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Identification of Transdominant-Negative Genetic Suppressor Elements Derived from hMSH2 That Mediate Resistance to 6-Thioguanine

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ABSTRACT

Using random screening for genetic suppressor elements, we sought to identify portions of hMSH2 important to the ability of the mismatch repair system to recognize and process DNA adducts that mimic mismatches. All recovered candidate genetic suppressor elements were derived from the region containing amino acids 782 to 844. Expression of a peptide corresponding to this region partially disabled mismatch repair as evidenced by 1.5- to 3.3-fold resistance to 6-thioguanine, cisplatin, and *N*-methyl-*N*′-nitrosoguanidine, an increase in the rate of generation of drug resistant variants, and the appearance of microsatellite instability. Even low-level expression of this protein was sufficient to partially impair mismatch repair. The results suggest that this region is important to the ability of the mismatch repair system to mediate drug sensitivity and to maintain genomic stability.

The human mismatch repair (MMR) system plays an important role in maintaining genomic stability by mediating postreplication repair and limiting recombination between mismatched sequences. Six proteins have been shown to participate in human MMR, including hMSH2, hMSH3, hMSH6, hMLH1, hPMS2, and hPMS1. Current evidence indicates that MMR is initiated through the binding of a heterodimer of hMSH2 with either hMSH6 (hMutSα) or hMSH3 (hMutSβ) to the site of the mismatch (Drummond et al., 1995; Fishel and Kolodner, 1995; Palombo et al., 1995; Acharya et al., 1996; Palombo et al., 1996; Alani et al., 1997). This is followed by binding of a heterodimer consisting of hMLH1 and hPMS2 (hMutLα), removal of the mismatch, filling of the gap by DNA polymerase δ, and ligation that renders the strands whole again (Li and Modrich, 1995).

Loss of MMR can alter the sensitivity of a tumor to chemotherapy in at least two ways. First, the MMR system can recognize certain types of drug-induced adducts in DNA and activate proapoptotic signals (Nehme et al., 1997; D'Atri et al., 1998). Loss of this putative detector function of MMR directly produces high-level resistance to 6-thioguanine (6TG) and lower levels of resistance to methylating agents such as *N*-methyl-*N*′-nitrosoguanidine (MNNG) and to the platinum-containing drugs cisplatin (DDP) and carboplatin (Andrews et al., 1990; Kat et al., 1993; Aebi et al., 1996; Carethers et al., 1996; Duckett et al., 1996; Fink et al., 1996; Mello et al., 1999). Second, loss of MMR increases the rate of mutation in the coding or regulatory sequences of other genes whose products regulate sensitivity to the cytotoxic effects of some drugs (Malkhosyan et al., 1996; de las Alas et al., 1997), resulting in the more rapid appearance of tumor cell variants resistant to other chemotherapeutic agents (Lin et al., 1999).

Among the MMR proteins, hMSH2 is of particular interest because of its pivotal role in initiating the repair process (Fishel et al., 1994; Fishel and Kolodner, 1995). The importance of hMSH2 is documented by the fact that mutations in the *hMSH2* gene are found in a large percentage of all families with hereditary nonpolyposis colorectal cancer (HNPCC) (reviewed in de la Chappelle and Peltomaki, 1995; Fishel and Kolodner, 1995). Recently, regions of the hMSH2 protein that actually contact its dimeric partners hMSH3 and hMSH6 have been identified (Guerrette et al., 1998). However, the regions of hMSH2 that contact the other proteins involved in MMR have not been accurately defined, and the region of hMSH2 involved in the interactions required for recognition of drug-induced DNA adducts that mimic mismatches re-

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ABBREVIATIONS: MMR, mismatch repair; 6TG, 6-thioguanine; MNNG, *N*-methyl-*N*′-nitrosoguanidine; DDP, cisplatin; HNPCC, hereditary nonpolyposis colorectal cancer; GSE, genetic suppressor element; bp, base pair(s); PCR, polymerase chain reaction; HAT, hypoxanthine/aminopterin/thymidine; EV, empty vector.

mains unknown. Identification of the parts of the protein that yield 6TG resistance when they are targeted by a transdominant-negative peptide is one approach to elucidating the function of regions critical to this protein's function.

In this study, we used genetic suppressor element (GSE) technology to identify regions of hMSH2 likely to be essential to its ability to maintain sensitivity to the adducts produced by 6TG. GSEs are short, biologically active cDNA fragments that encode dominantly acting peptides or inhibitory antisense RNAs (Roninson et al., 1995; Gudkov, 1996). Because generation of the expressed fragments is random, if transdominantly acting GSEs are recovered, they provide information about the regions of the full-length protein that can disrupt function. In this study, putative GSEs were generated from hMSH2 cDNA by randomly fragmenting the full-length cDNA, isolating fragments of 200 to 800 bp, and cloning them into a retroviral vector designed to express them in mammalian cells. After infection with this retroviral library, the population of tumor cells was subjected to selection with 6TG, and candidate GSEs were recovered from the resistant clones. We found that all the putative GSEs recovered were derived from a single region of hMSH2 encompassing amino acids 782 to 844, suggesting that this part of the protein is essential to maintain sensitivity to 6TG.

Materials and Methods

Cell Lines. The human head and neck squamous cell carcinoma cell line UMSCC10b was cultured in RPMI 1640 medium supplemented with 2 mM L-glutamine, 100 units/ml of penicillin, 100 units/ml streptomycin, and 10% regular fetal bovine serum (Krause et al., 1981). Phoenix cells are a high-titer amphotropic retrovirus packaging cell line derived from 293T cells stably transfected with a Moloney GagPol-IRES-Lyt2 construct with an RSV promoter and pPGK hygromycin selectable marker. The cells contain a Moloney amphotropic envelope gene driven by a cytomegalovirus promoter and the diphtheria toxin resistance gene (pPHED-7) (Kinsella and Nolan, 1996). The cells were obtained from the American Type Culture Collection (Manassas, VA) with permission from Dr. Garry P. Nolan of Stanford University. These cells were cultured in Dulbecco's modified Eagle's medium/low glucose supplemented with 2 mM L-glutamine, 100 units/ml penicillin, 100 units/ml streptomycin, and 10% heat-inactivated fetal bovine serum. All cell lines tested negative for *Mycoplasma* species.

