Cell Type-Dependent Regulation of hMLH1 Promoter Activity Is Influenced by the Presence of Multiple Redundant Elements

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Abstract

Immunohistochemical analysis confirmed the presence of MLH1 protein in A2780 ovarian cancer cells and its absence in this same cell line on acquired resistance to cisplatinum (A2780/CP). Transfection of a ~1781-bp hMLH1 promoter construct into either A2780 or A2780/CP cells produced similar (30-fold) induction of luciferase, an indication that the transcriptional machinery for hMLH1 expression remains intact. hMLH1-luciferase activity was also unaffected by re-expression of hMLH1 following treatment of A2780/CP cells with the methylase inhibitor 2-deoxy-5-azacytidine. Serial 5′-deletion studies of the hMLH1 promoter region in ovarian cancer cells localized transcriptional enhancers to a region (~250 to ~151 bp) that excludes the previously identified CCAAT element (~282) active in HeLa cells. When these same deletion constructs were transfected into HeLa cells, deletion of the CCAAT-containing region caused a significant loss of promoter activity, an indication of cell-specific use of enhancer elements. Finally, a series of internal deletion and linker mutation studies of the ~250 to ~151 bp ovarian enhancer region revealed that the hMLH1 promoter contains multiple redundant enhancer elements capable of independent promoter activation and may explain the association of this region with methylation silencing of hMLH1.

Introduction

In humans, the mismatch repair system maintains the fidelity of the cellular genome through excision and repair of mismatched DNA bases (1). The hMSH2 protein forms the core of a complex responsible for the initial recognition and targeting of mismatched nucleotides while the hMLH1 protein forms another complex that performs excision/repair (2). Defects in the hMLH1 and hMSH2 genes have been recognized as potential mediators of tumorigenesis (3), presumably the result of their crucial role in DNA repair and cellular genome integrity.

Studies suggest that the mismatch repair proteins play an important role in cisplatinum-induced tumor cell death (4, 5). The absence of hMLH1 expression has been correlated with acquired resistance of tumor cells to a number of DNA-damaging agents including cisplatinum and doxorubicin (6, 7). Clinically, the loss of hMLH1 expression has been associated with acquired resistance to chemotherapy in patients with ovarian cancer (6). Evidence for a direct link between hMLH1 expression and drug resistance was demonstrated by reintroducing the hMLH1 gene into MLH1 null mouse fibroblasts (8) or the MLH1 deficient ovarian cancer cell line, A2780/CP (9), to produce re-expression of MLH1 and concurrent cisplatinum resensitization.

Mutations have been identified in hMLH1 that account for the lack of expressed protein in several tumors (10, 11). However, in a significant number of cancers, where there are no detectable levels of the hMLH1 protein, no deletions or mutations of hMLH1 were detectable (12), leading to speculation that alterations in transcriptional activation of hMLH1 may be responsible.

DNA methylation silencing of genes, such as hMLH1, has emerged as the leading mechanism to account for the lack of detectable protein in tumors lacking mutations (13, 14). The precise mechanism responsible for methylation of normally unmethylated CpG sites in neoplastic cells is poorly understood. Current speculation is centered on chronic exposure to DNA-methyltransferase activity and/or the loss of normal protective mechanisms (15). Methylation of CpG sites appears to silence gene expression through interference with transcription factor-mediated trans-activation. The mechanism by which CpG methylation interferes with transcription is an area of intensive investigation. Initial studies indicated that CpG methylation could directly interfere with transcription factor binding (16). However, recently, attention has centered on transcriptionally repressive chromatin resulting from the recruitment of histone deacetylases (HDACs) (17, 18). The focus on alterations in chromatin has been prompted by evidence that deacetylation of the nucleosomal core histone proteins produces a repressive chromatin complex that prevents transcriptional activation of genes (19, 20).

