5.0000	5.0000	8.0000	12.0000	11.0000	13.0000	6.0000	7.0000
Total	5.0000	5.0000	7.6400	1.7170	0.1686	0.0268	6.0000	5.0200
Min	0.0000	1.0000	0.6000	7.0000e-3	3.0000e-3	2.5000e-5	1.0000	0.5000
Max	2.0000	1.0000	1.6400	0.3800	0.0600	9.7000e-3	1.0000	0.8700
Min.Pos	0.5000	1.0000	0.6000	7.0000e-3	3.0000e-3	2.5000e-5	1.0000	0.5000
Missing	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
Other	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
Skewness	0.0000		0.9313	1.0067	2.1428	1.6361		-0.4796

Column Statistics - N:\DATA\FIG1.SPW

	-9- mlh1 1	-10- mlh1 1.5	-11- mlh1 2	-12- msh6	-13- msh6 0.5	-14- msh6 1	-15- msh6 1.5	-16- msh6 2
Mean	0.3938	0.1065	0.0294	1.0000	0.7680	0.5587	0.1216	0.0386
Std.Dev	0.1486	0.0484	0.0213	0.0000	0.2060	0.3084	0.0986	0.0208
Std.Err	0.0525	0.0197	7.5218e-3	0.0000	0.0921	0.1259	0.0441	9.2930e-3
95% Conf	0.1242	0.0508	0.0178	0.0000	0.2557	0.3237	0.1224	0.0258
99% Conf	0.1839	0.0796	0.0263	0.0000	0.4240	0.5077	0.2029	0.0428
Size	8.0000	6.0000	8.0000	5.0000	5.0000	6.0000	5.0000	6.0000
Total	3.1500	0.6390	0.2353	5.0000	3.8400	3.3520	0.6080	0.1930
Min	0.1800	0.0270	3.0000e-4	1.0000	0.5500	0.1900	0.0380	0.0170
Max	0.5600	0.1700	0.0700	1.0000	1.0900	1.0700	0.2800	0.0700
Min.Pos	0.1800	0.0270	3.0000e-4	1.0000	0.5500	0.1900	0.0380	0.0170
Missing	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
Other	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	1.0000
Skewness	-0.1911	-0.4755	0.5811		0.6717	0.6123	0.8313	0.5656

Column Statistics - N:\DATA\FIG1.SPW

	-17- msh2	-18- msh2 0.5	-19- msh2 1	-20- msh2 1.5	-21- msh2 2	-22- pms	-23- pms 0.5	-24- pms 1
Mean	1.0000	1.2320	0.6060	0.1740	0.0510	1.0000	0.6900	0.2937
Std.Dev	0.0000	0.4827	0.2475	0.1102	0.0191	0.0000	0.1273	0.2821
Std.Err	0.0000	0.2159	0.1107	0.0493	8.5499e-3	0.0000	0.0636	0.1411
95% Conf	0.0000	0.5993	0.3074	0.1368	0.0237	0.0000	0.2025	0.4488
99% Conf	0.0000	0.9936	0.5096	0.2268	0.0394	0.0000	0.3711	0.8224
Size	3.0000	5.0000	5.0000	5.0000	5.0000	3.0000	4.0000	4.0000
Total	3.0000	6.1600	3.0300	0.8700	0.2550	3.0000	2.7600	1.1750
Min	1.0000	0.4900	0.3200	0.0930	0.0370	1.0000	0.5500	0.1320
Max	1.0000	1.6000	0.8500	0.3620	0.0770	1.0000	0.8500	0.7160
Min.Pos	1.0000	0.4900	0.3200	0.0930	0.0370	1.0000	0.5500	0.1320
Missing	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
Other	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
Skewness		-0.7652	-0.3133	1.1886	0.5306		0.2341	1.1399

Column Statistics - N:\DATA\FIG1.SPW

	-25- pms 1.5	-26- pms2	-27- msh3	-28- msh3-0.5	-29- msh3 1	-30- msh3 1.5	-31- msh3 2	-32- mlh2
Mean	0.0173	8.0000e-5	1.0000	1.1200	0.6900	0.2140	0.0610	1.0000
Std.Dev	0.0124	2.8284e-5	0.0000	0.2137	0.2740	0.0559	0.0180	0.0000
Std.Err	6.2214e-3	1.4142e-5	0.0000	0.1068	0.1226	0.0250	8.0561e-3	0.0000
95% Conf	0.0198	4.4997e-5	0.0000	0.3400	0.3403	0.0695	0.0224	0.0000
99% Conf	0.0363	8.2459e-5	0.0000	0.6230	0.5641	0.1152	0.0371	0.0000
Size	4.0000	4.0000	4.0000	4.0000	5.0000	5.0000	5.0000	4.0000
Total	0.0693	3.2000e-4	4.0000	4.4800	3.4500	1.0700	0.3050	4.0000
Min	3.0000e-4	4.0000e-5	1.0000	0.9400	0.4000	0.1400	0.0400	1.0000
Max	0.0280	1.0000e-4	1.0000	1.4100	0.9600	0.2900	0.0780	1.0000
Min.Pos	3.0000e-4	4.0000e-5	1.0000	0.9400	0.4000	0.1400	0.0400	1.0000
Missing	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
Other	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000	0.0000
Skewness	-0.6530	-0.8165		0.6247	-0.2229	0.0598	-0.3563	

Column Statistics - N:\DATA\FIG1.SPW

	-33- mlh2 0.5	-34- mlh2 1	-35- mlh2 1.5	-36- mlh2 2
Mean	0.9060	0.8160	0.2575	0.1168
Std.Dev	0.2228	0.2839	0.0499	0.0280
Std.Err	0.0996	0.1269	0.0250	0.0125
95% Conf	0.2766	0.3525	0.0794	0.0348
99% Conf	0.4586	0.5843	0.1455	0.0577
Size	5.0000	5.0000	4.0000	5.0000
Total	4.5300	4.0800	1.0300	0.5840
Min	0.5700	0.5600	0.1900	0.0800
Max	1.1600	1.1700	0.3100	0.1470
Min.Pos	0.5700	0.5600	0.1900	0.0800
Missing	0.0000	0.0000	0.0000	0.0000
Other	0.0000	0.0000	0.0000	0.0000
Skewness	-0.4883	0.3718	-0.4979	-0.3400

9.2. *scMLH1* gene sequence and cloning primers

F-BamHI Primer

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 51  TGCAGGTGAG ATCATAATAT CCCCCGTAAA TGCTCTCAAA GAAATGATGG

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151  GGAGGAATTA AGGTACTTCA AATAACAGAT AACGGATCTG GAATTAATAA

201  AGCAGACCTG CCAATCTTAT GTGAGCGATT CACGACGTCC AAATTACAAA

251  AATTCGAAGA TTTGAGTCAG ATTCAAACGT ATGGATTCCG AGGAGAAGCT

301  TTAGCCAGTA TCTCACATGT GGCAAGAGTC ACAGTAACGA CAAAAGTTAA

351  AGAAGACAGA TGTGCATGGA GAGTTTCATA TGCAGAAGGT AAGATGTTGG

401  AAAGCCCCAA ACCTGTTGCT GGAAAAGACG GTACCACGAT CCTAGTTGAA

451  GACCTTTTTT TCAATATTCC TTCTAGATTA AGGGCCTTGA GGTCCCATAA

501  TGATGAATAC TCTAAAATAT TAGATGTTGT CGGGCGATAC GCCATTCATT

551  CCAAGGACAT TGGCTTTTCT TGTAAAAAGT TCGGAGACTC TAATTATTCT

601  TTATCAGTTA AACCTTCATA TACCGTCCAG GATAGGATTA GGACTGTGTT

651  CAATAAATCT GTGGCTTCGA ATTTAATTAC TTTTCATATC AGCAAAGTAG

701  AAGATTTAAA CCTGGAAAGC GTTGATGGAA AGGTGTGTAA TTTGAATTTC

751  ATATCCAAAA AGTCCATTTC ACCAATTTTT TTCATTAATA ATAGACTAGT

801  GACATGTGAT CTTCTAAGAA GAGCTTTGAA CAGCGTTTAC TCCAATTATC

851  TGCCAAAGGG CAACAGACCT TTTATTTATT TGGGAATTGT TATAGATCCG

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1201 AGAATAGATG CTTCACAAGC TAAAATTACG TCATTTTTAT CCTCAAGTCA

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1301 AGGTAACAAA TGTAAGCCAC TCCCAAGAGG CAGAAAAGCT GACACTAAAT

1351 GAAAGCGAAC AACCGCGTGA TGCCAATACA ATCAATGATA ATGACTTGAA

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1851 TAGTAAAATA TGGGACATGA GCAGTATGCT AAATGAGTAC TATTCCATAG

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2001 ATTTTTTATA TATCGCCTGG GTAAAGAAGT TGATTGGGAG GATGAACAAG

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2101 ATGGTTCCGA AAGTCGATAC ATCTGATGCA TCGTTGTCAG AAGACGAAAA

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2201  TTCTCTTCCC TTGTATCAAA CGAAGGTTCC TGGCCCCTAG ACACATTCTC
                                                  R-MluI Primer
2251  AAGGATGTCG TGGAAATAGC CAACCTTCCA GATCTATACA AAGTTTTTGA

2301  GAGGTGTTAA
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(sequence number U07187_1.cds from Saccharomyces Genome Database). Underlined sequences show annealing primer sites.

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DEFINITION  Saccharomyces cerevisiae DNA mismatch repair (MLH1) gene, complete
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ACCESSION   U07187
NID         g460626
KEYWORDS    .
SOURCE      baker's yeast.
  ORGANISM  Saccharomyces cerevisiae
            Eukaryotae; mitochondrial eukaryotes; Fungi; Ascomycota;
            Hemiascomycetes; Saccharomycetales; Saccharomycetaceae;
            Saccharomyces.
REFERENCE   1  (bases 1 to 3218)
  AUTHORS   Prolla,T.A., Christie,D. and Liskay,R.M.
  TITLE     Dual Requirement in Yeast DNA Mismatch Repair for MLH1 and PMS1,
            Two Homologs of the Bacterial mutL Gene
  JOURNAL   Mol. Cell. Biol. 14, 407-415 (1994)
  MEDLINE   94088538
REFERENCE   2  (bases 1 to 3218)
  AUTHORS   Liskay,R.M.
  TITLE     Direct Submission
  JOURNAL   Submitted (25-FEB-1994) Robert Michael Liskay, Oregon Health
            Sciences University, Portland, OR 97201-3098, USA
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ORIGIN

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     151  CTAGCATGCT AGGACAATTT AACTGCAAAA TTTTGATACG ATAGTGATAG

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3001 TTTTGAACCG AATATTGTGG GCAGATTTGC AGTAAGTTCA GTTAGATCTA

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3101 ATGAAAGAGA CCTCGCGCGT AATGGTTTGC ATCACCATCG GATGTCTGTT

3151 GAAAAACTCA CTTTTTGCAT GGAAGTTATT AACAATAAGA CTAATGATTA

3201 CCTTAGAATA ATGTATAA

SGD

Sequence for a region of U07187_1.cds

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yeast MLH-1 +/- 20 bp.

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BLAST | FASTA | Sacch3D | Design Primers | SGD Home

Send questions or suggestions to SGD | DNA Sequence Retrieval Form

This display was generated at SGD using a licensed version of the Wisconsin Sequence Analysis Package® from the Genetics Computer Group. For more information about this package, see GCG®.

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             cds.
ACCESSION    U07187
NID          g460626
KEYWORDS     . . . .

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     201  AACGGATCTG GAATTAATAA AGCAGACCTG CCAATCTTAT GTGAGCGATT

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2251  TGGCCCCTAG ACACATTCTC AAGGATGTCG TGGAAATAGC CAACCTTCCA
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BESTFIT of: 212R1.consensus check: 155 from: 1 to: 2622

to: u07187 check: 690 from: 1 to: 3218

OCUS SCMLHI 3218 bp DNA PLN 17-MAR-1994
EFINITION Saccharomyces cerevisiae DNA mismatch repair (MLH1) gene, complete cds.
CCESSION U07187
ID g460626
EYWORDS . . . .

Symbol comparison table: /software/gcg9/gcgcore/data/rundata/swgapdna.cmp
CompCheck: 2335

Gap Weight: 50 Average Match: 10.000
Length Weight: 3 Average Mismatch: -9.000

Quality: 21503 Length: 2369
Ratio: 9.197 Gaps: 25
Percent Similarity: 98.886 Percent Identity: 98.757

Match display thresholds for the alignment(s):
| = IDENTITY
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212R1.consensus x u07187 February 3, 1998 14:03 ..

