Study of the *Escherichia coli* Vsr Endonuclease and its Interaction with MutL

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Abstract

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Kathy M.J. Doiron, Ph.D.

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In all living organisms, DNA repair proteins are essential for maintaining the accuracy of genetic material. In *Escherichia coli*, methyl-directed mismatch repair (MMR) corrects most types of misinsertion errors that arise during DNA replication. In contrast, very short patch (VSP) repair, corrects T/G mismatches caused by the deamination of 5-methylcytosine to thymine in C(T/G)WGG (W = A or T) sequences. Initial experimental data had revealed that the over production of the Vsr endonucleaseresulted in a spectrum of transition and frameshift mutations similar to that produced by MMR deficient strains. This spectrum of mutations was also observed for two mutant Vsr proteins known to be defective in VSP repair. These results strongly suggested that the inhibition in MMR repair was due to an interaction between Vsr and one or more of the MMR proteins. The finding of an interaction between Vsr and MutL using both the yeast and bacterial two-hybrid systems further strengthened this hypothesis. Utilization of the two-hybrid systems further revealed that Vsr mutants, with the amino-terminal truncated, still showed an interaction. Conversely even the smallest deletion of the Vsr carboxyl terminal resulted in loss of interaction.

A new purification protocol for Vsr yielded a more stable protein that did not precipitate, nor lose its amino terminus and preserved its activity even after long-term storage at -80°C. *In vitro* endonuclease assays revealed that the activity is equivalent to established values. Single turnover, time course experiments confirmed that MutL does not have a stimulatory effect on Vsr under these conditions. In contrasrst, under conditions where Vsr is not limiting, MutL does have a stimulatory effect. In addition, Vsr was found to be stimulated to the same extent by MutL mutants deficient in nucleotide binding and/or hydrolysis as it is by wild type MutL. Vsr stimulation in the presence of MutL was unaffected by the presence or absence of ATP or ADP. These results lead us to conclude that the interaction between Vsr and MutL is not nucleotide dependent.

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projects. All the sacrifices you made to get me through this challenge were always noticed and truly appreciated.

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List of abbreviations

Abbreviation	Definition
°C	Celsius.
A	Adenine.
a.a.	Amino acid.
aBSA	Acetylated bovine serum albumin.
AD	Activation domain.
ADP	Adenosine diphosphate.
AMPpnp	5'-adenylyl-beta, gamma-imidodiphosphate.
ATP	Adenosine triphosphate.
ATPase	Protein that hydrolyses ATP.
BD	Binding domain.
bp	Base pairs.
C	Cytosine.
cAMP	Cyclic adenosine monophosphate.
CAP	Catabolite Activator Protein.
cm	Centimetre.
CTD	Carboxy terminal domain.
DMSO	Dimethyl sulfoxide.
DNA	Deoxyribonucleic acid.
dNTP	Deoxynucleotide triphosphates.
dsDNA	Double stranded DNA.
DTT	Dithiothreitol.
E. coli	*Escherichia coli.*
EDTA	Ethylenediamine tetraacetic acid.
fmol	Femtomoles.
G	Guanine.
g	Gram.
GHKL	Gyrase, HSP90, Histidine Kinase and MutL.
H69A	Histidine 69 mutated to alanine.
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.
His	Histidine.
HNPCC	Hereditary nonpolyposis colorectal cancer.
hr	Hour.
IPTG	Isopropyl β-D-1-thiogalactopyranoside.
IR	Infrared.

kb	Kilobases.
kDaa	Kilodaltons.
L	Litre.
Lac^+	Can metabolize lactose.
LB	Luria broth.
Leu	Leucine.
LiAc	Lithium acetate.
LN40	Amino terminal fragment of MutL, only a.a. 1-349.
M	Molar.
MCS	Multi cloning site.
min	Minute.
ml	Millilitre.
mM	Millimolar.
mm	Millimetre.
MMR	Methyl-directed mismatch repair.
ng	Nanogram.
nM	Nanomolar.
NTD	Amino terminal domain.
OD	Optical density.
ONPG	O-nitrophenyl β-D-galactopyranoside.
PAGE	Polyacrylamide gel electrophoresis.
PCR	Polymerase chain reaction.
PEG	Polyethylene glycol.
RNA	Ribonucleic acid.
rpm	Revolutions per minute.
rt	Room temperature.
S. cerevisiae	*Saccharomyces cerevisiae.*
SD	Synthetic drop out.
SDS	Sodium dodecyl sulphate.
SS or ss	Single stranded.
SSB	Single stranded binding protein.
SSC	Sodium chloride and sodium citrate.
ssDNA	Single stranded DNA.
T	Thymine.
TAE	Tris, Acetate, EDTA.
TBE	Tris, Boric acid, EDTA.
TE	Tris, EDTA.
TEMED	Tetramethylethylenediamine.
trc	Fusion of the tryptophan and lactose promoters.

Trp	Tryptophan.
UAS	Upstream activating sequence.
v	Volts.
V/V	Volume / volume.
VSP	Very short patch.
W	A or T.
Wt	Wild type.
W/V	Weight / volume.
x g	Gravity.
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside.
YPD	Yeast, peptone, dextrose.
µl	Microlitre.
µM	Micromolar.

Contribution of Authors

All the data presented in this thesis is the work of the author, except for the following:

- Ms. C. Mansour: using the bacterial two-hybrid system, she analyzed the interactions among the wild type mismatch repair proteins (MMR) and between the MMR proteins and the wild type Vsr. Mansour *et al.* 2001.
- Dr. Y. Polosina performed the trypsin experiments on MutL and the MutL mutants in order to test their conformation and ATP binding affinity.

1. Introduction

1.1 Maintaining genome integrity

From the point of view of evolution, both small and large mutations in the DNA are a good thing; they allow for the possibility of improving existing traits or even developing new ones. However, they also have the potential to be detrimental, even lethal. In addition to being implicated in the ageing process, mutations sustained by DNA have been found to be the leading cause of heritable diseases as well as cancers (Modrich, 1994). Consequently, all organisms normally strive to maintain the integrity of their genetic material. They do so by means of two key processes: accurate DNA replication and DNA repair. Even bacteria such as *Escherichia coli* (*E. coli*) live according to this cornerstone.

In all organisms, the foremost step to mutation avoidance is through DNA replication fidelity; which is achieved by two different functions of the DNA polymerase holoenzyme (Kunkel *et al.* 2000):

- First, there is base selection:

  In *E. coli,* the DNA polymerase III holoenzyme, discovered by Kornberg and Gefter in 1971, moves at an astounding rate of approximately 1000 base/second (Kornberg *et al.* 1972 and 1992). Consequently, bases must be selected very swiftly but as efficiently as possible. Nevertheless, the DNA polymerase holoenzyme is not 100 % efficient and mistakes do slip through. This occurs *in vitro* at a rate of approximately one in every 10^5 to 10^6 bases

(Radman *et al.* 1981). So, for *E. coli,* with a genome size of 4.7×10^6 bases, this means there would be approximately 5 to 50 mutations for every round of replication.

- Secondly, there is proofreading:

  Proofreading is where the polymerase actually edits out bases that were erroneously inserted (Livingston *et al.* 1975), functioning as the cellular equivalent of "spell-checking". Proofreading improves accuracy by a further two orders of magnitude.

Even with base selection and proofreading, mutation rates remain high. With every mutation amassed, there lies the potential for very harmful consequences, and that is unacceptable to the organism. For this reason, the second step in maintaining the genetic integrity of an organism is through a large defensive team of DNA repair systems. One line of defence is post replication repair, where its role is to detect and specifically repair any mispairs left behind by the DNA polymerase holoenzyme. Post replication repair, in conjunction with an efficient DNA polymerase, lowers the probability of a mutation occurring in the genome to 1 in every 10^{10} bases (Kornberg *et al.* 1992). In other words, *E. coli* could go through many rounds of replication without incorporating a single mutation. On the other hand, with population sizes of 10^9 cells per ml, there will always be mutants in the population.

However, the DNA polymerase is not the only source of DNA lesions in a cell. Lesions in the DNA can arise after replication. They can occur due to

environmental stressors (e.g. ultraviolet radiation, pollutants) as well as from the cell's own metabolic processes (e.g. deamination, oxidative damage). Consequently, the second line of repair includes a wide variety of systems that are responsible for the detection and correction of these numerous non replicative lesions.

For this thesis, the focus will be on two DNA rectification systems found in *E. coli*, specifically: 1) methyl-directed mismatch repair (MMR) and 2) very short patch (VSP) repair.

1.2 Methyl-directed mismatch repair

The post replication DNA repair pathway in *E. coli* is the methyl-directed mismatch repair system (MMR). This system is conserved, with some degree of homology from simple bacteria all the way up to humans. So far, the only organisms known not to possess a MMR system are Archaea (Grogan 2004) and Mycobacteria (Springer *et al.*, 2004). Interestingly, Archaea must have an alternative to the otherwise ubiquitous MutS/MutL mismatch repair pathway since mutational frequency rates are comparable with those of other microorganisms, despite their harsh environmental conditions (Grogan *et al.* 2001 and 2004).

In all other organisms, cells containing mutations in MMR genes characteristically display a mutator phenotype where the frequency of spontaneous mutation can be elevated by as much as 1000 fold (Modrich *et al.*

1996 and Kunkel *et al.* 2005). In mammals, including humans, defects in MMR result in elevated cancer frequencies, particularly hereditary nonpolyposis colorectal cancer (HNPCC) (Kolodner *et al.* 1994 and Lynch *et al.* 1999). The attractive aspect of studying MMR in *E. coli* is that the repair pathway is less complex, containing far fewer proteins than in eukaryotes. However, this "simple" system still has plenty of secrets to surrender and all knowledge gained from *E. coli* provides valuable clues to understanding the far more complicated eukaryotic systems.

As the polyvalent repair system in *E. coli,* MMR is responsible for correcting mis-incorporations and strand slippage errors that arise during DNA replication, as well as mismatches that occur from homologous recombination. Of the eight possible mispairings that can occur during DNA replication, C-C mispairs are the only ones that are refractory to being corrected by MMR (Dohet *et al.* 1985, Jones *et al.* 1987 and Kramer *et al.* 1984). The other seven types of mismatches are all corrected, albeit with variable efficiency: G-T and A-C are very efficiently corrected, while G-G and A-A are corrected efficiently, however T-T, C-T and G-A mispairs are repaired with lower and variable efficiencies (Dohet *et al.* 1985, Jones *et al.* 1987, Kramer *et al.* 1984 and Kunkel *et al.* 2005). Insertion and deletion loops of one to three bases in length are fully repaired (Dohet *et al.* 1986 and Fishel *et al.* 1986); lops of four bases are marginally repaired; but non-homologies of five bases and larger are not repaired (Dohet *et al.* 1987 and Parker *et al.* 1992).

In addition to mispairs, MMR is also involved in recognizing certain DNA lesions caused by both intracellular metabolism (Wyrzykowski *et al.* 2003) and by external environmental insults (Rydberg 1978, Harfe *et al.* 2000 and Kunkel *et al.* 2005). However, when the damage is too great for repair in eukaryotic cells, MMR is involved in triggering apoptosis (Harfe *et al.* 2000 and Kunkel *et al.* 2005). In mammals, MMR proteins are also involved in generating antibody diversity (Bellacosa *et al.* 2001).

Moreover, during homologous recombination of heteroduplex DNA, MMR plays a dual role. It can proceed with error correction of the heteroduplex or, if the sequences are too dissimilar, it can function as an anti-recombinator and cause the abortion of the recombination process (Claverys *et al.* 1986 and Harfe *et al.* 2000). As a result, this function of MMR prevents interspecies recombination (Rayssiguier *et al.* 1989).

For MMR to function properly, strand specificity is required to target repair only to the newly synthesized DNA strand (Wagner *et al.* 1976). In *E. coli*, this specificity is provided by the Dam adenine methylase, an enzyme which catalyses the methylation of adenines in sequence-specific GATC sites, but lags behind DNA replication (Lyons *et al.* 1984 and Marinus 1976). Thus, for a few minutes immediately after replication, the newly synthesised daughter strand is under-methylated relative to the parental one (Herman *et al.* 1981 and Marinus *et al.* 1984). This hemimethylated status of the DNA duplex allows MMR the window of opportunity required to target the newly synthesized strand for

correction (Lu *et al.* 1983 and Pukkila *et al.* 1983). Furthermore, since hemimethylated GATC sites are not always conveniently located near the mispair, the strand signal is capable of directing correction efficiently even several kilo basepairs (kb) away. However, when the separation exceeds two kb, the signal seems to be relatively weak in promoting repair (Lahue *et al.* 1987 and Bruni *et al.* 1988).

In order to achieve the multi-step process of strand discrimination, error detection, error excision and DNA synthesis, the MMR system engages DNA, ATP and thirteen separate proteins in a very intricate dance (Table 1). The three core proteins of this repair system are: MutS, MutL and MutH (Cox 1976, Pukkila *et al.* 1983, Lu *et al.* 1983 and 1984). The first protein to open this molecular dance is MutS, a DNA binding protein specific for heteroduplex DNA (Figure 1). It contains an ATPase (ATP binding cassette) domain that regulates its activities, and it has been observed in both homodimeric and homotetrameric forms (Lamers *et al.* 2000 and Bjornson *et al.* 2003).

The crystal structure of the *E. coli* MutS reveals an asymmetric dimer with the DNA mismatch binding (N-terminal) and ATPase (C-terminal) domains at opposite ends of the molecule (Figure 2). DNA containing a mismatch, or an insertion/deletion loop, is kinked at approximately 60° and held in place by the mismatch-binding domain of one subunit and the clamp domain of the other subunit (Lamers *et al.* 2000 and 2003).

Protein	Interaction partners during MMR	Known function during MMR
Dam methylase	DNA	Methylation of adenine in a GATC sequences.
MutS	DNA, MutL, MutS, β-clamp	Mispair recognition.
MutL	ssDNA, MutH, MutL, MutS, Helicase II, β-clamp	Molecular coordinator and regulator of the MMR system.
MutH	DNA, MutL	Single stranded endonuclease; recognises the hemimethylated GATC site and cleaves the DNA 5' of the adenine on the unmethylated strand.
Helicase II	DNA, MutL	Unwinds the DNA prior to excision.
Exonuclease I & exonuclease X	DNA	Performs 3'→ 5' excision.
Exonuclease VII and RecJ	DNA	Performs 5'→ 3' excision.
ssDNA binding protein	ssDNA	Protects ssDNA from degradation.
β-clamp	MutL, MutS, DNA polymerase III*	Processivity subunit of the DNA polymerase III holoenzyme.
DNA polymerase III*	β-clamp, MutL	Polymerizes the segment of DNA that has been excised.
Ligase	DNA	Repairs the nick left in the DNA backbone.

Table 1: List of proteins involved in methyl-directed mismatch repair and the role they play in the system.

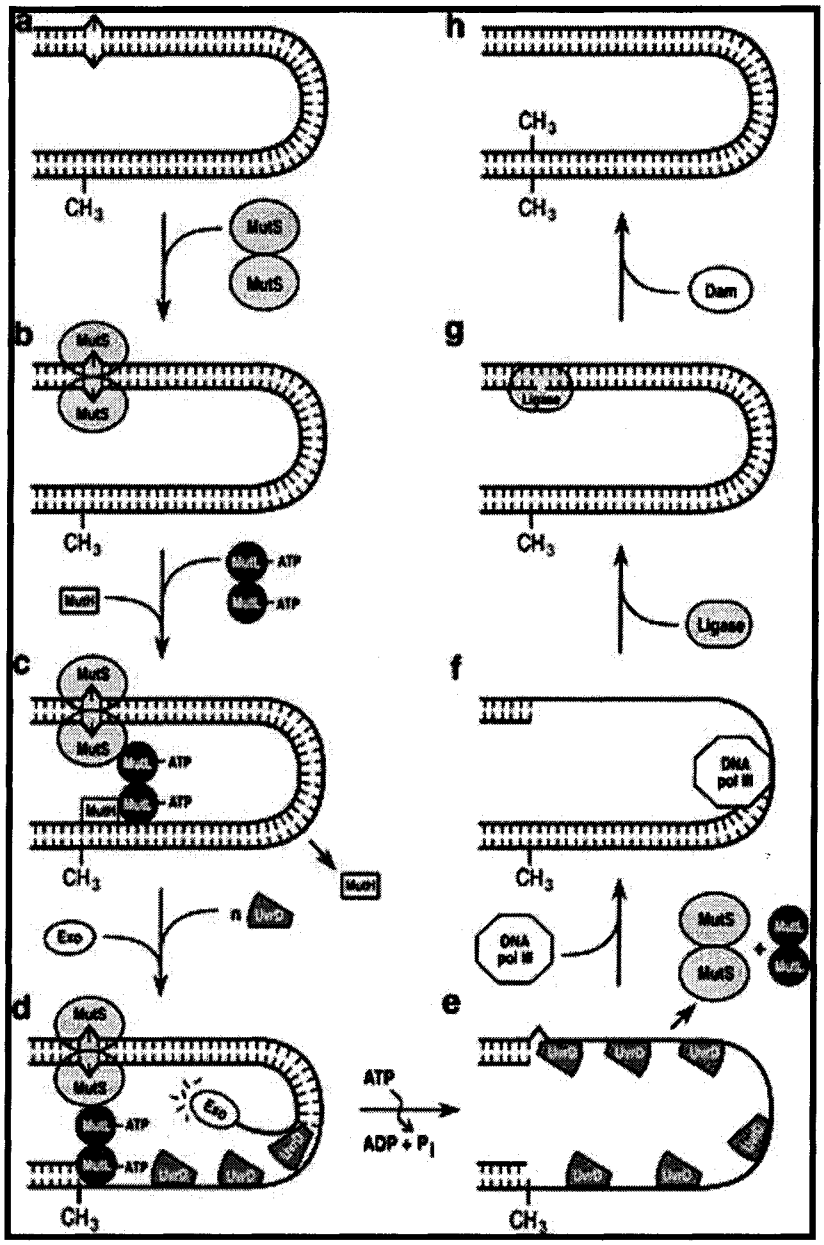


Figure 1: MMR repair model (Figure taken from Robertson *et al.*, 2006). The mispair is recognized and bound by a MutS homodimer. Dimeric MutL, with ATP bound, mediates communication between MutS that is bound at the mismatch and MutH that is bound at the nearest hemimethylated d(GATC) site. MMR is bidirectional, but only one direction is shown in this model. Once the DNA is nicked MutL loads multiple molecules of UvrD to unwind the damaged strand, which is then degraded by one of four exonucleases with an appropriate polarity. Removal of the damaged strand extends past the error, and presumably, MutL and MutS are displaced by an unknown mechanism. ssDNA binding proteins coat the parental strand to protect it from degradation. DNA polymerase III resynthesizes the missing DNA, the resulting nick is sealed by DNA ligase and Dam methylase ultimately methylates the d(GATC) on the repaired DNA strand.

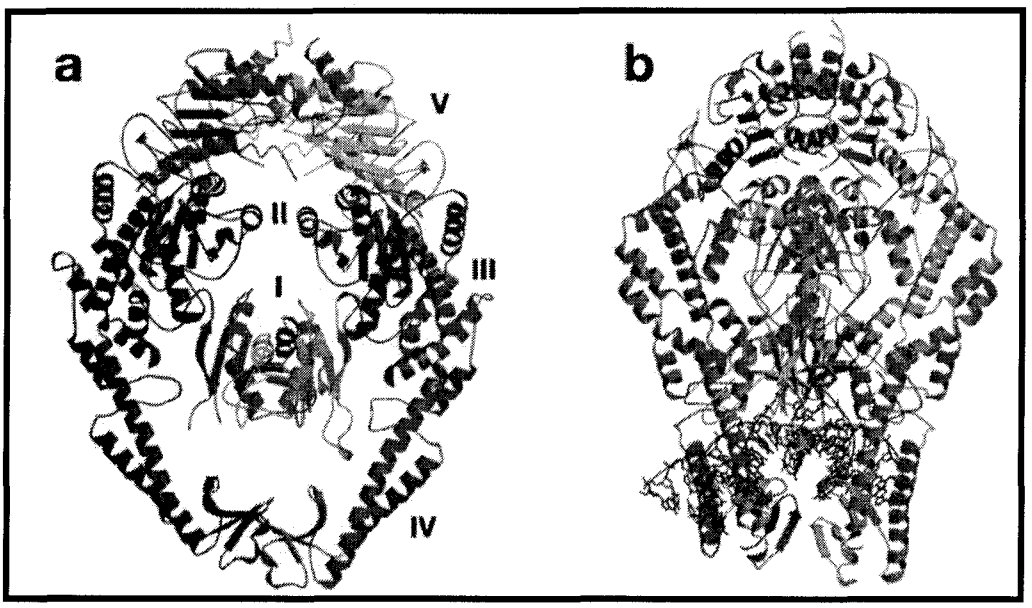


Figure 2: Crystal structure of MutS. Figure taken from Kunkel *et al.*, 2005. **A.** MutS is a homodimer and each subunit has five domains. **B.** MutS binds asymmetrically to duplex DNA with each subunit making numerous but different contacts to the DNA. The lower channel contains mismatched DNA that is kinked by 60◦.