Methylation silencing of the hMLH1 5′ proximal promoter region is not uniform, but instead, appears to be confined to several targeted regions (21). DNA sequencing-based methylation analysis has localized the region of methylation associated with the loss of hMLH1 expression in colon cancer cell lines to sequences between ~248 and ~178 bp (21). The importance of methylation of the hMLH1 gene in silencing its expression has
been substantiated by the observation that cell lines suspected of methylation-suppressed expression of hMLH1 can be induced to re-express the gene by addition of an inhibitor of DNA methylation, 2′-deoxy-5-azacytidine (5-Aza) (22).

To date, studies aimed at the identification of enhancer elements within the hMLH1 promoter have been restricted to HeLa cells, making it difficult to ascertain the relationship, if any, between the methylation silencing observed in cisplatinum-resistant cells and transcription factor binding sites. In addition, the HeLa cell data are somewhat equivocal. The initial report on the cloning of the hMLH1 promoter indicated that full promoter activity in HeLa cells was confined to −184 bp of the 5′-upstream proximal promoter (23). However, subsequent studies identified a CCAAT binding site located further upstream (−282 bp) that also appears to be important for transcriptional activation in HeLa cells (24, 25).

In the present studies, we examined the transcriptional activity of the 5′ proximal promoter region of the hMLH1 gene in HeLa cells as well as a cisplatinum-sensitive ovarian cancer cell line, A2780, and its cisplatinum-resistant subline, A2780/CP (26). Transfection studies in these two ovarian cell lines clearly demonstrate that the transcriptional machinery required for hMLH1 expression remains intact in the cisplatinum-resistant A2780/CP cells, even though expression of the endogenous gene is silenced. Furthermore, re-expression of the endogenous hMLH1 gene by pre-treating the cells with the methylase inhibitor 5-Aza also had no effect on hMLH1 transgene expression. Finally, a series of deletion and mutation studies in both A2780 and HeLa cells indicated that the hMLH1 promoter contains multiple, redundant enhancer elements that have cell-specific activities. The presence of multiple, redundant enhancers in the hMLH1 promoter region may explain the observed silencing of hMLH1 expression in cancer cells associated with methylation of multiple CpG sites.

Results

Before initiating our studies on the regulation of the hMLH1 promoter, we performed a detailed immunohistochemical analysis of hMLH1 expression in both A2780 and cisplatinum-resistant A2780/CP ovarian cancer cell lines (Fig. 1). A2780 cells showed abundant expression of hMLH1 localized primarily to the nucleus in 91.7 ± 2.7% of the cells. In contrast, in cisplatinum resistance A2780/CP cells, expression of hMLH1 was virtually absent, with only 7.3 ± 1.7% of the cells exhibiting detectable levels of hMLH1 protein.

Our analysis of the 5′-upstream promoter region of hMLH1 began with the isolation of a 1781-bp fragment of the MLH1 proximal promoter and its insertion into the pGLO-luciferase reporter vector (−1781 hMLH1-pGL3). A series of 5′ deletions of the −1781-bp fragment was subsequently generated and transfected into the hMLH1-expressing A2780 cells, to identify important regulatory regions (Fig. 2A). When the entire −1781-bp 5′-upstream promoter region from hMLH1 was ligated upstream of the pGLO-luciferase reporter, a significant (P < 0.01) 50-fold increase in luciferase activity was observed consistent with the presence of the hMLH1 promoter in this construct. Serial 5′-deletions to −914, −577, −294, and −250 bp produced no significant reduction in promoter activity, with the activity of the −250-bp construct equal to 105% of the full-length −1781 fragment. Further 5′ deletions to −195 bp produced a significant (P < 0.05) 80% decrease in luciferase activity while deletion to −150 bp resulted in an additional loss of activity to 6.6% of that observed in the −250-bp fragment (P < 0.01). No further reduction in activity was observed when the promoter region was reduced from −150 to −100 bp, whereas, deletions below −100 bp resulted in a complete loss of promoter activity (data not shown), an indication that the −150- and −100-bp fragments contain only the minimal (basal) hMLH1 promoter.

