Human mismatch-repair protein MutL homologue 1 (MLH1) interacts with *Escherichia coli* MutL and MutS *in vivo* and *in vitro*: a simple genetic system to assay MLH1 function

Barbara QUARESIMA*, Pietro ALIFANO†, Pierfrancesco TASSONE*, Enrico V. AVVEDIMENTO‡, Francesco S. COSTANZO*¹ and Salvatore VENUTA*

*Dipartimento di Medicina Sperimentale e Clinica 'G. Salvatore', Università degli Studi di Catanzaro 'Magna Græcia', Via Tommaso Campanella 115, 88100 Catanzaro, Italy, †Dipartimento di Scienze e Tecnologie Biologiche ed Ambientali, Università degli Studi di Lecce, Via Monteroni, 73100 Lecce, Italy, and ‡Dipartimento di Biologia e Patologia Cellulare e Molecolare 'L. Califano', Università degli Studi di Napoli 'Federico II', Via S. Pansini 5, 80131 Napoli, Italy

A simple genetic system has been developed to test the effect of over-expression of wild-type or mutated human MutL homologue 1 (hMLH1) proteins on methyl-directed mismatch repair (MMR) in *Escherichia coli*. The system relies on detection of Lac⁺ revertants using MMR-proficient or MMR-deficient *E. coli* strains carrying a lac + 1 frameshift mutation expressing hMLH1 proteins. We report that expression of wild-type hMLH1 protein causes an approx. 19-fold increase in mutation rates. The mutator phenotype was due to the ability of hMLH1 protein to interact with bacterial MutL and MutS proteins, thereby interfering with

the formation of complexes between MMR proteins and mismatched DNA. Conversely, expression of proteins encoded by alleles deriving from hereditary-non-polyposis-colon-cancer (HNPCC) families decreases mutation rates, depending on the specific amino acid substitutions. These effects parallel the MutL- and MutS-binding and ATP-binding/hydrolysis activities of the mutated proteins.

Key words: ATP binding/hydrolysis, genetics, hereditary non-polyposis colon cancer (HNPCC), mismatch repair, mutation.

INTRODUCTION

Hereditary non-polyposis colorectal cancer (HNPCC), an autosomal dominant tumour-predisposing condition, is caused by deficient DNA mismatch repair (MMR). Six human MMR genes have been identified that, when mutated in the germ line, cause susceptibility to this syndrome. The database established by the International Collaborative Group (ICG) on HNPCC (<http://www.nfdht.nl>) has led to the characterization of more than 300 mutations. The majority of these mutations affect the human MutL homologue 1 (hMLH1) (approx. 60%), human MutS homologue 2 (hMSH2) (approx. 35%) and 6 (MSH6) (approx. 5%) genes [1–5]. A variety of nonsense, mis-sense and splicing mutations have been identified in the hMLH1 gene from HNPCC families with clinical features meeting the 'Amsterdam 1 criteria' or 'Amsterdam 2 criteria', which includes extracolonic neoplasms associated with Lynch II syndrome, a hereditary predisposition to the development of cancer (specifically of the breast, colon, ovary, pancreas, uterine and gastric systems) [6]. Moreover, a number of DNA alterations have been reported in hMLH1 from kindreds with tumour predisposition not fulfilling these criteria [7]. Because of the large and increasing number of MMR gene mutations, and the absence of correlation between genotype and phenotype in most of MMR gene mutations, development of biochemical or genetic systems to test mutated MMR gene mutations may be extremely useful in the management and counselling of HNPCC patients.

The evolutionary conserved hMLH1 protein is homologous with the *Escherichia coli* MutL gene product, which is involved in two pathways for DNA MMR. The first pathway, methyl-

directed MMR, enhances the fidelity of chromosomal replication 100–1000 fold and requires the concerted action of the MutS, MutH and UvrD proteins [8,9]. The second pathway, 'very short patch repair', serves to correct G–T mispairing that arise from the spontaneous deamination of 5-methylcytosine residues [10].

More recently, structural/functional studies have clarified some aspects of the biochemistry of the MutL protein. The MMR protein has been shown to belong to the GHF [gyrase b/heat-shock protein 90 (hsp90)/MutL] ATPase superfamily that includes DNA topoisomerase II and hsp90. MutL exists in solution as a homodimer, and ATP binding induces conformational changes promoting direct contact between the N-terminal domains of the monomers, thereby modulating interactions between MutL and other components of the repair machinery [11,12]. The ATP binding/hydrolysis site lies in a 40 kDa N-terminal fragment of MutL that retains all of the conserved residues in the MutL family. The same region of the protein is sufficient to interact with DNA and MutS in a dynamic ternary complex [13], and with MutH, although activation of MutH by MutL may depend also on the C-terminal region [11,12]. The C-terminal region, which is not evolutionarily conserved, has been shown to physically interact with UvrD, a protein conferring UV-radiation resistance [14,15], and to induce MutL dimer formation [11,12]. On the basis of this evidence it has been hypothesized that the ATPase activity of MutL acts as a switch to co-ordinate MMR. Significantly, mutations in the MutL family that cause deficiencies in DNA MMR and a predisposition to cancer mainly occur in the putative ATP-binding site [11].

In eukaryotes, the homologues of the MutS and MutL proteins act in the form of heterodimers that have different mispair-

Abbreviations used: FEN1, flap structure-specific endonuclease 1; GHF, gyrase b/hsp90/MutL; hMLH1, human MutL homologue 1; hMSH2 and hMSH6, human MutS homologues 2 and 6; HNPCC, hereditary non-polyposis colon cancer; hsp90, heat-shock protein 90; IPTG, isopropyl β -D-thiogalactoside; MMR, mismatch repair; NER, nucleotide excision repair; Ni-NTA, Ni²⁺-nitrilotriacetate; ORF, open reading frame; PCNA, proliferating-cell nuclear antigen; PMS1, postmeiotic segregation increased 1; RFC, replication factor C; RPA, replication protein A; S93G (etc.), Ser⁹³ → Gly mutation (etc.).