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    213 AAAAATAACATAGACCTAT....CAATAAGCAATGTCTCTCAGAATAAAA 258

    111 GCACTTGATGCATCAGTGGTTAACAAAATTGCTGCAGGTGAGATCATA.A 159
        |||||||||||||||||||||||||||||||||||||||||||||||||
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    160 TATCCCCCGTAAATGCTCTCAAAGAaATGATGGAGAATTCCATCGATGCG 209
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        |||||||||||||||||||||||||||||||||||||||||||||||||
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        |||||||||||||||||||||||||||||||||||||||||||||||||
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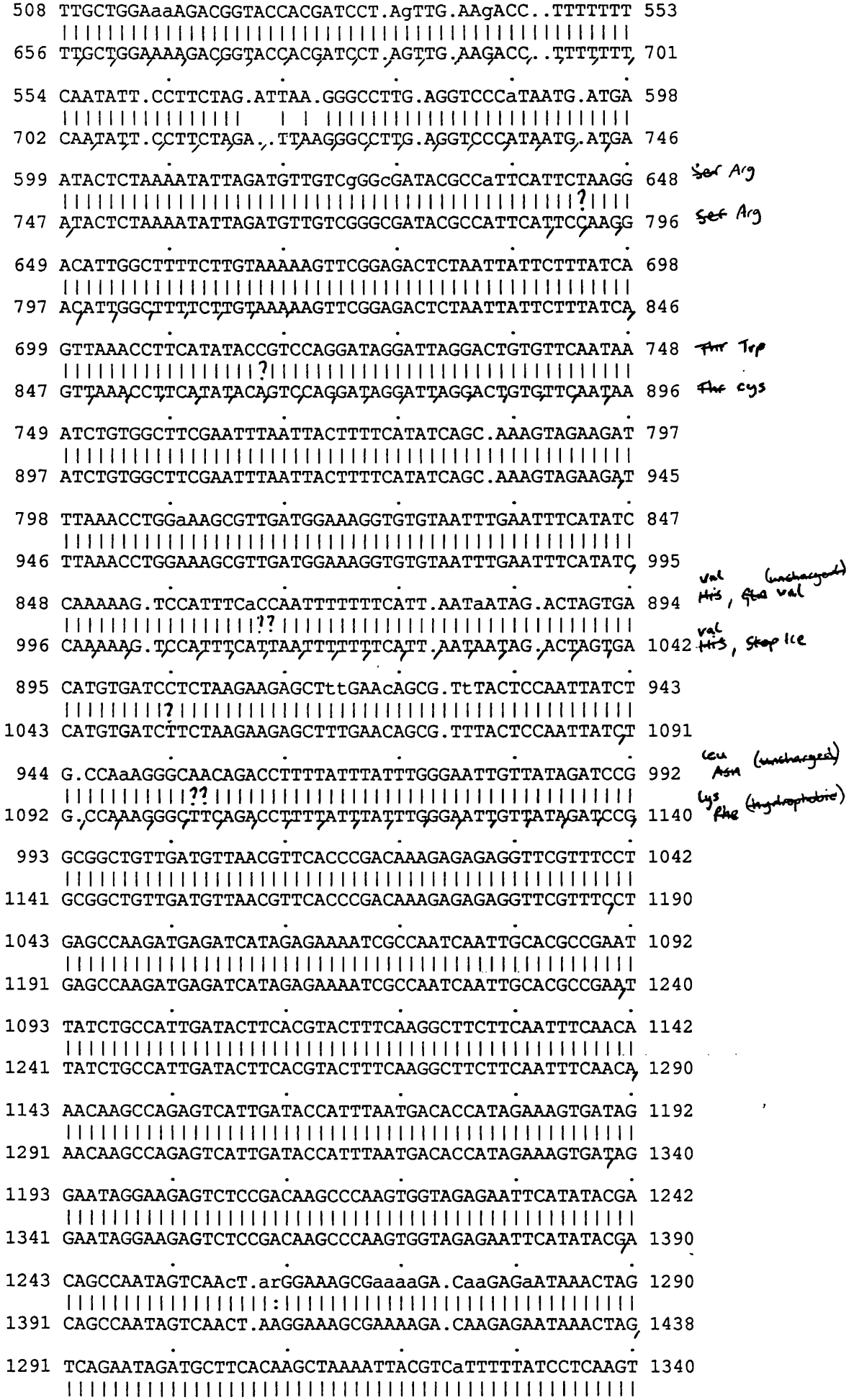
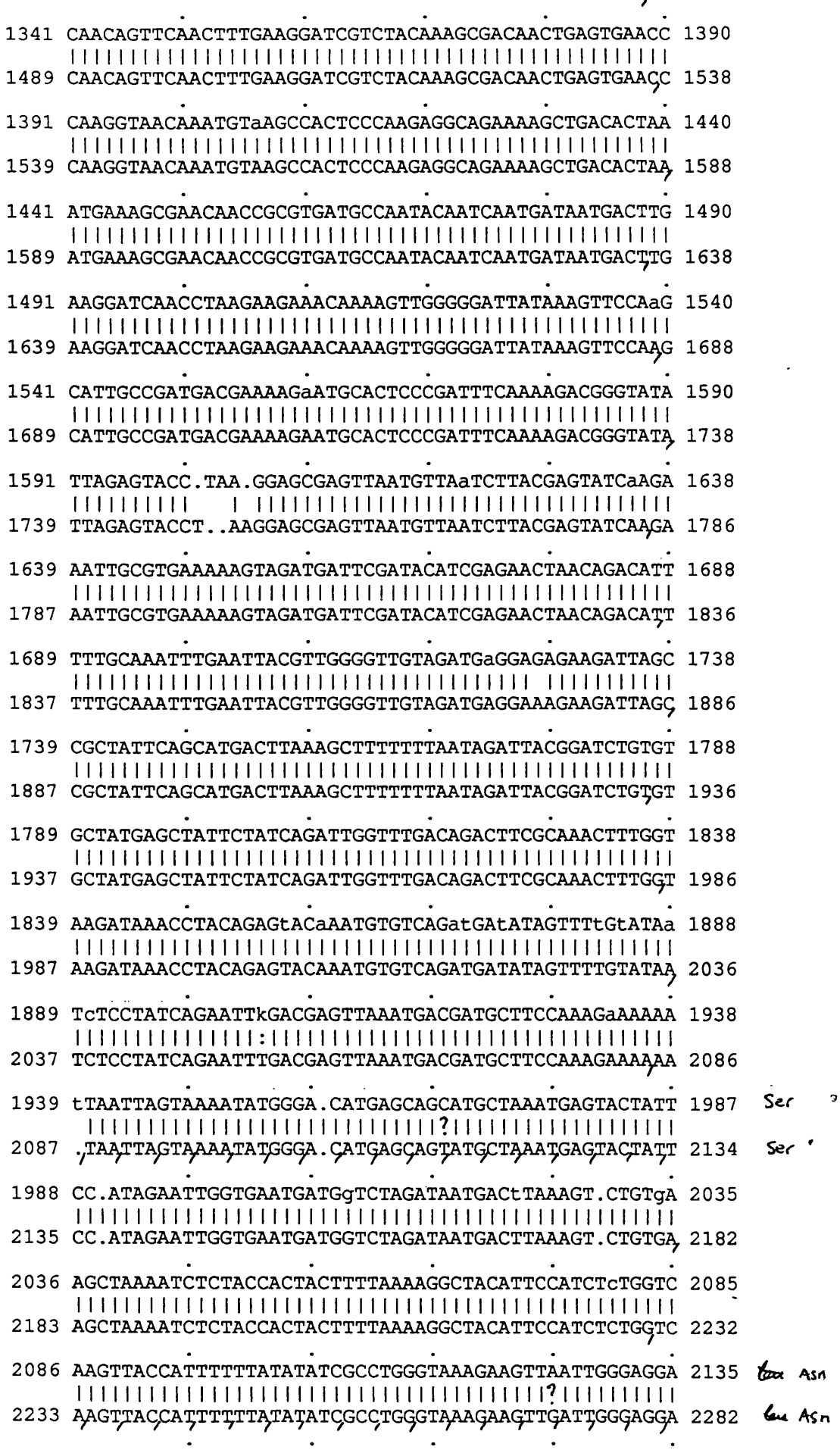

Ser Arg
Ser Arg
Thr Trp
Thr Cys
val (uncharged)
His, Glu val
val
His, Stop Ile
Leu (uncharged)
Asn
Lys (hydrophobic)
Phe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1988 CC.ATAGAATTGGTGAATGATGgTCTAGATAATGACtTAAAGT.CTGTgA 2035
2135 CC.ATAGAATTGGTGAATGATGGTCTAGATAATGACTTAAAGT.CTGTGA 2182
2036 AGCTAAAATCTCTACCACTACTTTTAAAAGGCTACATTCCATCTcTGGTC 2085
2183 AGCTAAAATCTCTACCACTACTTTTAAAAGGCTACATTCCATCTCTGGTC 2232
2086 AAGTTACCATTTTTTATATATCGCCTGGGTAAAGAAGTTAATTGGGAGGA 2135 Asn
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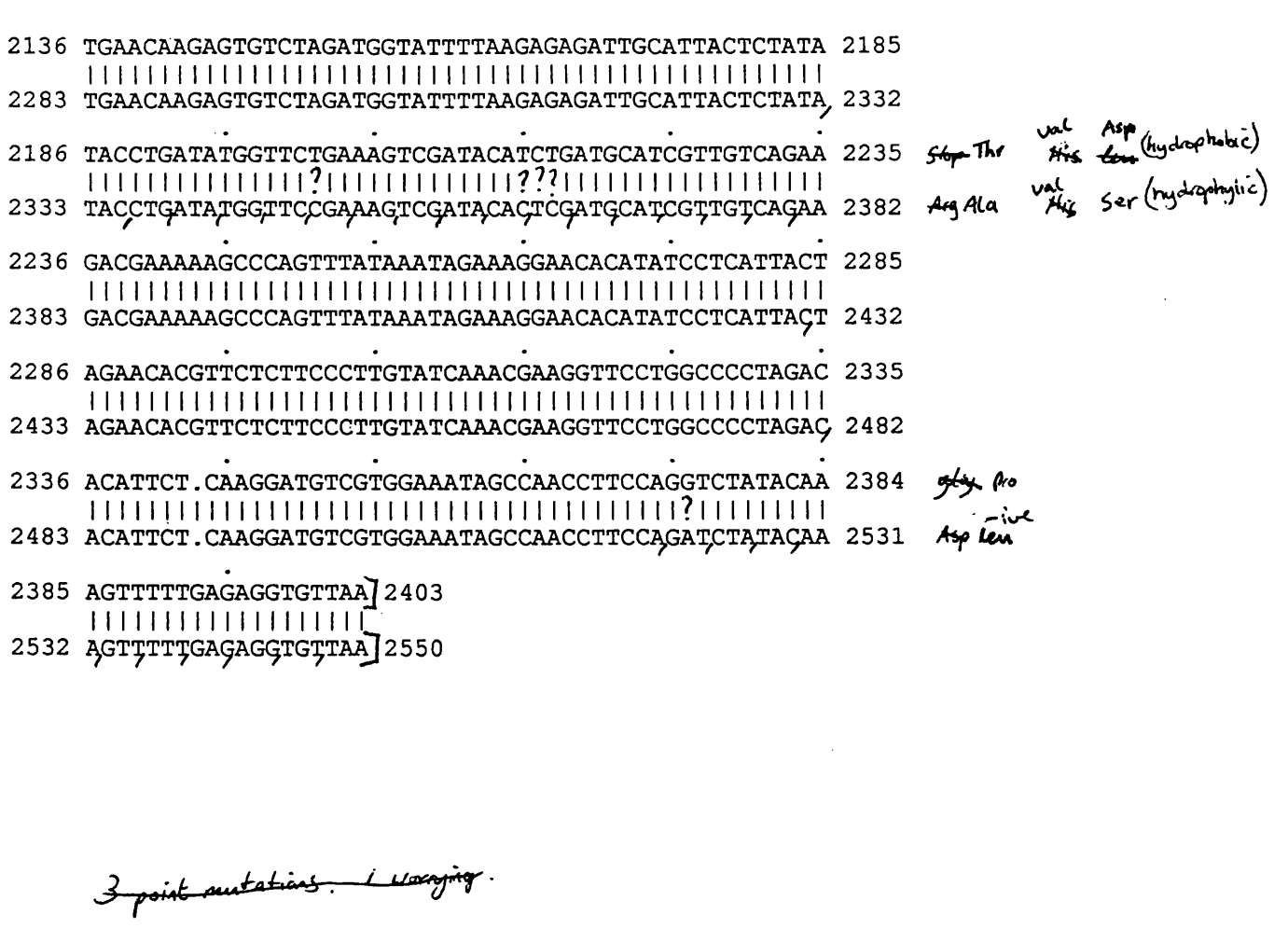
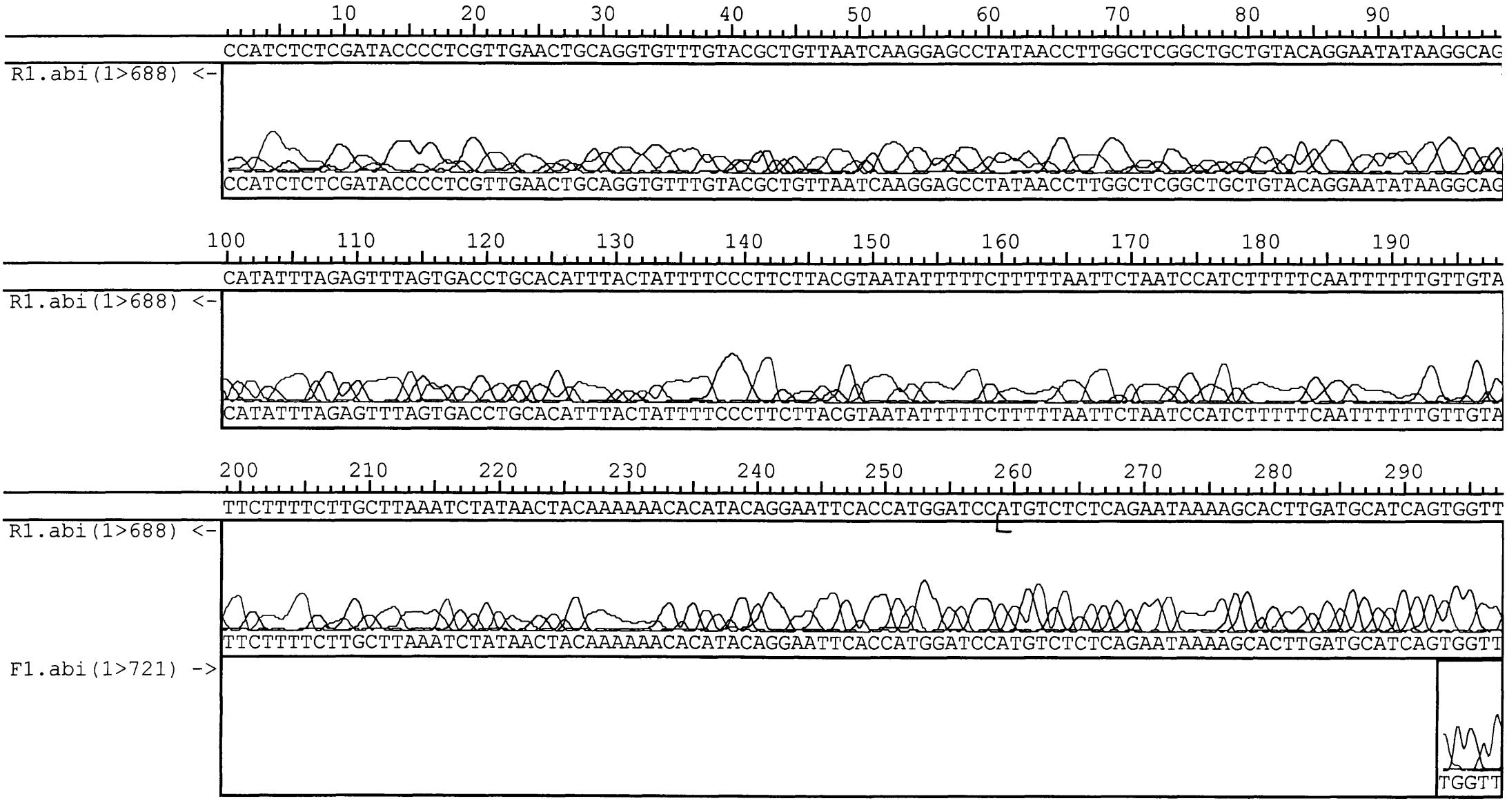