To test whether silencing of hMLH1 in A2780/CP cells observed in Fig. 1 affects the activities of our hMLH1-pGLO3 constructs, we transfected the −1781 and −150 MLH1-luciferase constructs into both A2780 and A2780/CP cells (Fig. 2B). As depicted in the figure, transfection of either the −1781- or −150-bp construct into either cell line produced a significant (P < 0.05) increase in reporter gene activity above that seen with the empty pGLO3 vector control. Furthermore, a comparison of either the fold-increase or relative luciferase activity indicates that both the −1781- or −150-bp constructs produced similar luciferase expression in either cell line and thus, appear to be unaffected by the methylation silencing of hMLH1 in A2780/CP cells.

![Image](image-url)
The lack of hMLH1 expression in tumor cells has been attributed to hypermethylation silencing of the hMLH1 promoter region (14). To examine whether re-expression of hMLH1 through the use of inhibitors of DNA methylation affects the activity of our transfected hMLH1-luciferase constructs, we transfected A2780/CP cells that had been pretreated with the methylase inhibitor 5-Aza (Fig. 3). Immunohistochemical analysis confirmed that 5-Aza pretreatment produced a significant \((P < 0.05)\) re-expression of hMLH1 from 7.7 \(\pm\) 1.9% of control cells to 62.1 \(\pm\) 6.1% following 5-Aza (Fig. 3A). When we transfected these cells, we found that 5-Aza-induced reactivation of hMLH1 expression produced no discernable affect on the expression of the \(-1781\) or \(-250\)-bp hMLH1-luciferase (Fig. 3B) with similar luciferase activity as well as similar fold-induction.

A thorough examination of the activities of our 5‘-deletion constructs in either A2780 or A2780/CP cells revealed an unanticipated result. As is apparent in Figs. 2A and 3B, in ovarian cancer cells, transcriptional activity of the hMLH1 proximal promoter region is contained within the first \(-250\)-bp fragment. This observation is somewhat at odds with reports from two independent laboratories that have identified a CCAAT regulatory element within the hMLH1 promoter at position \(-282\), using HeLa cells (24, 25).

In an attempt to reconcile this discrepancy, we transfected our hMLH1 deletion constructs into HeLa cells (Fig. 4). Immunohistochemical staining showed similar hMLH1 expression in HeLa cells, 90.8 \(\pm\) 6.3% (Fig. 4A), to that observed previously in the hMLH1-expressing A2780 cells in Fig. 1. When transfected, HeLa cells showed no loss of promoter activity when 5‘ deletions...
are made between \(-1781\) and \(-294\) (data not shown), similar to our previous result in A2780 cells. However, unlike A2780 cells, in HeLa cells, when the fragment between \(-294\) and \(-251\) containing the \(-282\) CCAAT sequence was removed, there was a significant \((P < 0.05)\) loss of luciferase reporter activity (Fig. 4B), a result consistent with the activity associated with the previously identified CCAAT element (24, 25).

To examine whether the CCAAT element-containing region that is active in HeLa cells has any activity in our A2780 cells, we generated the following two constructs: The \(-150\)-bp basal promoter was used as the starting construct; upstream of this fragment, we inserted two different sequences: (a) the \(-294\) and \(-251\) region that is active in HeLa cells and contains the previously identified CCAAT site or (b) the sequence between \(-914\) and \(-295\). Both the \(-294\) to \(-251\) CCAAT-containing region and the \(-914\) to \(-295\) regions were selected because our 5'-deletion studies showed loss of these sequences produced no change in promoter activity when the \(-250\)-to \(-151\)-bp region was present (Figs. 2A and 3B). Transfected these constructs into A2780 cells (Fig. 5) resulted in a significant \((P < 0.05)\) 3-fold induction of hMLH1 promoter activity above that seen with the \(-150\)-bp minimal promoter fragment, a finding consistent with the presence of additional "redundant" enhancer elements within both of these upstream regions.