¹ To whom correspondence should be addressed (e-mail fsc@unicz.it).

recognition properties and different abilities to support MMR [16]. In baker's yeast (*Saccharomyces cerevisiae*), MSH2 forms heterodimers with MSH6 [17,18], and studies of the specificity of MMR have led to the conclusion that the MSH2–MSH6 heterodimer (MutS α) primarily recognizes and corrects single base-pair mismatches, while the MSH2–MSH3 heterodimer (MutS β) acts to correct heteroduplex DNA containing small loops formed by frameshift mutations [17–19]. The major MutL homologue is a heterodimeric complex of MLH1 and postmeiotic segregation increased 1 (PMS1; MutL α) in yeast [20,21], whereas an additional MutL activity involves an MLH1–MLH3 (MutL β) heterodimer and acts in conjunction with MutS β [22]. ATP-dependent assembly of a ternary complex consisting of DNA mismatch and the yeast MutS α and MutL α has been demonstrated [23]. More recently, functional studies on yeast MutL α have confirmed the essential role of the ATPase domains of both MLH1 and PMS1, supporting the view that ATP induces conformational changes in the N-terminal regions that may serve to co-ordinate downstream events during DNA MMR [24].

In human cells, hMLH1 forms a heterodimer with hPMS2 and this complex, referred to as hMutL α , has been shown to interact with the human MutS equivalent hMutS α (hMSH2–hMSH6) and replication factors [25]. Indeed, a number of other proteins have been implicated in MMR, including DNA polymerase δ , RPA (replication protein A), PCNA (proliferating-cell nuclear antigen), RFC (replication factor C), exonuclease 1, flap structure-specific endonuclease 1 [FEN1; 'RAD27' (required for cell-cycle arrest following DNA damage)] and the DNA polymerase- δ - and - ϵ -associated exonucleases. Eukaryotic MMR proteins have also been shown to function in other types of repair and recombination that appear distinct from MMR. MMR proteins function in these processes in conjunction with components of nucleotide excision repair (NER) and recombination [16].

On the basis of these data and on the high degree of similarity in structure and function between the MutL homologues in both eukaryotes and prokaryotes [24,26], we tested the effect of expression of hMLH1 proteins in MMR-proficient and -deficient *E. coli* cells on DNA repair, in an attempt to develop a simple genetic assay to screen hMLH1 alleles.

EXPERIMENTAL

Bacterial strains and growth conditions

E. coli strains used in this study were: SMR506 (*rif^r thi⁻ proB⁻ F'*), the congenic derivatives SMR3406 (*mutS201::Tn5*) and SMR3428 (*mutL211::Tn5*), which were a gift from Dr S. M. Rosenberg (Department of Molecular and Human Genetics, Baylor College of Medicine, Houston, TX, U.S.A.). The strain DH5 α [*F⁻ Φ 80d lacZ Δ M15 endA1 recA1 hsdR17 supE44 thi-1 λ -gyrA96 Δ (lacZYA-argF) U169]* was used in cloning procedures and the strain BL21 λ DE3 (*F⁻ ompT r_B⁻ m_B⁻*) was used to overexpress recombinant proteins [27]. *E. coli* strains were grown in Luria–Bertani broth or Luria–Bertani agar supplemented with 50 μ g/ml ampicillin when requested. MacConkey–lactose agar was used in the test of Lac papillae [28] (the test of Lac papillae, to assay mutator phenotype, is performed by counting papillae in a zone of clearing around the spot of dry nalidixic acid dropped on 0.1 ml of saturated culture spread on Luria–Bertani plates).

Plasmids and cloning procedures

The entire open reading frame (ORF) of hMLH1 was a gift of Professor Dr Josef Jiricny, Medizinische Fakultät, Institut für

Medizinische Radiobiologie (IMR) der Universität Zürich und des Paul Scherrer Instituts, Zurich, Switzerland, and was cloned between the *Bam*HI and *Kpn*I sites of pUC19 (pUMLH1 wild-type) translationally in-frame with the start codon of the vector *lacZ*, under control of the inducible *lac* promoter. Site-directed mutagenesis of the hMLH1 cDNA insert was obtained using the QuickChange Kit (Stratagene). The pUMLH1 mutants were generated with the following primers:

For pUMLH1 S93G: forward: 5'-CCTTTGAGGATTTAGC-CGGTATTTCTACCTATGGC-3'; reverse: 5'-GCCATAGG-TA GAAATACCGGCTAAATCCTCAAAGG-3'

For pUMLH1 K84E: forward: 5'-TCACTACTAGTGAAGT-GCAGTCCTTT-3'; reverse: 5'-AAAGGACTGCAGTTCAC-TAGTAGTGA-3'

For pUMLH1 V716M: forward: 5'-TGGAAGTGGACTAT-GGAACACATTGT-3'; reverse: 5'-ACAATGTGTTCCATA-GTCCACTTCCA-3'

The strain DH5 α was used in cloning procedures.

All these constructs were verified by DNA sequencing to confirm that both the mutation and the remainder of the hMLH1 cDNA insert were correct.

The plasmids pMQ393 and pMQ395, expressing histidine-tagged MutL and MutS proteins used in the present study were a gift from Professor Martin G. Marinus, Department of Biochemistry and Molecular Pharmacology, University of Massachusetts Medical School, Worcester, MA, U.S.A. [29].

Lac⁺ reversion test

The *E. coli* strains SMR506 (*rif^r thi⁻ proB⁻ F'*) and the congenic derivatives SMR3406 (*mutS201::Tn5*) and SMR3428 (*mutL211::Tn5*) were transfected with plasmids engineered to express wild-type (pUMLH1) or mutated (pUMLH1 S93G, pUMLH1 K84E and pUMLH1 V716M) hMLH1 proteins. The Lac papillae reversion test was performed essentially as described in [28]. Lac⁺ reversion rates were determined by fluctuation analysis as previously described [30].