3 point mutations - 1 warping.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100 110 120 130 140 150 160 170 180 190
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R1.abi(1>688) <-
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200 210 220 230 240 250 260 270 280 290
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R1.abi(1>688) <-
TTCTTTTCTTGCTTAAATCTATAACTACAAAAAACACATACAGGAATTCACCATGGATCCATGTCTCTCAGAATAAAAGCACTTGATGCATCAGTGGTT
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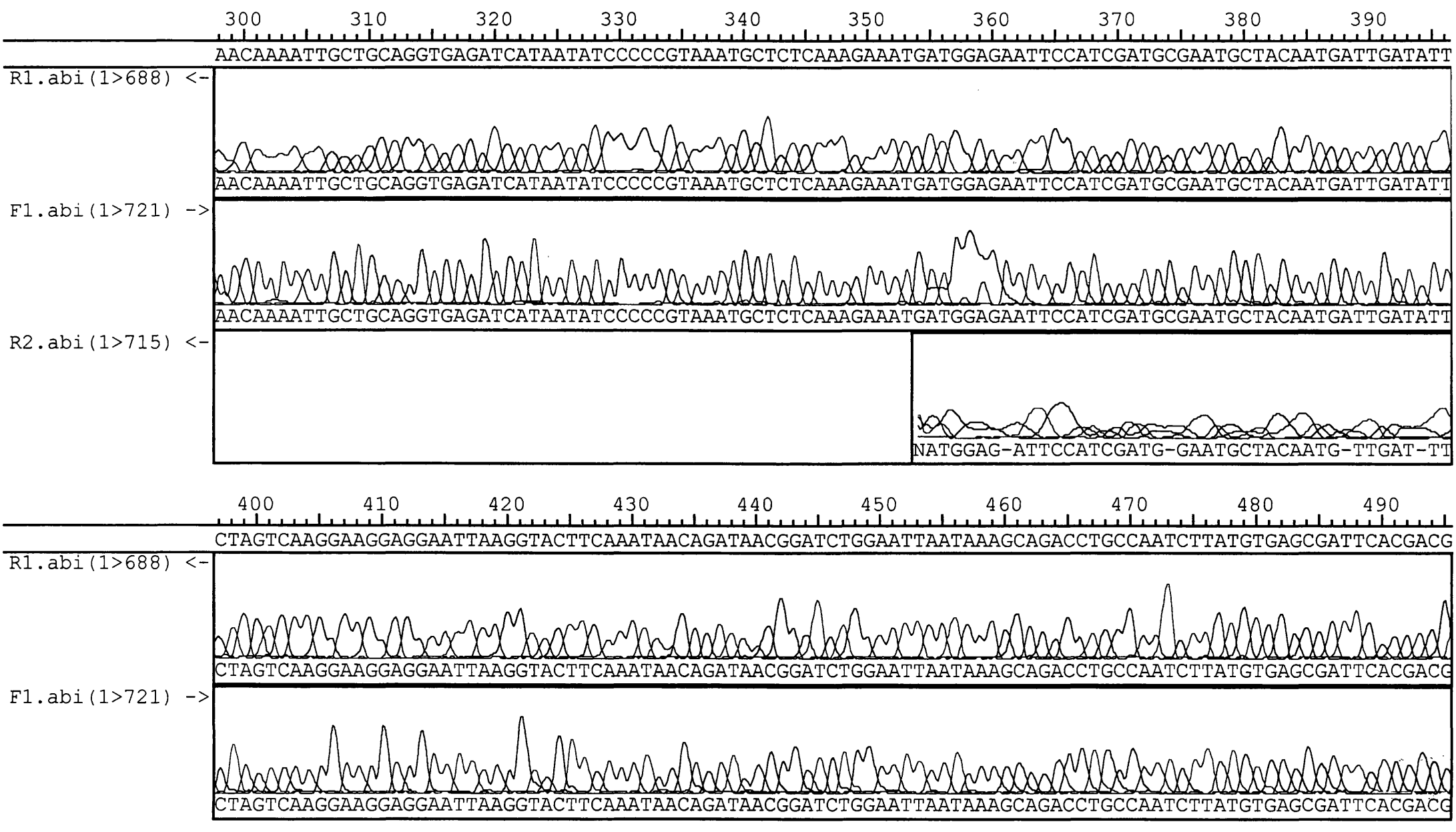
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F1.abi(1>721) ->
AACAAAATTGCTGCAGGTGAGATCATAATATCCCCCGTAAATGCTCTCAAAGAAATGATGGAGAATTCCATCGATGCGAATGCTACAATGATTGATATT
R2.abi(1>715) <-
NATGGAG-ATTCCATCGATG-GAATGCTACAATG-TTGAT-TT
400 410 420 430 440 450 460 470 480 490
CTAGTCAAGGAAGGAGGAATTAAGGTACTTCAAATAACAGATAACGGATCTGGAATTAATAAAGCAGACCTGCCAATCTTATGTGAGCGATTCACGACG
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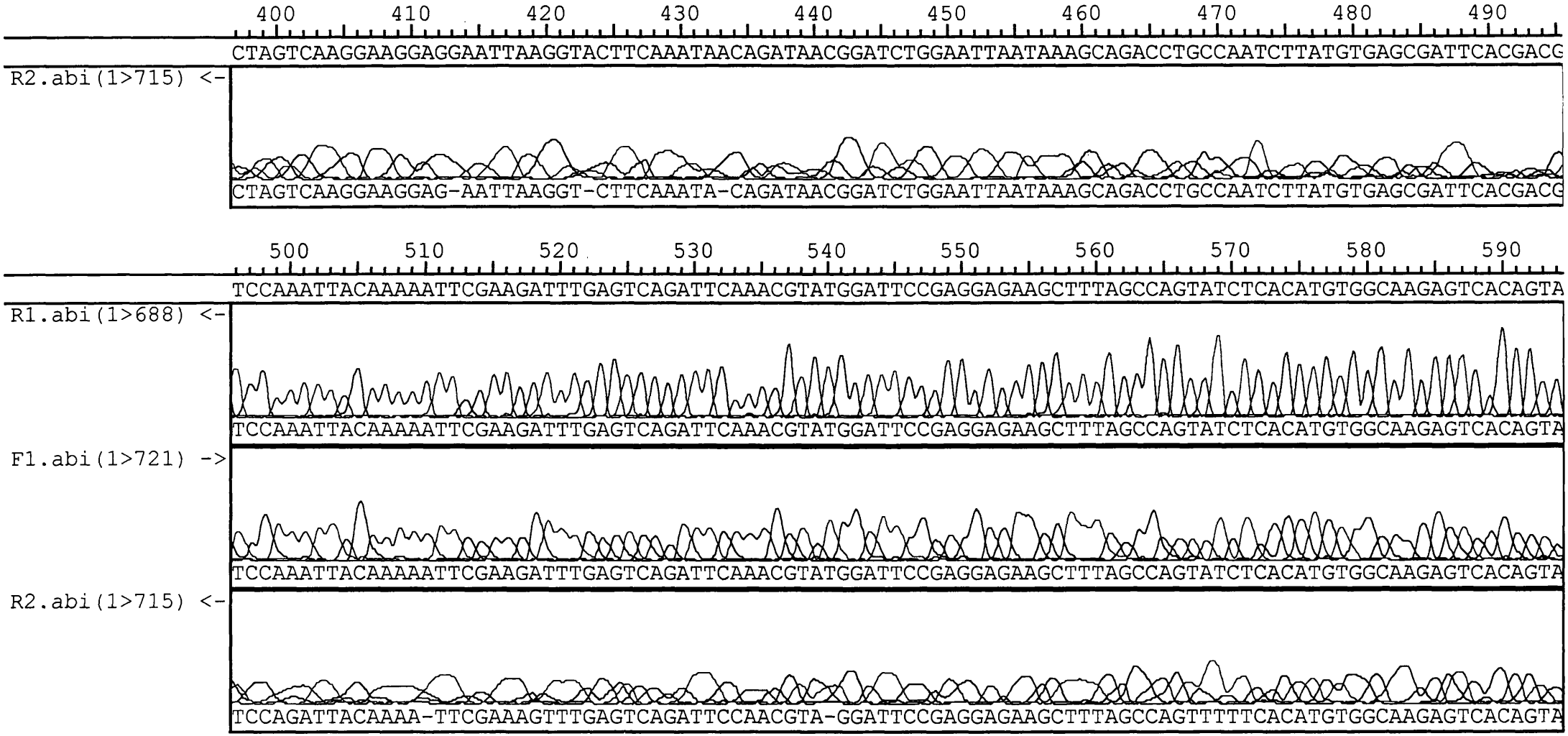
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500 510 520 530 540 550 560 570 580 590
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R1.abi(1>688) <-
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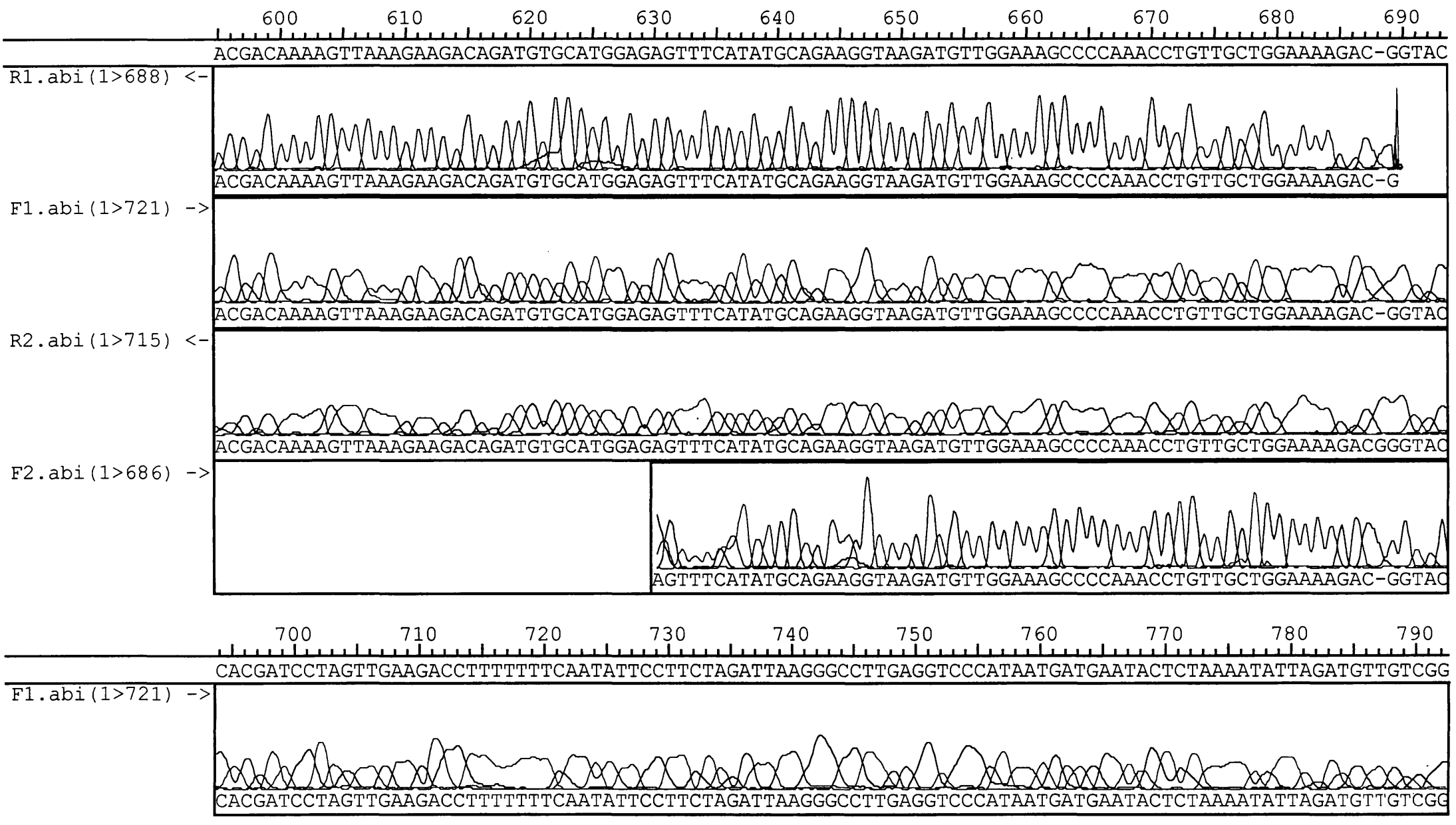
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ACGACAAAAGTTAAAGAAGACAGATGTGCATGGAGAGTTTCATATGCAGAAGGTAAGATGTTGGAAAGCCCCAAACCTGTTGCTGGAAAAGAC-GGTAC
R2.abi(1>715) <-
ACGACAAAAGTTAAAGAAGACAGATGTGCATGGAGAGTTTCATATGCAGAAGGTAAGATGTTGGAAAGCCCCAAACCTGTTGCTGGAAAAGACGGGTAC