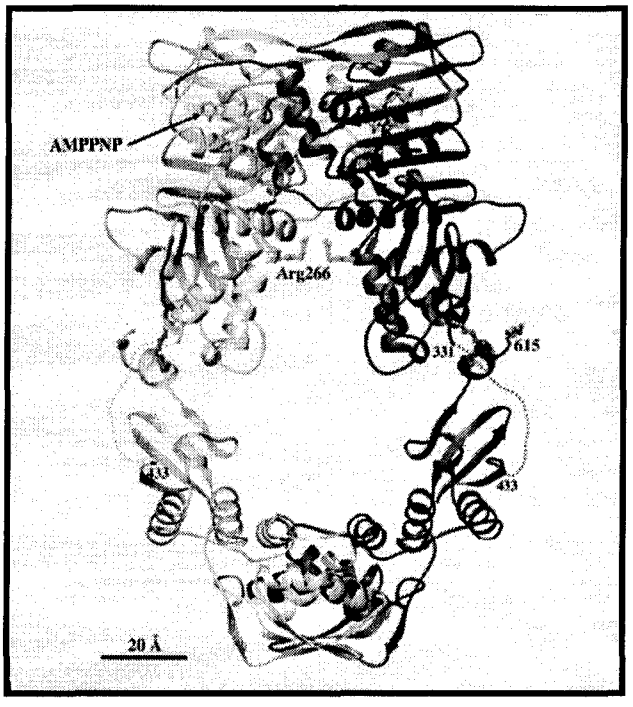


Figure 3: One postulated crystal structure of MutL. Figure taken from Guarne *et al.*, 2004. MutL contains a C-terminal dimerization region and an N-terminal ATPase region that has DNA-binding activity.

The ATPase domains also have a corresponding asymmetry; only the mismatch-binding subunit binds to ADP in the crystal structure, while the other ATPase site remains vacant. Even though it is recognized that the ATP binding and hydrolysis functions of MutS are crucial for protein activity (Wu *et al.* 1994), it is still not clearly understood exactly how ATP modulates its effect (Kunkel *et al.* 2005). However, what is known is that MutS recognizes damaged DNA and consequently initiates the downstream process of mismatch repair (Su *et al.* 1986 and 1988).

The following step in the cascade of events to repair the lesion is directed by MutH, a single stranded endonuclease protein with an extremely weak activity (Ban *et al.* 1998a and Hall *et al.* 1999) (Figure 1). It is responsible for the recognition of the hemimethylated GATC site and for the cleavage of the DNA 5' of the adenine on the unmethylated strand (Langle-Rouault *et al.* 1987 and Welsh *et al.* 1987). However, for MutH to do its job, it must be part of the quaternary complex (Allen *et al.* 1997 and Grilley *et al.* 1989). The MutL protein, in the presence of ATP, will stimulate MutH to nick the target DNA (Ban *et al.* 1998a and Hall *et al.* 1999). This stimulatory effect of MutL on MutH makes perfect sense, since the presence of an endonuclease capable of cutting all hemimethylated GATC sites in an indiscriminate manner would definitely not be desirable.

Once MutH has incised the DNA, helicase II (UvrD), with the assistance of MutL, unwinds the DNA so excision of the tract can proceed from the nick to

shortly past the mispair (Matson, 1986; Modrich, 1989; Runyon, 1990; Hall *et al.* 1998; and Yamaguchi *et al.* 1998) (Figure 1). Amazingly, this system is capable of bidirectional repair, a function provided by the exonucleases. Excision in the 5'→3' direction is performed by either the exonuclease VII or the RecJ exonuclease (Chase *et al.* 1974 and Lovett *et al.* 1989). As for the 3'→5' excision, it is performed by either exonuclease I or X (Lahue, 1989). Finally, after the DNA has been excised, the DNA polymerase III holoenzyme resynthesizes the gap and DNA ligase seals the nick (Lahue *et al.* 1989).

Of predominant interest, is the elusive *E. coli* MutL protein (Figure 3). Crystallographic and biochemical studies have shown that MutL contains a C-terminal dimerization region and an N-terminal ATPase region that has DNA-binding activity (Ban and Yang, 1998; Ban *et al*, 1999). Since the linker that connects the two domains has not yet been crystallized, the model shown in the figure is only a hypothesis. An alternative model was proposed by Kosinski *et al.* (2005). The ATPase region is conserved among all MutL homologs and was found to be part of the GHKL (Gyrase, HSP90, Histidine Kinase and MutL) ATPase/kinase super-family (Ban *et al.* 1998b and 1999 and Dutta *et al.* 2000). What these proteins all have in common is an unconventional Bergerat ATP binding motif (Dutta *et al.* 2000). Other than ATP hydrolysis (Ban and Yang, 1998b) and ssDNA binding capabilities (Grilley *et al.* 1989), no other measurable activity was found for MutL. It is theorized that MutL actually functions as a molecular co-ordinator and regulator in the MMR pathway (Grilley *et al.* 1989 and

Ban *et al.* 1999). Several studies have now shown that MutL does indeed assist other proteins to carry out their reactions.

The presence of MutL is observed to increase the efficiency of MutS binding to heteroduplex DNA (Allen *et al.* 1997, Drotschmann *et al.* 1998 and Selmane *et al.* 2003). It is also responsible for activating the otherwise extremely weak endonuclease activity of MutH (Ban *et al.* 1998 and Hall *et al.* 1999). In addition, MutL is observed to bind to ssDNA and to help load helicase II onto the nick of the DNA substrate, consequently stimulating the rate of initiation of unwinding (Mechanic *et al.* 2000 and Yamaguchi *et al.* 1998). Both MutL and MutS are further found to interact with multiple subunits of the β-clamp (Lopez de Saro *et al.* 2006; Li *et al.* 2008); the β-clamp is the processivity subunit of the *E. coli* DNA polymerase III holoenzyme. It is interesting to note that the β-clamp interacts with MutL only in the presence of ssDNA (Lopez de Saro *et al.* 2006). The loading of the clamp consequently allows targeting of the DNA polymerase III holoenzyme to the ssDNA tract that has been created. Lastly, MutL was also found to increase the endonuclease activity (Monastiriakos *et al.*, 2004) and the DNA binding capability (Drotschmann *et al.* 1998) of the Vsr protein. Interestingly these proteins can all function independently, albeit some extremely weakly, but all are enhanced by the presence of MutL.

Even though the various components of the mismatch repair system are known, it is still not yet completely understood how the system actually recognises the various mispairs it corrects. It is not known exactly how the repair

complex assembles after error recognition has occurred, how the complex gets to the hemimethylated site, how the helicase and exonucleases are loaded on, what determines where the exonucleases terminate activity nor how and when the complex interacts with the β-clamp.

1.3 Very short patch repair.

In *E. coli K-12*, the Dcm methylase enzyme is responsible for the methylation of the second cytosine in a CCWGG (W = A or T) DNA sequence context (Schlagman *et al.* 1976 and Bhagwat *et al.* 1986) (Figure 4). These 5-methylcytosines are especially unstable and have been found to be up to 21 times more susceptible to spontaneous hydrolytic deamination than their cytosine counterparts (Coulondre *et al.* 1978). When deamination of one of these modified bases occurs, the result is a thymine (Figure 4). Consequently, this event produces a T/G mismatch that can lead to a C→T transition mutation. The function of VSP repair system is the recognition of these T/G mismatches and their correction back to C/G (Glasner *et al.* 1995 and Lieb *et al.* 1995) (Figure 5).

The VSP repair system is initiated by the Vsr endonuclease, which cleaves the DNA at the T/G mismatch and results in a single stranded nick 5' of the T (Figure 5). Subsequently, DNA polymerase I removes and replaces a very short stretch of the DNA 3' of the nick so the DNA ligase can then complete the repair (Lieb and Bhagwat, 1996). Interestingly, even though Vsr is required to avoid

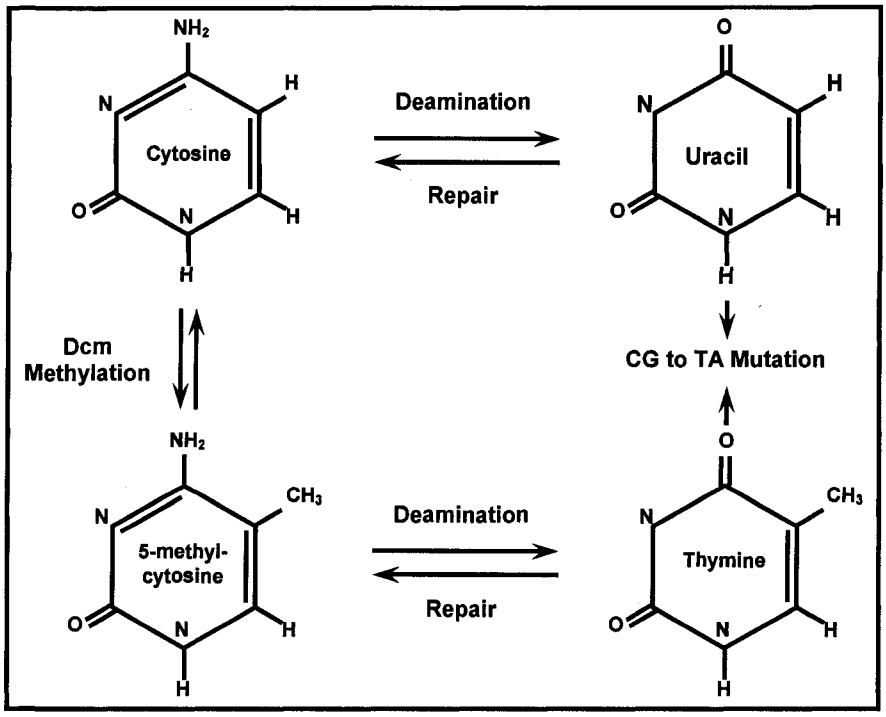


Figure 4: Schematic illustrating, at the molecular level, a C being methylated by Dcm followed by its spontaneous deamination. The result of this event is the production of a T, which consequently yields a C -> T transition mutation. Also shown is the deamination of a C, which results in a U.

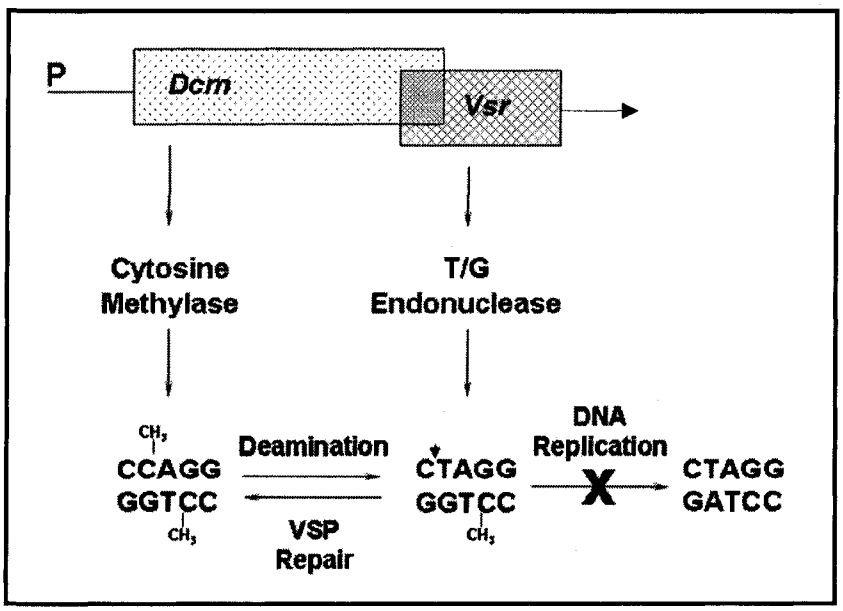


Figure 5: Chromosomal arrangement of *dcm/vsr* and VSP repair correction schematic. VSP repair of T/G mismatches resulting from 5-methylcytosine deamination events.

mutations due to 5-methylcytosine deamination, it was observed that Vsr over expression stimulates transition and frame shift mutations throughout the genome (Doiron *et al.* 1996 and Macintyre *et al.* 1997).

The gene for the Vsr endonuclease has an unusual chromosomal arrangement with the gene for the Dcm methylase (Sohail *et al.* 1990). The 5' end of the *vsr* gene overlaps by 20 base pairs the 3' end of *dcm* in a +1 reading frame (Sohail *et al.* 1990) (Figure 5). These two genes share a single promoter, 5' of *dcm,* and are thought to be co-transcribed as a single mRNA (Dar and Bhagwat 1993). Since both genes are expressed from the same promoter, one would intuitively think that their cellular levels would be identical. It is interesting to observe that the levels of Dcm are constant throughout the growth phase while those of Vsr show growth phase dependency (Macintyre *et al.* 1999). The endonuclease disappears rapidly upon dilution in fresh growth media and remains undetectable until late in log phase where it progressively appears (Macintyre *et al.* 1999). Even when the operon is placed under the control of the synthetic, high expression *trc* promoter, the pattern of protein expression remains identical (Macintyre *et al.* 1999). Furthermore, it could be thought that it is the unusual genetic arrangement of the operon that may lead to a decrease in the efficiency of *vsr* translation. However, when *vsr* alone is also put under the control of the synthetic *trc* promoter, the expression pattern stays identical (Macintyre *et al.* 1999). The growth phase-dependent expression of Vsr, therefore, seems independent of the *dcm* promoter as well as the operon structure; consequently, the regulation is post-transcriptional or post-translational

(Macintyre *et al.* 1999). However, the rate at which Vsr disappears from the cells upon subculturing suggests active protein degradation and that Vsr might be targeted for proteolysis during log phase (Macintyre *et al.* 1999).

Uncoupling the expression of Vsr from that of Dcm consequently permits the monitoring of Vsr effects. Studies show that high levels of Vsr increase the competition between VSP and MMR for T/G mispairs in a CT/GAGG context (Doiron *et al.* 1996 and Macintyre *et al.* 1997) (Figure 6). As observed in a post DNA replication context, a T/G replication error (of a TA base pair) is "corrected" to a C/G by VSP, instead of being repaired back to its original T/A by MMR.

The competition between both systems may consequently explain the over representation of the d(CCWGG) pentamer as compared to the d(CTWGG) in *E. coli* (Bhagwat *et al.* 1992 and Merkle *et al.* 1992). Interestingly, the results from studies on the over expression of Vsr also show that Vsr-stimulated mutagenesis is not limited to T/G mismatches in a restricted sequence context (Doiron *et al.* 1996, and Macintyre *et al.* 1997). Over expressing Vsr actually resulted in elevated levels of transition and frame shift mutations (Doiron *et al.* 1996, and Macintyre *et al.* 1997). The spectrum and magnitude of the mutations produced by this over expression of Vsr (Doiron *et al.* 1996 and Macintyre *et al.* 1997) is similar to that produced by MMR deficient strains (Cupples *et al.* 1989 and 1990).

Therefore, it is possible that excess Vsr causes mutations by interfering with the MMR system. Macintyre *et al.* (1997) examined this possibility and observed that the levels of Vsr stimulated mutations are decreased upon the

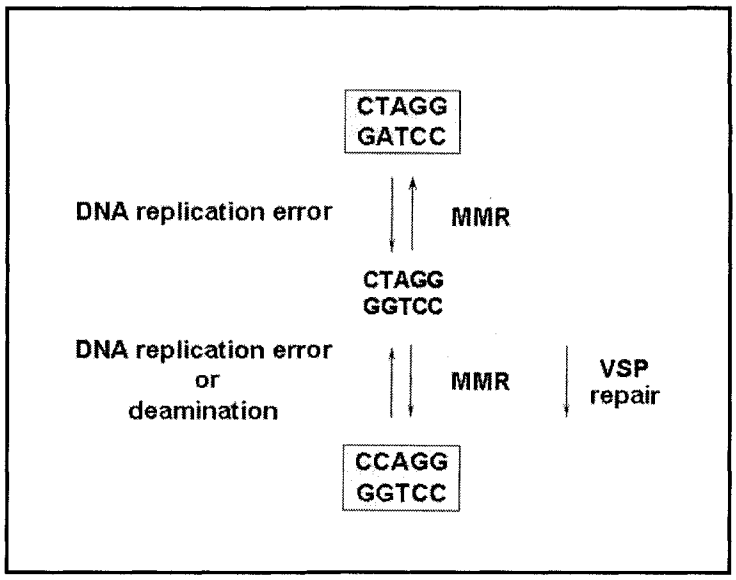


Figure 6: Competition between VSP and MMR for T/G mispairs in a CCWGG context. Two types of replication errors can give a T/G mismatch, both of which can be repaired by MMR. However, deamination of a 5-methylcytosine can also result in a T/G mispair. By adding VSP into the equation, the potential for problems can be seen immediately. In the case of the top parental duplex, MMR will repair it appropriately, but VSP repair will cause a transition mutation. As for the bottom parental duplex, both systems can correct the mutation, regardless of whether the mispair is from replication or deamination.

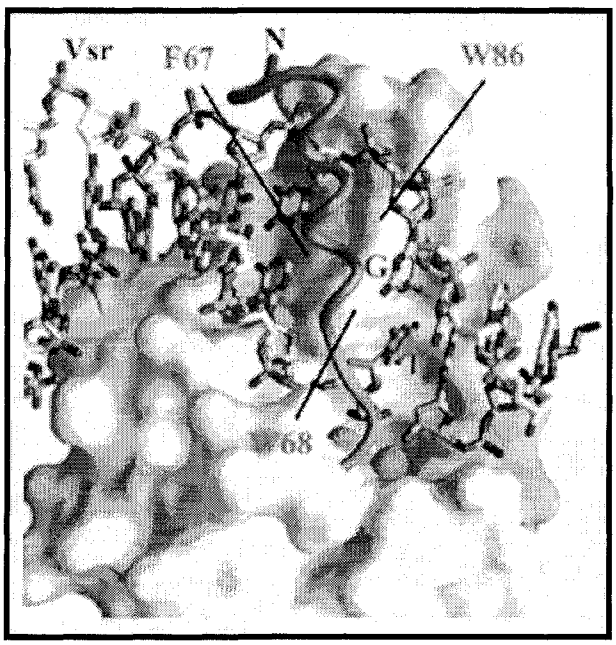


Figure 7: Crystal structure of the Vsr endonuclease with DNA heteroduplex. Amino-terminal residues 1-20 are in wire form and the three aromatic residues that intercalate the DNA can be seen. Figure taken from Tsutakawa *et al.*, 1999a.

addition of extra MutH or MutL proteins. However, when extra MutS is added, the mutation levels increase (Macintyre *et al.* 1997). It was further observed that VSP repair is partially defective in strains that are *mutS*$^-$ or *mutL*$^-$ (Bell and Cupples, 2001), although not completely defective, as was originally thought (Lieb 1987; Jones *et al.* 1987; and Zell *et al.* 1987).

To understand whether the Vsr stimulated mutagenesis has any dependency upon VSP repair activity, two mutant proteins were studied, H69A and H71A. These two proteins are defective in VSP repair (Monastiriakos *et al.* 2004) due to a lack of DNA binding and cleaving ability *in vitro* and *in vivo* (Drotschmann K. unpublished and Tsutakawa *et al.* 1999). With the structure of Vsr available, it is now possible to see that both mutated histidine residues are involved in the co-ordination of essential metal cations: His-69 with magnesium and His-71 with zinc (Tsutakawa *et al.* 1999a and 1999b) (Figure 7). If VSP repair is disabled and mutagenesis is still observed, this would suggest that Vsr is causing mutations by interacting with other proteins and not with the DNA. When examined for potential mutagenic activity these proteins showed the same spectrum and intensity of mutations as the wild type (Doiron 1998 and Monastiriakos *et al.* 2004). It has been shown that these Vsr mutants are being expressed (Monastiriakos *et al.* 2004) so one can make the assumption that the structure of these proteins is still similar enough to the wild type so as to stimulate mutagenesis. Results imply that the DNA binding and endonuclease capabilities of Vsr are not required for the expression of a mutagenic phenotype

of Vsr. They further suggest that the inhibition of repair may be due to the interaction between Vsr and one or more of the MMR proteins.

The crystal structure of Vsr, when bound to its target C(T/G)AGG substrate, further reveals that the DNA is actually held by a pincer-like structure. This structure is composed of three aromatic residues that intercalate into the major groove and of the amino-terminal alpha helix that lies across the minor groove (Tsutakawa *et al.* 1999a and 1999b) (Figure 7). In contrast to that of the wild type protein, deletion of the first 14 amino acids of this α-helix reveals, *in vivo,* that the mutant protein is still rather efficient in mediating very short patch repair but shows a considerable decrease in its mutagenic effect (Monastiriakos *et al.* 2004). However, *in vitro,* the endonuclease activity of this mutant is significantly reduced and cannot be stimulated by MutL (Monastiriakos *et al.* 2004). This suggests that the amino-terminus is required for ¨strong¨ binding to DNA, but not for target recognition, and appears important for the completion of repair. It also suggests that this structure plays a role in the relationship between Vsr and MutL.