Western-blot analysis

E. coli strain SMR506 was transfected with pUMLH1 wild-type, pUMLH1 S93G, pUMLH1 K84E, pUMLH1 V716M or control vector pUC19, and grown to early exponential phase. After incubation with 1 mM isopropyl β -D-thiogalactoside (IPTG) for 2 h at 37 °C, bacteria were lysed by sonication in buffer H (20 mM Hepes/100 mM KCl/5 mM MgCl₂/1 mM CaCl₂/0.1 % Nonidet P40/1 mM PMSF, pH 7.1), and protein products were purified from the soluble-extract fraction by centrifugation for 12 min at 12000 g. The proteins were electrophoresed on SDS/10 % -PAGE gels and transferred to a nitrocellulose filter (Immobilon™ P; Millipore). After addition of the blocking mixture [5 % (w/v) BSA in PBS, pH 7.4], the membrane was incubated with a 1:200 dilution of rabbit anti-(hMLH1) antibody (Santa Cruz Biotechnology) for 2 h at room temperature. Bound antibody was detected with anti-rabbit horseradish-peroxidase-conjugated secondary antibody (1:5000) and by enhanced chemiluminescence (Santa Cruz Biotechnology).

MutL–hMLH1 and MutS–hMLH1 interaction *in vitro*

E. coli strains SMR506 harbouring pUMLH1 wild-type, pUMLH1 S93G, pUMLH1 K84E, pUMLH1 V716M or pUC19,

and BL21 λ DE3 harbouring pMQ393 or pMQ395 plasmids were grown to early exponential phase and induced with IPTG as described above. Bacteria were lysed by sonication in buffer H, and protein products were purified from the soluble-extract fraction by centrifugation. The supernatants normalized to the amounts of the recombinant proteins [histidine-tagged MutL (from plasmid pMQ393) or MutS (from plasmid pMQ395)] were incubated with Ni²⁺-nitrilotriacetate (Ni-NTA)-agarose for 2 h at 4 °C. Histidine-tagged proteins were collected by centrifugation, washed four times with buffer H and incubated for 2 h at 4 °C with the supernatants from bacteria expressing pUMLH1 wild-type, pUMLH1 S93G, pUMLH1 K84E, pUMLH1 V716M or control vector pUC19, normalized to the amount of expressed recombinant proteins. Samples were centrifuged and washed four times with the buffer H before addition of SDS/PAGE sample buffer. The samples were then heated at 95 °C and loaded on to a SDS/10 % -PAGE gel.

ATP binding/hydrolysis assay

E. coli strain SMR506 harbouring pUMLH1 wild-type, pUMLH1 S93G, pUMLH1 K84E, pUMLH1 V716M or pUC19 were grown to early-exponential phase and induced with IPTG. Bacteria were then lysed by sonication in buffer A [50 mM Tris/HCl (pH 7.5)/150 mM NaCl/1 % Nonidet P40/protease inhibitor cocktail (Roche)]; after centrifugation, the supernatant was incubated with mouse anti-(hMLH1) (BD Biosciences Pharmingen, San Diego, CA, U.S.A.) for 2 h at 4 °C. The samples were subsequently incubated for 1 h at 4 °C with Protein A-Sepharose. To determine ATP hydrolysis, the pellets were collected by centrifugation, washed four times with buffer A and incubated with 0.25, 0.5 or 1 mM of [α -³²P]ATP in 15 μ l of buffer B (20 mM Tris/30 mM KCl/5 mM MgCl₂/1 mM dithiothreitol) at 37 °C for 5, 15 or 30 min. A 2 μ l portion of each reaction mix was spotted on a polyethyleneimine TLC plate. Labelled ATP and ADP were separated by developing the TLC plate in 1 M formic acid/0.4 M LiCl₂.

To quantify the amounts of ATP and ADP bound to the hMLH1 proteins at a steady state, double-filter binding assays were performed in the presence of [α -³²P]ATP or [γ -³²P]ATP, as previously described [31]. Nitrocellulose (Hybond-C; Amersham) was used to retain all proteins, including free and nucleotide-associated proteins. Nylon (Hybond-N+; Amersham) was used to retain free nucleotides. Filter papers were pre-soaked in the reaction buffer and stacked on to a 96-well dot-blot apparatus in the order: 3 MM gel-blot paper, nylon and nitrocellulose membrane. hMLH1 proteins were affinity-purified from the *E. coli* extracts using mouse anti-(hMLH1) and Protein A-Sepharose as described above. After centrifugation and washing with buffer A, ATP binding was performed in the same buffer system (buffer B) used for ATP hydrolysis, and 0.1 mM [α -³²P]ATP or [γ -³²P]ATP in parallel at room temperature. Samples (25 μ l) were withdrawn and applied to the blot apparatus at various time points from 2 to 60 min. The steady state was reached in 15 min. Following drawing of the samples through the membranes by vacuum, wells were washed by adding 100 μ l of the reaction buffer. The membranes were removed and air-dried, and ³²P-free and protein-associated nucleotides were quantified using the PhosphorImager (Molecular Dynamics) system.

RESULTS

Effects of expression in *E. coli* of hMLH1 alleles on MMR activity

A simple genetic system was developed to test the effect of over-expression of wild-type or mutated hMLH1 proteins on MMR in

Figure 1 Western-blot analysis of recombinant hMLH1 proteins

E. coli cells (strain SMR506) expressing wild-type hMLH1 ('wt'), mutated hMLH1(S93G) ('S93G'), hMLH1(K84E) ('K84E') and hMLH1(V716M) ('V716M') or control vector ('control'), were induced with IPTG and lysed by sonication to recover protein products from the soluble-extract fraction by centrifugation. Proteins were electrophoresed on an SDS/10 % -PAGE gel and analysed by Western blot using a rabbit anti-hMLH1 antibody.