Preparation of Library of Random hMSH2 Fragments. A pBluescript $SK^{+/-}$ vector (Stratagene, La Jolla, CA) containing the full-length hMSH2 cDNA was obtained from the laboratory of Dr. Bert Vogelstein (Department of Pharmacology & Molecular Sciences, Johns Hopkins University School of Medicine, Baltimore, MD). The cDNA was isolated from the vector, randomly fragmented with DNase I, and the ends were rendered blunt with DNA polymerase and Klenow fragment. Two different adaptors were prepared by annealing two sets of complementary synthetic oligonucleotides (GENSET Inc., San Diego, CA): 5′-AAATCTCGAGATGTA-ATATGG-3′ (N60) to 5′-CCATATTACATCTCGAGA-3′ (N61) and 5′-AAAGCAGGATCCTAACTAACTA-3′ (N62) to 5′- TAGTTAGTTAG-GATC-CTGC-3′ (N63). The upstream adaptor contains an ATG translation initiation codon, whereas the downstream adaptor incorporates translation termination codons in all three possible reading frames. In addition, these adaptors also have *Xho*I and *Bam*HI restriction sites at the 5′ and 3′ ends, respectively, to facilitate cloning into the retroviral vector pLXSN (Miller and Rosman, 1989). After overnight ligation of the adaptors to the blunt-ended hMSH2 fragments at 16°C, the cDNA was size-fractionated by agarose gel electrophoresis, and gel-purified 200- to 800-bp fragments were amplified by PCR (thermocycler; PerkinElmer Life Science, Boston, MA) using N60 and N62 as primers. The PCR cycles were 65°C for 15 min to remove nicks, 94°C for an initial 3 min, then 30 cycles of 94°C for 1 min, 52°C for 1 min, and 72°C for 1 min, followed by a 7-min extension at 72°C and storage at 4°C. The PCR products were then electrophoresed on a 1% agarose gel and purified using the pet wool method (Sambrook et al., 1989); phenol/chloroform was extracted, and ethanol was precipitated. The purified products were then digested with *Xho*I and *Bam*HI at 37°C for 1 h, concentrated using Centricon100 filters (Millipore Corporation, Bedford, MA), and cloned into the pLXSN vector that had previously been digested with *Xho*I and *Bam*HI.

Library Transduction and 6TG Selection. Phoenix amphotropic packaging cells were transfected with the purified hMSH2 library of random fragments in pLXSN or insert-free pLXSN (control) by a standard calcium phosphate procedure (Sambrook et al., 1989). Forty-eight hours later, the cell culture media containing the viral supernatant was aspirated, passed through a 0.22-μm filter, and used to infect UMSCC10b cells that had been cleansed of *HGPRT* mutants by growth in 1× HAT medium (1 mM sodium hypoxanthine, 4 μM aminopterin, and 160 μM thymidine). The cells were selected with 500 μg/ml G418 for 1 week to ensure the presence of a copy of the integrated vector in the cell. The surviving cells were then exposed to 100 μM 6TG for a minimum of 2 weeks, and the surviving individual clones were then allowed to grow without drug for 2 more weeks. Genomic DNA was recovered from individual clones (Sambrook et al., 1989), and inserts rescued by PCR using primers corresponding to sequences in the pLXSN vector approximately 100 bp external to the insert: 5′-AAGCCCTTTGTACACCCCT-AAGC-3′ (sense1) and 5′-TGGGGACTTTTCCACACCTGGTT-3′ (antisense1). A secondary PCR was then done on these products using nested primers overlapping a portion of the pLXSN vector and a portion of the adaptors ligated onto the original hMSH2 cDNA fragments: 5′-TTCGTTAACTCGAGACAATGG-3′ (sense2) and 5′-ACAGCCG-CATCCTAACTAACTA-3′ (antisense2). These products were then sequenced to locate their position within the hMSH2 cDNA.

GSE Characterization. Primers that overlap the bases corresponding to amino acids 782 to 789 in the 5′ end and 836 to 844 in the 3′ end of the coding region of the hMSH2 cDNA were used to amplify a consensus region corresponding to the putative GSEs rescued from 6TG-resistant clones. An overhang sequence was added to the 5′ end of each primer equivalent to the original nested primers used in the secondary PCR to rescue the putative GSE inserts. These nested primers overlap part of the pLXSN vector and a portion of the original adaptors as well as contain restriction sites for *Xho*I and *Bam*HI. The primers were 5′-TTCGTTAACTCGAGACAATGGCT-CATTTTCATGAACTTACTGCCTTG-3′ (sense3) and 5′-ACAGCCG-GATCCTAACTAACTTTTAGCACACTCTATTACATGCTTAGG-3′ (antisense3). The region of hMSH2 cDNA corresponding to the putative GSEs was amplified by PCR starting with 95°C for 3 min, followed by 35 cycles of 95°C for 1 min, 56°C for 1 min, and 72°C for 1 min. A final extension was performed for 5 min at 72°C. A portion of the fresh PCR product was run on a 3% agarose gel to document the correct size of the fragment. Another aliquot was cloned into the pCR 2.1 vector (Invitrogen, Carlsbad, CA) according to manufacturer's instructions. This construct was then amplified in chemically competent INVαF′ bacteria. An aliquot of the GSE-containing pCR2.1 construct was digested with *Xho*I and *Bam*HI to release the putative GSE fragment, which was then ligated into a previously digested pLXSN vector. This construct was used to generate infectious virus from Phoenix packaging cells. Infected UMSCC10b cells were selected with G418 and individual colonies were isolated for use in clonogenic drug sensitivity and mutation rate assays.

Determination of Drug Sensitivity by Clonogenic Assay. The drug sensitivity of clones expressing the putative GSE was determined by plating 200 cells into each well of a six-well plate. A clone of UMSCC10b cells infected with empty vector and selected for G418 resistance was included as a control. Triplicate wells were

exposed continuously to six graded concentrations of drug. MNNG was tested at concentrations of 0, 1, 2, 3, 4, and 5 μM. Cells were exposed to DDP at concentrations of 0, 0.2, 0.4, 0.6, 0.8, and 1 μM. A survival curve was also obtained for 6TG at concentrations of 0, 2, 4, 6, 8, and 10 μM. To confirm that the effect of the GSE was not caused by clonal variability, the drug sensitivity of six additional clones containing the empty vector were also compared with six other clones containing the putative GSE. Colonies containing at least 50 cells were counted by hand after fixing with 70% ethanol and staining with Giemsa 8 to 10 days later. Percentage survival was calculated as the quotient of the number of colonies in the drug-treated and control wells.

Confirmation of the Representativeness of the Library of Random hMSH2 Fragments. Four portions of the hMSH2 cDNA corresponding to four different regions of the protein were isolated for use as probes with which to analyze the library of randomly generated fragments. Two portions were obtained by digesting the full-length hMSH2 cDNA in pBluescript with *Xba*I and *Eco*RI to recover a 528-bp fragment corresponding to nucleotides 0 to 528, and with *Eco*RI alone to recover a 737-bp fragment corresponding to nucleotides 663 to 1399. Fragments corresponding to nucleotides 1983 to 2366 and 2344 to 2529 were amplified by PCR using appropriate primers. The original library of random hMSH2 fragments in the pLXSN vector was PCR-amplified using the N60 and N62 primers with a 1:1000 dilution of the plasmid, and subjected to Southern blot analysis using the four ^{32}P-labeled fragments as probes.

Measurement of Resistant Variant Generation Rate. The rate of generation of resistant variants was determined as described previously (Glaab and Tindall, 1997). One million cells were plated in media containing 3 μM etoposide at a density of 10×10^4 cells per 10-cm dish; 5×10^2 cells were plated simultaneously in triplicate to determine the plating efficiency. The remaining cells were re-seeded at a density of 3×10^6 cells per 175-cm^2 flask and grown for 3 days. The total cell number was again determined, and aliquots were removed for determination of the frequency of etoposide-resistant variants and for plating efficiency, and the remaining cells were reseeded at 3×10^6 cells per 175-cm^2 flask and grown for another 3 days. This series of steps was repeated at least four times. The frequency of drug-resistant variants was calculated from the number of surviving clones in the 10-cm diameter dishes at each time point using the following formula: variant frequency = $a/10^6 \times (b/1.5 \times 10^3)$, where a is the total number of etoposide-resistant colonies and b is the total number of colonies on all three plating efficiency plates. The population doubling is given by (ln[total number of cells] − ln[number of cells plated × plating efficiency])/ln2. The rate of generation of etoposide-resistant variants (variants/cell/generation) is equal to the slope of the plot of etoposide-resistant variant frequency versus population doubling.