1.4 Role of MutL ATPase function in relation to Vsr

Various lines of evidence point to the possibility that an interaction between the VSP repair and MMR repair systems does exist, more specifically between Vsr and MutL. As discussed earlier, MutL is capable of stimulating the

endonuclease and DNA binding activity of Vsr, thereby making it a good prospective candidate (Drotschmann *et al.* 1998 and Monasteriakos *et al.* 2004). We suggest that the functional interaction between Vsr and MutL causes a conformational change in the amino-terminus of Vsr, which consequently enhances Vsr activity, but also causes a decrease in the ability of MutL to carry out mismatch repair. We propose that the cause for MutL inactivation may reside in the hydrolysis of its ATP, which results in an internal conformational change. The crystal structure of the amino-terminus of MutL (LN40 fragment, only a. a. 1-349) shows that the binding of an ATP molecule causes significant reordering within the protein, especially around the ATP binding site (Ban *et al.* 1998 and 1999) (Figure 8). When ATP binds to MutL, the two domains come closer together and the disordered regions slowly reorder themselves (Ban *et al.* 1998 and 1999). This slow, rate-limiting conformational change throughout the protein, consequently allows MutL to dimerize (Ban *et al.* 1998 and 1999).

Using MutH as a model, it is observed that MutL and ATP are required to activate the endonuclease and as a consequence of this reaction, ATP is ultimately hydrolysed (Ban *et al.* 1998 and 1999). The ATP hydrolysis therefore results in MutL returning to its disordered state. Our premise is that the interaction of MutL with Vsr has parallels with the interaction of MutL with MutH. An interaction between MutL and Vsr may result in MutL hydrolysing its ATP and therefore becoming disorganised. This event would consequently render MutL incapable of interacting with the other Mut proteins and therefore, of participating in MMR for a significant period of time.

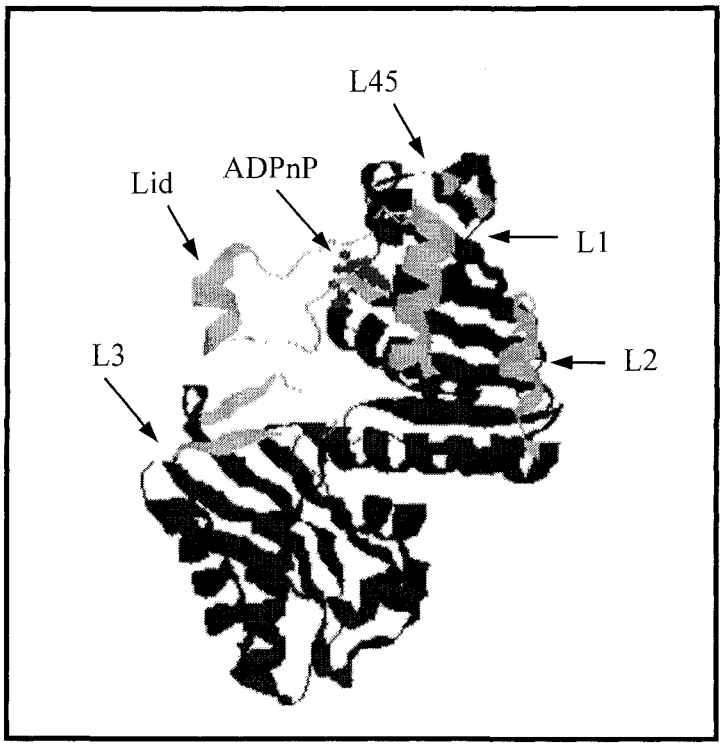


Figure 8: Rasmol rendition of the MutL amino-terminal with ADPnP. Areas subject to conformational reorganization are coloured pale grey and labelled L1-L5 and lid (part of the ATP binding motif).

1.5 Hypothesis

Our hypothesis to explain Vsr mutagenesis was that over expression of Vsr causes mutagenesis indirectly by disabling MMR. One attractive possibility is that excess Vsr inactivates MMR by sequestering or incapacitating MutL. Consequently, the objectives of this project were:

1. To study the interaction between the proteins of the VSP and MMR repair systems, using both the yeast and bacterial two and three-hybrid systems.
2. To study the influence of MutL ATP binding and hydrolysis on the stimulation of Vsr activity, using an *in vitro* endonuclease assay.

2.0 Materials and Methods

2.1 Yeast two-hybrid assay

2.1.1 Description of the system

The yeast two-hybrid system, first described by Fields and Song (1989), is an ingenious *in vivo* protein-protein interaction test system. It is based on the modular nature of the *Saccharomyces cerevisiae* GAL4 transcriptional activator, which contains separate DNA binding and activation domains (Keegan *et al.* 1986). The DNA binding domain (amino acids 1-147) functions to localise the transcription factor to specific nucleotide sequences present in the upstream regions of the genes that it regulates. The function of the activation domain (amino acids 768-881) on the other hand, is to come into contact with other components of the transcription machinery in order to initiate transcription.

These two domains can be separated and used to create chimeric proteins consisting of the DNA binding domain, or the activation domain, fused to putatively interacting proteins (Figure 9). If the two test proteins interact, they will allow the reconstitution of a functional GAL4 within *S. cerevisiae*. This complex will consequently be capable of activating a *lacZ* reporter gene whose regulatory region contains GAL4 binding sites, the result being the identification of the interacting pair. Consequently, this system has enabled us to examine interactions between various combinations of Vsr and the MMR proteins.

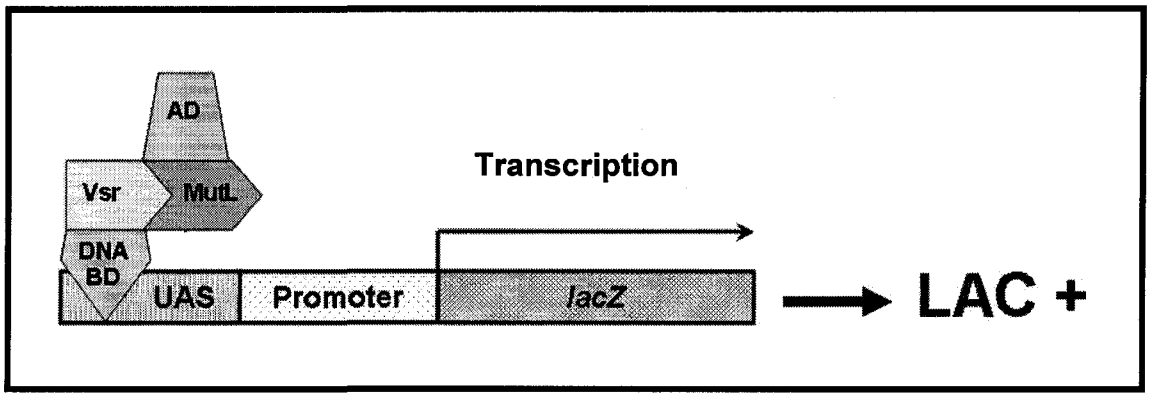


Figure 9: Yeast two-hybrid system schematic (based on Fields and Song 1989). The *S. cerevisiae* Gal4 transcriptional activator is separated into its DNA binding and activation domains. These two domains can be used to create chimeric proteins consisting of one of the Gal4 domains fused to one of a putatively interacting protein pair. If test proteins interact, they allow the reconstitution of a functional GAL4 molecule. This complex will activate a *lacZ* reporter gene whose regulatory region contains GAL4 binding sites, resulting in the identification of the interacting pair.

A clever variation on the yeast two-hybrid system, the yeast three-hybrid system, actually permits the investigation of tertiary protein complexes. In this system, the pGBT9 vector is replaced by the pBridge vector, which harbours two distinct multiple cloning sites. The first of these MCS is identical to that of the pGBT9 vector and allows the expression of the DNA-BD fusion protein. However, the ingenuity of this system resides in the second distinct MCS. This second site actually allows for the independent expression of another protein under the control of a conditional methionine promoter. The use of this promoter allows expression to be switched on or off by a simple replica plating step. Hence, for the protein to be expressed, methionine must be absent from the media.

As a result, the three-hybrid system permits the investigation of more complex protein interactions than would ever be possible with the standard two-hybrid system. The new player to the system can therefore participate in the interaction between the two fusion proteins in several ways (Figure 10). It can:

- Act as a bridge.
- Modify one or both of the pairing proteins.
- Act as a competitor and inhibit the two-hybrid interaction.

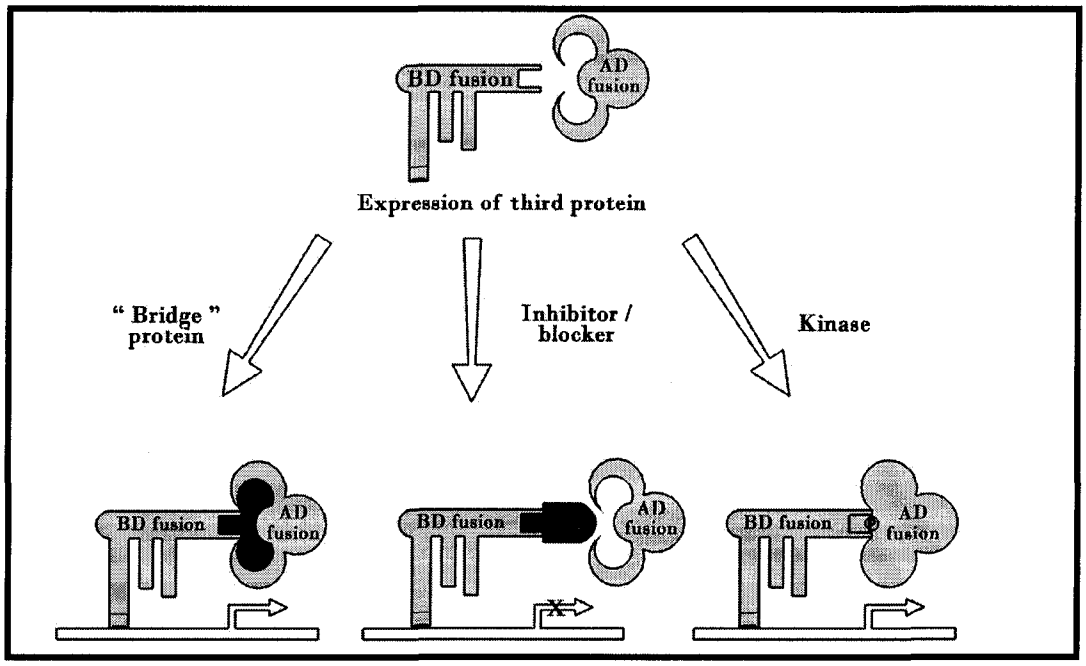


Figure 10: Yeast three-hybrid system schematic (*Clontech*). The incorporation of a third protein to the system allows studying how this new protein interacts with the two-hybrid pair, as a bridge, an inhibitor or a modifier.

By using the pBridge vector we were thus able to look at:

- The competition between the Vsr and MutH for MutL.
- The possible tertiary complex of MutS-MutL-MutH.

2.1.2 Strains, plasmids and oligos

***E. coli* strain**	
P90C (CSH162)	*ara, Δ(gpt-lac)5, thi, pro, F⁻*
LJ2809	*fruR11::Tn10, xyl-7, ΔcyaA854, ΔargH1*
***E. coli* plasmids**	
pH69A	pMAL-c with *vsr* inserted as a StuI-EcoRI fragment. Changed codon 69 from H to A. Amp^r. Gift from K. Drotschmann.
pH71A	pMAL-c with *vsr* inserted as a StuI-EcoRI fragment. Changed codon 71 from H to A. Amp^r. Gift from K. Drotschmann.
***S. cerevisiae* strains**	
Y153	MATa, URA3-52, LEU2-3, HIS3-200, ADE2-101, TRP1-901, ΔGAL4, ΔGAL80, GAL1::HIS3, GAL1::LACZ
***S. cerevisiae* plasmids**	
pGBT9	Amp^r,TRP1, GAL4 DNA-binding domain with MCS. *Clontech.*
pB-mutH	Derived from pGBT9 with *mutH.* Gift from S. Matson.
pB-mutL	Derived from pGBT9 with *mutL.*
pB-mutS	Derived from pGBT9 with *mutS.*
pB-vsr	Derived from pGBT9 with *vsr.*
pB-v-H69A	Derived from pGBT9 with *vsr-H69A.*
pB-v-H71A	Derived from pGBT9 with *vsr-H71* A.
pGAD424	Amp^r, LEU2, GAL4 activation domain. *Clontech.*
pA-mutH	Derived from pGAD424 with *mutH.* Gift from S. Matson.
pA-mutL	Derived from pGAD424 with *mutL.*
pA-LN-293	Derived from pGAD424 with *mutL.* Gift from S. Matson.
pA-LN-344	Derived from pGAD424 with *mutL.* Gift from S. Matson.
pA-LN-397	Derived from pGAD424 with *mutL.* Gift from S. Matson.
pA-LN-438	Derived from pGAD424 with *mutL.* Gift from S. Matson.
pA-LC-59	Derived from pGAD424 with *mutL.* Gift from S. Matson.
pA-mutS	Derived from pGAD424 with *mutS.*
pA-vsr	Derived from pGAD424 with *vsr.*
pA-v-H69A	Derived from pGAD424 with *vsr-H69A.*
pA-v-H71A	Derived from pGAD424 with *vsr-H71* A.
pBridge	Amp^r, TRP1, GAL4 DNA-binding domain with MCS, second MCS under the control of the MET25 promoter. *Clontech.*
pR vsr	Derived from pBridge with *vsr.*
pR vsr/mutH	Derived from pBridge with *vsr* and *mutH.*
pR mutH	Derived from pBridge with *mutH.*
pR mutH/vsr	Derived from pBridge with *mutH* and *vsr.*

Table 2: List of strains and plasmids.

Oligo #	Sequence	CS created
mutS: pB-mutS, pA-mutS		
147	GCAATAGAATTCTTCGACGCC	*Eco*RI
148	CGGGAATTCTTATTACACCAG	*Eco*RI
mutL: pB-mutL and pA-mutL		
149	CGATTGATGCGGATCCAGGTCTTA	*Bam*HI
150	AATCGCCTTCTGCAGGCTCGC	*Pst*I
mutH: pR mutH and pR mutH/vsr		
159	TATCATGAATTCTCCCAACCT	*Eco*RI
160	CCGGAAAGCTGCAGGTCAAAG	*Pst*I
mutH: pR vsr/mutH		
175	TAATCAAGGTATGCGGCCGCGTCCCAACCT	*Not*I
176	GGTCAAAGGGATCCCTACTGGATC	*Bam*HI
vsr: pB-vsr, pA-vsr, pB-vH69A, pA-vH71A, pR vsr and pR vsr/mutH		
151	AGGCACAACACTGCAGACGTTCAC	*Pst*I
152	GCGCCAGTTCTGCAGGACGCA	*Pst*I
vsr: pR mutH/vsr		
174	TGCGTCAGCAAGGCGGCCGCCATGGCCGAC	*Not*I
81	GCGGATCCGGACTGGGTGGAGAAACAC	*Bam*HI

Table 3: List of oligos used for the cloning of *vsr* and the mismatch repair genes into the yeast two-hybrid vectors.

Legend: Restriction cut sites introduced into the primers so as to allow in frame fusion of the mismatch repair genes into the yeast two-hybrid vectors.

2.1.3 Protocols

2.1.3.1 Vector construction

Note: All of the steps of vector construction for the yeast two-hybrid took place in *E. coli*. Only the assaying for β-galactosidase activity was performed in *S. cerevisiae*.

All of the plasmids used in the yeast assays are derivatives of pGAD424, pGBT9, and pBridge (*Clontech*). The genes to be studied were amplified by PCR (Expand long PCR kit; *Roche*) using primers that allowed for restriction sites on either end and maintained the reading frame of the fusions. Digestions, dephosphorylation and ligation of all DNA molecules were done according to the manufacturer's individual instructions. All enzymes were purchased from *Fermentas, NEB,* or *USB*. Agarose gel electrophoresis was done in 1 x TAE (based on Sambrook *et al.* 1989). DNA band extractions were done using the Geneclean kit (*Bio101*).

Genes of interest were fused 3' of either the DNA binding domain or the activation domain of the GAL4 transcriptional activator. (See table 2 and 3 for the list of all oligos used and plasmids created.) Note that Dr. S. Matson (Hall et al. 1998 and 1999) graciously provided the plasmids for mutH, as well as those for the mutL truncations.

Chemically competent *E. coli* cells were transformed with individual ligation reactions and plated on appropriate selective media (based on Sambrook

et al. 1989). Transformants were screened for the presence of recombinant clones. Plasmid DNA was extracted using an alkaline lysis method (based on Sambrook *et al.* 1989) or the *Qiagen* mini-prep kit.

2.1.3.2 *S. cerevisiae* transformation and assays

Yeast strain Y153 was co-transformed with a pGBT9 and a pGAD424 derived plasmid and plated on appropriate selective media (based on Schiestl *et al.* 1989). Transformants were then assayed for β-galactosidase activity in two different ways: first, using the quick and qualitative filter assay (based on Bartel *et al.* 1993); followed by the quantitative liquid assay (based on Greenwood *et al.* 1986). Liquid assays were done in duplicates of triplicate cultures and each assay was done at least twice.

2.2 Bacterial two-hybrid assay

2.2.1 Description of the system

Like the yeast two-hybrid, the bacterial two-hybrid is a genetic system used to study protein-protein interactions (Karimova *et al.* 1998). For this study however, it has the added advantage that protein interactions are observed in their native *E. coli* environment and not in a eukaryotic one. This system utilises the fact that the catalytic domain of the *Bordetella pertussis* adenylate cyclase can be separated into two complementary fragments: T25 (amino acids 1-224) and T18 (amino acids 225-399) (Karimova *et al.* 1998). Fusion proteins can be constructed consisting of the test proteins and either of the adenylate cyclase fragments. The interacting proteins therefore allow for the reconstitution of the adenylate cyclase catalytic domain within a strain that has a *cyaA*$^-$ background (LJ2809). This, in turn, allows the production of cAMP, the activation of the CAP binding protein and consequently the stimulation of the *lacZ* gene (Karimova *et al.* 1998) (Figure 11). Other sugar operons, such as maltose and arabinose, are also stimulated by the presence of cAMP; however, their effects are not as easily quantifiable.

In order to identify which part of Vsr interacts with MutL, we constructed five truncation fragments: two amino-terminal deletions VsrN14 and VsrN19 and three carboxy-terminal deletions Vsr-C124, Vsr-C112, and Vsr-C27 (Figure 12). The decision to construct the three carboxy terminal truncations was made on the basis of cloning facility. We made use of internal blunt end cleavage sites,

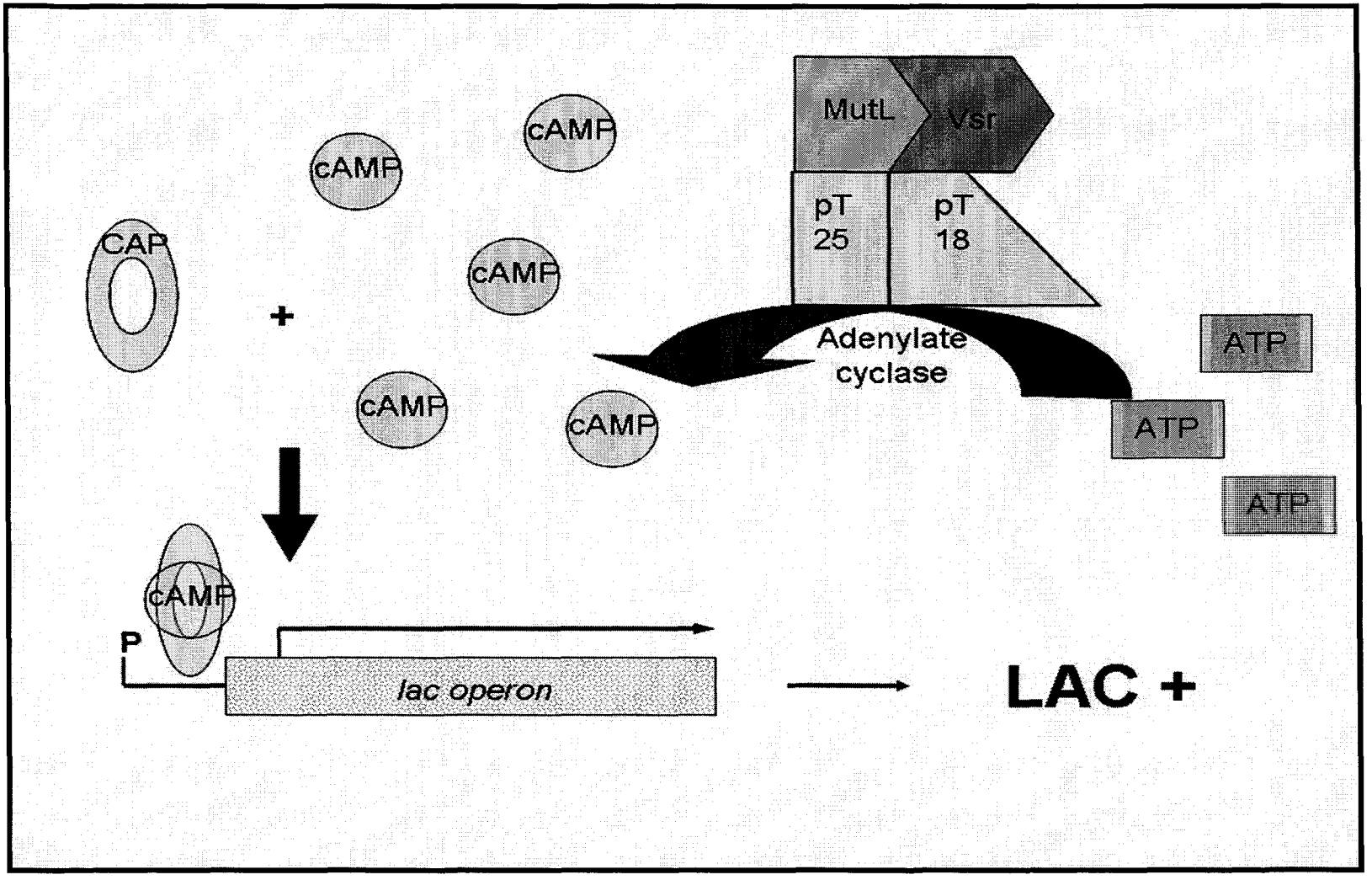


Figure 11: Bacterial two-hybrid system schematic.

which were in frame with an *Eco*RV site located within the multi-cloning area, between the genes for *vsr* and the T18 fragment.