Table 1 Mutation rates in MMR-proficient and MMR-deficient *E. coli* strains expressing hMLH1 proteins

Mutation rates (column 4) were determined by fluctuation assay as described in the Experimental section. Data were obtained from five independent experiments. In each experiment, 20 independent cultures of each strain were tested. Means were determined between the values obtained by fluctuation analysis in each set of experiments. Mutation rates and S.D. values (in parentheses) are shown relative to the rate observed for SMR506 harbouring pUC19 (8.7×10^{-10}). Different quantitative levels of mutator phenotype in strains carrying different mismatch repair-defective mutations, such those observed here in SMR3428 and SMR3406 transformed with pUC19, have been previously found in the same strains. A possible explanation for the higher mutation frequencies in the MutS strain than in MutL has been suggested in [30].

Strain	Relevant genotype	Plasmid	Relative mutation rate to Lac ⁺
SMR506	Wild-type	pUC19	1.0 $\pm$ 0.3
		pUMLH1 wt	19.4 $\pm$ 2.4
		pUMLH1 S93G	2.1 $\pm$ 0.7
		pUMLH1 K84E	31.2 $\pm$ 3.7
		pUMLH1 V716M	9.9 $\pm$ 1.6
SMR3428	<i>mutL211::Tn5</i>	pUC19	19.1 $\pm$ 2.1
		pUMLH1wt	19.2 $\pm$ 1.2
		pUMLH1S93G	18.7 $\pm$ 1.1
		pUMLH1 K84E	29.1 $\pm$ 3.5
		pUMLH1 V716M	24.1 $\pm$ 2.1
SMR3406	<i>mutS201::Tn5</i>	pUC19	240.1 $\pm$ 28.1
		pUMLH1wt	246.3 $\pm$ 19.7
		pUMLH1S93G	224.8 $\pm$ 40.8
		pUMLH1 K84E	238.1 $\pm$ 11.3
		pUMLH1 V716M	238.1 $\pm$ 11.3

E. coli. The system relies on congenic MMR-proficient or MMR-deficient *E. coli* strains carrying a *lac* +1 frameshift mutation (CCC to CCCC) within a *lacI-lacZ* fusion residing on an F' episome. The mutation is polar on *lacZ*, making the cell Lac⁻. Lac⁺ reversion mutations can restore the reading frame by deletion or addition anywhere between two out-of-frame stop codons in a window of about 70 bp. In many cases, Lac⁺ revertants are detected as a consequence of -1 deletions in the small nucleotide repeat [28]. The *E. coli* strains were transfected with plasmids expressing wild-type or mutated hMLH1 proteins. The vectors were pUC19 derivatives containing the coding sequences from wild-type hMLH1 or mutated hMLH1(S93G), hMLH1(K84E), hMLH1(V716M) alleles translationally in frame with the start codon of the vector *lacZ*, under control of the inducible *lac* promoter (see the Experimental section) (S93G, as an example, means a Ser⁹³ → Gly mutation). Because of the presence of many codons in the hMLH1 sequence, which are poorly translated in *E. coli*, expression of the heterologous protein in bacteria upon IPTG induction was tested by Western-blot analysis (Figure 1). A polyclonal antibody directed against the wild-type hMLH1 protein detected comparable amounts of specific peptides of the expected molecular mass in all transfected strains that were not present in the untransfected cells.

Figure 2 Interaction of hMLH1 proteins with histidine-tagged MutL

(A) Amounts of histidine-tagged MutL protein were immobilized on Ni-NTA-agarose (lower panel), and challenged with *E. coli* extracts expressing wild-type ('wt') or mutated hMLH1 proteins ('S93G', 'K84E' and 'V716M') as described in the Experimental section. After extensive washing, the amounts of hMLH1 proteins interacting with MutL were determined by Western blot using a rabbit anti-hMLH1 antibody (upper panel). The expected molecular masses of hMLH1 and MutL proteins are indicated on the left. (B) Quantitative analysis of the interaction between hMLH1 proteins and histidine-tagged MutL. Values are means for at least five independent experiments shown in (A) and they represent percentages ($\pm$ S.E.M.) of densitometric values.

Since it is known that the MMR repair capacity is repressed in nutritionally stressed bacteria by depletion of MutS and MutH proteins [32,33], the Lac reversion test was performed under non-selective growth conditions in order to detect spontaneous (non-adaptive) reversions. The results of this analysis (Table 1) indicated that reversion frequencies to Lac⁺ were approx. 19-fold higher in MMR-proficient *E. coli* cells harbouring the recombinant plasmid expressing the wild-type hMLH1 protein than in a control strain transfected by a vector plasmid. A comparable increase in mutation rates (about 10-fold) was detected in *E. coli* cells expressing the mutated hMLH1(V716M) protein, and even more (about 31-fold) in bacteria expressing hMLH1(K84E). Conversely, the increase was not detected in *E. coli* cells producing the mutated hMLH1(S93G) protein.

We also measured Lac⁺ reversion rates in MMR-deficient *E. coli* strains transfected with the different hMLH1 expressing plasmids (Table 1). At variance with the MMR-proficient strain, MutS-deficient bacteria, transfected with the vector plasmid or with the different hMLH1-expressing plasmids, all exhibited higher rates of Lac⁺ reversion with similar frequencies (about 250-fold higher than in the Mut⁺ control strain). Similar results were obtained with MutL-deficient bacteria. However, these cells exhibited a lower increment in mutation rates (about 19-fold higher than in the Mut⁺ control strain) that was the same either in the absence or in the presence of wild-type hMLH1 or mutated hMLH1(S93G) or hMLH1(V716M) proteins. This increment was comparable with that observed in Mut⁺ strain when transformed with the wild-type-hMLH1-expressing plasmid. Note that this value was a little higher (29-fold) in MutL-deficient bacteria expressing hMLH1(K84E).