Microsatellite Instability Analysis with Plasmid Shuttle Vector pZCA29. The vector pZCA29 (Diem and Runger, 1998) was constructed by inserting the PCR-amplified fragment containing parts of the multiple cloning site of pBluescript KS$^+$ and two $(CA)_{14}$ repeats from the vector pCAR2 (Parsons et al., 1993) with the primers T3 and T7 into the multiple cloning site of pBluescript KS$^+$. The resultant construct was cleaved with *Hae*II, the fragment harboring the β-galactosidase gene and the inserted two $(CA)_{14}$ repeats was cloned into the *Eco*RI site of the episomally replicating shuttle vector pZ189. A plasmid mutated in the repeat tracts resulting in 29 CA repeats was selected and named pZCA29. Four million empty vector (EV) and GVC8 cells were transfected with 2 μg of pZCA29 by electroporation. The cells were then incubated at 37°C for 48h to allow for replication of pZCA29. Replicated pZCA29 was recovered from the transfected cells on days 3, 5, 7, 9, and 11 by a rapid alkali lysis procedure. Unreplicated input plasmid DNA was removed by digestion with *Dpn*I, which cleaves the methylated DNA from bacteria. *Escherichia coli* XL1-Blue MRF′ (Stratagene) was transformed with recovered pZCA29 and then selected on LB agar plates containing 5-bromo-4-chloro-3-indolyl-β-galactosidase, isopropyl-β-D-thiogalactoside, and ampicillin. The total number of white and blue colonies was counted. The mutation frequency was calculated and analyzed by Student's t test and χ^2 test in 2×2 tables to test for the differences.

Preparation of 6xHis-GSE Protein. A putative GSE extending from amino acids 782–844 was synthesized by PCR using the sense3 primer and an antisense primer consisting of 5′-CAGCCAAGCTTTAACTAAACTTTTAGCACACTCTATTACATGCTTAGG-3′ (antisense4), to incorporate a *Hin*dIII cloning site downstream of the putative GSE fragment. This product was cloned into the pCR2.1 vector, recovered by digestion with *Xho*I and *Hin*dIII, and ligated into the pRSETB (Invitrogen) expression vector which places the 6xHis affinity tag at the N terminus of the recombinant protein. BL21-DE3 *E. coli* expression hosts were transformed with the expression constructs and grown overnight at 37°C. A preliminary test indicated that induction of the protein with isopropyl-β-D-thiogalactoside was not necessary, the bacteria were lysed and protein eluted using the B-PER 6xHis Elution Kit (Pierce, Rockford, IL) according to manufacturer's instructions. Protein concentrations were determined by a protein assay (Bio-Rad, Hercules, CA) for each elution aliquot. Ten micrograms of denatured eluted 6xHis protein from two aliquots were detected by Western Blot analysis using 6xHis monoclonal, hMSH2 monoclonal, and hMSH2 polyclonal antibodies (Oncogene Science, Cambridge, MA).

Western Blot Analysis. Cells were harvested during exponential growth, washed in phosphate-buffered saline, and lysed on ice in buffer (0.15 M NaCl, 5 mM EDTA, 1% Triton X-100, 10 mM Tris-Cl, pH 7.4) with freshly added 0.1 mM phenylmethylsulfonyl fluoride and 5 mM ϵ-aminocaproic acid. The lysate was clarified by centrifugation and stored at −70°C. Twenty micrograms of denatured total cell extract were loaded into each well of a 15% Tris-HCl precast acrylamide gel (Bio-Rad). One microgram of 6xHis-tagged putative GSE protein was also loaded as a positive control. The extracts and 6xHis GSE protein were electrophoresed at 200 V for 30 min alongside a protein ladder (Bio-Rad) and were then transferred onto a nitrocellulose membrane at 100 V for 1 h, after which the blot was incubated overnight in 3% bovine serum albumin with 0.05% Tween 20. A polyclonal anti-hMSH2 antibody (Oncogene Science) was used as the primary antibody for detection of the presence of the putative GSE protein in the whole-cell extracts. It was used at a dilution of 1:100 in blocking buffer, and the incubation time was 1 h. The blot was then washed three times for 10 min each with phosphate-buffered saline/0.05% Tween 20 and exposed to secondary anti-rabbit antibody at a dilution of 1:3000 (Amersham Biosciences, Piscataway, NJ) for 45 min. The presence of the protein was detected by chemiluminescence using the enhanced chemiluminescence detection kit (Amersham Biosciences) for 2 min.

Results

Preparation and Characterization of GSE Library. Two types of putative GSEs can be made from a cDNA of interest: those that generate an antisense RNA and those that generate a sense transcript that is translated into a dominant-negative fragment of the protein (Gudkov, 1996). The GSE technology allows one to screen for sequences within the cDNA that produce a phenotype by either of these mechanisms. Random 200- to 800-bp fragments of the full-length hMSH2 cDNA were cloned into a retroviral vector (pLXSN) capable of expressing them as either an antisense RNA or as a short portion of the hMSH2 protein while at the same time coexpressing the neo gene.

To document that all portions of the MSH2 cDNA were represented in the GSE library, the inserts from the pLXSN library were PCR-amplified and subjected to Southern blot analysis using probes directed to four different regions of the

hMSH2 cDNA. The extent of hybridization to two fragments containing the full-length hMSH2 cDNA was measured concurrently to control for variations in the affinity of the different probes. As shown in Fig. 1, relative to hybridization to the control fragments, there was equal hybridization for each of the four probes to the library as quantified by filmless autoradiographic analysis. Thus, there was equal representation of each region of the MSH2 cDNA in the DNase I digested hMSH2 library.

Isolation of Putative GSEs by 6TG Selection. The pLXSN library was transfected into Phoenix packaging cells (http://www.uib.no/mbi/nolan/N-L-Homepage.html) to produce infectious virus with a titer of 10^5 particles/ml, and this virus was used to infect human UMSCC10b head and neck carcinoma cells that had previously been cultured in HAT medium to remove any pre-existing *HGPRT* mutants. These cells were then selected first with G418 to isolate cells in which successful integration of the retrovirus had occurred, and then screened for the presence of a functional putative GSE by continuous exposure to 100 μM 6TG. Survival in the presence of this high concentration of 6TG is expected to reflect either a new mutation in *HGPRT* or loss of the ability of the MMR system to recognize the 6TG adduct or process it to produce an apoptotic signal. Candidate GSE fragments from surviving clones were recovered by PCR using primers directed at the viral sequences flanking the putative GSE with genomic DNA as the template.