The rationale for the construction of the amino-terminal deletion mutants was two fold: the structure of the amino-terminal domain (Tsutakawa *et al.* 1999b), and a study by Monastiriakos *et al.* (2004). The *in vitro* endonuclease experiments in that study demonstrated that removal of the amino-terminal α-helix produced truncated proteins capable of cutting a DNA heteroduplex, albeit less efficiently than the wild type protein (Monastiriakos *et al.* 2004). Additionally, the *in vivo* results showed: 1) Reduced levels of VSP repair for the VsrN14 and even more so for the VsrN19 truncation. 2) Decreased mutagenesis levels for the VsrN14 and even more so for the VsrN19 truncation (Monastiriakos *et al.* 2004). It was therefore of interest to see if these fragments could interact with MutL in the bacterial two-hybrid system.

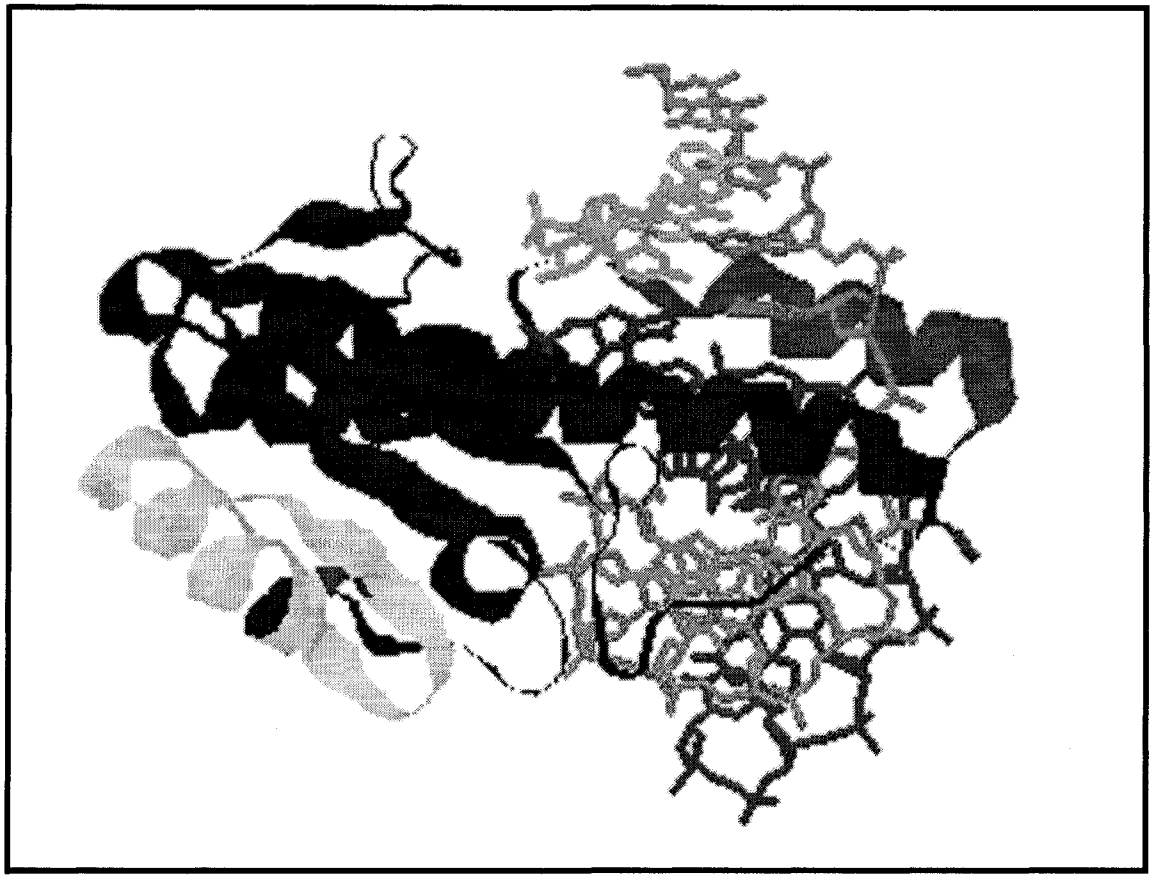


Figure 12: Crystal structure of the Vsr endonuclease with DNA heteroduplex. Amino-terminal residues 1-20 are labelled in medium grey, while carboxy-terminal residues 129-156 are in pale grey.

2.2.2 Strains and plasmids

Strain	
P90C	*ara, Δ(gpt-lac)5, thi, pro, F⁻*
LJ2809	*fruR*11::Tn10, *xylA*7, Δ*cyaA*854, Δ*argH*1
Plasmids	
pT18	Amp^r, contains the T18 fragment from *B. pertussis* with MCS. Gift from D. Ladant & Hybrigenics.
pT18-zip	Derived from pT18 with the gene for leucine zipper domain in the MCS. Positive control. Gift from D. Ladant & Hybrigenics.
pT18-mutH	Derived from pT18 with *mutH.* Made by C. Mansour.
pT18-mutL	Derived from pT18 with *mutL.* Made by C. Mansour.
pT18-mutS	Derived from pT18 with *mutS.* Made by C. Mansour.
pT18-vsr	Derived from pT18 with *vsr.* Made by C. Mansour.
pT18-vsr N14	Derived from pT18 with Δ*vsr N14.*
pT18-vsr N19	Derived from pT18 with Δ*vsr N19.*
pT18-vsr C124	Derived from pT18 with Δ*vsr C124.*
pT18-vsr C112	Derived from pT18 with Δ*vsr C112.*
pT18-vsr-C27	Derived from pT18 with Δ*vsr C27.*
pT25	Cm^r, contains the T25 fragment from *B. pertussis* with MCS. Gift from D. Ladant & Hybrigenics.
pT25-zip	Derived from pT25 with the gene for leucine zipper domain in the MCS. Positive control. Gift from D. Ladant & Hybrigenics.
pT25-mutH	Derived from pT25 with *mutH.* Made by C. Mansour.
pT25-mutL	Derived from pT25 with *mutL.* Made by C. Mansour.
pT25-mutL-N	Derived from pT25 with *mutL N* terminus
pT25-mutL-C	Derived from pT25 with *mutL C* terminus
pT25-mutS	Derived from pT25 with *mutS.* Made by C. Mansour.
pT25-vsr	Derived from pT25 with *vsr.* Made by C. Mansour.
pT18-mutH vsr	Derived from pT18 with *mutH* and *vsr* cloned into *BamHI.* Made by C. Mansour.
pT18-mutL vsr	Derived from pT18 with *mutL* and *vsr* cloned into *BamHI.* Made by C. Mansour.
pT18-mutS vsr	Derived from pT18 with *mutS* and *vsr* cloned into *BamHI.* Made by C. Mansour.

Table 4: list of all strains and plasmids used.

Oligo #	Sequence	CS created
***mutL*: pT25-mutL N (Δ C-265)**		
181	AACTAAGCCCGGGTGATGCC	*Sma*I
220	TTCCGGTACCGAACGCGGTGCAGG	*Kpn*I
***mutL*: pT25-mutL C (Δ N-350)**		
221	CCTGCCCGGGGTTCCATTCCGGTT	*Sma*I
182	TTAGGCAGGTACCCCTTACT	*Kpn*I
***vsr*: pT18-vsr ΔN14**		
218	CAGGGGCCCTATGCGCGCGATTGCC	*Apa*I
194	GGACGAAGCTTGCGAGTAAAT	*Hind*III
***vsr*: pT18-vsr ΔN19**		
219	GATGGGCCCGCGTGATACGGCGATA	*Apa*I
194	GGACGAAGCTTGCGAGTAAAT	*Hind*III

Table 5: List of oligos used for the cloning, of both *vsr* and *mutL* truncations, into the bacterial two-hybrid vectors.

Legend: Restriction cut sites introduced into the primers so as to allow in frame fusion of the mismatch repair genes into the yeast two-hybrid vectors.

Plasmid	Digestions performed
pT18-vsr ΔC124	Digested with *Hinc*II and *Eco*RV; religated blunt ends together. Removed the last 124 amino acids.
pT18-vsr ΔC112	Digested with *Bsh*1365I and *Eco*RV; religated blunt ends together. Removed the last 112 amino acids.
pT18-vsr ΔC27	Digested with *Eco*47III and *Eco*RV; religated blunt ends together. Removed the last 27 amino acids.

Table 6: List of digestions performed on the pT18-vsr vector in order to produce truncation mutants.

2.2.3 Protocols

2.2.3.1 Vector construction and transformation

All the plasmids used were derivatives of pT18 and pT25. (Dr. D. Ladant and Hybrigenics graciously provided parental vectors, pZip positive controls and technique.) The construction of the bacterial two-hybrid plasmids was similar to that of the yeast two-hybrid ones; refer to sections 2.1 and 5.1. For the list of strains, plasmids and oligos used in the cloning, see tables 4, 5 and 6. *E. coli* strain LJ2809 was co-transformed with the pT18 and a pT25 derived plasmid.

2.2.3.2 β-galactosidase plate and liquid assays

Qualitative β-galactosidase assays were done on x-gal plates. Quantitative liquid assays were done as triplicates of triplicate cultures and each was done at least twice.

2.3 Protein purification

2.3.1 Description of the system

In vitro assays allow us to remove individual proteins from their native environments and study them in a very controlled milieu where one variable can be altered at a time, and the effects monitored. These techniques allow the possibility of understanding subtle details of protein function while being aware that what occurs in a test tube may sometimes differ *in vivo*.

As discussed earlier, *E. coli* normally produces the Vsr endonuclease in very small amounts. To get around this problem, the *vsr* gene is cloned into the pET15b vector (*Novagen*) in frame with an amino terminus hexa-histidine tag and under the control of an inducible T7 promoter. The plasmid is then transformed into BL21 (DE3) (*Novagen*). The strain carries the gene for the T7 RNA polymerase, which is under the control of the *lac* promoter. This consequently allows for the control of Vsr expression. Cells can therefore be grown and the polymerase induced with IPTG, ultimately resulting in the production of a hexa-histidine tagged Vsr.

The protein is purified from cell extracts by using a cobalt affinity resin (*Clontech*) that binds the histidine tag. The column is then washed to remove unbound protein and subsequently, Vsr is eluted with buffer containing imidazole. This tag, which allows for easy protein purification, can be removed by thrombin cleavage. However, this step is not necessary since the protein is fully active

even with the tag is present (Monastiriakos *et al.* 2004). Note that wild type and mutant MutL were purified in a similar manner.

The instability of the purified Vsr protein has been noted in several instances (Turner *et al.* 2000, Gonzalez-Nicieza *et al.* 2001, Monastiriakos 2002 and Elliott *et al.* 2005). During purification, Vsr was observed to aggregate, lose its amino terminus and lose activity upon long-term storage. It was therefore essential to have a good purification protocol that reduces aggregation, and thereby increases the final yield as well as the stability of the protein.

2.3.2 Strains and plasmids

Strain	
BL21 DE3	F^- *dcm/vsr ompT hsdS*(r_B^- m_B^-) *gal* λ(DE3). *Novagen.*
Plasmids	
pH69A	pMAL-c with *vsr* inserted as a *StuI-EcoR*I fragment. Changed codon 69 from H to A. Amp^r. Gift from K. Drotschmann.
pH71A	pMAL-c with *vsr* inserted as a *StuI-EcoR*I fragment. Changed codon 71 from H to A. Amp^r. Gift from K. Drotschmann.
pET 15b	Amp^r, *lacI*^{q}. Vector contains: N-terminal 6-His•Tag sequence, thrombin cleavage site and MCS. Transcription controlled by T7 RNA polymerase and inducible by IPTG. *Novagen.*
pET vsr	Derived from pET15b with *vsr*; made by G. Macintyre.
pET mutL	Derived from pET15b with *mutL*; gift from M. Winkler.
pET mutL E29A	Derived from pET15b with *mutL E29A*; gift from P. Hsieh.
pET mutL E32K	Derived from pET15b with *mutL E32K*; gift from P. Hsieh.
pET mutL R95F	Derived from pET15b with *mutL R95F*; gift from W. Yang.
pET mutL R95F/N302A	Derived from pET15b with *mutL R95F/N302A*; gift from W. Yang.
pET mutL R266E	Derived from pET15b with mutL *R266E*; gift from P. Hsieh.
pET mutL N302A	Derived from pET15b with mutL *N302A*; gift from W. Yang.
pET mutL K307A	Derived from pET15b with mutL *K307A*; gift from P. Hsieh

Table 7: list of all strains and plasmids used.

2.3.3 Protocols

Vsr was purified and stored at pH 7.8 in 20 mM Hepes, 110 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT, 10 % glycerol and protease inhibitors instead of the previously published protocol (Cupples and Macintyre 1999). MutL was purified

in the same manner. All protein samples were resolved by 12.6 % SDS PAGE. Samples were also quantified by nano-spectrophotometry using extinction coefficients.

2.4 Trypsin digestions

2.4.1 Description of the system

When MutL binds ATP, it is known to change conformation (Ban *et al.* 1998 & 1999). Trypsin digestion was used as an indirect way to see if purified preparations of the MutL and MutL mutants were behaving as expected with regards to their nucleotide binding properties. MutL without ATP bound has a more disorganised conformation (Ban *et al.* 1998 & 1999) and is therefore more susceptible to trypsin digestion than MutL in the presence of ATP. Consequently, mutants that show a decrease or incapability to bind ATP will be more susceptible to digestion than the wild type protein in the presence of the nucleotide.

Mutated residue	Function of the residue
MutL E29A	This residue helps co-ordinate a crucial water molecule in the ATP binding site. It is thought that this residue is also essential for ATP hydrolysis. Proteins are proficient for ATP binding, but deficient for ATP hydrolysis (Selmane *et al.* 2003).
MutL E32K	This residue it not conserved, but plays a role in hydrolysis. Proteins proficient for ATP binding, but deficient for ATP hydrolysis (Selmane *et al.* 2003).
MutL R95F	This residue is located in the ATP lid and occupies the ATP pocket in the absence of nucleotide. The mutation from an arginine to phenylalanine at this site results in an increased affinity for the nucleotide but in a decreased ATPase activity (Ban *et al.* 1999).
MutL R95F/N302A	A combination of both the R95F and N302A mutants.
MutL R266E	Mutation in the middle of the groove largely abolishes the DNA-binding activity of the full-length MutL. Reduced DNA binding activity. Has ATP activity (Selmane *et al.* 2003).
MutL N302A	This residue is distant from the ATP binding site but is essential for dimerization. This mutant binds ATP well but hydrolyses it poorly (Ban *et al.* 1999).
MutL K307A	May play a role in stabilizing the γ-phosphate. Reduced ATP binding and hydrolysis (Ban *et al.* 1999).

Table 8: Description of the MutL mutants used.

2.4.2 Protocols

Trypsinolysis reaction: 30 μM of MutL protein, 0.15 μM trypsin in Hepes reaction buffer with or without 5 mM ATP. At 0, 1, 5, 10 and 20 min, 20 μl aliquots were removed. Each aliquot was stopped by adding 6 μl sample buffer and boiling immediately. Samples were run on a 12.6% SDS PAGE gel. Digestions patterns are then compared to analyze the rate of digestion in response to the various conditions.

2.5 Endonuclease assay

2.5.1 Description of the system

As discussed previously, the function of VSP repair is the correction of T/G mispairs arising from the deamination of 5-methylcytosines in a (CCWGG) context. Vsr nicks DNA 5' of the T and provides a site for polymerase I to remove and replace a short stretch of DNA 3' of the nick. The *in vitro* assay used is a useful tool that allows direct monitoring of the Vsr endonucleolytic activity on heteroduplex DNA containing a (C(T/G)WGG) sequence. As compared to *in vivo* assays that measure repair (or lack thereof); this assay permits the measurement of the endonuclease function, separate from downstream events. The simplicity of this assay lies in the fact that Vsr requires no accessory proteins or cofactors, permitting the direct monitor of the endonucleolytic capabilities of Vsr. It also permits measurement of the effect of MutL and MutL mutants on Vsr.

2.5.2 DNA oligomers

Li-cor labelled oligos:	
Hetero	5' GGTCGCTGGGGAAC[A/T]AGGGTGGCCACGGCG 3'
Homo	5' GGTCGCTGGGGAACCAGGGTGGCCACGGCG 3'
Unlabelled oligos:	
Hetero cold	5' GGTCGCTGGGGAAC[A/T]AGGGTGGCCACGGCG 3'
Hetero complementary	5' CCAGCGACCCCTT[A/T]GTCCCACCGGTGCCGC 3'
Homo cold	5' GGTCGCTGGGGAACCAGGGTGGCCACGGCG 3'

Table 9: List of oligonucleotides used in the endonuclease assay.

Legend:

- 5' end labelled with IR_{700} dye for Li-cor detection.
- Target cc(a/t)gg site.
- [redacted]

2.5.3 DNA substrates used:

- **T/G heteroduplex labelled**

```
*5' GGTCGCTGGGGAAC AGGGTGGCCACGGCG 3'
3' CCAGCGACCCCTTGGTCCCACCGGTGCCGC 5'
```

- **T/A homoduplex labelled**

```
*5' GGTCGCTGGGGAACCAGGGTGGCCACGGCG 3'
3' CCAGCGACCCCTTGGTCCCACCGGTGCCGC 5'
```

2.5.4 Protocols

An IR_{700} labelled oligonucleotide containing the 5'-CTAGG-3' sequence was annealed to an unlabelled oligonuleotide containing either a 3'-GGTCC-5' (heteroduplex) or 3'-GATCC-5' (homoduplex control) sequence. This labelled dsDNA is incubated with purified Vsr in reaction buffer. Experimental conditions are listed in the results for each experiment. (See section 3.5.) The reaction is then denatured and loaded on a 20 % denaturing PAGE. The gel is then placed onto the Li-cor Odyssey scanner to allow detection of the reaction products. If the Vsr protein cuts, we observe a species of DNA that is smaller than the full-length oligomer (Figure 13). For single turnover assays, the reaction products are quantified using the *Odyssey* software. In order to calculate the k_{cat}, the data was then graphed and fitted to a first order rate equation using GraFit (Erithacus software) version 6.