The possible presence of redundant enhancer elements in the hMLH1 5'-proximal promoter led us to analyze in greater detail the sequence between \(-250\) and \(-196\) bp where the majority of enhancer activity in ovarian cell lines resides (Fig. 2A). An examination of this 55-bp sequence revealed the presence of several CpG methylation sites (Fig. 6A), which have previously been shown in colon cancers to be hypermethylated (21). Analysis of the methylation status of the cytosine bases present within this 55-bp sequence isolated from hMLH1 silenced A2780/CP cells, as measured by protection from sodium bisulfite modification, indicates that greater than 90% of the cytosine residues contained within these five CpG sites are methylated. In contrast, analysis of this same region isolated from A2780 cells revealed only 50% cytosine methylation, a value that accords with previous observations that in hMLH1-expressing A2780 cells, a single hMLH1 allele is methylated (22).

In their previous identification of the CCAAT element at \(-282\) bp, Deng et al. (24) also showed that methylation of a CpG site 2 bp upstream inhibited binding of the transcription factor CBF to this site. Therefore, to simultaneously examine any potential relationship between the CpG methylation sites within the \(-250\)-to \(-196\)-bp region and also to refine the location of transcriptional enhancer activity, we systematically generated a series of small contiguous mutations encompassing

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**FIGURE 3.** hMLH1 expression and transient transfection of control or 5-Aza pretreated A2780/CP. A2780/CP cells were pretreated in the absence (control) or presence of 2 \(\mu \text{M}\) 5-Aza for 48 h. A. Photomicrographs of immunohistochemical stained cells for hMLH1 expression. The percentage of cells stained for hMLH1 protein is depicted directly below each photomicrograph as determined using an ACIS (Chromavision). B. Transient transfection of control and 5-Aza pretreated cells with hMLH1, \(-1781\), \(-250\), and \(-150\), pGL3 luciferase reporter constructs. The relative promoter activity of each construct in both cell lines is indicated in the graph. The length of the 5' proximal hMLH1 promoter ligated to luciferase is indicated immediately beneath each bar on the graph. Data are expressed as relative luciferase activity. Columns, mean of two independent experiments; bars, SE; four plates transfected for each construct \((n = 8)\).
the CpG sites. To accomplish this, a series of four contiguous 10- to 15-bp scanning-linker mutations was generated (Fig. 6B, left panel) within the −250- to −196-bp region, and transfected into A2780 cells (Fig. 6B, right panel). The −250 pGL3 and −195 pGL3 constructs were also transfected to provide a reference to compare the effects of the mutations. As is apparent from the figure, none of the four contiguous mutations produced any significant loss in promoter activity normally associated with the loss of enhancer elements, making it impossible to ascertain what, if any, relationship exists between enhancer activity and CpG methylation sites. On the other hand, since combining the four linker mutation constructs encompasses the entire region deleted in the −195-bp fragment, it seems likely that this small 55-bp region of the hMLH1 promoter also contains multiple redundant enhancer elements.

**FIGURE 4.** Relative enhancer activity of 5’ serial deletion constructs of the hMLH1 promoter in HeLa cells. A, Photomicrographs of immunohistochemical stained HeLa cells for hMLH1 expression. B, Transient transfection of HeLa cells with deletion constructs of the hMLH1 proximal promoter region ligated to a luciferase reporter. The relative promoter activity of each construct in both cell lines is indicated in the graph. The length of the 5’ proximal hMLH1 promoter ligated to luciferase is indicated immediately beneath each bar on the graph. Data are expressed as relative luciferase activity. Columns, mean of two independent experiments; bars, SE; four plates transfected for each construct (n = 8).

### Discussion

Evidence suggests that MLH1 proteins may provide a crucial link in the cells’ ability to recognize cisplatinum-induced DNA damage and mediators of apoptosis such as p53 (5, 27). This is supported by studies that show that loss of hMLH1 expression due to DNA methylation is associated with acquired resistance to cisplatinum in ovarian cancer cells (22) while reintroduction of the hMLH1 gene into these MLH1-deficient cells leads to resensitization to cisplatinum (9).