F2.abi(1>686) ->
AGTTTCATATGCAGAAGGTAAGATGTTGGAAAGCCCCAAACCTGTTGCTGGAAAAGAC-GGTAC
700 710 720 730 740 750 760 770 780 790
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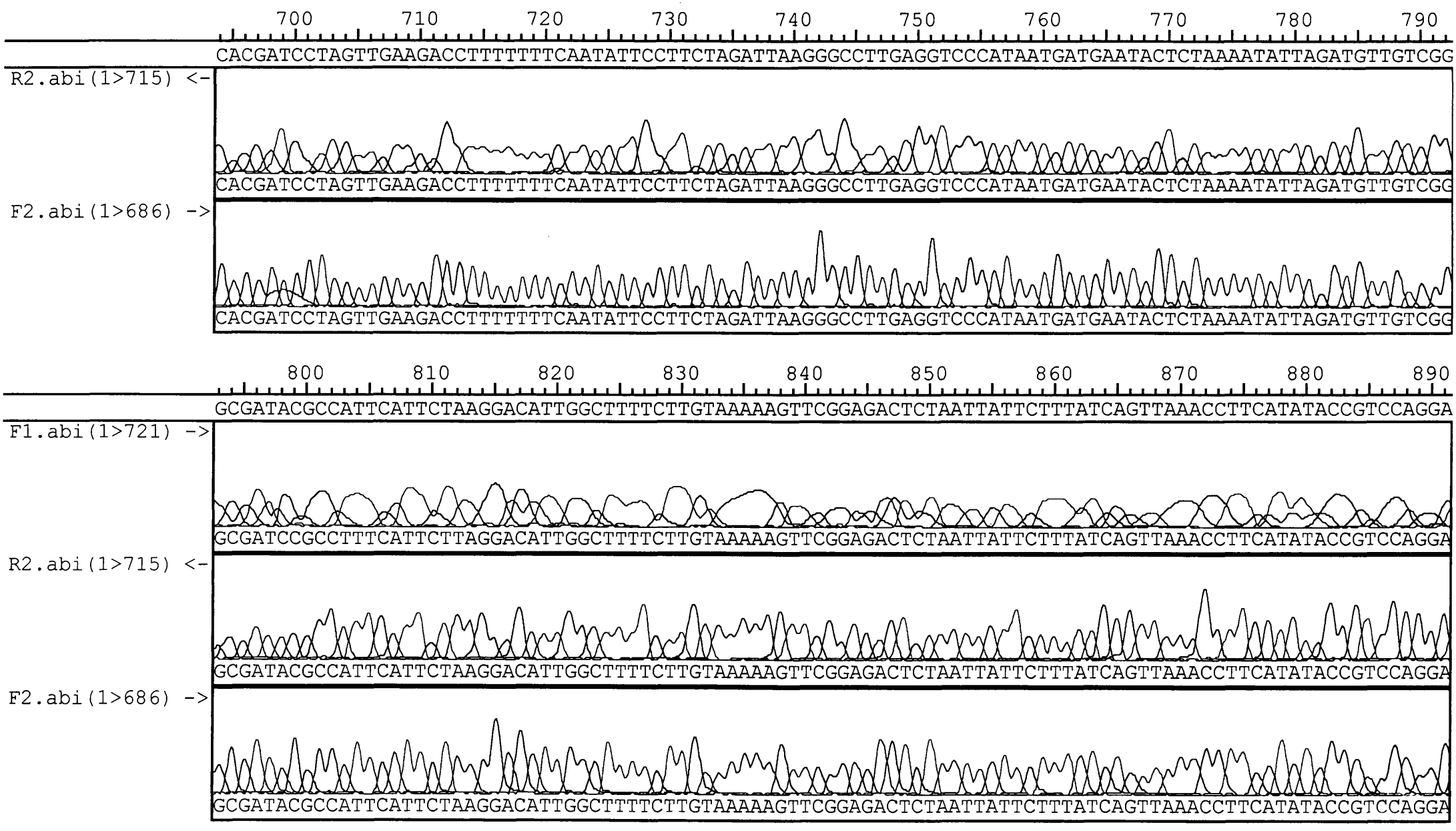
700 710 720 730 740 750 760 770 780 790
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R2.abi(1>715) <-
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F2.abi(1>686) ->
CACGATCCTAGTTGAAGACCTTTTTTTCAATATTCCTTCTAGATTAAGGGCCTTGAGGTCCCATAATGATGAATACTCTAAAATATTAGATGTTGTCGG
800 810 820 830 840 850 860 870 880 890
GCGATACGCCATTCATTCTAAGGACATTGGCTTTTCTTGTAAAAAGTTCGGAGACTCTAATTATTCTTTATCAGTTAAACCTTCATATACCGTCCAGGA
F1.abi(1>721) ->
GCGATCCGCCTTTCATTCTTAGGACATTGGCTTTTCTTGTAAAAAGTTCGGAGACTCTAATTATTCTTTATCAGTTAAACCTTCATATACCGTCCAGGA
R2.abi(1>715) <-
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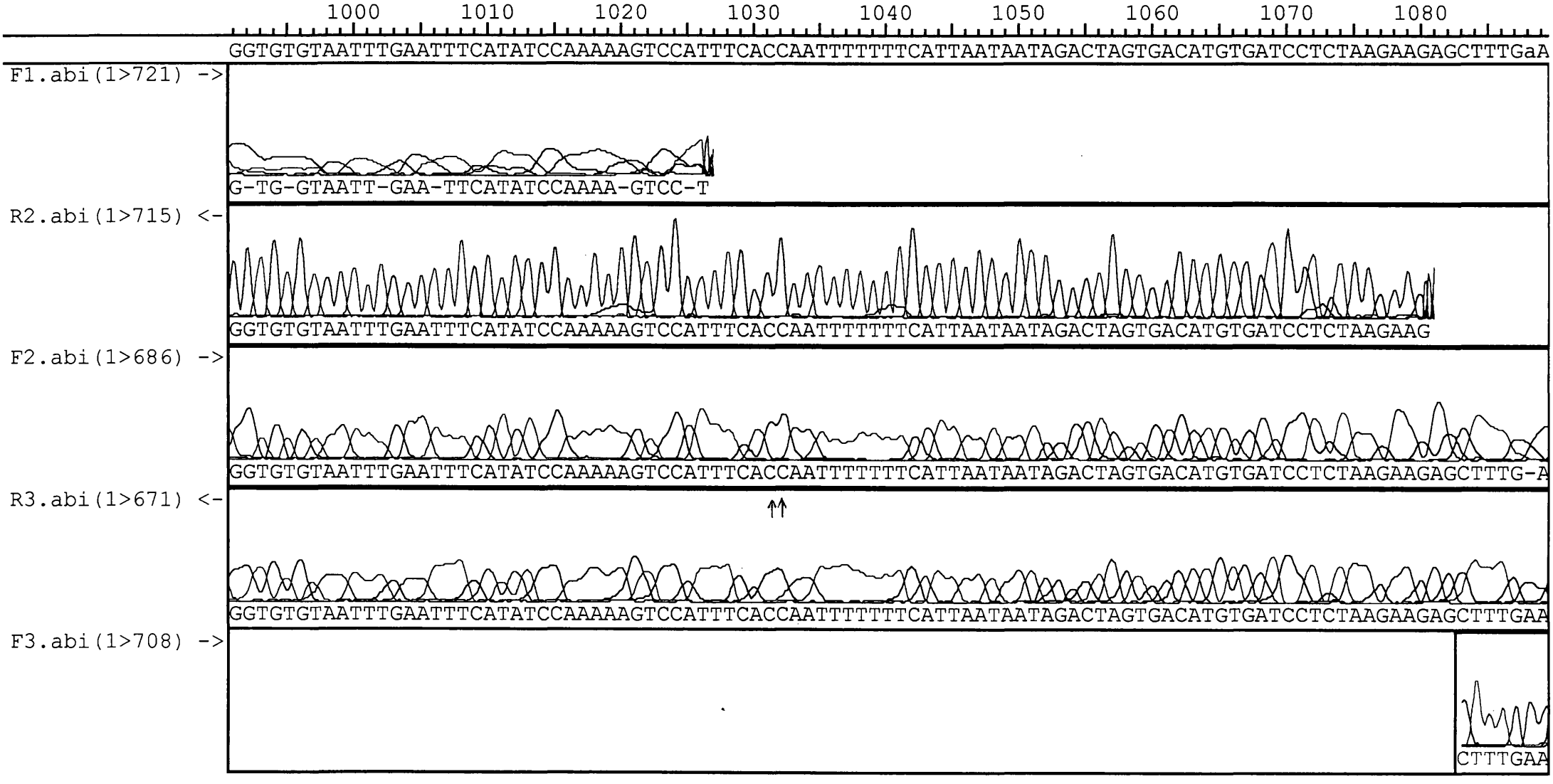
1000
1010
1020
1030
1040
1050
1060
1070
1080
GGTGTGTAATTTGAATTTCATATCCAAAAAGTCCATTTCACCAATTTTTTTCATTAATAATAGACTAGTGACATGTGATCCTCTAAGAAGAGCTTTGaA
F1.abi(1>721) ->
G-TG-GTAATT-GAA-TTCATATCCAAAA-GTCC-T
R2.abi(1>715) <-
GGTGTGTAATTTGAATTTCATATCCAAAAAGTCCATTTCACCAATTTTTTTCATTAATAATAGACTAGTGACATGTGATCCTCTAAGAAG
F2.abi(1>686) ->
GGTGTGTAATTTGAATTTCATATCCAAAAAGTCCATTTCACCAATTTTTTTCATTAATAATAGACTAGTGACATGTGATCCTCTAAGAAGAGCTTTG-A
R3.abi(1>671) <-
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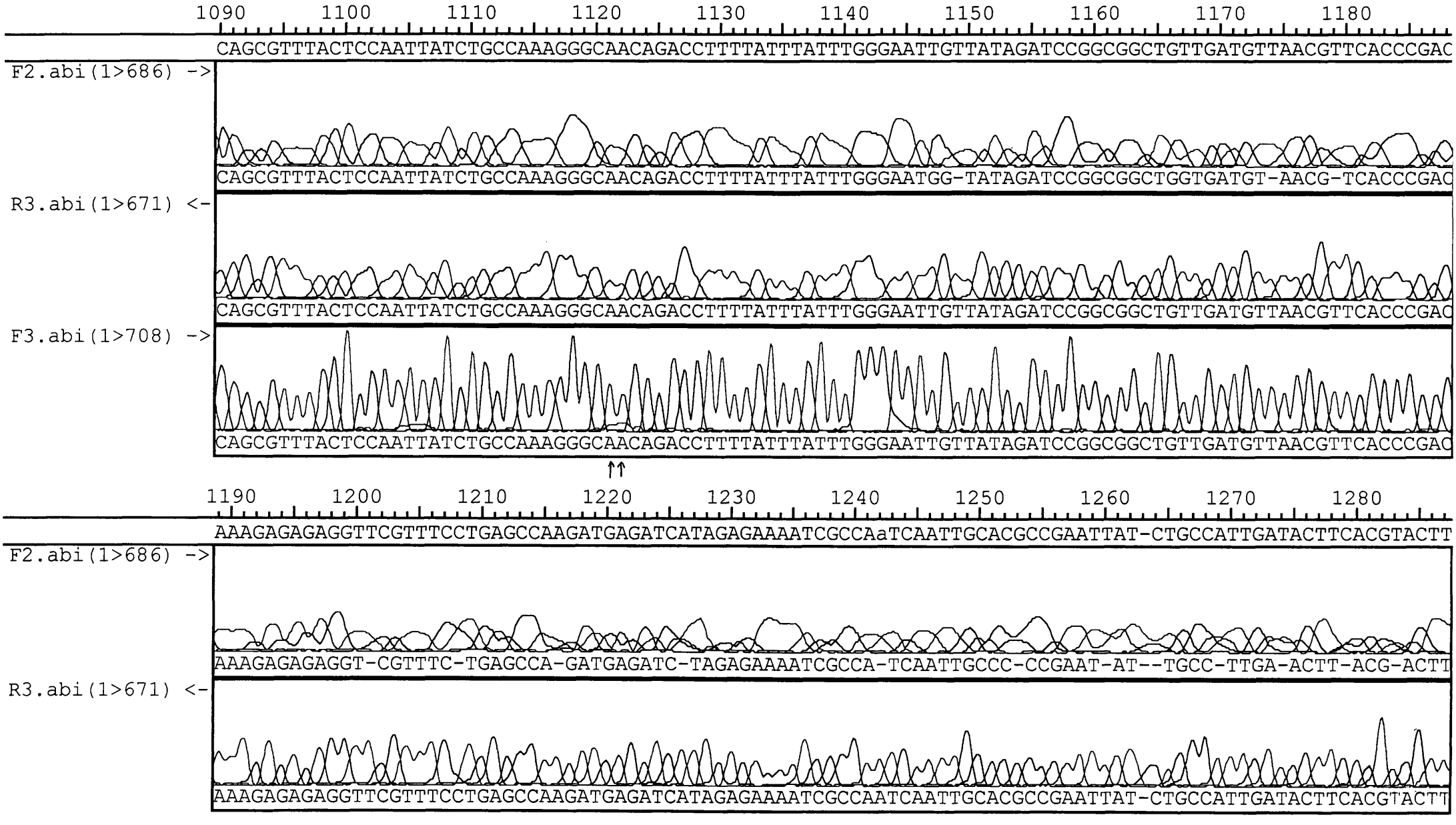
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F2.abi(1>686) ->