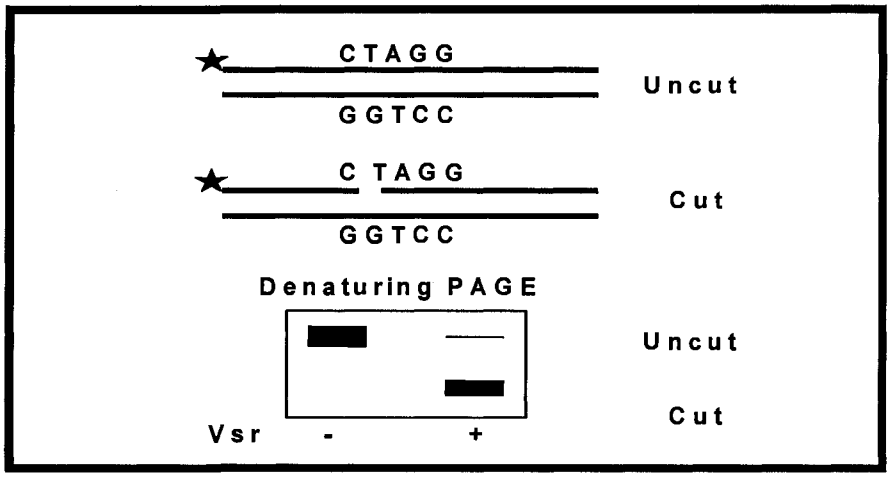


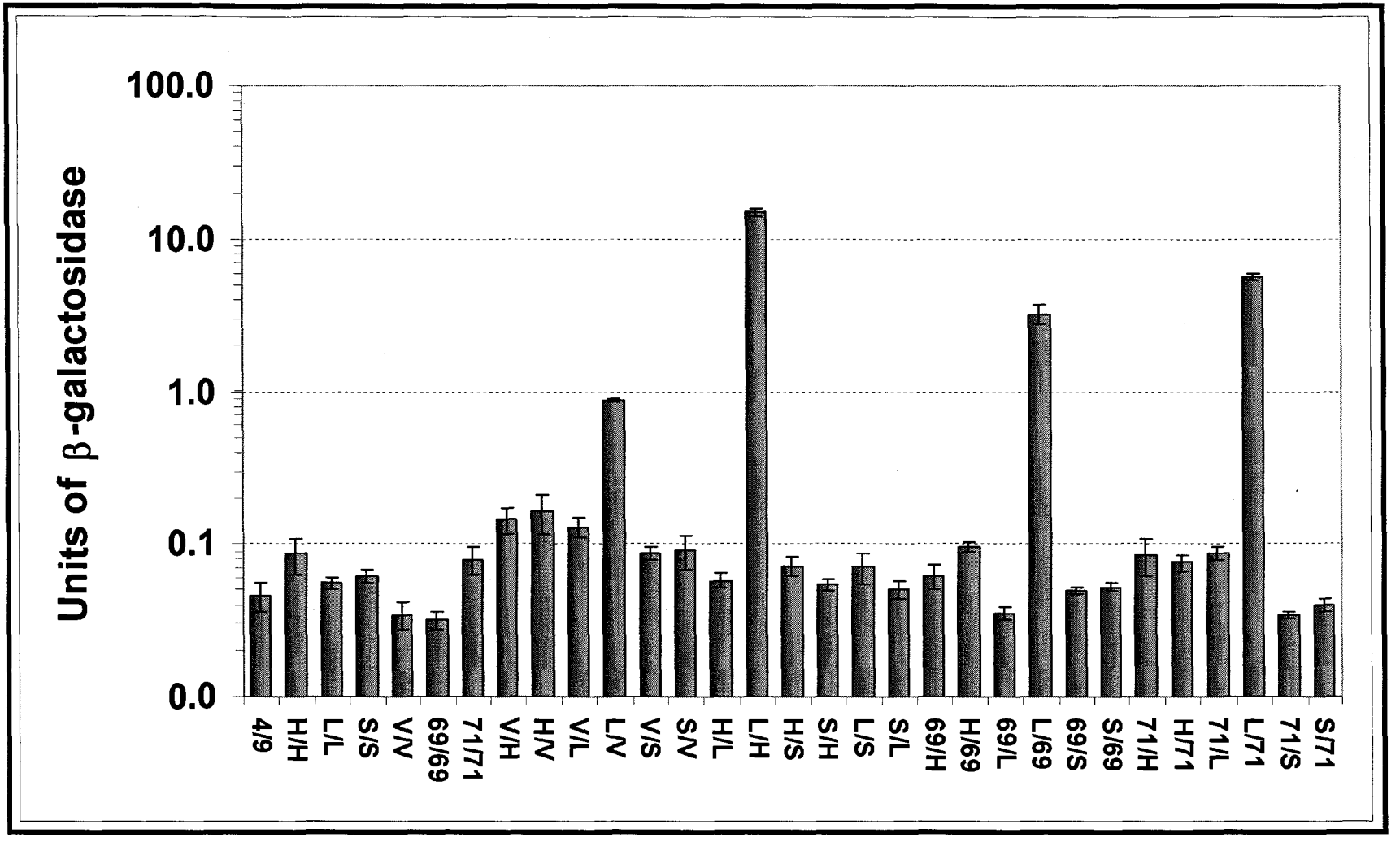
Figure 13: Schematic of the endonuclease reaction. When Vsr is present in the reaction mixture, the heteroduplex DNA is nicked. The short product is then visualized by running the DNA on denaturing PAGE.

3.0 Results

3.1 Yeast two-hybrid assay

3.1.1 Study of interactions between Vsr and MMR proteins

All possible pair-wise combinations of Vsr, MutH, MutL, and MutS were tested to identify potential interactions involving mismatch repair proteins and Vsr. Hall *et al.* (1998 and 1999), in parallel to our lab performed, a yeast two-hybrid screen of *uvrD* (helicase II) and MMR proteins. Both parties identified a physical interaction between MutL and MutH. However, none of the other interactions predicted by *in vitro* results was observed, such as the MutL homodimer, the MutS homodimer, and the MutL-MutS pair (Hall *et al.* 1999). The screen we performed supported the findings of Hall *et al.* (1998 and 1999). Additionally, we observed a novel interaction between Vsr and MutL (Mansour *et al.* 2001). Like the MutL/MutH and the MutL/UvrD interactions, the MutL/Vsr interaction was also found to be unidirectional; interactions took place only with MutL fused to the pGAD424 activation domain and Vsr fused to the pGBT9 DNA binding domain. Furthermore, we observed a unidirectional interaction between the two Vsr mutants (H69A and H71A) and MutL (Mansour *et al.* 2001) (Graph 1).



Graph 1: β-galactosidase activity assay of all possible pair wise combinations between MMR proteins and Vsr. In all cases, the first symbol refers to the pGAD424 fusion, while the second symbol stands for the pGBT9 fusion.

3.1.2 Interaction domain localization between Vsr and MutL

In their screen, Hall *et al.* (1999) observed that the carboxy-terminal 218 amino acids of MutL are sufficient for the two-hybrid interaction with MutH. However, removal of a small number of residues from either terminus of the MutH endonuclease eliminates the interaction with MutL (Hall *et al.* 1999). Likewise, the deletion analysis revealed that the carboxy terminal 218 amino acids of MutL were sufficient for the two-hybrid interaction with UvrD, and that both termini of UvrD are required for interaction with MutL (Hall *et al.* 1998).

We further used the yeast two-hybrid system to study the interaction between Vsr (156 A.A.) and MutL (674 A.A.) in an attempt to localise MutL domains essential for Vsr interaction. The series of MutL truncations were graciously provided by Matson (Hall *et al.* 1998 and 1999) (Figure 14). The results of our initial qualitative filter assay showed that only the carboxy terminal 59 deletion mutant interacted with full length Vsr as well as with the H69A mutant. However, it is important to mention that the color intensity of this interaction was very faint (filter data not shown). The liquid β-galactosidase assay performed corroborates the qualitative observation (Graph 2).

3.1.3 Yeast three-hybrid: interference study

As previously discussed, it is hypothesised that when Vsr is over expressed, transition and frame shift mutations appear to be caused by an interference with the MMR system. Hence, the yeast three-hybrid system was

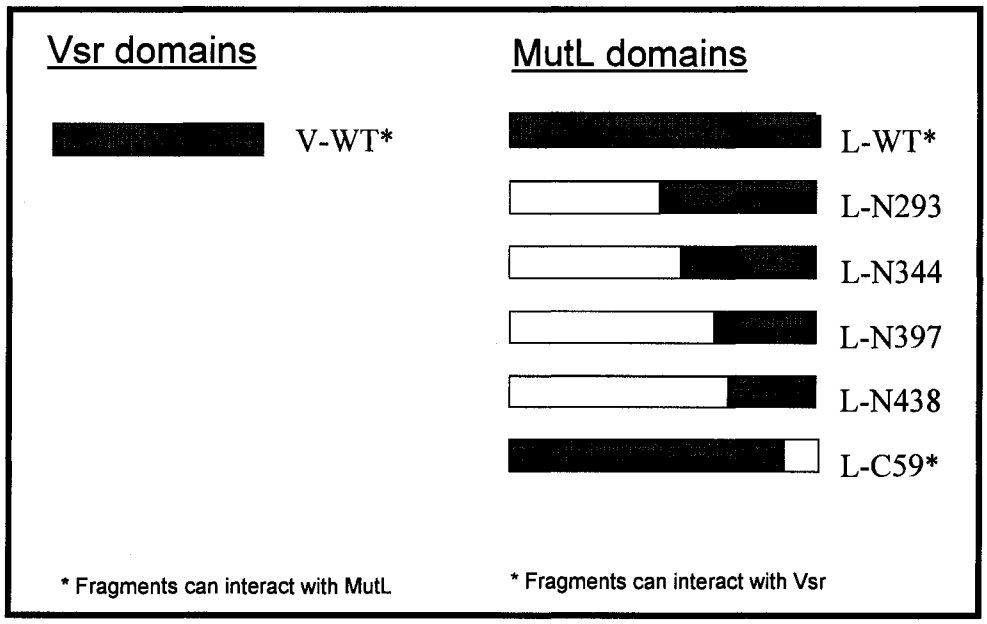
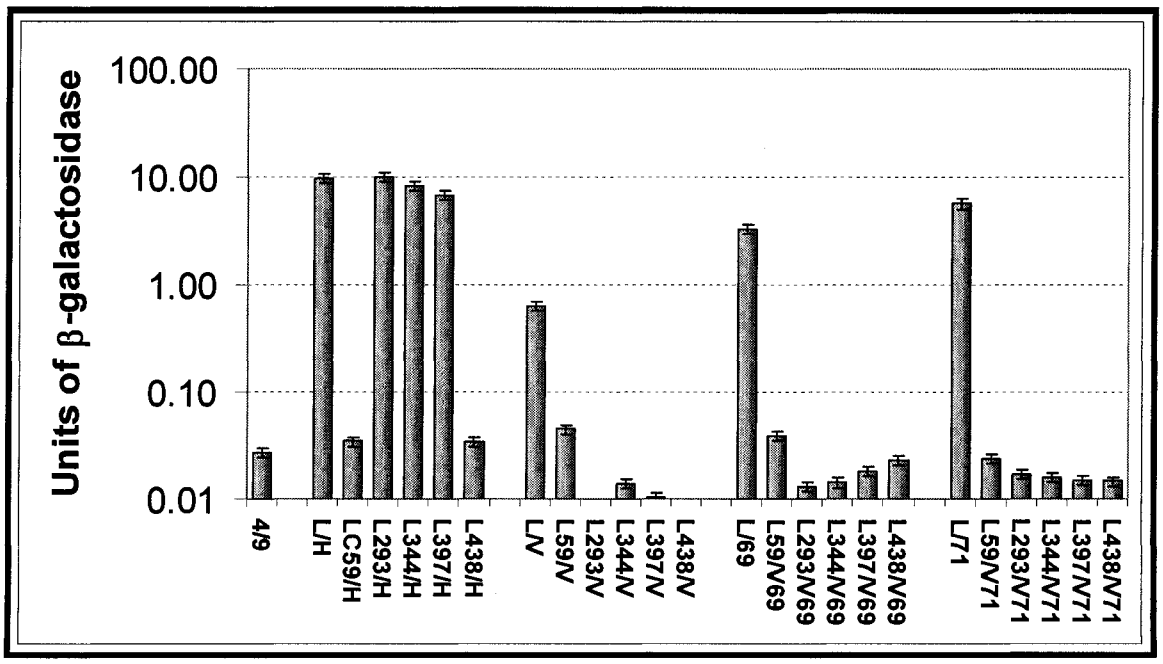


Figure 14: Graphic representation of the MutL truncations used in the localization study. Asterisks depict interactions.



Graph 2: β-galactosidase activity assay, domain localization. The first symbol refers to the pGAD424 fusion, while the second symbol refers to the pGBT9 fusion. Except for the control in lane 1, the first symbol also refers to the MutL fragment.

used in an attempt to investigate the disruption of the known and observable MutL interactions: MutL/MutH, and MutL/Vsr. To test for this disruption, either *vsr* or *mutH* (under the control of a conditional methionine promoter) were cloned into the second site on the pBridge vector.

The separate addition of extra Vsr or MutH to the test system did not yield the anticipated results (Graph 3). There was no observed difference in the interaction pattern between MutL/MutH and MutL/Vsr, with or without the presence of a third protein.

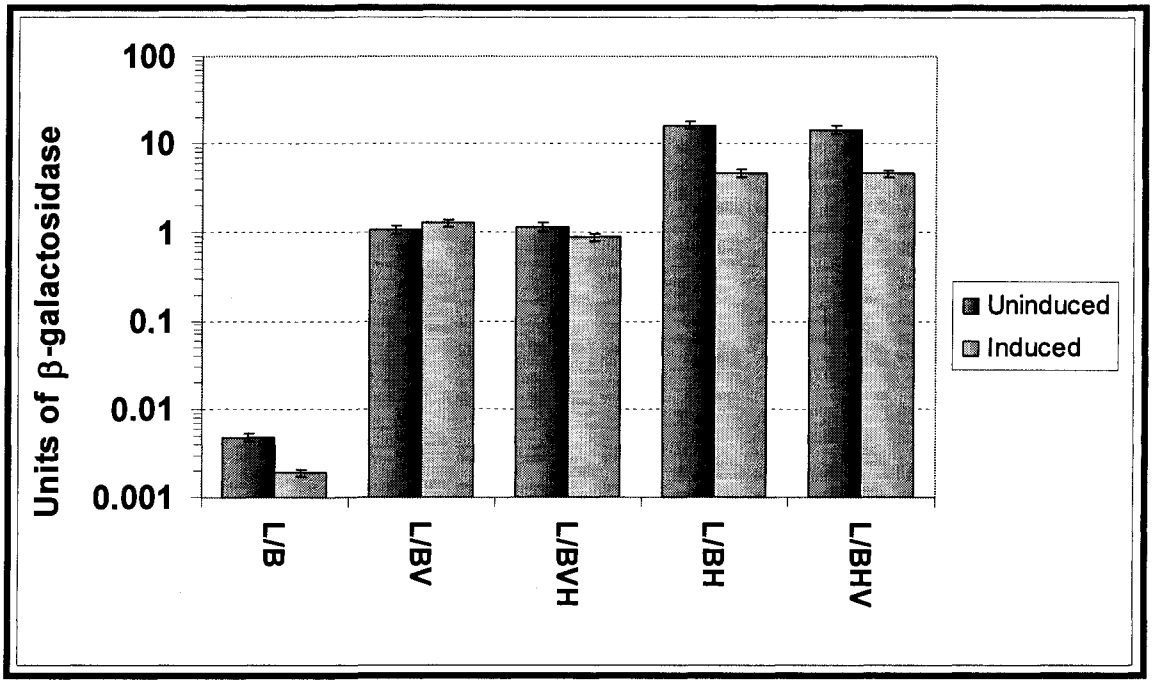
At this point, it was decided not to use the yeast three-hybrid system further to probe the MutS-MutL-MutH interaction. The decision was based on the fact that both our data and Hall's failed to show the expected interaction between MutL and MutS. Further analysis of this particular tripartite interaction was probed using the bacterial two- and three-hybrid systems instead.

3.2 Bacterial two-hybrid assay

3.2.1 Study of interactions between Vsr and MMR proteins

All possible pair-wise combinations of Vsr, MutH, MutL, and MutS were tested with the bacterial two-hybrid assay to identify potential interactions involving the mismatch repair proteins and Vsr. The two-hybrid screen was performed by Mansour *et al.* (2001) and confirmed the Vsr/MutL interaction previously seen in the yeast test system (Graph 4). The assay further revealed MutH/MutL,

MutL/MutL, MutS/MutL and MutS/MutS interactions as predicted from *in vitro* studies reported in the literature, and confirmed the interaction between Vsr and MutL which we observed in the yeast two-hybrid assay. It is interesting to note that the results also show a new putative interaction between MutS/MutH, which had never been observed *in vitro* (Mansour *et al.* 2001).



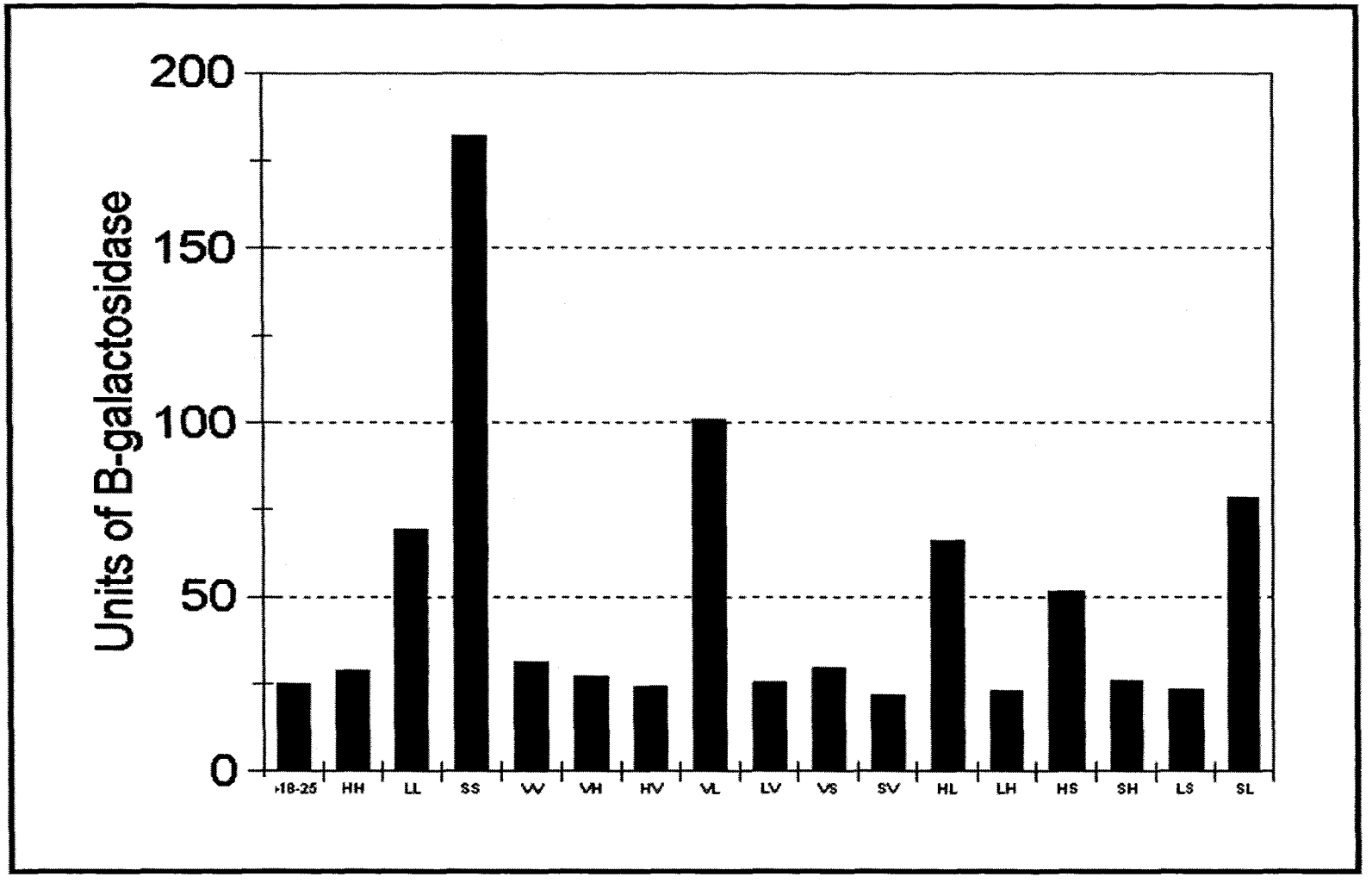
Graph 3: β-galactosidase activity assay, effect of extra MutH or Vsr on yeast two-hybrid interactions. The first symbol refers to the pGAD424 fusion, while the second symbol refers to the pBridge fusion.

3.2.2 Interaction domain localization between Vsr and MutL

As with the yeast two-hybrid, the bacterial system was further used to study the interaction between Vsr and MutL in an attempt to localise both Vsr and MutL domains essential for interaction. Crystallographic data shows that MutL possess two separate domains in the wild type protein: an amino terminus (amino acids 1-350) conserved amongst the MutL protein family and a carboxy terminus (amino acids 350-615) (Ban *et al* 1998 and Guarne *et al* 2004). These domains are linked by a non-conserved, flexible domain, which has not yet been crystallized. Vectors were therefore constructed to reproduce this natural division in MutL (Figure 15). Results showed that Vsr did not interact with either of the protein fragments (Mansour *et al.* 2001).

As predicted by the yeast two-hybrid data obtained from Hall *et al.* (1999), the MutH protein interacted with the amino terminus of MutL (Graph 5). Also predicted by the literature (Ban *et al.* 1999), was the interaction between the full-length MutL protein and the MutL amino terminus (Mansour *et al.* 2001) (Graph 5).

In order to identify which domain of Vsr interacts with the MutL protein, we constructed five truncation fragments: two amino terminal deletions (VsrN14 and VsrN19) and three carboxy terminal deletions (VsrC124, VsrC112, and VsrC27) (Figure 15). We were interested to see if these fragments could interact with MutL in the bacterial two-hybrid system.



Graph 4: β-galactosidase activity assay of all pair wise combinations between MMR proteins and Vsr. In all cases the first symbol refers to the pT18 fusion, while the second symbol stands for the pT25 fusion.

The results in graph 5 showed that the removal of the first 14 and 19 amino acids from the amino terminus of Vsr improved the interaction with the MutL protein (Mansour *et al.* 2001). However, deletion of even 27 amino acids from the carboxy terminus of Vsr completely abolishes the interaction with MutL.

3.2.3 Bacterial three-hybrid: interference study

As previously discussed, when Vsr is over expressed, the resulting appearance of transition and frame shift mutations are hypothesised to be caused by interference with MMR. The explanation put forth is that by interacting with MutL, Vsr causes a disruption in MMR, which results in the mutagenic spectrum observed. To test for this disruption, *vsr* (under the control of a synthetic, constitutive *trc* promoter) was cloned into a second site on the pT18 vector. We then observed with particular interest that the addition of extra Vsr to the test system actually abolished all interactions, even the MutH/MutS, with the sole exception of the MutS dimer (Mansour *et al.* 2001) (Graph 6).