Putative GSE fragments were successfully recovered from nine 6TG-resistant clones. The length of each fragment differed slightly. These PCR products were sequenced to identify the position of the recovered putative GSE within the hMSH2 cDNA. Figure 2 shows that eight of these nine were in the sense orientation and corresponded to a region encompassing amino acids 782 to 844. Four of these eight had deletions of from one to nine amino acids. The one putative GSE in the antisense orientation (clone 19) spanned amino acids 772 to 884. The fact that all of the putative GSEs in the sense orientation recovered from the library of random hMSH2 fragments corresponded to a rather limited portion of the hMSH2 protein identifies this region as pivotal for the ability of the GSE-derived peptide to disable the ability of MMR system to recognize and/or process the 6TG adduct.

Characterization of a Consensus Candidate GSE. A consensus candidate GSE corresponding to amino acids 782

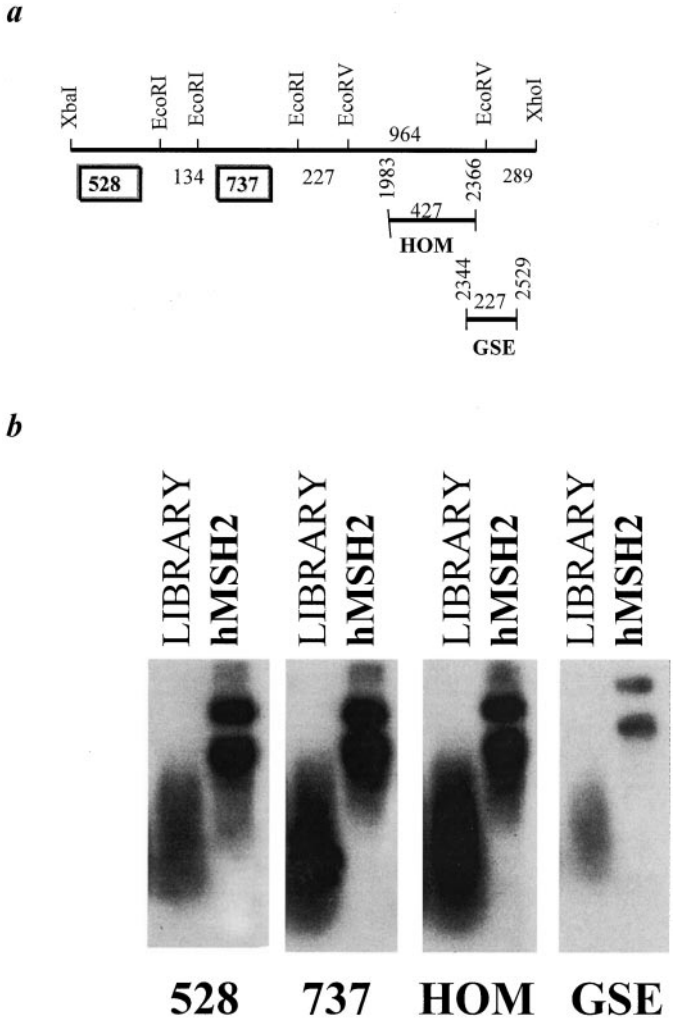


Fig. 1. Documentation of the representation of all regions of the MSH2 cDNA in the library of random fragments. A, portions of hMSH2 that were used as probes in hybridizations to the library of random hMSH2 fragments, and their corresponding region on the full-length hMSH2 cDNA. B, Southern blots of the library of random hMSH2 fragments hybridized with probes directed against 4 different regions of the cDNA. Lanes 1, 3, 5, and 7, random fragment library; lanes 2, 4, 6, 8, MSH2 cDNA control. The probes used were as follows: lanes 1 and 2, 528-bp fragment; lanes 3 and 4, 737-bp fragment; lanes 5 and 6, 427-bp fragment; and lanes 7 and 8, 227-bp fragment. The MSH2 cDNA controls consisted of two fragments digested out of a pBluescript vector containing the full-length cDNA.

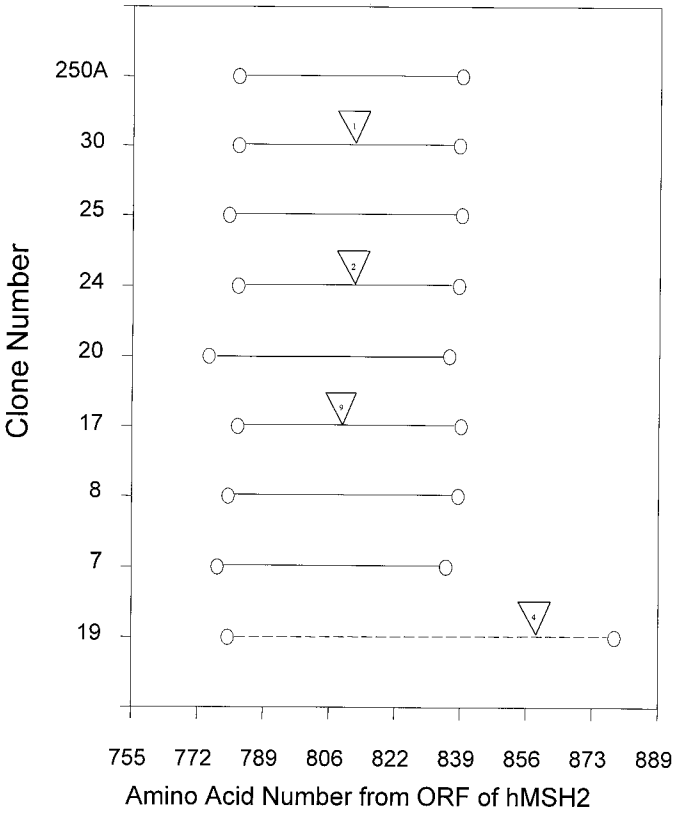


Fig. 2. GSEs recovered from the genomic DNA of clones resistant to 6TG. Solid lines indicate sense orientation; dashed lines indicate antisense orientation. Arrows indicate deletions found in the GSEs when mapped against the hMSH2 sequence. Numbers in arrows indicate number of amino acids deleted.

to 844 was generated by PCR and cloned into the pLXSN retroviral vector; this vector was then used to generate infectious virus from Phoenix packaging cells. UMSCC10b cells were first cleansed of pre-existing 6TG-resistant clones by growth in HAT medium, and then infected with the virus. Sixteen G418-resistant colonies were isolated and tested for their sensitivity to DDP, 6TG, and MNNG, using clonogenic assays with continuous drug exposure. A clone of UMSCC10b cells that had been infected with an EV and selected the same way with G418 served as a control. Relative to the sensitivity of EV cells, 7 of 16 clones containing the putative GSE demonstrated >1.5-fold resistance to 6TG, 2 of 16 demonstrated this degree of resistance to DDP, and 1 of 16 was >1.5-fold resistant to MNNG. The two clones that were >1.5-fold resistant to DDP were among the seven that were >1.5-fold resistant to 6TG, and the single clone that was resistant to MNNG was also resistant to 6TG and DDP. As shown in Fig. 3, the GVC8 clone was 3.3-fold resistant to 6TG, 2.2-fold resistant to DDP, and 1.7-fold resistant to MNNG. The GVC8 clone was selected for further genomic stability analysis, along with a clone demonstrating no drug resistance (GVC1).