CAGCGTTTACTCCAATTATCTGCCAAAGGGCAACAGACCTTTTATTTATTTGGGAATGG-TATAGATCCGGCGGCTGGTGATGT-AACG-TCACCCGAC
R3.abi(1>671) <-
CAGCGTTTACTCCAATTATCTGCCAAAGGGCAACAGACCTTTTATTTATTTGGGAATTGTTATAGATCCGGCGGCTGTTGATGTTAACGTTCACCCGAC
F3.abi(1>708) ->
CAGCGTTTACTCCAATTATCTGCCAAAGGGCAACAGACCTTTTATTTATTTGGGAATTGTTATAGATCCGGCGGCTGTTGATGTTAACGTTCACCCGAC
1190 1200 1210 1220 1230 1240 1250 1260 1270 1280
AAAGAGAGAGGTTCGTTTCCTGAGCCAAGATGAGATCATAGAGAAAATCGCCAaTCAATTGCACGCCGAATTAT-CTGCCATTGATACTTCACGTACTT
F2.abi(1>686) ->
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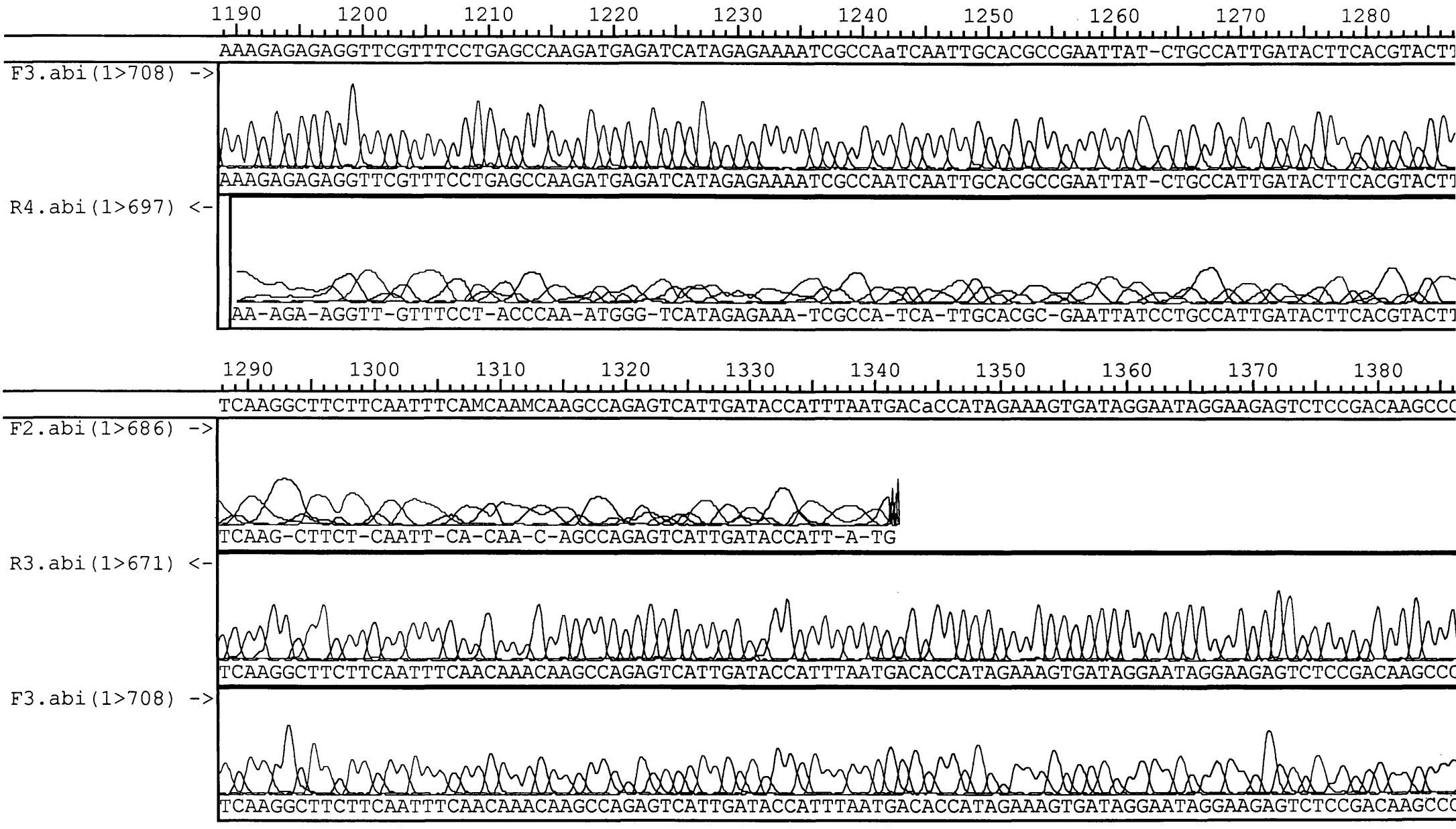
1190
1200
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1220
1230
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1250
1260
1270
1280
AAAGAGAGAGGTTCGTTTCCTGAGCCAAGATGAGATCATAGAGAAAATCGCCAaTCAATTGCACGCCGAATTAT-CTGCCATTGATACTTCACGTACTT
F3.abi(1>708) ->
AAAGAGAGAGGTTCGTTTCCTGAGCCAAGATGAGATCATAGAGAAAATCGCCAATCAATTGCACGCCGAATTAT-CTGCCATTGATACTTCACGTACTT
R4.abi(1>697) <-
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1290
1300
1310
1320
1330
1340
1350
1360
1370
1380
TCAAGGCTTCTTCAATTTCAMCAAMCAAGCCAGAGTCATTGATACCATTTAATGACaCCATAGAAAGTGATAGGAATAGGAAGAGTCTCCGACAAGCCC
F2.abi(1>686) ->
TCAAG-CTTCT-CAATT-CA-CAA-C-AGCCAGAGTCATTGATACCATT-A-TG
R3.abi(1>671) <-
TCAAGGCTTCTTCAATTTCAACAAACAAGCCAGAGTCATTGATACCATTTAATGACACCATAGAAAGTGATAGGAATAGGAAGAGTCTCCGACAAGCCC
F3.abi(1>708) ->
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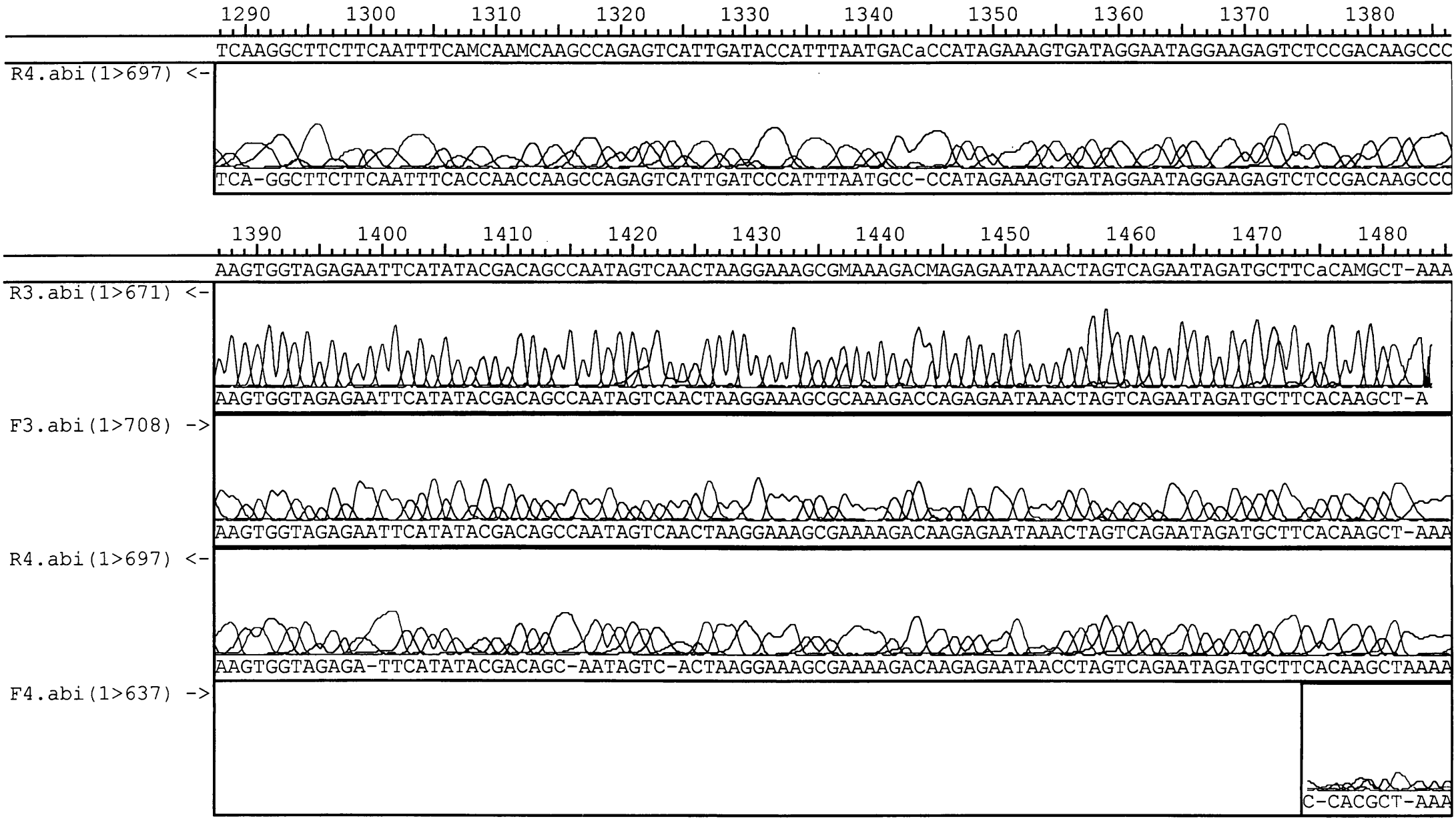
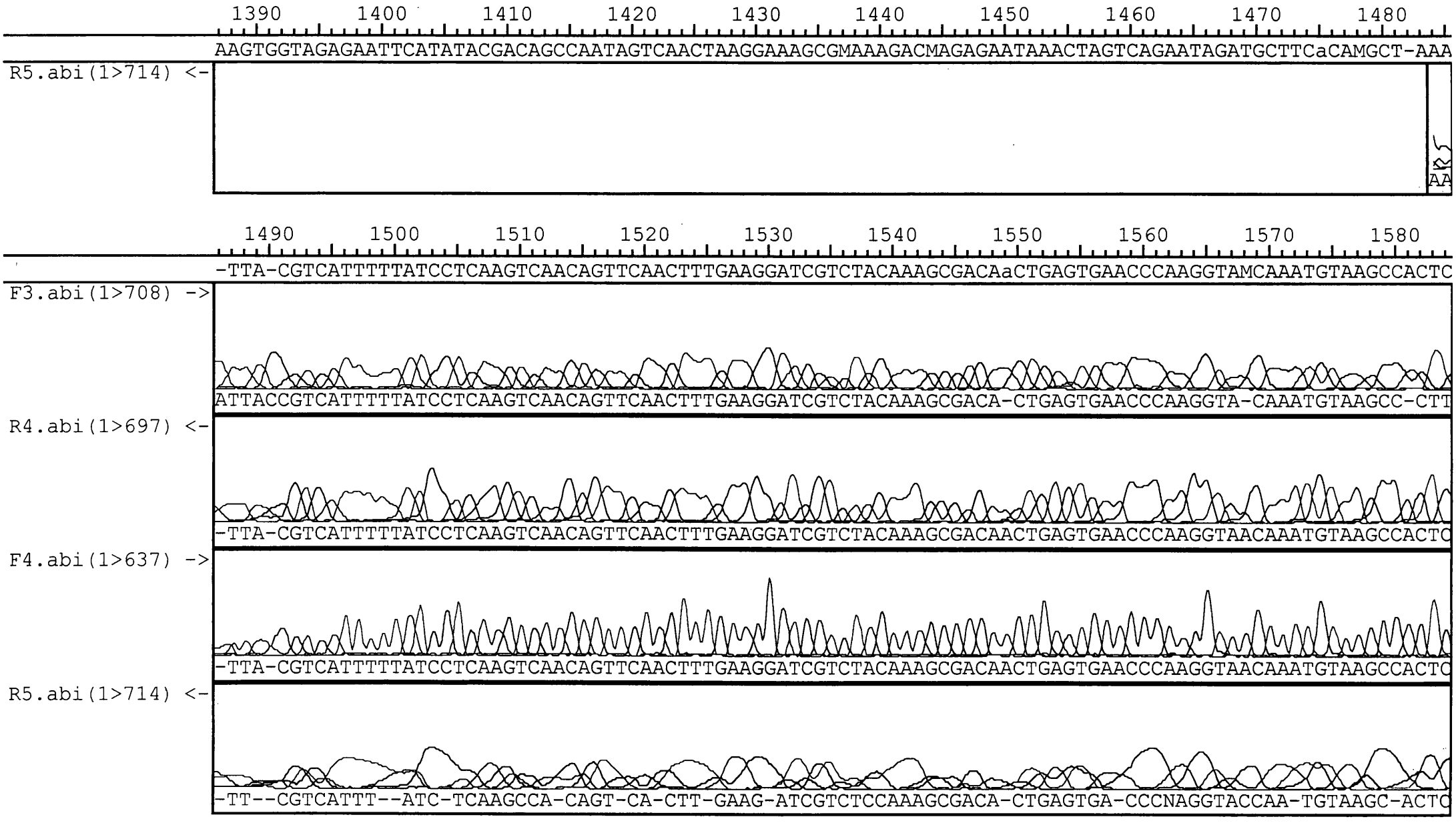
Project: 2ndmlh1.SQD Contig 1