Several laboratories have reported that CpG hypermethyl-ation silencing occurs in the hMLH1 gene (13, 14, 21), and that hypermethylation is associated with acquired cisplatinum resistance in the ovarian cancer cell line A2780/CP (22). In our present studies, coordinate transfection of both hMLH1-expressing A2780 and non-expressing (silenced) A2780/CP cells (Fig. 1) revealed that both cell lines were able to induce similar levels of hMLH1-luciferase activity (Fig. 2B). Furthermore, re-expression of hMLH1 in A2780/CP cells by pretreatment with the DNA methylase inhibitor 5-Aza (Fig. 3A) produced no change in hMLH1-luciferase expression (Fig. 3B). Taken together, these data provide convincing evidence that the transcriptional machinery required for hMLH1 expression remains intact in hypermethylated A2780/CP cells and is unaffected by changes in the methylation status of the hMLH1 gene.

Deletion studies of the hMLH1 proximal promoter uncovered an apparent difference in the regulation of this gene in ovarian versus HeLa cells. In ovarian cancer cells, deletion of the region between −295 and −251 bp had no effect on hMLH1-luciferase expression (Figs. 2A and 3B). This was a surprising result given the fact that two independent laboratories have identified a CCAAT element (−282 bp) within this same region, which is active in HeLa cells (24, 25). Subsequent transfection of our hMLH1-luciferase constructs into HeLa cells produced a result consistent with these previous studies, as deletion of the −294- to −251-bp region containing the CCAAT element results in a significant loss of promoter activity (Fig. 4B).

Our deletion studies in ovarian versus HeLa cells appear to indicate that hMLH1 contains cell-specific enhancer elements. However, when we ligated the −294- to −251-bp region to the −150 hMLH1 minimal promoter, we discovered that it is also capable of enhancing promoter activity, as is the −914- to −295-bp region (Fig. 5). Therefore, the absence of a detectable fall in luciferase expression in ovarian cancer cells when the −294- to −251- or −914- to −295-bp regions were deleted could also be due to the presence of redundant enhancer elements within the hMLH1 proximal promoter.

The presence of redundant enhancer elements in the hMLH1 promoter was further verified by closer examination of the enhancer activity contained within the −250- to −196-bp region where a majority of enhancer activity in ovarian cells resides. Utilizing 10–15 bp scanning-linker mutations of this region, we found no change in promoter activity, even though combining these 10- to 15-bp mutations formed a contiguous deletion of the entire region between −250 and −196 (Fig. 6B); again, consistent with the presence of redundant enhancer elements.

If, as it appears, hMLH1 contains redundant enhancer elements, the loss of one or possibly two of these elements...
within the −250- to −196-bp region via linker mutation would have little or no effect on overall luciferase expression, as long as the remaining elements were sufficient to produce maximal transcriptional activity. Only when enough elements were removed (ex. 5′ deletion to −195 bp), that those remaining can no longer induce maximal hMLH1 promoter activity, do you see a reduction in luciferase. Additional studies designed to identify and characterize individual enhancer element contained within the hMLH1 promoter region will be required to confirm the presence of redundant enhancers.

Recently, studies have shown that a small region within the hMLH1 promoter (−248 to −178) exhibits hypermethylation that correlates with the lack of expression in colorectal cancer cell lines (21). Follow-up studies in several gastric cancer cell lines also indicate that methylation of a similar sequence (−270 to −199) was associated with silencing of MLH1 expression (28). Our deletion studies (Fig. 2A) indicate that a comparable region (−250 to −151 bp) contains regulatory elements important for hMLH1 expression in ovarian cancer cells. DNA methylation analysis also identified additional CpG sites upstream of the −248- to −178-bp region, which are often hypermethylated in both colon (21) and gastric (28) cancer cell lines. The methylation status of these upstream sites, relative to hMLH1 silencing, was weak with no effect on hMLH1 expression in the absence of coordinate methylation of the −248 to −178 region. The lack of effect methylation of these upstream sequences has on hMLH1 silencing accords with our transfection studies, which show that deletion of sequences upstream of −250 bp has little effect on hMLH1 promoter activity when the −250- to −151-bp fragment is present (Figs. 2A and 3B).