Because of the degree of resistance produced by the GSE was only modest, further analysis was performed to confirm that the phenotype was due to the effect of the GSE and not an artifact of clonal variability. Twelve additional clones, six containing the empty vector and six containing the putative GSE, were isolated and tested for their sensitivity to 6TG, MNNG, and DDP as described previously. As shown in Table 1, the average IC_{50} values for clones containing the putative GSE were statistically significantly higher ($p < 0.05$) than the IC_{50} values for EV-containing clones for DDP. In addition, all clones containing the putative GSE were more resistant to 6TG and MNNG than all the empty vector clones ($p < 0.001$). To further define the specificity of the effect of the GSE-derived peptide, the same clones were tested for their sensitivity to a drug, oxaliplatin, for which loss of MMR function does not result in drug resistance. As also shown in Table 1, there was no difference in the oxaliplatin IC_{50} values for the empty-vector and GSE-expressing clones.

A histidine-tagged version of the protein coded for by the consensus putative GSE was produced by expressing it in bacteria and subsequently purifying it on a nickel column. A polyclonal rabbit anti-hMSH2 antibody visualized this purified protein on Western blot analysis (data not shown). As shown in Fig. 4, this antibody detected native hMSH2 in lysates of three clones by Western blot analysis. In clone GVC8, it also visualized a protein of 8.8 kDa, which is the expected size of the protein coded for by the putative GSE. Thus, one of the clones that exhibited a consistent drug-resistant phenotype also expressed the putative GSE at measurable levels. However, in other clones, the level of expression was too low to be detected by Western blotting, and thus an association between the level of expression of the GSE-derived peptide and drug resistance could not be established.

If the consensus putative GSE was capable of disabling MMR, one would expect to observe the types of genomic instability that accompany loss of MMR function, including an increase in mutation rate and microsatellite instability. Cells that have lost MMR function have an increased rate of mutation to 6TG resistance, and we have demonstrated previously that they also have an increased rate of generation of variants resistant to etoposide (de las Alas et al., 1997), suggesting that genes involved in the control of cellular sensitivity to etoposide are also targets for mutation when the loss of MMR destabilizes the genome. The rate of generation of etoposide- and 6TG-resistant variants was measured for GVC8, EV, and the parental UMSCC10b cells. Starting with a population of 10^6 cells, measurements were made of the number of resistant variants in the population after every three doublings as it expanded. The variant generation rate was then determined from the slope of a plot of the number of resistant variants versus the number of population doublings (Glaab and Tindall, 1997). A resistant variant was defined on

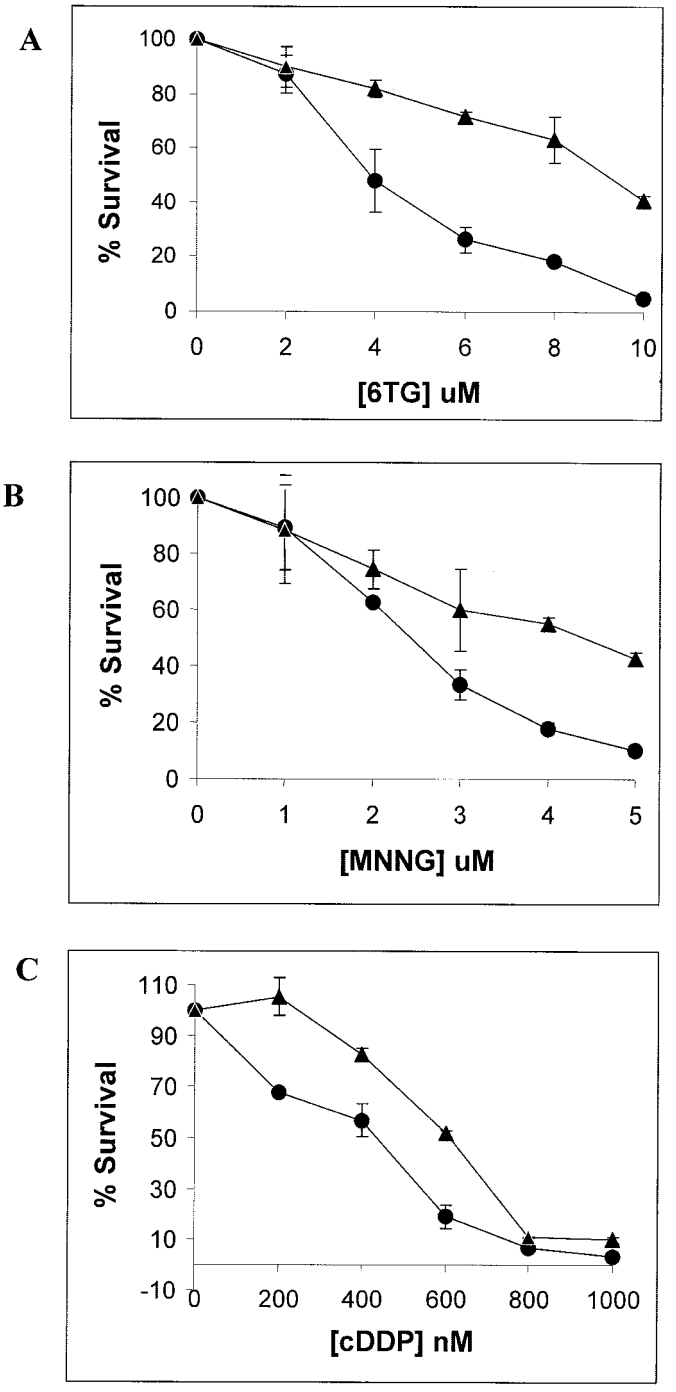


Fig. 3. Dose-response curves of GVC8 (▲) versus empty vector (●) clones to exposure in 6-thioguanine (A), cisplatin (B), and MNNG (C).

the basis of its ability to form a colony in the presence of a concentration of drug that reduced the surviving fraction of the overall population to 2×10^{-5}. Figure 5 shows that the rate of variant generation was similar for the parental and EV cells, but that in the GVC8 clone, the rate was increased 4.3-fold for resistance to etoposide and 6.3-fold for resistance to 6TG.

To determine whether microsatellite sequences were unstable in the clone expressing the putative GSE, we transfected EV and GVC8 cells with the shuttle vector pZCA29 containing an out-of-frame 94-bp insertion with a 28-bp CA repeat tract and a 30-bp GT repeat tract arranged palindromically with the coding sequences of a β-galactosidase reporter gene. The vector contained simian virus 40 T antigen, *ori*, and an enhancer to allow the episomal replication in the human cells. Insertions or deletions in the repeat tract during replication of the vector in the recipient human cells could therefore result in correction of the reading frame of the β-galactosidase gene and the expression of β-galactosidase when the plasmids were recovered from the human cells and transduced into permissive bacteria. Figure 6 shows the frequency of blue colonies compared with the total colonies as a function of incubation time for EV and GVC8 cells. Plasmids recovered after 3 days of pZCA29 growth in the GVC8 cells demonstrated a mutant frequency 3.6-fold higher than plasmids recovered from EV cells ($p < 0.01$). The revertant frequency increased as a function of time the pZCA29 vector spent in the GVC8 cells and was higher than that for the EV cells at all the time points tested. With both cell lines, the mutation frequency was above that observed in bacteria transformed directly with control that had not been transfected into mammalian cells pZCA29 (~0.8%).