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R5.abi(1>714) <-
1490 1500 1510 1520 1530 1540 1550 1560 1570 1580
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F3.abi(1>708) ->
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R4.abi(1>697) <-
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F4.abi(1>637) ->
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R5.abi(1>714) <-
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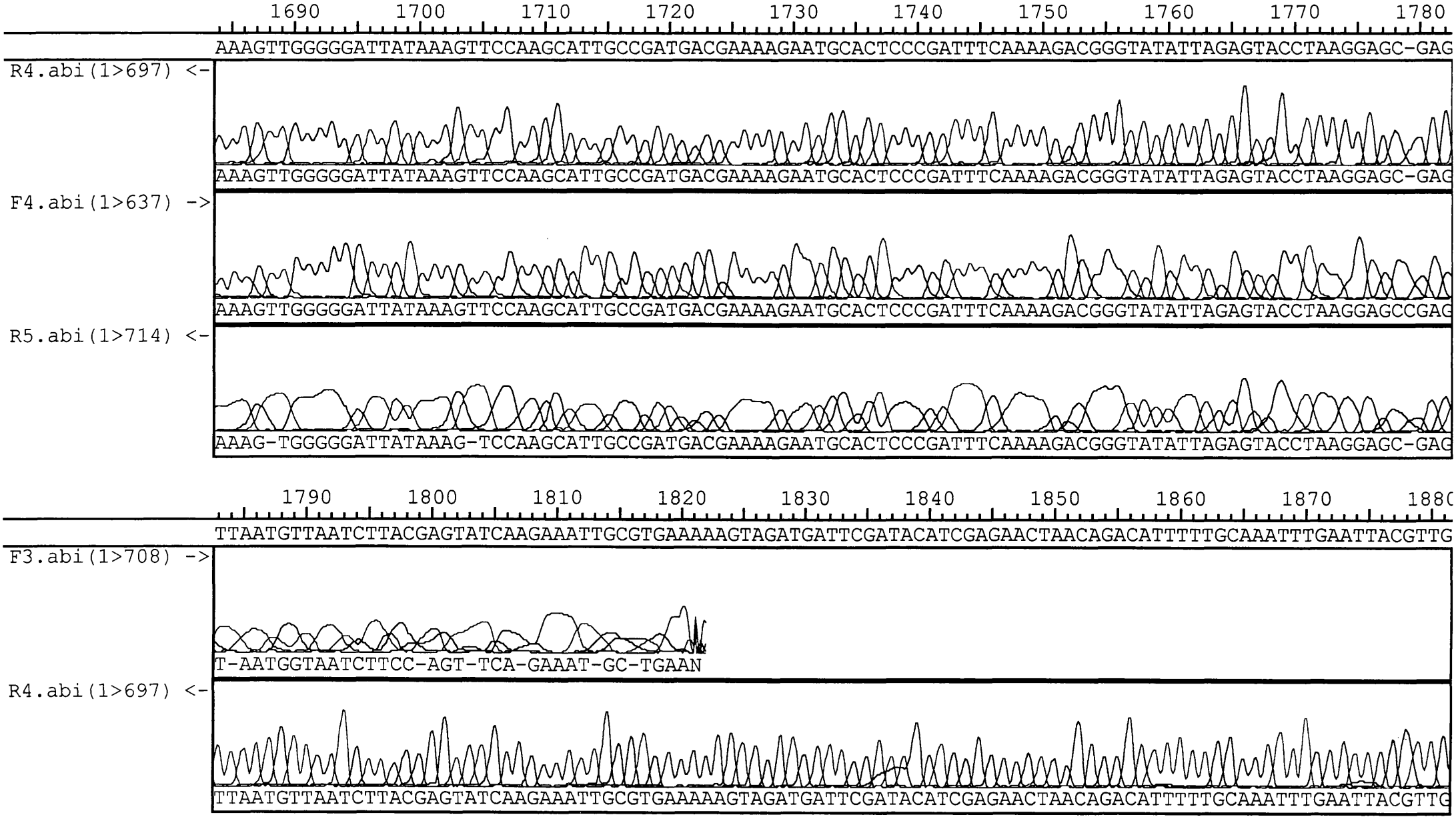
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R4.abi(1>697) <-
AAAGTTGGGGGATTATAAAGTTCCAAGCATTGCCGATGACGAAAAGAATGCACTCCCGATTTCAAAAGACGGGTATATTAGAGTACCTAAGGAGC-GAG
F4.abi(1>637) ->
AAAGTTGGGGGATTATAAAGTTCCAAGCATTGCCGATGACGAAAAGAATGCACTCCCGATTTCAAAAGACGGGTATATTAGAGTACCTAAGGAGCCGAG
R5.abi(1>714) <-
AAAG-TGGGGGATTATAAAG-TCCAAGCATTGCCGATGACGAAAAGAATGCACTCCCGATTTCAAAAGACGGGTATATTAGAGTACCTAAGGAGC-GAG
1790 1800 1810 1820 1830 1840 1850 1860 1870 1880
TTAATGTTAATCTTACGAGTATCAAGAAATTGCGTGAAAAAGTAGATGATTCGATACATCGAGAACTAACAGACATTTTTGCAAATTTGAATTACGTTG
F3.abi(1>708) ->
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R4.abi(1>697) <-
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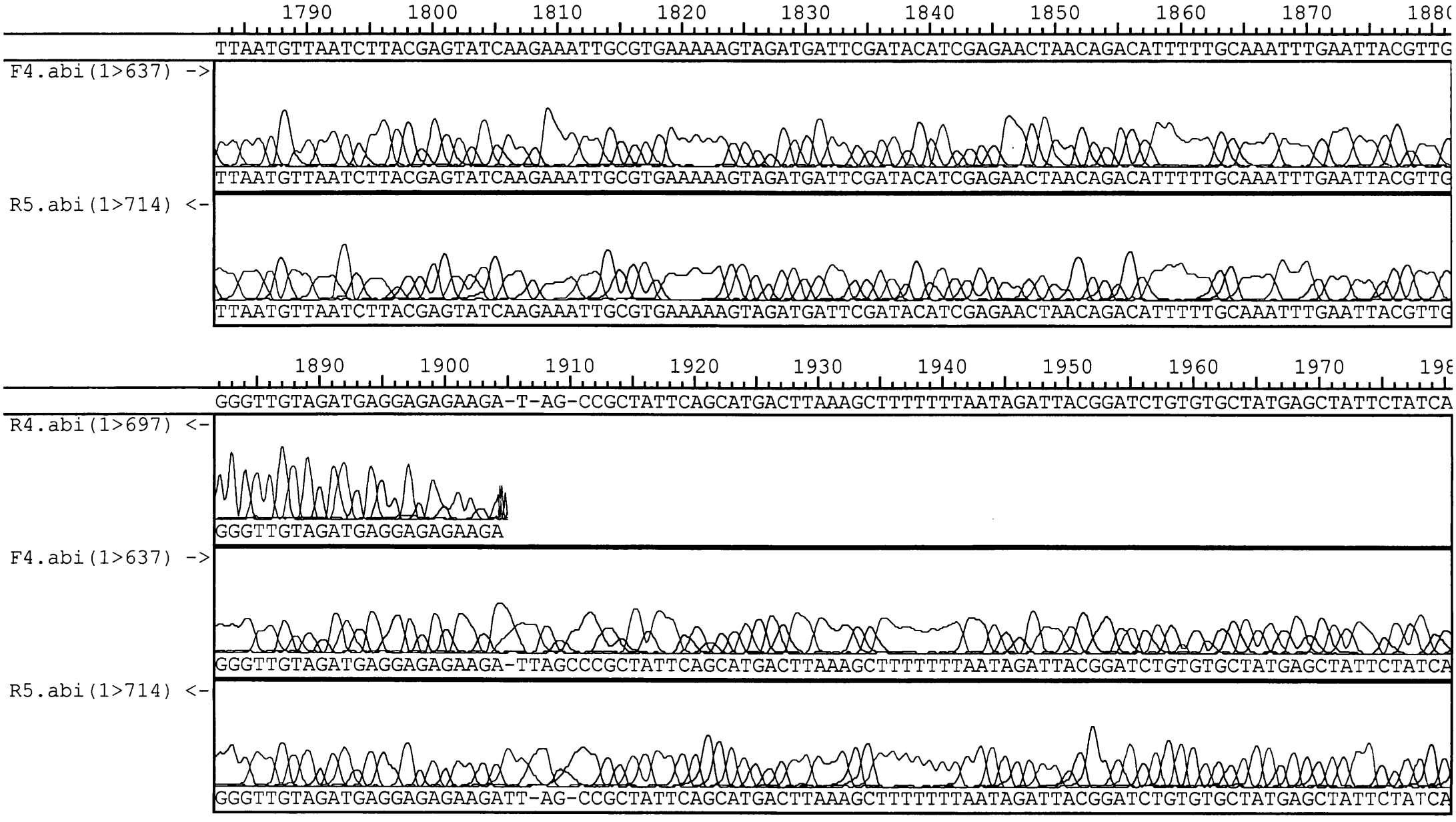
1790 1800 1810 1820 1830 1840 1850 1860 1870 188
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F4.abi(1>637) ->
TTAATGTTAATCTTACGAGTATCAAGAAATTGCGTGAAAAAGTAGATGATTCGATACATCGAGAACTAACAGACATTTTTGCAAATTTGAATTACGTTG
R5.abi(1>714) <-
TTAATGTTAATCTTACGAGTATCAAGAAATTGCGTGAAAAAGTAGATGATTCGATACATCGAGAACTAACAGACATTTTTGCAAATTTGAATTACGTTG
1890 1900 1910 1920 1930 1940 1950 1960 1970 198