How methylation of the hMLH1 promoter interferes with transcriptional activity is not currently understood. Deng et al. (24) as part of the characterization of the hMLH1 CCAAT site showed that methylation of a CpG site 2 bp upstream prevented binding of the CBF transcription factor, in vitro. However, data from other systems indicate that a direct effect on transcription factor binding is probably not the primary mechanism responsible for methylation silencing; instead, silencing may be the result of a more generalized alteration in chromatin structure (29, 30). The exact dynamic linking CpG methylation to alterations in chromatin structure is not well understood. It appears to involve methyl-CpG binding proteins (MeCPs) (31, 32), which recruit HDAC enzymes to the promoter region of genes to produce repressive chromatin structures (33, 34). In recent years, considerable evidence has emerged that transcriptionally repressive chromatin is formed by HDACs through deacetylation of the core histone proteins, altering nucleosome DNA binding (20, 35).

Studies have also begun to address the relationship between the position and length of methylated sequences of DNA relative to their impact on promoter activity. Initial data seem to indicate that the location of a methylated region adjacent to the promoter, preferably within the “transcriptional unit”, may be key to gene silencing (36, 37). Related studies have also shown that methylation-induced alterations in histone acetylation appears to be a local effect, covering one or possibly two nucleosomes, and does not propagate along the DNA strand (37, 38). If substantiated, localized regions of methylation-induced repressive chromatin capable of interfering with the activity of transcription factors might explain the apparent association between our strong transcriptional enhancer region (−250 to −151 bp) and methylation of this region in hMLH1 silenced cancer cells. Clearly, additional studies involving the identification and characterization of individual enhancer elements within the −250- to −151-bp region, coupled with mutation and/or translocation of these elements relative to the CpG methylation sites, will be required to provide a definitive resolution of the relationship between enhancer elements and methylation silencing of the hMLH1 promoter.

Finally, the physiological significance of multiple redundant elements within the hMLH1 promoter raises a number of interesting questions. Previous studies have identified multiple transcription factor binding sites in the mouse HTF9 housekeeping gene, only a subset of which are necessary for transcriptional activation (39). Sequence data obtained at the time of the original cloning of the hMLH1 5′-upstream region indicates it as a TATA-less promoter (23) similar to those found in many “housekeeping” genes, which tend to be ubiquitously expressed in most if not all cells. A limited computer search for “potential” transcription factor binding sites within the −250- to −196-bp fragment of hMLH1 promoter using OMIGA software (Accelrys, Princeton, NJ) and a modified NASITE database revealed several sequences with 85–100% homology to binding sites, for SP-1, SF-1 STAT 5, and MAZ.

The presence of multiple enhancer elements opens the possibility that different combinations of transcription factors can induce expression of hMLH1 in different cell types (e.g., ovarian versus HeLa; Figs. 2A and 4). Multiple redundant elements may also provide an adaptive advantage by allowing
hMLH1 expression to remain elevated when faced with a changing intracellular environment resulting from cellular stress, replication, or injury, consistent with the role of hMLH1 in repairing damaged DNA.

Materials and Methods

Cell Culture and Reagents
The human ovarian cancer cell lines were obtained from the European Collection of Cell Cultures [A2780, A2780/CP] (ECACC; Salisbury, United Kingdom) and HeLa cells from the American Type Culture Collection [SK-OV-3] (ATCC, Manassas, VA). A2780 and its subline, A2780/CP, were selected due to the fact that acquired resistance to cisplatinum in A2780/CP cells has previously been shown to be associated with hypermethylation of the MLH1 gene (22) and is reversible on reintroduction via gene transfer of a non-methylated copy of hMLH1 (9). A2780, A2780/CP cells were cultured in RPMI 1640 and HeLa cells in Eagle’s MEM with Earls BSS and 2 mM L-glutamine. All media were supplemented with 10% fetal bovine serum. Culture were grown at 37°C in a humid 95% air/5% CO2 chamber and were periodically tested to ensure they remained free of mycoplasm infection during the course of the experiments. When indicated, A2780/CP cells were pretreated with 5-Aza (2 μM) in the culture media.