Table 2 Features of hMLH1 proteins

hMLH1 allele	Relative mutation rate to Lac ⁺⁺	Interaction with MutL†	Interaction with MutS†	ATP binding/hydrolysis†
Control	1.0	—	—	—
hMLH1 wild-type	19.4	+	+	+
hMLH1 S93G	2.1	+	+	+
hMLH1 K84E	31.2	+/-	+	+
hMLH1 V716M	9.9	+	+	+

* Values are reported from Table 1.

† The different abilities of wild-type and mutated hMLH1 proteins to bind either histidine-tagged MutL or MutS, or to bind/hydrolyse ATP are represented as symbols (+, —). The assignment of these symbols is consistent with the quantitative analyses shown in Figures 2–4.

Figure 3 Interaction of hMLH1 proteins with histidine-tagged MutS

(A) The interactions between histidine-tagged MutS and hMLH1 proteins were analysed as described in the legend to Figure 2(A). The expected molecular masses of hMLH1 (upper panel) and MutS (lower panel) proteins are indicated on the left. (B) The quantitative analysis of the interaction between hMLH1 proteins and histidine-tagged MutS was performed as described in the legend to Figure 2(B).

hMLH1 interferes with the *E. coli* MMR machinery

The results described above suggested that expression of hMLH1 proteins interfered with the activity of the endogenous MMR machinery. We hypothesized that wild-type and several hMLH1 proteins might interact with specific bacterial MMR protein(s), thereby subtracting them from the damage lesions. To test this hypothesis we analysed the ability of hMLH1 proteins to interact with *E. coli* MMR proteins. The interaction between hMLH1 proteins and MutL was assayed *in vitro* by pull-down experiments (Figure 2). To this end, different amounts of histidine-tagged MutL protein were immobilized on Ni-NTA-agarose. Note that the histidine-tag at the N-terminus of MutL did not affect the normal activity of the protein [29]. The tagged MutL was challenged with *E. coli* extracts expressing wild-type or mutated

Figure 4 ATP binding/hydrolysis of hMLH1 proteins

(A) *E. coli* strain SMR506 harbouring pUMLH1 wild-type ('hMLH1', lane 2), pUMLH1 K84E ('hMLH1K84E', lane 3) pUMLH1 S93G ('hMLH1S93G', lane 4) or pUC19 ('control', lane 1) were grown to early-exponential phase, induced and lysed. The soluble-extract fractions were incubated first with mouse anti-hMLH1, and then with Protein A–Sepharose. Insoluble immunocomplexes were then collected by centrifugation, re-suspended, washed and incubated with 0.5 mM [α - 32 P]ATP for 15 min as detailed in the Experimental section. Labelled ATP and ADP were separated by TLC. (B) ATP hydrolysis of hMLH1 proteins as a function of ATP concentrations. Values are means for at least ten independent experiments exemplified in (A), obtained by incubating with 0.25, 0.5 and 1 mM labelled ATP for 5 or 15 min, and they represent percentages ($\pm$ S.E.M.) of densitometric values. (C) Time course of ATP hydrolysis of hMLH1 proteins. Values are means for at least ten independent experiments exemplified in (A), obtained by incubating with 0.5 mM labelled ATP for 5, 15 and 30 min, and they represent percentages ($\pm$ S.E.M.) of densitometric values. (D) ATP binding of hMLH1 proteins at steady state. ATP binding at steady state was determined by double-filter binding assays with [α - 32 P]ATP or [γ - 32 P]ATP as described in the Experimental section. Values are means $\pm$ S.D. for at least five independent experiments.

hMLH1 proteins. After extensive washing, hMLH1 protein interacting with MutL was assayed by Western blotting using a rabbit anti-hMLH1 antibody (Figure 2A). The percentage of binding activity determined by densitometric scanning of the filters is reported in Figure 2(B). Bacterial MutL retained wild-type hMLH1 and, albeit to a lesser extent, mutated hMLH1 (V716M). The interaction of MutL with hMLH1 mutants was markedly decreased with hMLH1(K84E) (about 90 %) and to lesser extent with hMLH1(S93G) (about 70 %) compared with the wild-type hMLH1 (Figure 2B). The mutant at the C-terminus [hMLH1(V716M)] showed only a modest decrease in interaction (about 50 %) (Figure 2B). This finding explains the genetic data indicating that these proteins interfered with the *E. coli* MMR machinery. The effects elicited by wild-type and mutated hMLH1 proteins in the Lac⁺ reversion test (Table 1) were associated with the different ability of the hMLH1 proteins to interact with the *E. coli* MutL (Figures 2A and 2B). In fact, wild-type hMLH1

and hMLH1(V716M) interacted strongly with MutL and determined a relevant increase in mutation rates (Table 2). Conversely, hMLH1(S93G) did not affect substantially the frequency of Lac⁺ reversion, and, accordingly, its binding to MutL was weaker (Table 2). However, hMLH1(K84E) increased the mutation rates and interacted with MutL with a lower affinity compared with the wild-type hMLH1 (Table 2). This finding suggested that hMLH1(K84E) was interacting with another component of the complex to down-regulate MMR function. Because MutL was demonstrated to be part of a dynamic ternary nucleoprotein complex with MutS and mismatched DNA, we tested by Western blotting the ability of hMLH1 proteins to interact with histidine-tagged MutS (Figure 3A). Figure 3(B) shows that hMLH1 binds recombinant MutS and that the interaction is considerably stronger with hMLH1(K84E) (about 3-fold) than that found with the other hMLH1 proteins. These data indicated that the mutator phenotype determined by

expression of hMLH1(K84E) is dependent on its ability to interfere with bacterial MutS.