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R4.abi(1>697) <-
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F4.abi(1>637) ->
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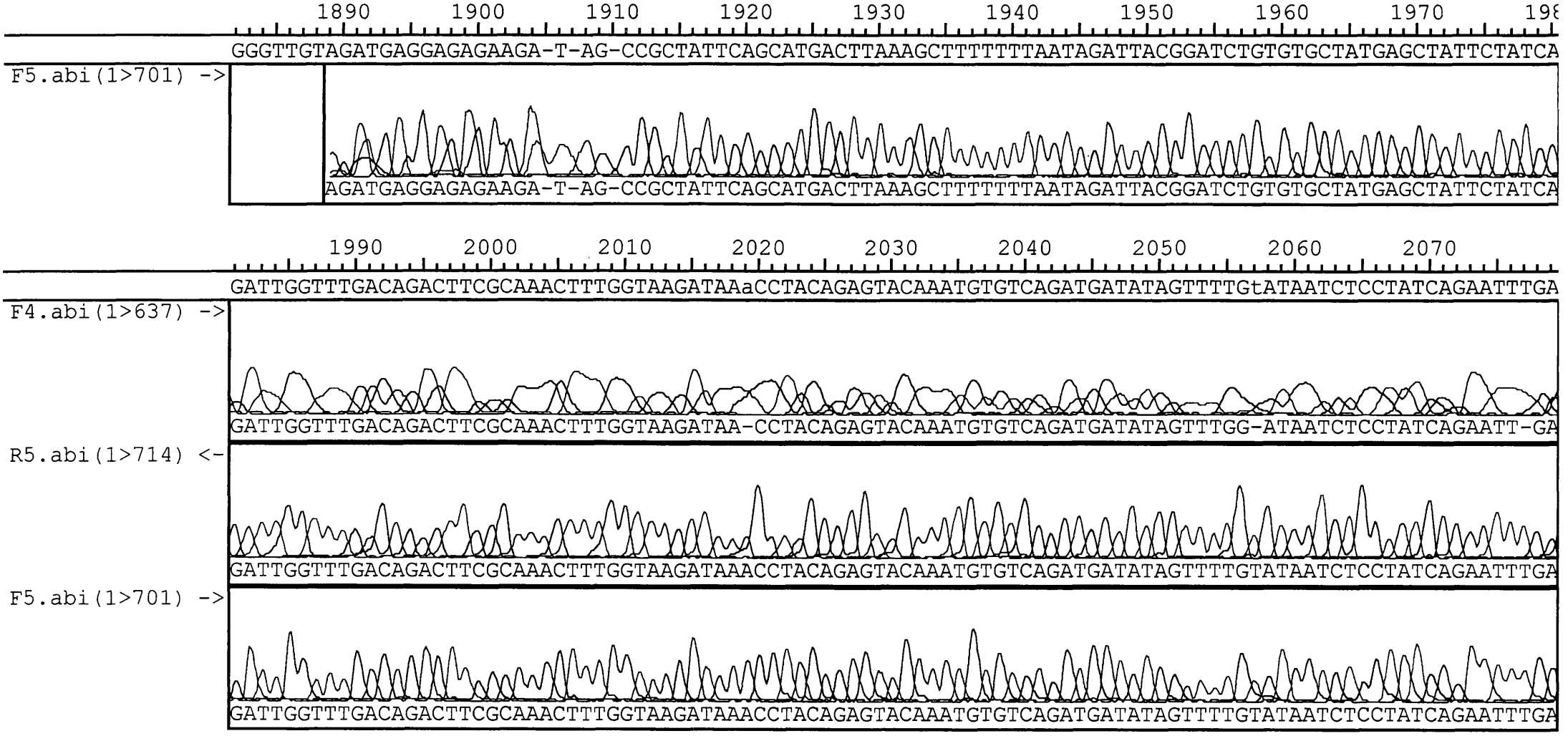
1890
1900
1910
1920
1930
1940
1950
1960
1970
GGGTTGTAGATGAGGAGAGAAGA-T-AG-CCGCTATTCAGCATGACTTAAAGCTTTTTTTAATAGATTACGGATCTGTGTGCTATGAGCTATTCTATCA
F5.abi(1>701) ->
AGATGAGGAGAGAAGA-T-AG-CCGCTATTCAGCATGACTTAAAGCTTTTTTTAATAGATTACGGATCTGTGTGCTATGAGCTATTCTATCA
1990
2000
2010
2020
2030
2040
2050
2060
2070
GATTGGTTTGACAGACTTCGCAAACTTTGGTAAGATAAaCCTACAGAGTACAAATGTGTCAGATGATATAGTTTTGtATAATCTCCTATCAGAATTTGA
F4.abi(1>637) ->
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R5.abi(1>714) <-
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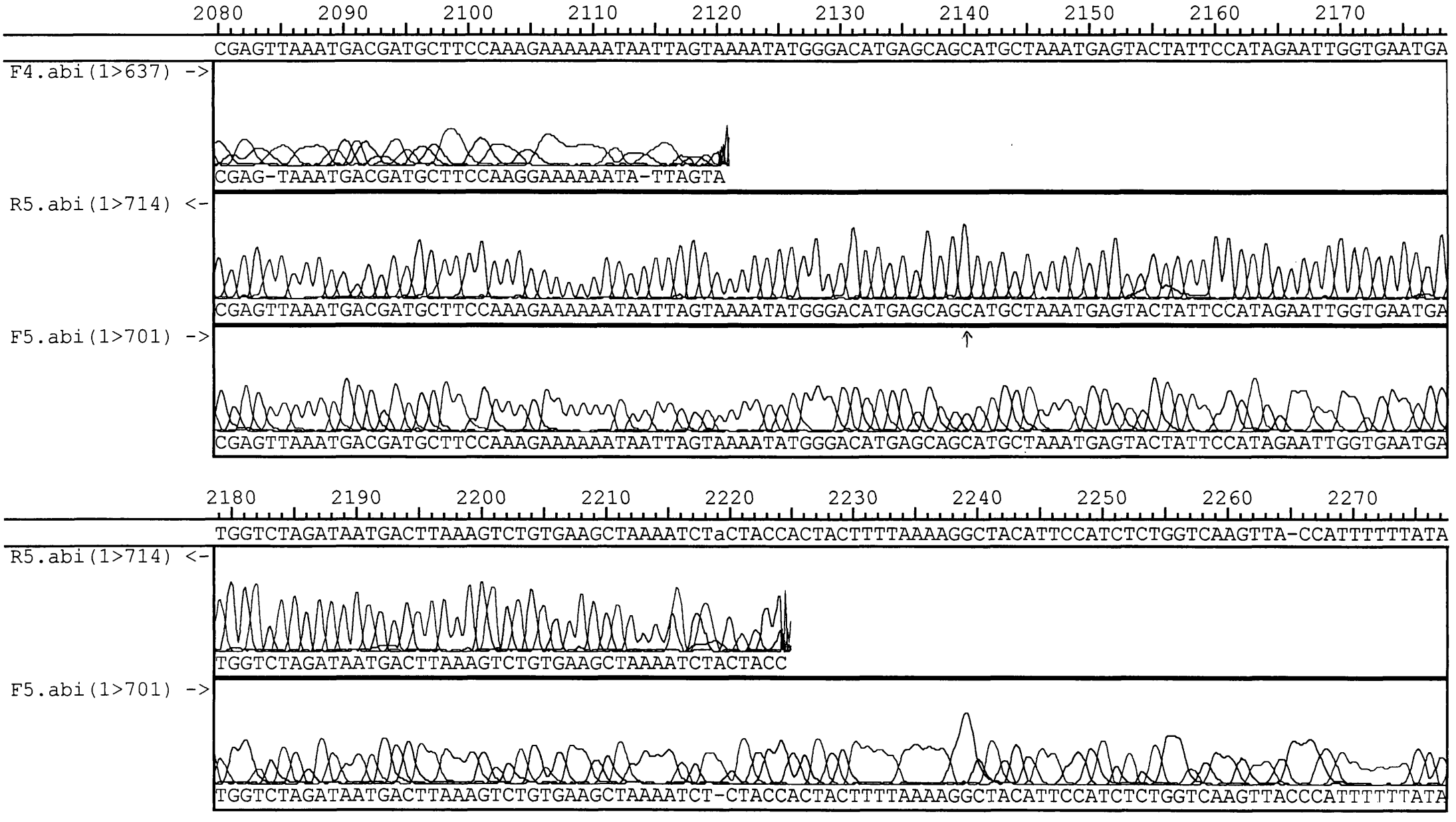
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F4.abi(1>637) ->
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R5.abi(1>714) <-
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F5.abi(1>701) ->
CGAGTTAAATGACGATGCTTCCAAAGAAAAAATAATTAGTAAAATATGGGACATGAGCAGCATGCTAAATGAGTACTATTCCATAGAATTGGTGAATGA
2180 2190 2200 2210 2220 2230 2240 2250 2260 2270
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R5.abi(1>714) <-
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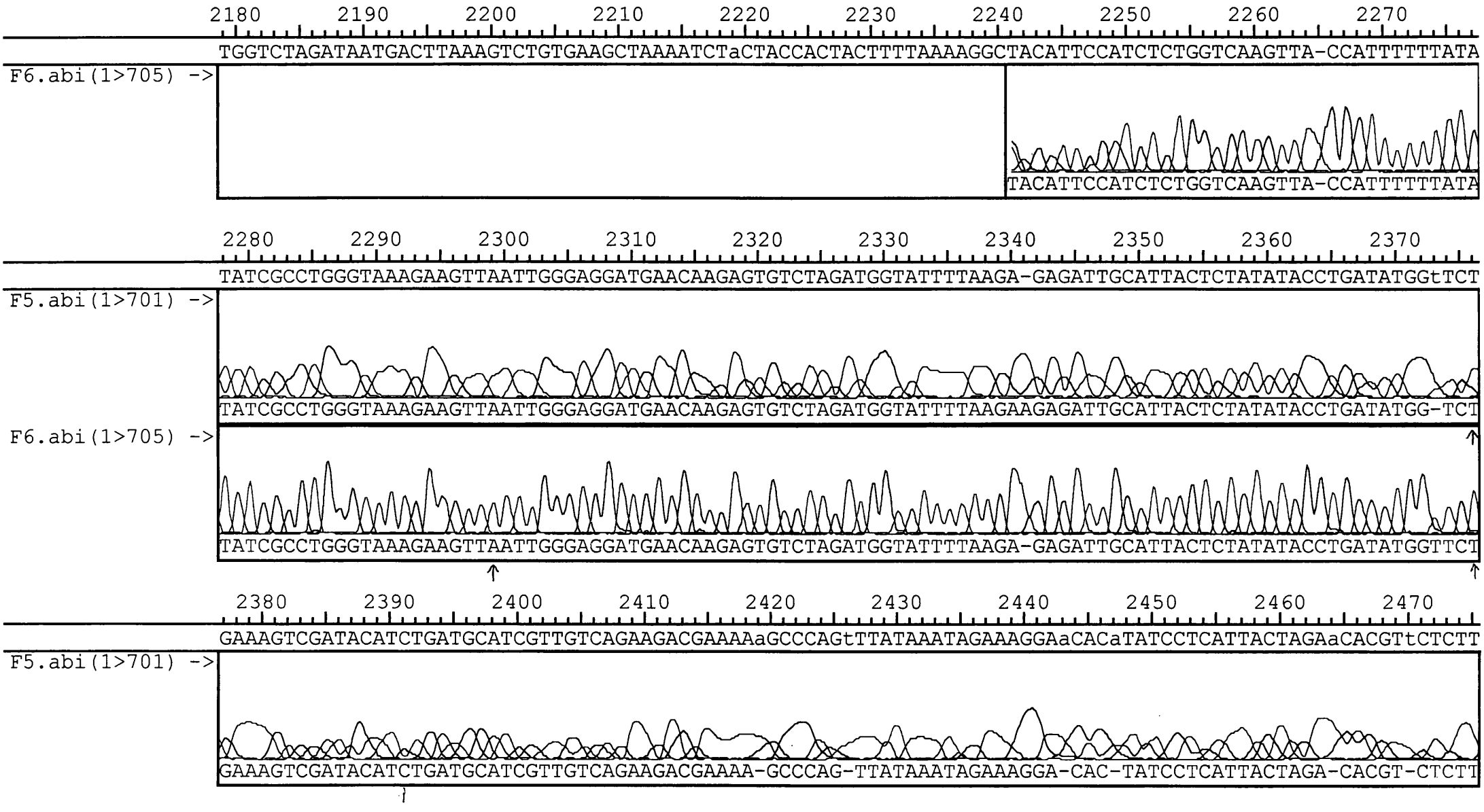
2180 2190 2200 2210 2220 2230 2240 2250 2260 2270
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F6.abi(1>705) ->
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2280 2290 2300 2310 2320 2330 2340 2350 2360 2370