TABLE 1.
Sensitivity to chemotherapeutic agents of empty vector and GSE-expressing clones

	IC_{50}			
	DDP	MNNG	6TG	Oxaliplatin
			μM	
Empty Vector Clones				
EV-1	0.23	4.41	0.94	0.74
EV-2	0.35	2.4	1.32	0.8
EV-5	0.24	4.13	3.48	0.79
EV-7	0.14	3.1	4.37	1.1
EV-9	0.13	3.18	4.01	0.64
EV-11	0.27	5.63	1.35	0.52
Mean	0.23	3.81	2.58	0.77
S.D.	0.08	1.15	1.54	0.18
GSE-Expressing Clones				
GSE-1	0.15	7.21	5.4	0.48
GSE-2	0.31	9.87	11.9	1
GSE-4	0.3	9.96	9.42	0.59
GSE-8	0.34	10.9	14.1	0.8
GSE-11	0.6	8.11	7.55	0.92
GSE-13	0.5	9.84	13.7	0.9
GSE-15	0.5	15.2	12.1	N.D.
Mean	0.39	10.16	10.60	0.78
S.D.	0.15	2.55	3.25	0.20
P value	0.041	0.0003	0.0003	0.89

N.D., not determined.

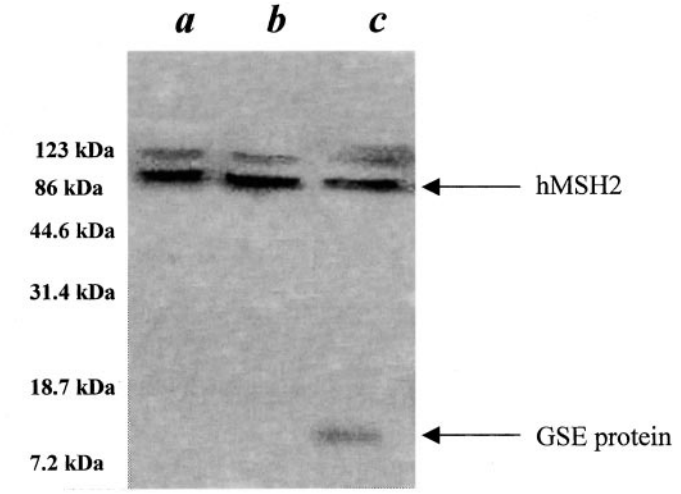


Fig. 4. Western blot analysis of total cell extracts for MSH2. Lane A, EV; lane B, GVC1; lane C, GVC8.

Discussion

Loss of MMR is an important mechanism of resistance to a variety of clinically used chemotherapeutic agents (reviewed in Fink et al., 1998). However, detailed information about the molecular mechanisms by which the MMR proteins function to recognize and remove mismatches and drug-induced adducts is not yet available. In this study we used the approach of recovering putative GSEs capable of mediating resistance to 6TG to identify regions of hMSH2 that, when expressed as short proteins, are capable of modifying the phenotype in a manner consistent with disruption of MMR function. The eight transdominant putative GSEs identified in this study all corresponded to the region of MSH2 extending from amino acids 782 to 844. Because the library from which these putative GSEs were recovered contained equal representation of random sequences from the entire hMSH2 cDNA, the possibility that this region was targeted because of a bias in the library is discounted, although it remains possible that not all putative GSEs are expressed equally well or stably. The fact that the candidate GSEs corresponded to a single region of the hMSH2 protein is consistent with the experience of other investigators who have found that putative GSEs isolated in this way correspond to a limited number of different regions in their cognate protein (Holzmayer et al., 1992; Gallagher et al., 1997; Ossovskaya et al., 1999).

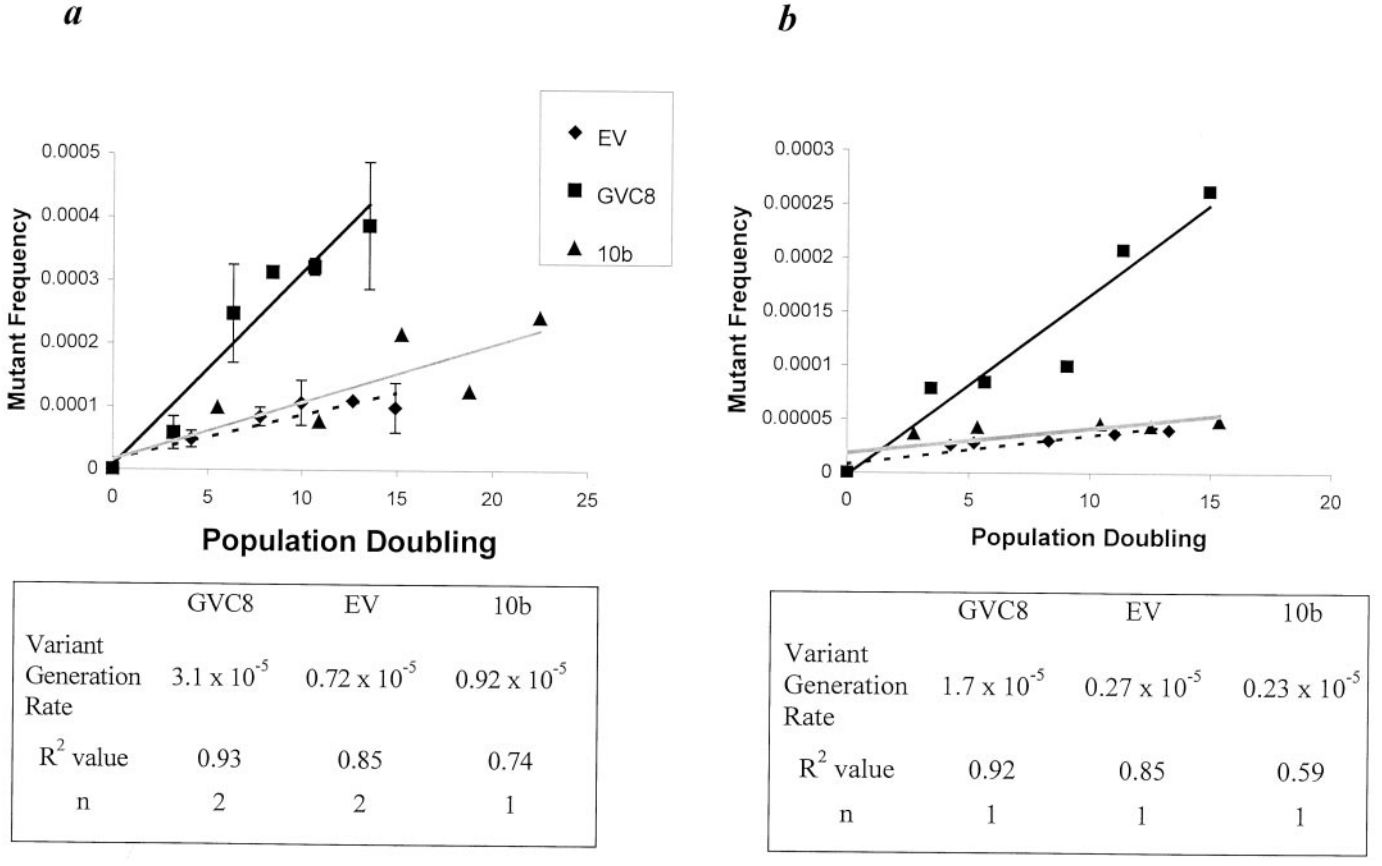
The eight transdominant putative GSEs identified in this study were isolated on the basis of their ability to produce resistance to 6TG. It is not known which steps in the sequence of events mediated by the MMR system is impacted by the putative GSEs. Several of the most obvious possibilities are that they could compete with hMSH2 for recognition of the adduct, they could bind to either hMSH6 or hMSH3 and prevent heterodimerization with MSH2, or they could interfere with the ability of these heterodimers to recruit hMLH1, hPMS1, or hPMS2 to the adduct. Current evidence suggests that loss of hMLH1 function is sufficient to yield 6TG resistance (Aebi et al., 1997), but because it is not known whether defects in the other proteins produce this phenotype, the finding of 6TG resistance by itself does not permit discrimination between several specific protein-protein interactions that might be targeted by the transdominant negative GSEs.