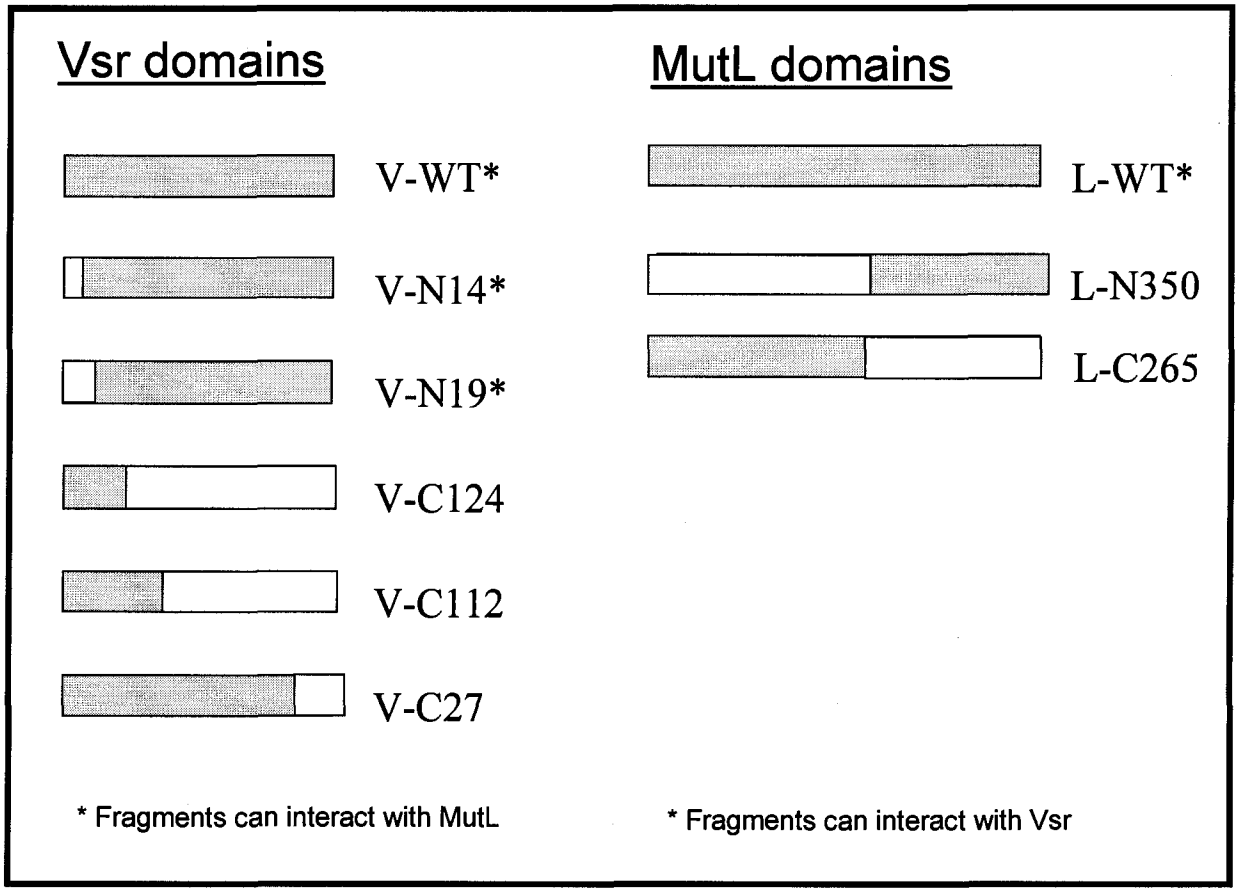
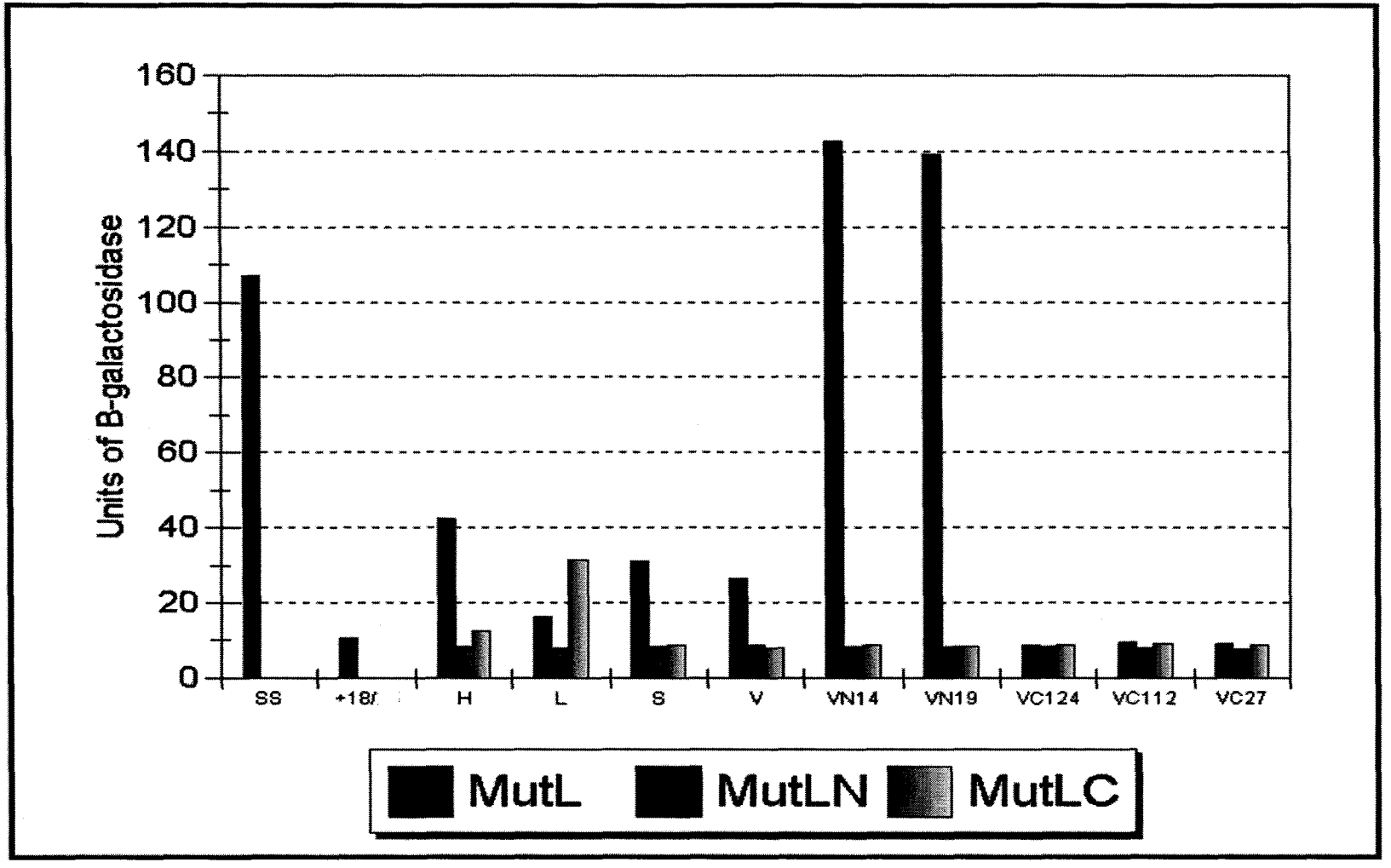
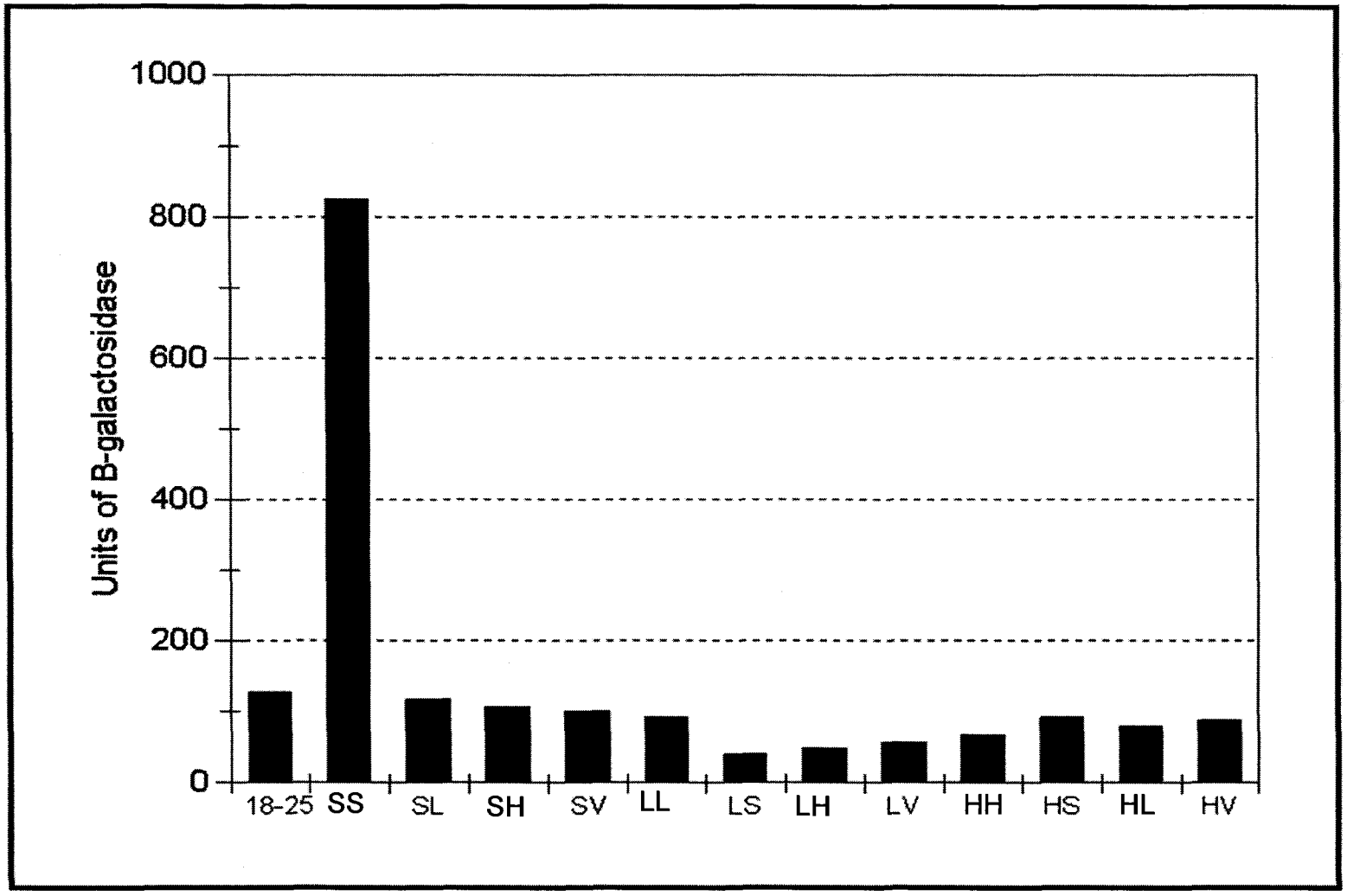


Figure 15: Graphic representation of the Vsr and MutL truncations used in the localization study. Asterisks depict interactions.



Graph 5: β-galactosidase activity assay: domain localization. Wt Vsr and truncation fragments (pT18) were tested against MutL and MutLN deletion and MutLC deletion (pT25). Columns 1 and 2 are the positive and negative controls. For all other columns, the symbol refers to the Mut protein or Vsr fragment while the block colour refers to the MutL fragment.



Graph 6: β-galactosidase activity assay: effect of extra Vsr on bacterial two-hybrid interactions. In all cases the first symbol refers to the pT18 fusion, while the second symbol stands for the pT25 fusion.

3.3 Protein purification

The two-hybrid and three-hybrid assays provided strong evidence for interactions between Vsr and MutL. We now wanted to test the functional nature of these interactions *in vitro*, using purified proteins and defined DNA substrates. From the literature, the protocols available for the purification of the Vsr endonuclease fell short of yielding a stable preparation of active protein. Substantial amounts of protein were lost using the original purification process (Cupples *et al.* 1999), mostly due to precipitation, while protein activity was a very serious issue with the Turner protocol (Turner *et al.* 2000).

The new protocol used (See section 2.3.) was derived from the purification schematic of MutL by Schofield *et al.* (2001) and was based on a HEPES buffer system. This new protocol yielded a protein that is stable, consistently active and can be stored at -80°C for a considerable length of time without adverse effect. The major protocol modifications that gave the best results were:

1. Performing all manipulations at 4°C.

2. Adding protease inhibitors to all of the buffers in order to prevent protein degradation.

3. Adding $MgCl_2$ to the lysis and exchange buffers so as to allow a better folding of the protein.

4. Increasing the sodium chloride concentration of the final buffer solution from [100mM] to [110mM].

5. Increasing the number of washes performed, thus helping to remove non-specific proteins.

6. Utilizing a desalting column to remove the imidazole, instead of dialyzing, thereby reducing the processing time.

7. Adding DTT to the exchange buffer in order to significantly reduce the multimerization and precipitation problem.

3.3.1 Protein induction

All protein samples were induced by adding IPTG to a final concentration of 1 mM. SDS PAGE gels were then run to ensure the proper induction of each protein. Figure 16 shows the induction of separate cultures of Vsr and MutL. The MutL mutants were expressed in a similar manner (data not shown).

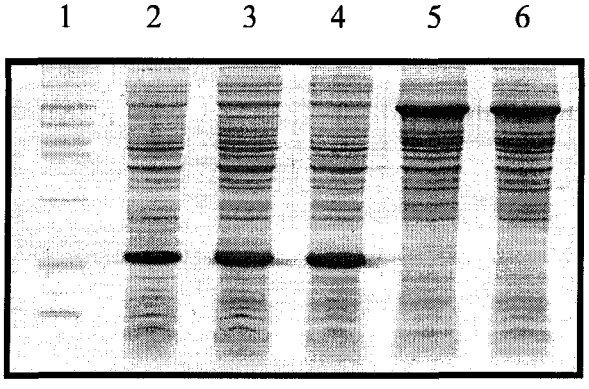


Figure 16: SDS PAGE gel of crude protein extract samples taken from *E. coli* BL21 DE3 cells containing pET15b derivative plasmids and induced with 1 mM IPTG. Lane 1: marker (97, 66, 45, 30, 20.1, and 14.4 kDa); lanes 2-4: Vsr crude extract of three separate cultures; lanes 5-6: MutL cell lysis extract of two separate cultures.

3.3.2 Protein purification

As demonstrated in figure 17, the modified purification protocol gave high yields of protein for Vsr. However, a small amount of contaminating protein co-eluted with Vsr. For MutL, not only did we obtain a good protein yield (Figure 18), we also observed very little degradation, a frequently reported problem for MutL (Hall and Kunkel 2001). The MutL mutants behaved in a similar manner (data not shown).

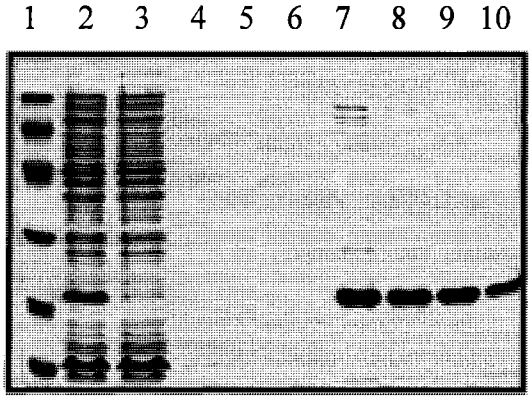


Figure 17: SDS PAGE gel of Vsr purification steps. Lane 1: marker (97, 66, 45, 30, 20.1, and 14.4 kDa); lane 2: crude lysate; lane 3: unbound fraction of lysate; lane 4: wash 3; lane 5: wash 5, overnight; lane 6: wash 8; lanes 7-10: elutions.

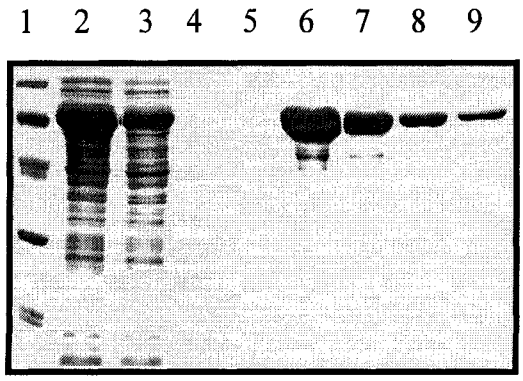


Figure 18: SDS PAGE gel of MutL purification steps. Lane 1: marker (97, 66, 45, 30, 20.1, and 14.4 kDa); lane 2: crude lysate; lane 3: unbound fraction of lysate; lane 4: wash 5, overnight; lane 5: wash 8; lanes 6-9: elutions.

3.3.3 Purified protein

The final protein preparations for both Vsr and MutL (Figure 19) were of good quality with very little contamination and only mild degradation product for MutL alone. Purity of preparations was of >95% purity as judged by SDS-PAGE (Figure 19). Preparations of MutL mutants were of similar quality. (Data not shown.) It is noteworthy to observe the stability of Vsr, where no loss of the amino terminus was detected during the purification of the protein as compared to Turner *et al.* (2000) and Monastiriakos (2002). Moreover, what cannot be seen from this figure, but was of paramount importance to us, was the striking reduction in aggregation of both proteins during the final steps of purification.

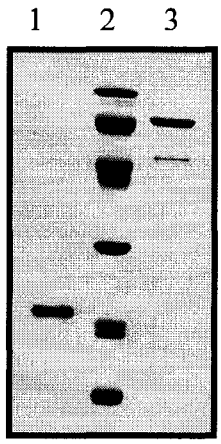


Figure 19: SDS PAGE gel of purified protein samples. Lane 1: Vsr; lane 2: marker (97, 66, 45, 30, 20.1, and 14.4 kDa); lane 3: MutL.

3.4. Trypsin analysis of MutL and its mutants

When MutL binds ATP, it is known to change conformation (Ban *et al.* 1998 and 1999). Trypsin digestion was used to determine whether purified preparations of the MutL and MutL mutants were behaving as expected with regards to their nucleotide binding properties. As expected, MutL without ATP bound has a more disorganised conformation (Ban *et al.* 1998 & 1999) and is therefore more susceptible to trypsin digest than MutL in the presence of ATP. This can be seen in figure 20 where lanes 3-6 show that MutL undergoes more extensive cleavage in the absence of ATP, as compared to lanes 7-10 in the presence of ATP.

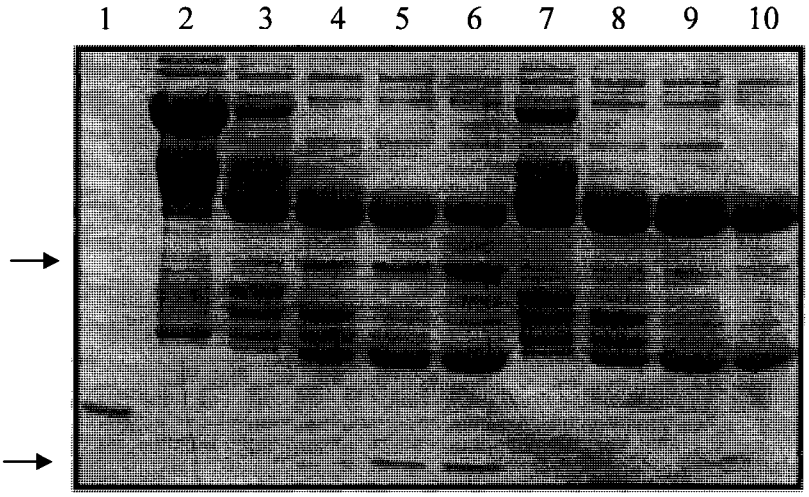


Figure 20: Tryptic digestion of wild type MutL without and with 5 mM ATP. Lane 1: Position marker (20 kDa). Lane 2: undigested control at 0 min. Lanes 3-6 without ATP. Lane 3: 1min. Lane 4: 5 min. Lane 5: 10 min. Lane 6: 20 min. Lanes 7-10 with ATP. Lane 7: 1 min. Lane 8: 5 min. Lane 9:10 min. Lane 10: 20 min. Arrows point to areas of differential cleavage.

Mutant MutL proteins were also treated with trypsin. The results obtained were as expected. Mutants E29A, E32K, R266E and N302A, which can bind ATP showed a digestion pattern similar to that of wild type MutL albeit with some gradation for mutants that bind ATP less well. (Data not shown.) As for mutants R95F/N302A and K307A that do not bind ATP, their digestion pattern was identical whether ATP was present or not. (Data not shown.)