TATCGCCTGGGTAAAGAAGTTAATTGGGAGGATGAACAAGAGTGTCTAGATGGTATTTTAAGA-GAGATTGCATTACTCTATATACCTGATATGGtTCT
F5.abi(1>701) ->
TATCGCCTGGGTAAAGAAGTTAATTGGGAGGATGAACAAGAGTGTCTAGATGGTATTTTAAGAAGAGATTGCATTACTCTATATACCTGATATGG-TCT
F6.abi(1>705) ->
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2380 2390 2400 2410 2420 2430 2440 2450 2460 2470
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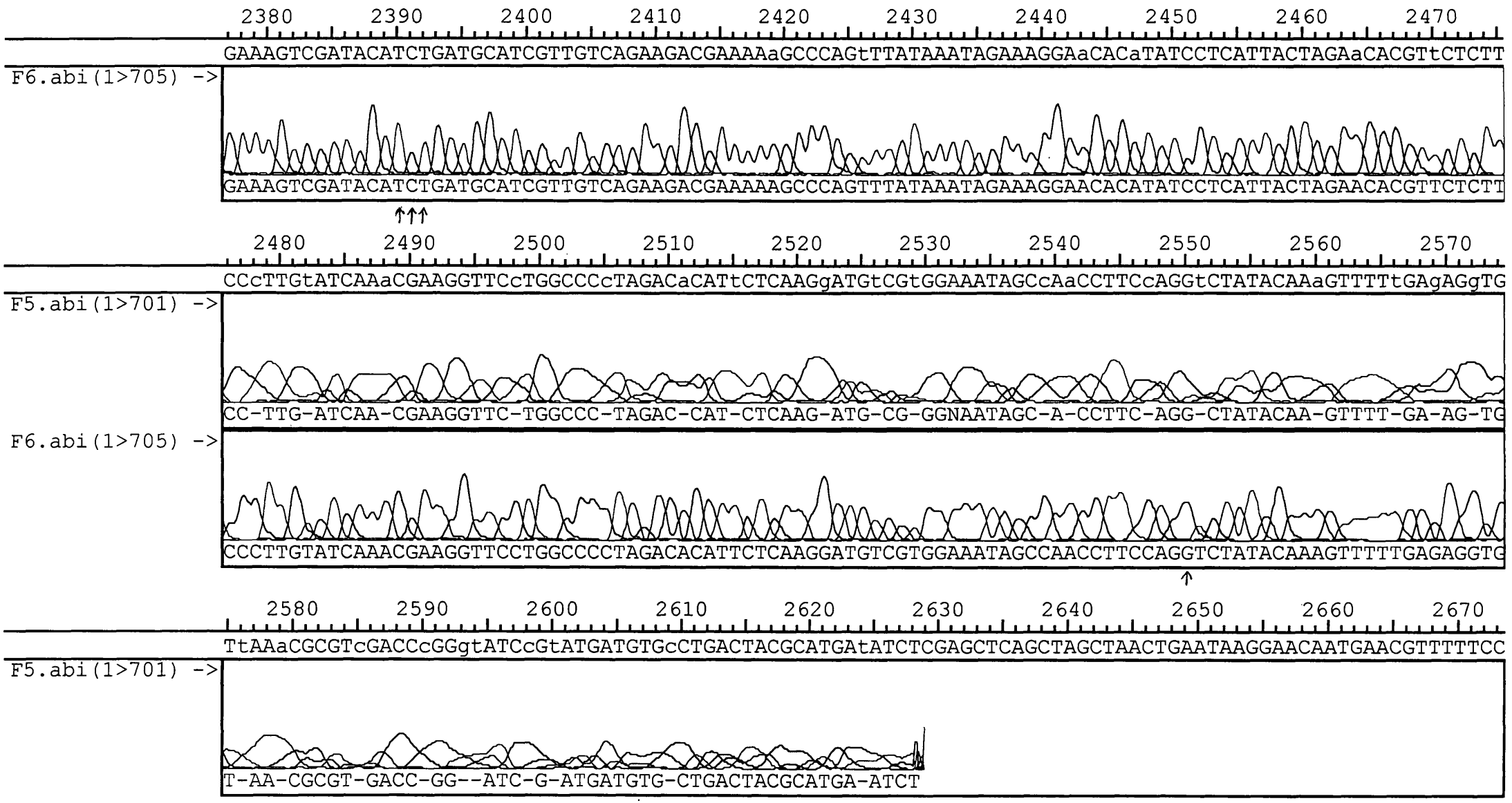
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F6.abi(1>705) ->
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2480 2490 2500 2510 2520 2530 2540 2550 2560 2570
CCcTTGtATCAAaCGAAGGTTCcTGGCCCcTAGACaCATtCTCAAGgATGtCGtGGAAATAGCcAaCCTTCcAGGtCTATACAAaGTTTTtGAgAGgTG
F5.abi(1>701) ->
CC-TTG-ATCAA-CGAAGGTTC-TGGCCC-TAGAC-CAT-CTCAAG-ATG-CG-GGNAATAGC-A-CCTTC-AGG-CTATACAA-GTTTT-GA-AG-TG
F6.abi(1>705) ->
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2580 2590 2600 2610 2620 2630 2640 2650 2660 2670
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F5.abi(1>701) ->
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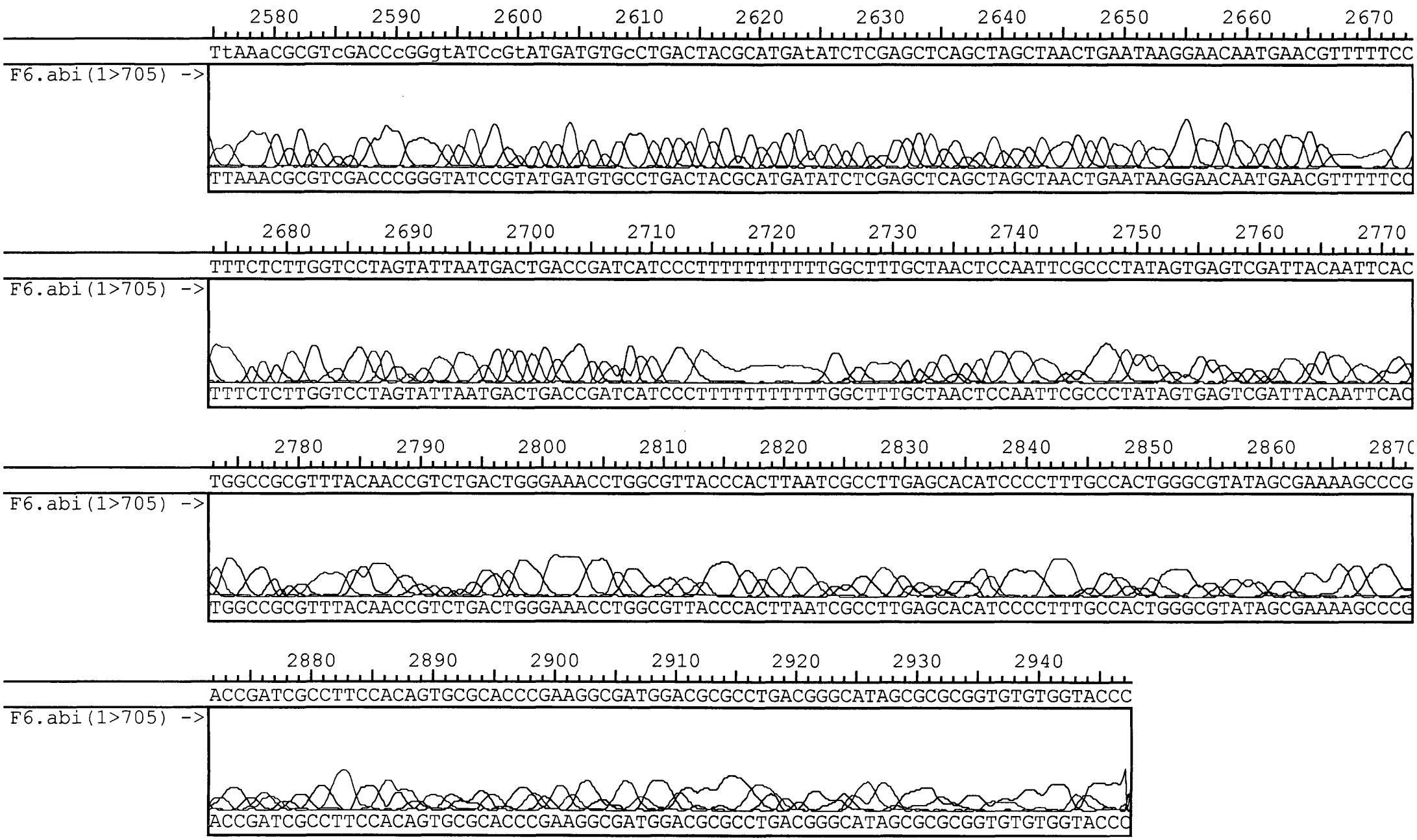
2580 2590 2600 2610 2620 2630 2640 2650 2660 2670
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2680 2690 2700 2710 2720 2730 2740 2750 2760 2770
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F6.abi(1>705) ->
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2780 2790 2800 2810 2820 2830 2840 2850 2860 2870
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F6.abi(1>705) ->
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2880 2890 2900 2910 2920 2930 2940
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