Several lines of evidence support the importance of the region defined by the putative GSEs in the function of the native hMSH2 protein. First, starting with a library of random fragments in which all parts of the hMSH2 cDNA were

equally represented, all the putative GSEs recovered after 6TG selection corresponded to this one region. Second, this region overlaps a portion of exon 15 (amino acids 819–878), an area of high mutability often leading to the deletion of this exon in HNPCC families (de la Chappelle et al., 1995; Liu et al., 1995). It also contains an alanine (amino acid 834) that has been found to be mutated to a threonine in other HNPCC families (J. Wijnen, unpublished data; see also http://www.nfdht.nl/). In addition, comparison of the human MSH2 sequence with that of other organisms, as shown in Fig. 7, demonstrates more conservation of sequence in this region than in the rest of the hMSH2 cDNA. This suggests that this part of the cDNA codes for a portion of the protein that serves a role crucial to its overall function. Also, the helix-turn-helix domain, previously identified in *E. coli* and *Saccharomyces cerevisiae* (Alani et al., 1997), overlaps this consensus putative GSE region in hMSH2 between amino acids 821 and 844. Finally, as shown in Fig. 8, when this region is mapped onto a theoretical model of hMSH2 (de las Alas et al., 1998), it turns out to correspond to an apparent protuberance that is well positioned to interface with other proteins; this is consistent with the notion that a transdominant-negative peptide consisting of this portion of the protein may interfere with essential interactions. Nevertheless, it remains possible that the 782 to 844 region of hMSH2 may have been identified by the GSE analysis because it is an accessible region of the protein rather than because of any function of this region critical to protein-protein interactions essential to MMR function.

The 6TG resistance observed in clones from which the original putative GSEs were recovered was reproduced by expressing a consensus GSE consisting of amino acids 782 to 844 as demonstrated in clone GVC8. However, as expected from the fact that the level of GSE expression varies between clones infected with the same viral GSE-expressing vector (Andrei Gudkov, personal communication), the level of resistance to 6TG varied among different clones. It is noteworthy that the magnitude of the 6TG resistance was quite modest relative to what has been reported for cells that express no hMSH2 or hMHL1 (Swann et al., 1996; Aebi et al., 1997). However, because no subline of UMSCC10b cells that completely lacks MMR function is available and because comparisons between different cell lines are complicated by their variable intrinsic sensitivity to 6TG, the level of 6TG resistance expected in the absence of MMR function in these cells is unknown. The putative GSE seems to produce only limited impairment of MMR function with respect to the recognition and processing of 6TG adducts. Despite this, the deficit in MMR function was sufficient to produce measurable degrees of resistance to both DDP and MNNG, demonstrating that the specific step in the sequence of repair events whose disability yields 6TG resistance is also involved in either the recognition or processing of DDP and MNNG adducts as well. In addition, compared with a panel of UMSCC10b clones containing the empty vector, all clones containing the putative GSE were more resistant than all the EV clones to MNNG and 6TG.

Despite the modest impairment of MMR function as evi-



	GVC8	EV	10b
Variant Generation Rate	3.1×10^{-5}	0.72×10^{-5}	0.92×10^{-5}
R^2 value	0.93	0.85	0.74
n	2	2	1

	GVC8	EV	10b
Variant Generation Rate	1.7×10^{-5}	0.27×10^{-5}	0.23×10^{-5}
R^2 value	0.92	0.85	0.59
n	1	1	1

Fig. 5. Rate of generation of etoposide-resistant (A) and 6TG-resistant variants (B). GSE-expressing clone GVC8 (■), empty vector clone EV (♦), and parental UMSCC10b cells (▲).

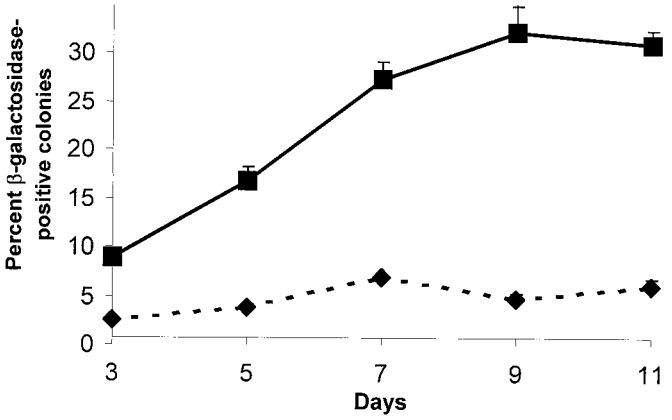
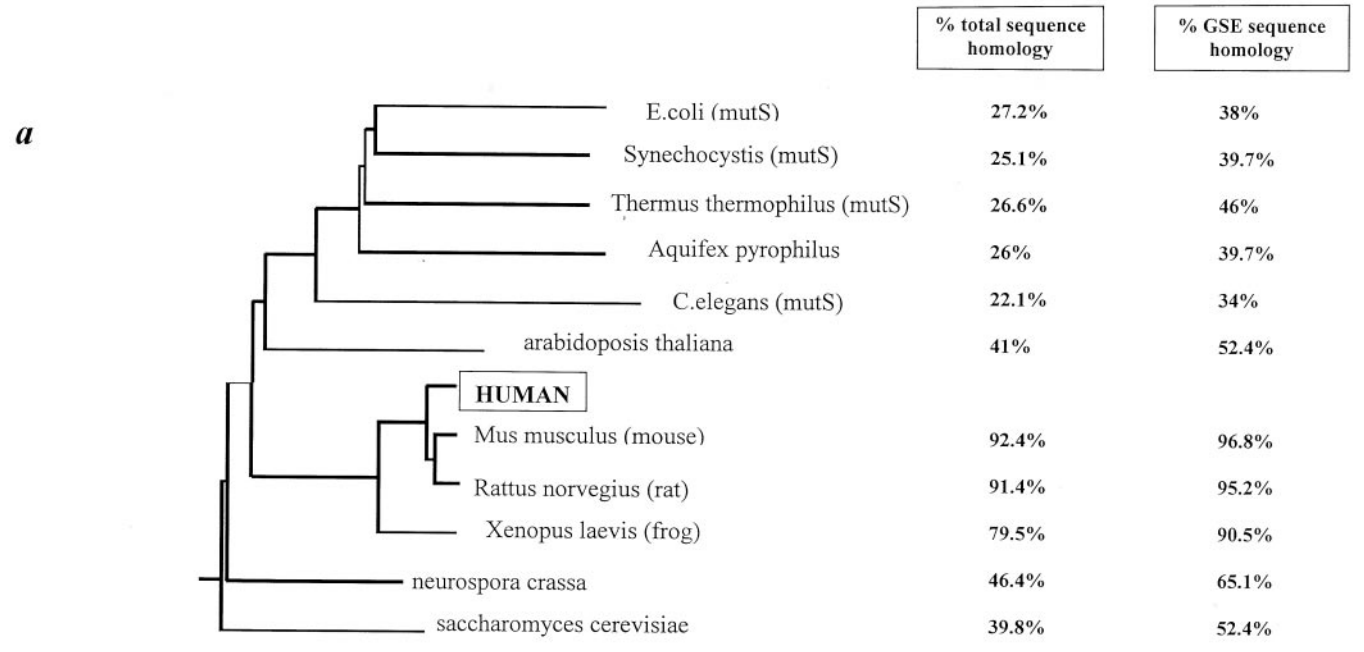