3.5 Endonuclease assay

3.5.1 Single turnover assays for Vsr, with and without MutL

Single turnover assays using 1 μM Vsr and 1 nM labelled DNA were done in order to compare the endonucleolytic activity of our protein, purified following the new protocol, with that of the established value. Assay results (Figure 21 and Graph 7) gave us a constant of k_{cat} = 3.6 +/- 0.21 min^{-1} which was comparable to the established value of k_{cat} = 2.9 +/- 0.43 min^{-1} (Monastiriakos *et al.* 2004).

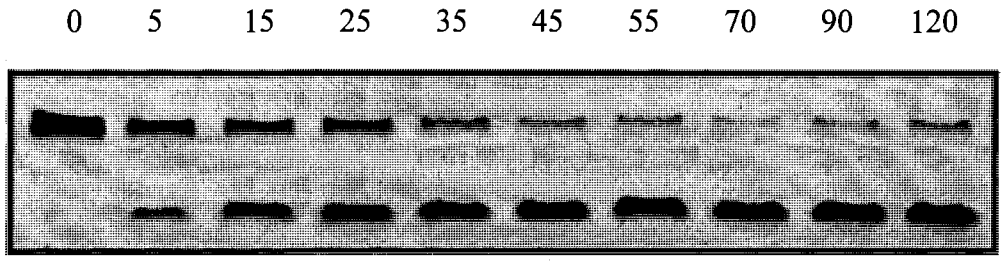
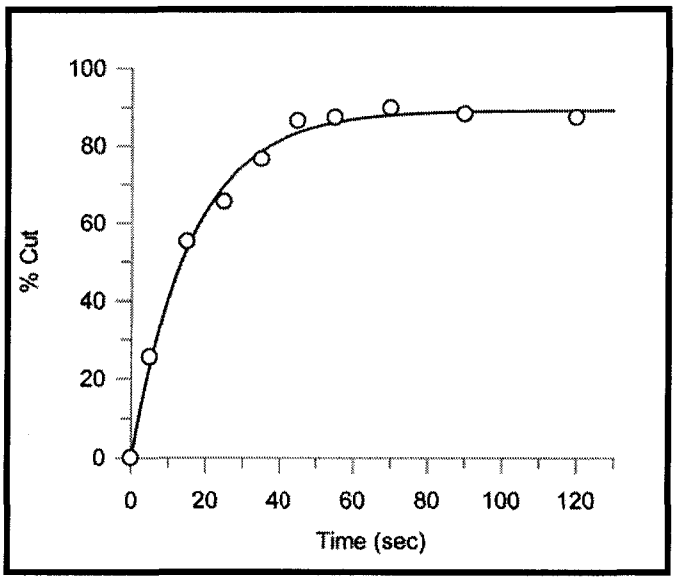


Figure 21: Single turnover reaction with 1 μM Vsr and 1 nM labelled DNA. Time points measured 0, 5, 15, 25, 35, 45, 55, 70, 90 and 120 seconds at rt.



Graph 7: Analysis of the data fitted to a first order rate equation using GraFit version 6 (Erithacus Software) with k_{cat} of 3.6 +/- 0.21 min^{-1}.

Since MutL has been observed to stimulate Vsr, (Monastiriakos *et al.* 2004) single turnover assays were done in order to measure the activity of the protein in the presence of MutL. When the ratio of MutL to Vsr was 1:10 or 1:1 no differences in endonclease activity were observed. (Figures 22, 23 and graph 8) If anything, when the ratio of MutL to Vsr increased to 2.5:1, we saw a slight decrease in the activity of Vsr. (Figure 24 and graph 8)

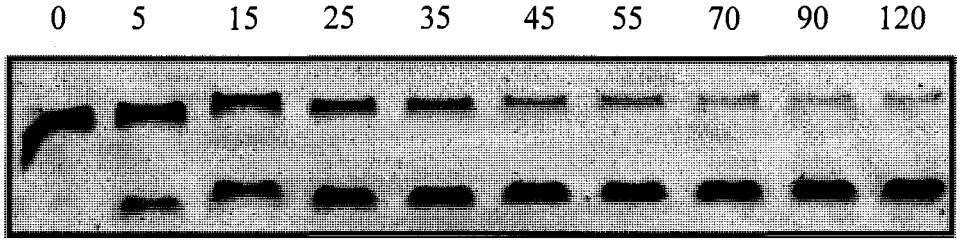


Figure 22: Single turnover with 1 μM Vsr, 0.1 μM MutL and 1nM labelled DNA. Time points measured 0, 5, 15, 25, 35, 45, 55, 70, 90 and 120 sec at rt.

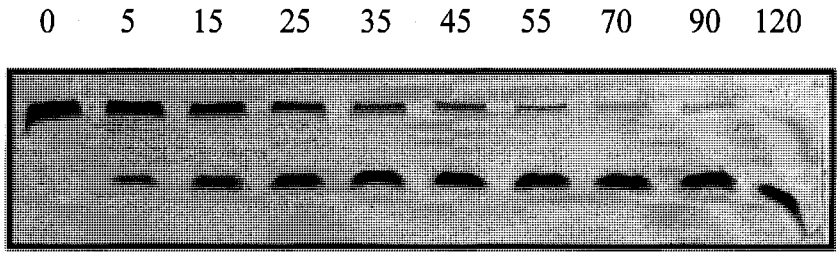


Figure 23: Single turnover with 1 µM Vsr, 1 µM MutL and 1nM labelled DNA. Time points measured 0, 5, 15, 25, 35, 45, 55, 70, 90 and 120 sec at rt.

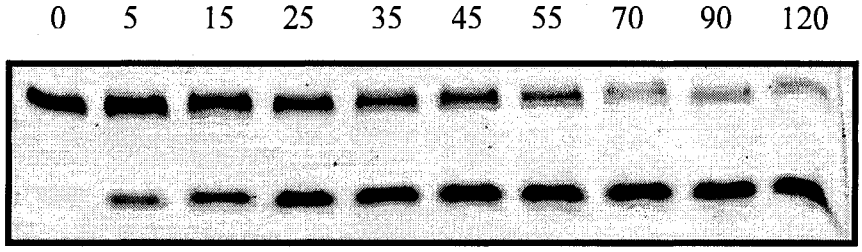
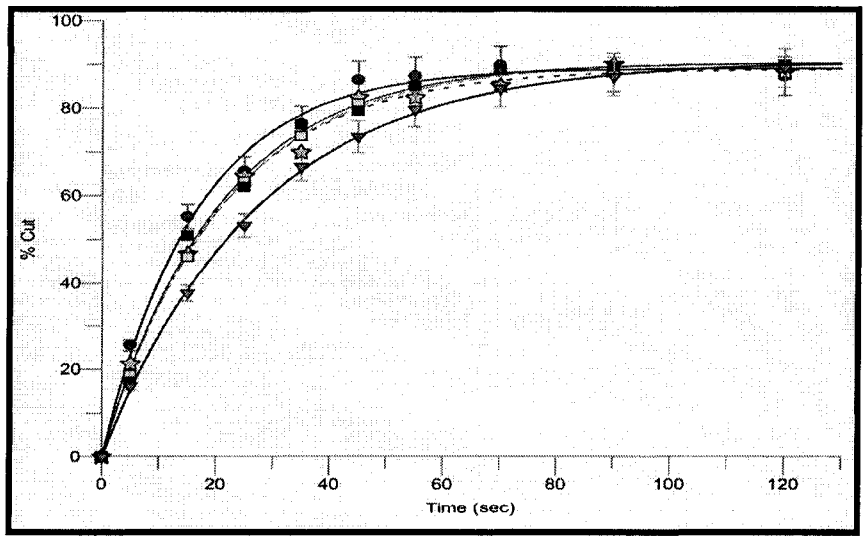


Figure 24: Single turnover with 1 µM Vsr, 2.5 µM MutL and 1nM labelled DNA. Time points measured 0, 5, 15, 25, 35, 45, 55, 70, 90 and 120 sec at rt.



Graph 8: Analysis of the data from figures 20, 21 and 22 fitted to a first order rate equation using GraFit version 6 (Erithacus Software). k_{cat} of: Vsr 3.6 +/- 0.21 min^{-1} (●), Vsr with 0.1 µM MutL 3.0 +/- 0.14 min^{-1} (□), Vsr with 1 µM MutL 3.0 +/- 0.14 min^{-1} (☆) and Vsr with 2.5 µM MutL 2.2 +/- 0.05 min^{-1} (▽).

3.5.2 Study of the stimulatory effect of MutL on Vsr activity

When we look at the effect of MutL on Vsr, with increased amounts of DNA instead of single turnover conditions, we can see a stimulatory effect of MutL upon Vsr (Figure 25). Under these conditions, Vsr undergoes multiple turnovers to cleave the substrate. This effect can be detected when the concentration of MutL is as low as 1/10 that of Vsr. However MutL had no stimulatory effect under single turnover conditions (Graph 8).

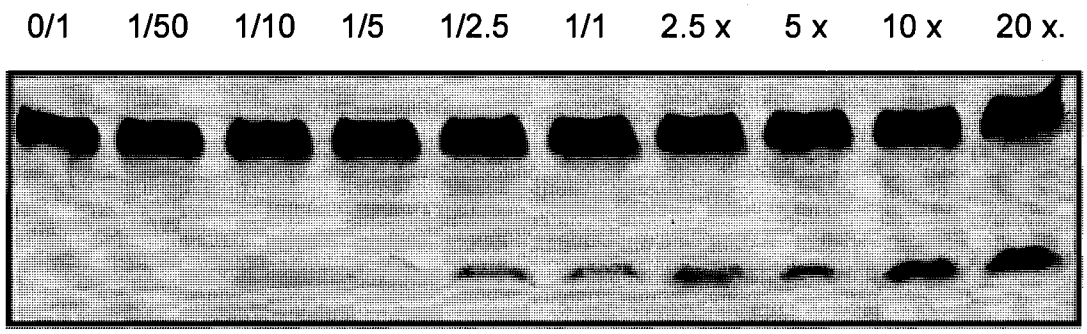


Figure 25: MutL stimulation. Effect of MutL on Vsr. 1: Vsr alone (37.5 nM); Vsr with increasing molecular concentrations of MutL 2: 1/50; 3: 1/10; 4: 1/5; 5: 1/2.5; 6: 1/1; 7: 2.5 x; 8: 5 x; 9: 10 x; 10: 20 x. Reaction proceeded at room temperature for 30 minutes. 40 nM DNA.

As previously discussed, MutL is an ATPase. As such, we wanted to look at the role of ATP hydrolysis on the stimulatory effect of MutL. This was investigated in a two fold approach, first by looking at the effect of adding ATP, ADP and novobiocin (hydrolysis inhibitor) to the reaction; and second, by looking at MutL mutants that cannot bind or hydrolyze ATP.

Figure 26 shows that ATP, ADP and novobiocin play absolutely no role on the stimulatory effect MutL has on Vsr. Furthermore, all of the binding and hydrolysis mutants showed a similar level of Vsr stimulation as the wild type MutL (Figure 27).

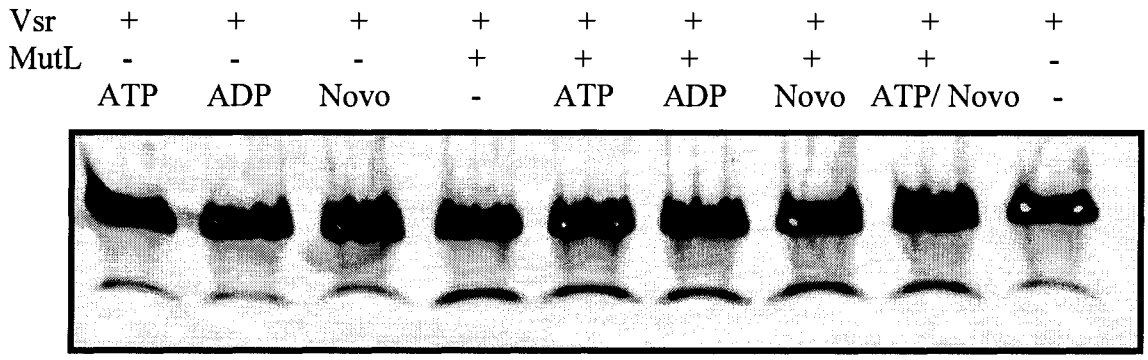


Figure 26: Effect of adding ATP (1.5 μM), ADP (1.5 μM) or novobiocin (1.5 μM) on Vsr (37.5 nM) in conjunction with MutL (187.5 nM; 5x). 1: Vsr with ATP; 2: Vsr with ADP; 3: Vsr with novobiocin; 4: Vsr with MutL; 5: Vsr with MutL and ATP; 6: Vsr with MutL and ADP; 7: Vsr with MutL and novobiocin; 8: Vsr with MutL, ATP and novobiocin; 9: Vsr alone. Reaction proceeded at room temperature for 30 minutes, 40 nM DNA.

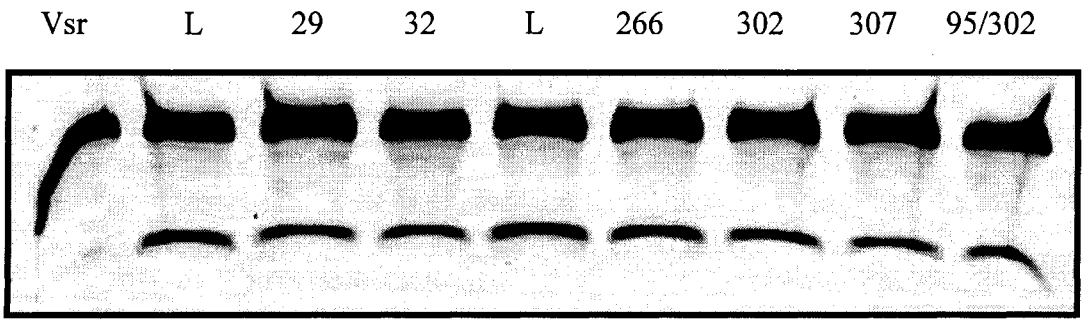


Figure 27: Effect of MutL Mutants (93.8 nM; 2.5 x) on Vsr (37.5 nM). Vsr alone, Vsr with MutL, 29, 32, 95, 266, 302, 307, 95/302. Reaction proceeded at room temperature for 30 minutes.

3.6.3 Controls: ATP, ADP, novobiocin & lysozyme

Control reactions were done in order to ascertain that ATP, ADP or novobiocin had no effect on Vsr. As can be seen in figures 28, 29 and 30 none of these three compounds had an effect, except at very high concentrations where it reduced the endonucleolytic capabilities of Vsr. This interference with Vsr may simply be because these molecules enter the DNA binding site of the protein and at high concentrations, interfere with DNA binding. Similar results were observed for MutH at high ATP concentrations (Hall *et al.* 1999). As for the lysozyme control, it is possible to conclude that molecular crowding does not play a role in the MutL stimulatory effect of Vsr.

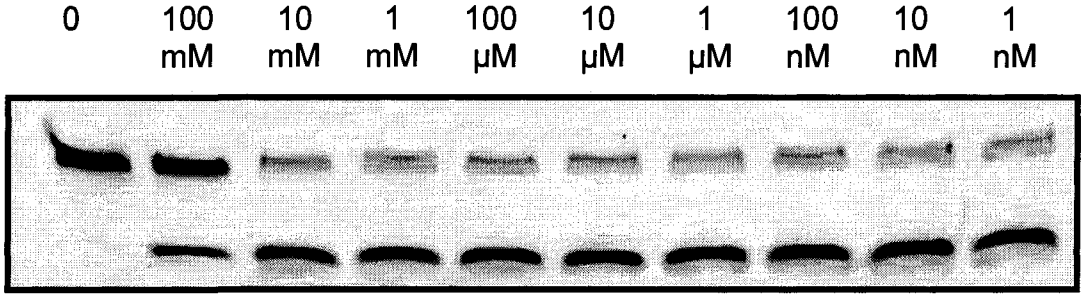


Figure 28: Effect of ATP on Vsr. 14 nM labelled DNA. 0.5 µM Vsr with 100 mM, 10 mM, 1 mM, 100 µM, 10 µM, 1 µM, 100 nM, 10 nM, 1 nM, or no ATP. Reaction proceeded at room temperature for 30 minutes.

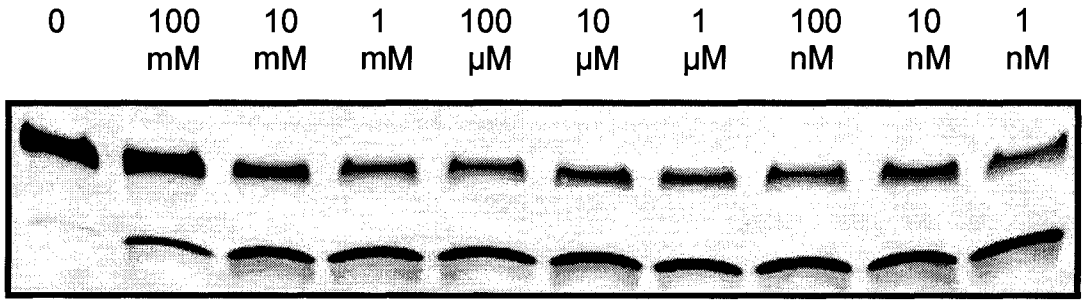


Figure 29: Effect of ADP on Vsr. 14 nM labelled DNA. 0.5 µM Vsr with 100 mM, 10 mM, 1 mM, 100 µM, 10 µM, 1 µM, 100 nM, 10 nM, 1 nM, or no ADP. Reaction proceeded at room temperature for 30 minutes.

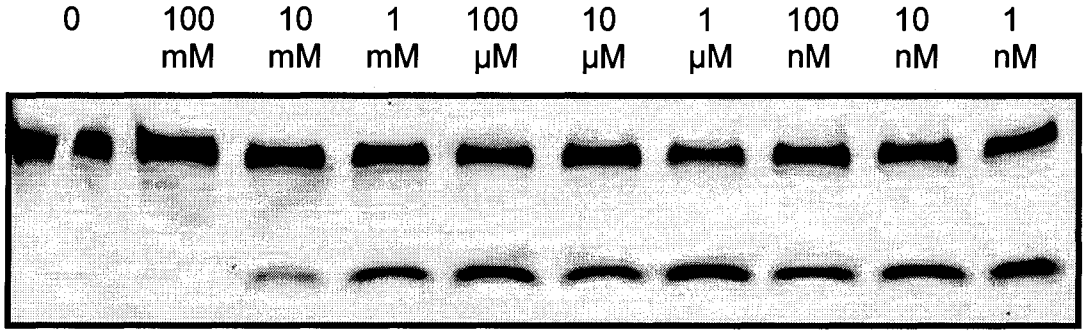


Figure 30: Effect of novobiocin on Vsr. 14 nM labelled DNA. 0.5 µM Vsr with 25 mM, 10 mM, 1 mM, 100 µM, 10 µM, 1 µM, 100 nM, 10 nM, 1 nM, or no novobiocin. Reaction proceeded at room temperature for 30 minutes.

A further control reaction was done in order to measure the effect of molecular crowding on the enzymatic activity of Vsr. The addition of lysozyme to Vsr only shows a minimal stimulatory effect at 10-20 times the concentrations used for Vsr (Figure 31). This establishes that any stimulatory effect caused by MutL is in fact due to this protein and not simply due to the molecular crowding of Vsr.

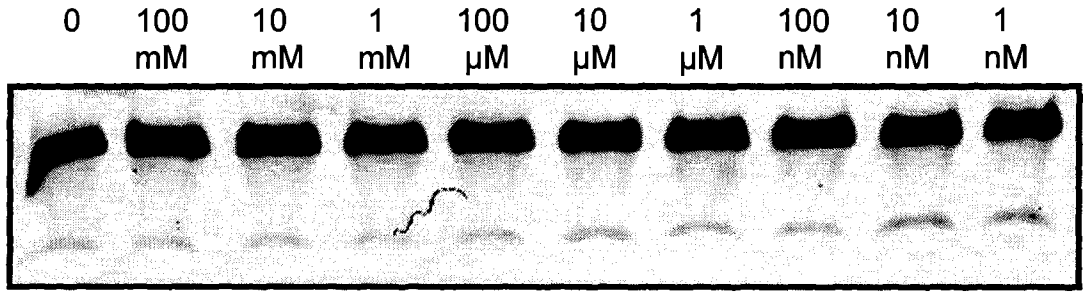


Figure 31: Effect of lysozyme on Vsr. 14 nM labelled DNA. Vsr alone (37.5 nM), Vsr with increasing molecular concentrations of lysozyme 1/50, 1/10, 1/5, 1/2.5, 1/1, 2.5x, 5x, 10x, 20x. Reaction proceeded at room temperature for 30 minutes.

4.0 Discussion

The original uncoupling of the expression of Vsr from that of Dcm permitted the monitoring of Vsr effects. Studies showed that high levels of Vsr increased the competition between VSP and MMR for T/G mispairs in a CT/GAGG context; resulting in an increase in C to T transition mutations (Doiron *et al.* 1996 and Macintyre *et al.* 1997) (Figure 6).

Further studies on the over expression of Vsr also showed that Vsr-stimulated mutagenesis was not limited to T/G mismatches in a restricted sequence context (Doiron *et al.* 1996, and Macintyre *et al.* 1997). It is noteworthy that the over expression of Vsr actually resulted in elevated levels of transition and frame shift mutations (Doiron *et al.* 1996, and Macintyre *et al.* 1997). The spectrum and magnitude of the mutations produced by this over expression of Vsr (Doiron *et al.* 1996 and Macintyre *et al.* 1997) was similar to that produced by MMR deficient strains (Cupples *et al*, 1989 and 1990).