ATP-binding/hydrolysis of wild-type and mutated hMLH1 proteins

Since ATP binding modulates interactions between MutL-like proteins and other components of the repair machinery, we reasoned that the different ability of the hMLH1 proteins to interact with the *E. coli* MutL and MutS might be dependent on different ATP-binding/hydrolysis properties. We therefore performed an *in vitro* ATP-binding/hydrolysis assay using *E. coli* extracts containing wild-type or mutated hMLH1 proteins and [α - 32 P]ATP (Figure 4). In this assay we used conditions that eliminated endogenous ATPase activity completely, thereby allowing detection of specific activity associated to the hMLH1 proteins (see the Experimental section). This is shown in Figure 4, where the hydrolysis product was only apparent when extracts expressing hMLH1 proteins (Figure 4A, lanes 2–4) were used, and was absent in the control extract (Figure 4A, lane 1). ATPase activity was determined at different ATP concentrations (Figure 4B) and as a function of time (Figure 4C). Figure 4 shows that the ATP-hydrolysis activities were different in the hMLH1 proteins. The activity was maximal in wild-type hMLH1, moderately decreased in hMLH1(V716M) (83.3% at 15 min of the reaction, using 0.5 mM ATP) and more markedly decreased in hMLH1(S93G) (50%) and hMLH1(K84E) (33.3%).

Since ATP binding must precede hydrolysis, we investigated the ATP-binding properties of the hMLH1 proteins. The results of double-filter binding assays using [α - 32 P]ATP and [γ - 32 P]ATP, which permits the quantification of the amounts of ATP and ADP bound to the proteins (see the Experimental section), demonstrated that decreased ATP hydrolysis of mutant hMLH1 proteins coincided with decreased ability to bind ATP (Figure 4D). This finding suggested that the ATPase defect is primarily due to inefficient binding of the nucleotide.

DISCUSSION

The original aim of the present study was to develop a simple genetic assay to test hMLH1 mutations from HNPCC kindreds. The high amino-acid-sequence homology between members of the MutL family prompted us to use *E. coli* as a control for the set-up of the assay. The sequence homology between *E. coli* MutL and hMLH1 is very impressive at their N-terminus, implying that the conserved N-terminal region carries out similar functions such as ATP binding/hydrolysis and interactions with other components of the repair machinery. It is not surprising that the majority of the reported mutations with dominant mutator phenotypes in *E. coli* MutL [34] are within the conserved N-terminal region, and that more than 50% of mis-sense mutations found in hMLH1 of HNPCC [35] are also within this equivalent conserved region.

Expression of wild-type hMLH1 in a MMR-proficient strain of *E. coli* results in a moderate mutator phenotype comparable with that of a MutL-deficient strain. Expression of N- or C-terminally mutated hMLH1 alleles had variable effects: hMLH1(S93G) did not enhance mutation rates significantly, whereas the effects produced by hMLH1(V716M) were similar to those of the wild-type hMLH1 gene. hMLH1(K84E) was the stronger mutator allele among those analysed (Table 1). The ability of eukaryotic MMR proteins to interfere with the normal bacterial MMR pathway is not novel. It has been reported that expression of hMSH2, a human homologue of MutS, in *E. coli* causes a dominant mutator phenotype, but the interaction with the

bacterial MMR proteins was not tested directly [36]. We have evidence that the mutator phenotype parallels with the ability of the hMLH1 proteins to interact with MutL and MutS *in vitro* (Table 2). Indeed, the N-terminal hMLH1(S93G) mutation greatly decreased (to about 30%) the interaction with MutL (Figure 2), whereas hMLH1(K84E), although interacting less efficiently with MutL (about 10%) than wild-type hMLH1, displayed the maximal MutS-binding activity (Figure 3).

In the Lac-reversion test the *E. coli* mutS mutant generally exhibited a significantly stronger mutator phenotype than the mutL mutant. It has been suggested that, in the mutL cells, MutS binds to mismatches and is able to kill or remove heteroduplexes that cannot be repaired by MMR [28]. Here we show that all the hMLH1 proteins are able to interact with *E. coli* MutS *in vitro*, albeit to different extents (Figure 3 and Table 2). However, all hMLH1 proteins save hMLH1(K84E) did not alter the mutation rates in mutL background (Table 1). It is possible that the weak MutS-binding activity of wild-type hMLH1 or mutated hMLH1(S93G) or hMLH1(V716M) proteins did not disturb substantially the function of MutS *in vivo*. Indeed, it has been reported that, in exponentially growing bacteria, MutS is present in slight excess over MutL and MutH [32]. In contrast, hMLH1(K84E) showed a considerably stronger affinity to MutS (Figure 3) and caused a higher mutation rate in mutL cells (Table 1).

These findings can be interpreted by taking into account recent results on the ATPase activity and associated structural transformations of proteins belonging to the MutL family [11,12,24]. The ATP-binding site of MutL is composed of constant and variable segments. The constant domain is well ordered, even in the absence of the nucleotide, and contains three of the four sequence motifs (I, II and IV) conserved in the GH1 ATPase superfamily. These motifs are: helix A, strands 2 and 6 and the loop following strand 2 in MutL, and make up the adenine-binding pocket [11]. The variable domain, which consists of the so-called ATP 'lid' (upon nucleotide association) and the L3 loop, becomes structurally ordered upon nucleotide binding. The ATP 'lid' contains motif III (residues 91–100 in MutL and 96–105 in hMLH1) and about 20 residues which precede this site and are conserved only in the MutL family. The ATP 'lid' and the L3 loop are sensors of the hydrolysis of γ -phosphate, and the conformational changes in the N-terminal regions of the MutL dimer may serve to modulate interactions with components of MMR machinery [12]. hMLH1 mutations K84E and S93G map within this region and affect two residues notably conserved among members of the MutL family. It is noteworthy that mutation S93G did not show any effect in an *in vitro* MMR complementing assay [37].