Fig. 6. Microsatellite instability in cells expressing a consensus GSE. The graph shows the frequency of blue bacterial colonies obtained after passage of pZCA29 through the GVC8 (■) and empty-vector containing UMSCC10b (♦) cell lines and subsequent transduction into permissive bacteria. Plasmid DNA was isolated from the human cells at the indicated times after transfection. The *x*-axis crosses the *y*-axis at the baseline value of spontaneous mutation frequency of pZCA29 in *E. coli* XL1-Blue MRF′. Each point represents the mean (± S.D.) of three experiments.

denced by the degree of drug resistance, expression of the consensus putative GSE in GVC8 was sufficient to produce genomic instability as measured by a change in the rate of generation of etoposide- and 6TG-resistant variants and an increase in microsatellite instability of the out-of-frame pZCA29 β-galactosidase vector. Interestingly, despite the fact that cells that have lost all MMR activity because of lack of expression of hMLH1 have only a 2.4-fold increase in the rate of generation of etoposide-resistant variants (de las Alas et al., 1997), the partial impairment in the GVC8 cells yielded variant generation rates that were 4.3- and 6.3-fold higher for etoposide and 6TG, respectively. This is consistent with the concept that small decrements in MMR function have a larger impact on genomic stability than on drug resistance.

In summary, the results of this study suggest that the region encompassing amino acids 782 to 844 in hMSH2 is important to the protein's ability to generate a proapoptotic signal from adducts produced by 6TG, DDP, and MNNG and to its ability to preserve genomic stability. The concept that the putative GSE-encoded protein acts to interfere with essential interactions of hMSH2 is supported by the predicted structure and location of this segment of the protein on a



b

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homo sapiens          AFCMFATHFHELTALANQIPTVNNLHVTALTTEETLT----------MLYQVKKGVCDQSFGIHVAELANFPKHVIECA
mus musculus          AFCMFATHFHELTALANQIPTVNNLHVTALTTEETLT----------MLYQVKKGVCDQSFGIHVAELANFPRHVIACA
rattus norvegius      AFCMFATHFHELTALASQIPTVNNLHVTALTTEETLT----------MLYQVKTGVCDQSFGIHVAELANFPRHVIECA
xenopus laevis        AFCMFATHFHELTALADQVPTVNNLHVTALTTEDTLIT---------MLYRIKKGVCDQSFGIHVAELANFPKHVIETA
neurospora crassa     CFALFATHFHELTALADQYPNVKNLHVTAHISGTDTDTDVITDEDEKAKKKREVTLLYKVEPGICDQSFGIHVAELVRFPDKVVRMA
arabid. Thaliana      APTLFATHFHELTALAQANSEVSGNTVGVANFHVSAHIDTESRK---------LTMLYKVEPGACDQSFGIHVAEFANFPESVVALA
s. cerevisiae         CFALFATHFHELTELSEKLPNVKNMHVVAHIEKNLKEQKHDDED---------ITLLYKVEPGISDQSFGIHVAEVVQFPEKIVKMA
E.coli (mutS)         ALTLFATHYFELTQLPEKMEGVANVHLDALEHGDTIA----------FMHSVQDGAASKSYGLAVAALAGVPKEVIKRA
T. thermophilus(mutS) CYTLFATHYFELTALA--LPRLKNLHVAAKEEEGGLV----------FYHQVLPGPASKSYGVEVAEMAGLPKEVVERA
Aquifex pyrophilus    AKTLLATHYLELTELERKVKGVKNYHMEVEETDEGIR----------FLYILKEGRAKGSFGIDVAKLAGLPEEVVREA
Synechocystis (mutS)  ARTIFATHYHELNELASLLENVANFQVTVKELPEEII----------FLHQVTPGGADKSYGIEAGRLAGLPSSVITRA
C.elegans (mutS)      PHIFLSSHFHALPNYIPLETNIATFLTFTVLREAGGKIK----------YLFRMTPGLVDCSFALSVAKEEGIPPPVIGRA
```

Fig. 7. A, dendrogram of MutS homologues found in various organisms. The percentage sequence homology for the total MSH2 sequence and for the sequence in the GSE region of each organism is compared with the human MSH2 sequence. B, comparison of the GSE region amino acid residues of human MSH2 and the corresponding residues of other MutS homologs. Conserved bases are white with black background. The helix-turn-helix domain of *E. coli* and *S. cerevisiae* are indicated by bold, italicized, and underlined letters.

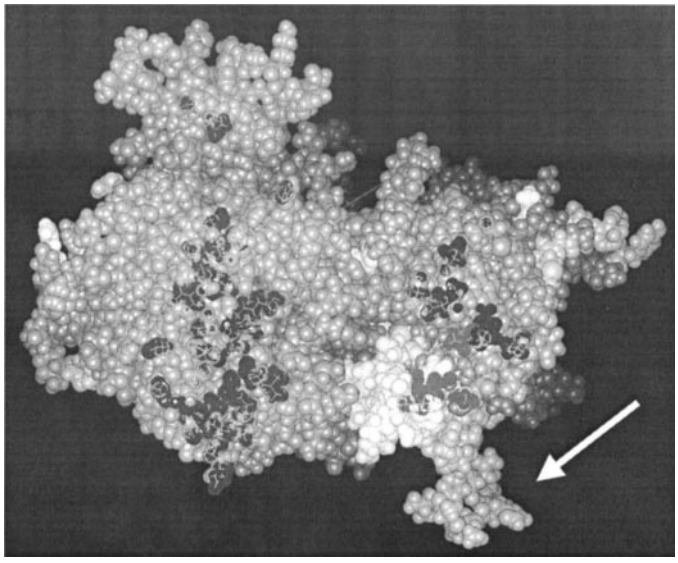

Fig. 8. Three dimensional theoretical model of the hMSH2 protein. The GSE region between amino acids 782 and 844 is indicated by an arrow.

theoretical model of hMSH2 and the fact that a portion of this segment overlaps the helix-turn-helix domain. Exactly what interactions are disrupted and what steps in the sequence of events that take place during adduct processing are affected by the putative GSE encoded protein are yet to be determined.

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