We hypothesised that Vsr may cause mutations by interfering with the MMR system. Macintyre *et al.* (1997) looked at this possibility and observed that the levels of Vsr stimulated mutations were decreased upon the addition of extra MutH or MutL proteins. However, when extra MutS was added, an increase in the levels of mutation was seen (Macintyre *et al.* 1997). It was further observed that VSP repair is partially defective in strains that are *mutS*$^-$ or *mutL*$^-$ (Bell and Cupples, 2001), although not completely dependent as was originally thought (Lieb, 1987; Jones *et al.* 1987; and Zell *et al.* 1987).

From there we explored the contribution of the Vsr structure to its mutagenic potential. The three aromatic intercalating residues F67A, W68A, W86A (Siponen, Biol 490 Thesis) and the two residues H69A and H71A (Doiron, Biol 490 Thesis), coordinating the zinc and magnesium ions respectively, were mutated. All five of these mutants were found to have no endonucleolytic capabilities and consequently no repair activity. They did however show a spectrum and level of mutagenesis similar to wild type MutL. Analysis of these mutants suggested that Vsr does not need to be enzymatically active in order to cause mutagenesis. Consequently, this added credence to the possibility that the mutator effect is due to an inhibition of protein-protein interactions. It is possible that Vsr interferes with the ability of MutS and/or MutH to interact with MutL.

By contrast, the two N-terminal truncations studied, Δ14 and Δ19, were found to have reduced endonucleolytic capabilities (Monastiriakos *et al.* 2004). Despite their low endonucleolytic activity, the truncations were still capable of carrying out VSP repair *in vivo*, with Δ14 being more efficient than Δ19, but almost as efficient as the wild type protein (Monastiriakos *et al.* 2004). This was unexpected given that the Δ14 truncated protein was previously reported to be inactive *in vivo* (Dar *et al.* 1993). However, the difference may lie in the fact that the N-terminal truncations in our system were highly expressed from a strong artificial promoter, independent of *dcm* (Monastiriakos *et al.* 2004). Consequently, the production of non-physiological amounts of these mutant proteins may give a misleading picture of their repair proficiency. In contrast to

the point mutants, the N-terminal truncations showed decreased levels of mutagenic activity with the Δ19 being less mutagenic than Δ14 (Monastiriakos *et al.* 2004). When combined, these results suggest that the N-terminal domain plays a role in the mutator phenotype shown by Vsr, and led to further investigation of the protein-protein interaction between Vsr and the MMR proteins.

Based on this collection of data, we decided to first use the *in vivo* yeast and bacterial two-hybrid systems in order to probe the interaction between VSP repair and MMR. We then used *in vitro* endonuclease assays to further understand Vsr and its relationship with the MMR system.

4.1 Yeast two-hybrid assay

4.1.1 Interaction study between Vsr and MMR proteins

Yeast two-hybrid results showed that MutL interacted with Vsr and MutH. However, none of the interactions predicted by the literature was observed, such as the MutL homodimer, the MutS homodimer, and the MutL-MutS pair (Hall *et al.* 1999 and Mansour *et al.* 2001) (Graph 1). Like the MutL/MutH and the MutL/UvrD interactions, the MutL/Vsr interaction was also unidirectional with MutL being fused to pGAD424 and Vsr to pGBT9. Furthermore, we observed a unidirectional interaction between the two Vsr mutants (H69A & H71A) and MutL (Mansour *et al.* 2001). These results therefore implied that there is an actual physical interaction

between the Vsr and the MutL proteins. Consequently, the results also suggested that VSP repair activity is not necessary for Vsr to stimulate mutagenesis. The structure of these two mutants remained similar enough to the wild type protein so as to still allow interaction with MutL.

Even though the yeast two-hybrid system is quite powerful, this technique does have its share of limitations. Being a eukaryotic test system, the environment provided for the *E. coli* proteins under study may not be optimal and they may not exhibit authentic behaviour. There is a possibility that the fusion proteins may not be appropriately folded or even that they have been degraded. Since chimeric proteins are required in this assay, there is also a possibility that the regions required for the interaction of the two test proteins may simply not be accessible.

4.1.2 Domain localization between Vsr and MutL

The yeast two-hybrid system was further used to study the interaction between Vsr (156 A.A.) and MutL (674 A.A.) in an attempt to localise MutL domains essential for Vsr interaction. The results of our deletion analysis showed that the 615 amino acids of the NH_2 terminal fragment (C59 deletion) are sufficient for the two-hybrid interaction with Vsr as well as with mutant H69A (Mansour *et al.* 2001) (Graph 2). It is interesting to note the decrease in the level of β-galactosidase activity of the truncated MutLC59/Vsr compared to the wild type MutL/Vsr. These results suggest that even though the amino terminal domain of MutL is sufficient for interaction, it may not be as efficient. This decrease may be

due to part of the interaction domain being gone or simply to the protein no longer being folded appropriately and preventing the interaction from occurring efficiently.

In analogy, the yeast-two hybrid analysis for MutH indicated that the CTD of MutL is sufficient for physical interaction with MutH (Hall *et al.* 1999), while *in vitro* the NTD of MutL in the presence of ATP is able to stimulate the latent endonuclease activity of MutH (Ban *et al.* 1999). Furthermore, crosslinking studies have shown linking between MutH and both the NTD and CTD of MutL (Ahrends *et al.* 2006). Preliminary crosslinking studies performed by the Friedhoff group have confirmed a link between Vsr and the N-terminal domain of MutL (Personal communication, Friedhoff).

4.1.3 Yeast three-hybrid

With the yeast three-hybrid assay, we intended to investigate the possible competition between Vsr and MutH for access to MutL. Unfortunately, the separate addition of extra Vsr or MutH to the test system did not yield the anticipated results (Graph 3). There was no observed difference in the interaction pattern between MutL/MutH and MutL/Vsr, with or without the presence of a third protein. Since the results were negative, it was not possible to say if the additional third protein actually plays no role or if the system simply cannot detect it.

4.2 Bacterial two-hybrid assay

4.2.1 Study of interaction between Vsr and MMR proteins

Unlike the yeast assay, the bacterial two-hybrid assay monitors protein interactions in their normal *E. coli* environment. We were therefore interested in using this alternate system to further probe the interaction between Vsr and the MMR proteins. The screen was performed and confirmed the Vsr/MutL interaction previously observed in the yeast test system (Mansour *et al.* 2001) (Graph 4). The assay further revealed interactions between MutH/MutL, MutL/MutL, MutS/MutL and MutS/MutS as predicted by the literature (Mansour *et al.* 2001). The lack of interaction between Vsr and either the MutH or the MutS proteins strengthened our hypothesis that Vsr inhibits MMR by interacting with MutL. These results therefore pointed to MutL playing a role in VSP repair and interestingly showed a new putative interaction between MutS/MutH, one that has never been observed previously (Mansour *et al.* 2001). However, the dynamics of MMR (Figure 1) make it very plausible that this is not a direct interaction, but one bridged by MutL (Mansour *et al.* 2001).

It is important to note, however, that the Vsr/MutL, MutH/MutL, MutS/MutL, and MutS/MutH interactions were only observed in a unidirectional manner; similar to what was seen in yeast. Complementation study of the chimeric proteins revealed that the pt25-Mut fusions complemented the mutator phenotype of the appropriate *mut* strain, but the pT18-Mut fusions did not (Mansour *et al.* 2001). Therefore, as in the yeast system, this suggests that some of the proteins may not

be folded properly or that due to the fusions, interacting surfaces may not be available. We concluded overall, that this system is more powerful than the yeast two-hybrid to test *E. coli* mismatch repair proteins.

4.2.2 Domain localization between Vsr and MutL

This system was further used to study the interaction between Vsr and MutL in an attempt to localise both Vsr and MutL domains essential for interaction. Crystallographic data shows that MutL possess two separate domains in the wild type protein: an amino terminal domain (amino acids 1-350) conserved amongst the MutL protein family and a carboxy terminal domain (amino acids 350-615) (Ban & Yang, 1998) (Figure 3). Regrettably, results showed that Vsr did not interact with either of the protein fragments (Mansour *et al.* 2001). The absence of interaction may be due to: missing a crucial part of the interaction surface, the surface not being available, a requirement for both domains, or the fusion protein not being appropriately folded (Graph 5).

In order to identify which part of Vsr interacts with the MutL protein, we looked at five truncation fragments: two NTD (VsrN14 and VsrN19) and three CTD deletions (VsrC124, VsrC112, and VsrC27). The results in graph 5 showed that the deletion of even 27 amino acids from the COOH-terminus of Vsr completely eliminated the interaction with MutL. Surprisingly however, results for the VsrN14 and VsrN19 showed an improved interaction with MutL (Mansour *et al.* 2001). This was counterintuitive since the NTD deletions are known to exhibit reduced

mutagenesis levels as compared to wild type Vsr (Monastiriakos *et al.* 2004). This consequently led us to think that even though MutL does not interact directly with the amino terminal domain of Vsr; the NTD of Vsr plays a role during the interaction of MutL with Vsr. It is possible that the loss of the NTD of Vsr will increase the stability of the protein, giving the increased interaction.

4.2.3 Bacterial three-hybrid

The premise of the bacterial three-hybrid system closely followed the approach used in yeast. However, unlike the yeast system that gave negative results, the bacterial system yielded results that are more significant. The addition of extra Vsr to the test system actually abolished all interactions, even the MutH/MutS, with the sole exception of the MutS dimer (Mansour *et al.* 2001) (Graph 6). This further points to the fact that Vsr interferes with the MMR system and more specifically with the ability of MutL to interact with other Mut proteins.

4.2.4 Summary from the two-hybrid systems (of *in vivo* work)

Previous data, together with our finding that Vsr interacts with MutL (Graph. 1 and 4) suggested a possible mechanism for Vsr-stimulated mutagenesis: physical competition between Vsr and MutH for limiting amounts of MutL. However, there are some pieces of evidence that argue against this:

dimerization (Ban *et al.* 1998; and Ban *et al.* 1999). Then, during MutH activation, ATP hydrolysis causes the dissociation of the MutL amino termini and consequently, the ADP-bound protein can no longer interact with the endonuclease (Ban *et al.* 1998; Ban *et al.* 1999; and Hall *et al.* 1999). The renewed dimerization of MutL is shown to involve a major conformational change and it is suggested that this step could be rate limiting in MMR (Ban *et al.* 1999).

Drawing from this explanation, if VSP repair also requires the MutL protein to hydrolyse its ATP, this would cause MutL to convert from its active form to its inactive ADP-bound form. This event would consequently render MutL incapable of interacting with the other Mut proteins and therefore, of participating in MMR. As discussed previously, Vsr levels are low during the log phase of normal cells and progressively increase during the stationary phase. Intuitively this makes sense since, if Vsr is present during growth phase, it would interfere with MMR. This is exactly what happened when we over-expressed Vsr, MMR was disrupted and the pattern of mutagenesis observed was similar to a *mut*$^-$ strain.

Through its association with Vsr, it is plausible that MutL also hydrolyses its ATP molecule. However, it is uncertain exactly how this happens. From the mutant Vsr data mentioned earlier, we know that VSP repair activity is not necessary for this protein to stimulate mutagenesis. These mutants, even though they are not cable of binding or cutting heteroduplex DNA, are still structurally similar enough to the wild type so as to be able to interact with MutL and stimulate mutagenesis. When the bacterial two-hybrid Vsr fusion proteins were

tested for both mutagenesis and repair activity (complementation in a *vsr*⁻ strain), it was observed that neither Vsr fusion possesses repair capabilities nor do they stimulate mutagenesis (Mansour *et al* 2001). It is conceivable that these proteins are not active since they are in fusions; however, we would have expected at least the T18 version to stimulate mutagenesis since it does interact with MutL. Consequently, this suggests that the interaction between the two proteins is not sufficient to disable MutL. Something, in addition to simple interaction between these two proteins, is responsible for triggering the mutagenesis effect and may have to do with the ATP binding and hydrolysis of MutL.

In addition to understanding what Vsr does to MutL, it is necessary to understand the reverse equation, which is what MutL does to Vsr. Again, by looking at the interaction between MutL and MutH proteins, we might gain some insights into Vsr. The crystal structure of the MutH endonuclease reveals that the enzyme consists of two domains capable of pivoting around each other to cleave the DNA (Ban *et al.* 1998). Ban and Yang (1998) suggested that MutL might stimulate the pivoting action of the protein by interacting with the carboxy terminus. Results from the deletion analysis showed that MutL also interacts with the carboxy terminus of the Vsr endonuclease. It is interesting to note that structurally, this region is far removed from the DNA binding site located in the NH_2-terminal portion of the molecule (Tsutakawa *et al.* 1999 a and b). Moreover, when Vsr is not bound to DNA, the amino terminus is reported as being highly disordered (Tsutakawa *et al.* 1999 a and b). Even though these regions are a distance apart, it is possible that MutL, through its interaction with the carboxy

terminus of Vsr, will facilitate the reordering of the amino terminus. *In vitro* results have shown that Vsr alone is capable of binding and cleaving DNA, but in the presence of MutL the efficiency is increased, i.e. less protein is needed to process a given amount of DNA (Drotschmann *et al.* 1998; Monastiriakos *et al.* 2004). Lastly, it is engaging to note that the removal of the first 19 amino acids of Vsr actually strengthens the two-hybrid interaction with MutL.

4.3 Protein purification

In order to study Vsr *in vitro,* pure protein was required. However, the protocols available for the purification of the Vsr endonuclease fell short of yielding a stable preparation of active protein. Substantial amounts of protein were lost during our original purification process (Cupples *et al.* 1999), mostly due to precipitation, and Vsr was also observed to lose its amino terminal domain (Monastiriakos 2002 and Turner *et al.* 2000). Furthermore, protein activity was a very serious issue with the Connolly protocol (Turner *et al.* 2000). The Connolly group suggested that Vsr had a very weak activity and that MutL converted Vsr from an inactive form into an active one. However, they subsequently found that their protein purification protocol was the cause of the inactivity (personal communication).

Under the old purification protocol, the Vsr prepared was unstable (Cupples *et al.* 1999):

- Was subject to losing its NTD.

- Was subject to protease degradation.
- Aggregated.
- Lost activity upon storage.

The new protocol developed has allowed us to purify a protein that:

- Is free from proteases.
- Does not lose its NTD.
- Demonstrates substantially less aggregation, both during purification and storage.
- Is consistently active. Preparations no longer just stop working.
- Can be stored at -80°C for a considerable length of time without adverse effect.

The activity of Vsr was similar whether purified with the old protocol or the new, (Graph 7) however, the quantity and quality of the new preparations was substantially higher. It is possible that the increased stability of the protein is due to a requirement for DTT as *E. coli* has a strong reducing environment.

4.4 Endonuclease assay

4.4.1 The stimulatory effect of MutL on Vsr activity

The function of MutL in MMR is understood much better than the role it plays in VSP repair. In MMR, MutL brokers interactions between MutS, MutH and UvrD in an ATP dependent manner. It is thought that MutL serves as a molecular

coordinator where it recruits various partner proteins at different steps in the pathway so as to coordinate MMR. The structural similarity of Vsr to Hsp90 suggested that MutL might function as a molecular chaperone, activating its partners by tweaking their three-dimensional structure. We had thought that this model might also apply to the interaction between MutL and Vsr. However, our results proved otherwise. The addition of ATP, ADP or the hydrolysis inhibitor novobiocin showed that these molecules played no role in the effect MutL has on Vsr (Figure 26). This was further substantiated by the data from the MutL mutants defective in ATP binding and/or hydrolysis: all the mutants showed the same effect on Vsr as the wild type protein (Figure 27). This lack of ATP dependence might partially explain why the interaction between Vsr and MutL is so debilitating when Vsr is over expressed. If Vsr can interact with MutL regardless of its conformation, the interaction can occur at all times when needed. However, this is not the case for the other interaction partners involved with MutL; there the MutL protein must be in a specific conformation. Furthermore, the interaction affinities of each of the binding partners are not known at the present time. If in addition to being able to interact with Vsr at all times, MutL has a stronger interaction affinity with Vsr, this would create serious difficulties for MutL's interactions with its other partners. However, this does not explain every thing.

How could the interaction function? When bound to its target C(T/G)AGG substrate, the crystal structure of Vsr reveals that the DNA is actually held by a pincer like structure. This structure is composed of three aromatic residues that

intercalate into the major groove and of the flexible amino-terminus alpha helix that lies across the minor groove (Tsutakawa *et al.* 1999a and 1999b) (Figure 7). The structure also revealed that unless Vsr is bound to its DNA substrate, the NTD is disorganised to the point that the protein could not be crystallized (Morikawa *et al.* 2000). The two-hybrid results suggest that MutL interacts with the CTD of Vsr (Graph 5). It is plausible that this interaction helps Vsr in the conformational change of its NTD, and that it is this process which affects MutL and ultimately results in the spectrum and levels of mutagenicity that we observe. However, the actual mechanism of this interaction is not known.

How does this interaction result in mutagenicity? The Vsr point mutants, which demonstrated mutagenicity levels at par with the wt protein, showed that the endonuclease activity was not required for protein-protein interaction to occur (Graph 1). On the other hand, the NTD deletions mutants, which demonstrated lowered levels of mutagenicity (Monastiriakos *et al.* 2004), not only interacted with MutL but exhibited an increased interaction signal (Graph 5). This decrease in mutagenicity could be explained by the fact that the NTD is deleted and that this causes an alteration to the interaction process and the conformational change of Vsr. As for the increased interaction signal, it is possible that it is simply due to a more stable fusion protein or a better access to the interaction domain. Furthermore, the simple interaction of Vsr with MutL is not responsible for mutagenesis. If this were the case, the NTD deletions and the pt18-Vsr fusion protein would also be mutagenic due simply to their interaction with MutL.

Where does MutL play its role in VSP repair, at the beginning or the end? Does it help Vsr bind to DNA or be released from the DNA? Several pieces of data suggested that MutL may actually play a role in releasing Vsr from the DNA:

1. Under single turn over conditions, MutL did not stimulate Vsr activity (Graph 9). Were MutL to help Vsr bind to DNA, we would have expected to see an increase in the endonucleolytic activity as compared to that of Vsr alone.

2. Vsr is stimulated by MutL in the presence of increased amounts of DNA, where Vsr undergoes multiple turnovers to cleave the substrate, but not under single turnover conditions. This effect was detected when the concentration of MutL is as low as 1/10 that of Vsr (Figure 25).

3. Trypsinolysis experiments of Vsr, with and without DNA, as well as with and without MutL (Polosina *et al.* 2008, in preparation). Results showed that:
   - Vsr was protected from trypsin cleavage by the addition of DNA substrate.
   - Vsr was not protected by the addition of MutL.
   - Vsr was not protected by the addition of MutL and DNA.
   - Vsr was not protected by the addition of MutL, DNA, and ATP or ADP.

Note that in the latter two cases, the Vsr had cleaved the DNA, suggesting that the MutL was causing Vsr to dissociate from the DNA after cleavage.

One piece of evidence that argues against this model.

1. Band shift results from another lab showed increased binding of Vsr to DNA in the presence of MutL (Drotschmann *et al.* 1998). However, the authors were concerned with the activity of their protein preparation and assumed that, since they saw an increase in binding of Vsr to DNA in the presence of MutL, their protein preparation was active. Furthermore, over the years, five different individuals in our lab have tried unsuccessfully to reproduce this experiment.

4.4.2 VSP repair cascade

What can we conclude about Vsr function? The data and results to date begin to reveal a possible cascade of events in the functioning of this protein. Thus far, the mechanism can be broken up into subsequent steps. Cumulative data suggests that the DNA cleaving ability of this protein may be separate from its DNA binding capability. 1- *In vitro,* studies with the wild type protein point to Vsr first recognising the mispair, then cleaving the DNA and subsequently remaining bound (Macintyre and Cupples unpublished). 2- In addition, the study with the NTD truncations showed that mutant Vsr proteins are capable of recognising the target site and cleaving it, but not of staying bound (Monastiriakos *et al.* 2002). Even though these truncations demonstrated reduced cleaving ability of their target, they still demonstrated repair capability. This suggests that the NTD is required for "strong" binding, but not for recognition.

It can then be concluded that after cleaving the DNA, Vsr would stay bound to the DNA in order to protect the nick from ligase simply resealing it. How would the repair then proceed? Could Vsr possibly be responsible for recruiting the DNA polymerase? Or an attractive possibility would be that MutL helps remove Vsr from the DNA and perhaps helps recruit DNA Polymerase I to the nick.

4.5 Conclusions

We know from *in vivo* work that Vsr inhibits MMR probably through MutL and we know that MutL stimulates Vsr *in vitro*. We were interested in the mechanism of both and interested to see if there was a functional link between the two. We were not able to reconcile the two but still found interesting results for both.

The continued study of the Vsr endonuclease will help shed more light on the intricacies of its functioning as well as on those of the MutL protein. It will hopefully also help us understand how the interaction between these two proteins results in the stimulation of Vsr function and in the disabling of MMR.

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