In addition to the regions directly involved in ATP binding/hydrolysis, the members of the MutL family share several regions potentially involved in N-terminal dimerization. The L2 loop (residues 150–162 in MutL and 155–167 in hMLH1) is the only conserved region in the MutL family that is not directly associated within nucleotide binding, but instead contributes prominently to the dimer interface. L2 loop is composed of residues that become ordered following ATP binding in LN40, a 40 kDa ATPase fragment of *E. coli* MutL. Conservation of this interface may account for the ability of wild-type hMLH1 to interact with *E. coli* MutL (Figure 2). Mutation S93G, which is located within a region (the ATP 'lid') that is not directly involved in ATP hydrolysis, severely affects ATP binding and hydrolysis (Figure 4), and markedly decreases its ability to bind MutL (Figure 2). Defective ATP binding is expected to prevent structuring of the interface. In fact, mutation G98A mapping close to S93G dramatically decreases ATP binding and the ability of yeast MLH1 to form heterodimers with PMS1 [24].

Similarly, mutation K84E also affects ATP binding (Figure 4) and decreases the MutL-binding activity of the protein (Figure 2). Indeed, there is evidence that the conserved K84 (Lys⁸⁴) residue, which corresponds to K79 in *E. coli* MutL, is directly involved in ATP binding by donating hydrogen bonds to the β -phosphate residue [12]. However, the phenotype displayed by K84E in the Lac reversion test is very different with respect to that of S93G (Table 1). Intriguingly, K84E enhances the ability of hMLH1 to interact with MutS (Figure 3). This unexpected property accounts for the strong mutator phenotype that is associated with hMLH1(K84E) expression in *E. coli*. To date, there is no evidence that this residue participates in the interaction with MutS. Even though the molecular basis for the interaction between MutS and MutL is elusive on the basis of crystallographic studies, the candidate region for this interaction is an interface that is composed mainly of the two L45 loops ('L45' stands for loop 45; it is one of the five loops (L1, L2, L3, L45 and the ATP lid) of the LN40-adenosine 5'-[β , γ -imido]-triphosphate complex). Formation of this interface depends on both nucleotide binding and association of the N-terminal region of MutL.

In conclusion, different N-terminal mis-sense mutations in hMLH1 are predicted to give different results in the *E. coli* assay, depending on their ability to interact with the components of the bacterial MMR system (Table 2). Testing these hMLH1 mutations in this simple genetic system would be therefore useful for genetic counselling of HNPCC kindreds by identifying defective alleles.

This work was supported by grants from Programma Operativo Plurifondo (POP, Regione Calabria) and from MURST (Ministero Università e Ricerca Scientifica e Tecnologica) (COFIN 1999 and CLUSTER C004). We thank Professor Dr Josef Jiricny for providing us with the entire ORF of hMLH1, Professor Martin G. Marinus for providing us with the MutL and MutS expression vectors and Professor Susan M. Rosenberg for providing us with the SMR506, SMR3406 and SMR3428 *E. coli* strains.

REFERENCES

- Buermeyer, A. B., Deschenes, S. M., Baker, S. M. and Liskay, R. M. (1999) Mammalian DNA mismatch repair. *Annu. Rev. Genet.* **33**, 533–564
- Jiricny, J. and Nystrom-Lahti, M. (2000) Mismatch repair defects in cancer. *Curr. Opin. Genet. Dev.* **10**, 157–161
- Lynch, H. T. (1999) Hereditary nonpolyposis colorectal cancer (HNPCC). *Cytogenet. Cell Genet.* **86**, 130–135
- Peltomaki, P. (2001) Deficient DNA mismatch repair: a common etiologic factor for colon cancer. *Hum. Mol. Genet.* **10**, 735–740
- Shibata, D. and Aaltonen, L. A. (2001) Genetic predisposition and somatic diversification in tumor development and progression. *Adv. Cancer Res.* **80**, 83–114
- Vasen, H. F., Mecklin, J. P., Khan, P. M. and Lynch, H. T. (1991) The International Collaborative Group on Hereditary Non-Polyposis Colorectal Cancer (ICG-HNPCC). *Dis. Colon Rectum.* **34**, 424–425
- Quaresima, B., Grandinetti, C., Baudi, F., Tassone, P., Barbieri, V., Conforti, S., Avvedimento, E. V., Costanzo, F. and Venuta, S. (1998) Hereditary nonpolyposis colorectal cancer: identification of novel germline mutations in two kindreds not fulfilling the Amsterdam criteria. *Hum. Mutat.* **12**, 433
- Modrich, P. (1987) DNA mismatch correction. *Annu. Rev. Biochem.* **56**, 435–466
- Modrich, P. and Lahue, R. (1996) Mismatch repair in replication fidelity, genetic recombination, and cancer biology. *Annu. Rev. Biochem.* **65**, 101–133
- Lieb, M., Allen, E. and Read, D. (1986) Very short patch mismatch repair in phage lambda: repair sites and length of repair tracts. *Genetics.* **114**, 1041–1060
- Ban, C. and Yang, W. (1998) Crystal structure and ATPase activity of MutL: implications for DNA repair and mutagenesis. *Cell* **95**, 541–552
- Ban, C., Junop, M. and Yang, W. (1999) Transformation of MutL by ATP binding and hydrolysis: a switch in DNA mismatch repair. *Cell* **97**, 85–97
- Galio, L., Bouquet, C. and Brooks, P. (1999) ATP hydrolysis-dependent formation of a dynamic ternary nucleoprotein complex with MutS and MutL. *Nucleic Acids Res.* **27**, 2325–2331
- Hall, M. C., Jordan, J. R. and Matson, S. W. (1998) Evidence for a physical interaction between the *Escherichia coli* methyl-directed mismatch repair proteins MutL and UvrD. *EMBO J.* **17**, 1535–1541
- Mechanic, L. E., Frankel, B. A. and Matson, S. W. (2000) *Escherichia coli* MutL loads DNA helicase II onto DNA. *J. Biol. Chem.* **275**, 38337–38346
- Kolodner, R. D. and Marsischky, G. T. (1999) Eukaryotic DNA mismatch repair. *Curr. Opin. Genet. Dev.* **9**, 89–96
- Johnson, R. E., Kovvali, G. K., Prakash, L. and Prakash, S. (1996) Requirement of the yeast MSH3 and MSH6 genes for MSH2-dependent genomic stability. *J. Biol. Chem.* **271**, 7285–7288
- Alani, E. (1996) The *Saccharomyces cerevisiae* Msh2 and Msh6 proteins form a complex that specifically binds to duplex oligonucleotides containing mismatched DNA base pairs. *Mol. Cell. Biol.* **16**, 5604–5615
- Marsischky, G. T., Filosi, N., Kane, M. F. and Kolodner, R. D. (1996) Redundancy of *Saccharomyces cerevisiae* MSH3 and MSH6 in MSH2-dependent mismatch repair. *Genes Dev.* **10**, 407–420
- Prolla, T. A., Christie, D. M. and Liskay, R. M. (1994) Dual requirement in yeast DNA mismatch repair for MLH1 and PMS1, two homologs of the bacterial *mutL* gene. *Mol. Cell Biol.* **14**, 407–415
- Prolla, T. A., Pang, Q., Alani, E., Kolodner, R. D. and Liskay, R. M. (1994) MLH1, PMS1, and MSH2 interactions during the initiation of DNA mismatch repair in yeast. *Science* **265**, 1091–1093
- Flores-Rozas, H. and Kolodner, R. D. (1998) The *Saccharomyces cerevisiae* MLH3 gene functions in MSH3-dependent suppression of frameshift mutations. *Proc. Natl. Acad. Sci. U.S.A.* **95**, 12404–12409
- Habraken, Y., Sung, P., Prakash, L. and Prakash, S. (1998) ATP-dependent assembly of a ternary complex consisting of a DNA mismatch and the yeast MSH2–MSH6 and MLH1–PMS1 protein complexes. *J. Biol. Chem.* **273**, 9837–9841
- Tran, P. T. and Liskay, R. M. (2000) Functional studies on the candidate ATPase domains of *Saccharomyces cerevisiae* MutL α . *Mol. Cell. Biol.* **20**, 6390–6398
- Gu, L., Hong, Y., McCulloch, S., Watanabe, H. and Li, G. M. (1998) ATP-dependent interaction of human mismatch repair proteins and dual role of PCNA in mismatch repair. *Nucleic Acids Res.* **26**, 1173–1178
- Pang, Q., Prolla, T. A. and Liskay, R. M. (1997) Functional domains of the *Saccharomyces cerevisiae* Mlh1p and Pms1p DNA mismatch repair proteins and their relevance to human hereditary nonpolyposis colorectal cancer-associated mutations. *Mol. Cell. Biol.* **17**, 4465–4473
- Studier, F. W. and Moffatt, B. A. (1986) Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. *J. Mol. Biol.* **189**, 113–130
- Longerich, S., Galloway, A. M., Harris, R. S., Wong, C. and Rosenberg, S. M. (1995) Adaptive mutation sequences reproduced by mismatch repair deficiency. *Proc. Natl. Acad. Sci. U.S.A.* **92**, 12017–12020
- Drotschmann, K., Aronsham, A., Fritz, H. J. and Marinus, M. G. (1998) The *Escherichia coli* MutL protein stimulates binding of Vsr and MutS to heteroduplex DNA. *Nucleic Acids Res.* **26**, 948–953
- Luria, S. E. and Delbruck, M. (1943) Mutations of bacteria from virus sensitivity to virus resistance. *Genetics* **28**, 491–511
- Wong, I. and Lohman, T. M. (1993) A double-filter method for nitrocellulose-filter binding: application to protein–nucleic acid interactions. *Proc. Natl. Acad. Sci. U.S.A.* **90**, 5428–5432
- Feng, G., Tsui, H. C. and Winkler, M. E. (1996) Depletion of the cellular amounts of the MutS and MutH methyl-directed mismatch repair proteins in stationary-phase *Escherichia coli* K-12 cells. *J. Bacteriol.* **178**, 2388–2396
- Harris, R. S., Feng, G., Ross, K. J., Sidhu, R., Thulin, C., Longerich, S., Szigety, S. K., Hastings, P. J., Winkler, M. E. and Rosenberg, S. M. (1999) mismatch repair is diminished during stationary-phase mutation. *Mutat. Res.* **437**, 51–60
- Aronsham, A. and Marinus, M. G. (1996) Dominant negative mutator mutations in the *mutL* gene of *Escherichia coli*. *Nucleic Acids Res.* **24**, 2498–2504
- Peltomaki, P. and Vasen, H. F. (1997) Mutations predisposing to hereditary nonpolyposis colorectal cancer: database and results of a collaborative study. The International Collaborative Group on Hereditary Nonpolyposis Colorectal Cancer. *Gastroenterology* **113**, 1146–1158
- Fishel, R., Lescoe, M. K., Rao, M. R., Copeland, N. G., Jenkins, N. A., Garber, J., Kane, M. and Kolodner, R. (1993) The human mutator gene homolog MSH2 and its association with hereditary nonpolyposis colon cancer. *Cell* **75**, 1027–1038
- Nystrom-Lahti, M., Herrera, C., Raschle, M., Panyushkina-Seiler, E., Marra, G., Curci, A., Quaresima, B., Costanzo, F., D'Urso, M., Venuta, S. and Jiricny, J. (2002) Functional analysis of MLH1 mutations linked to hereditary nonpolyposis colon cancer. *Genes Chromosomes Cancer* **33**, 160–167