Preparation of Paraffin Blocks From Cultured Cells
Cultured cell lines were fixed and embedded in paraffin blocks as previously described (40). Briefly, cultures were trypsinized and removed from the culture dish for 5 minutes, washed once in culture medium to remove the excess trypsin, and re-centrifuged. The final pellet was resuspended in 0.25 ml of medium, 0.5 ml of human plasma was added, followed by a similar volume of thromboplastin as previously described (41). The mixture was agitated for 2 min until coagulation occurred and followed by addition of 10% buffered-formalin for 5 min, washed once in culture medium to remove the excess trypsin, and re-centrifuged. The final pellet was re-centrifuged. The final pellet was re-centrifuged. The final pellet was re-

hMLH1/Luciferase Reporter Constructs
Determinations of the −1781 bp of the hMLH1 upstream/promoter region was amplified from human genomic DNA by the PCR. A high-fidelity Taq DNA polymerase with proofreading capabilities (Platinum Taq, Life Technologies, Rockville, MD) was used to minimize potential PCR errors. hMLH1-specific oligomeric primers were synthesized (Life Technologies); a 3′-hMLH1 primer 5′-ATAT{AAGCTT}TGGCGCCAGAAGAGCC-3′ and the −5′-hMLH1 primer 5′-ATAT{GCTAGC}TGAGGCAG-GAAAGTCTG-3′, using the previously published sequence for the 5′-upstream promoter region of the hMLH1 gene (23). The underlined sequence in each primer corresponds to sequences derived from the 3′- and 5′-ends of hMLH1 promoter region. For cloning purposes, a HindIII site was added to the

Immunohistochemistry
Fixed cells were immunostained for hMLH1 using an immunoperoxidase procedure as previously described (42). Slides were stained using the HRP, LSAB2 System (DAKO, Carpinteria, CA). Briefly, mouse monoclonal antibodies against human hMLH1 (BD PharMingen, San Diego, CA) were used as the primary antibody. The secondary antibody was a biotinylated goat anti-mouse (DAKO) which was then followed by Strepavidin-HRP incubation. Finally, the samples were counterstained with hematoxylin (DAKO), dehydrated, and mounted. The percentage of positively staining cells was determined from five separate areas of the slide using an ACIS (Chromavision), which combines automated microscopy to record and assemble hundreds of individual captured fields with computerized image analysis to simultaneously determine both staining intensity and percentage of stained cells for control and TSA-treated cells on the same slide.

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\begin{align*}
-250 & \quad -196 \\
{5′-GGAGGCA} & {5′-CCAGC} \\
TAXCCTCTA & \text{GCTAGCGGCCT} \\
AGTCGGCGCT & \text{TCAGGAGGAG} \\
ACCGAA & \text{A′}
\end{align*}
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Relative Luciferase Activity

0 50 100 150

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FIGURE 6. Mutation analysis of the −250 to −195 enhancer region. A. The sequence of the −250- to −195-bp region of the hMLH1 promoter region is shown. Methylated cytosines contained within CpG sites are shown in larger font/underlined. B. A series of four contiguous linker substitution mutations of the −250- to −195-bp hMLH1 proximal promoter was constructed as described in “Materials and Methods.” Schematic representations of the mutated regions are shown at the left. Open boxes indicate the regions mutated; numbers in brackets indicate the position of base pairs within the hMLH1 promoter substituted by the linker sequence. The −250- and −195-bp hMLH1 pGL3 constructs were transfected as controls. The relative luciferase activity of each construct, compared to the non-mutated −250-bp pGL3 (100%), is shown at the right. Columns, mean of two independent experiments of four plates transfected (n = 8); bars, SD.
3′-hMLH1 primer and an NheI site to the 5′-hMLH1 primer (shown in brackets). The 4-bp sequence ATAT was added immediately adjacent to both restriction enzyme sites to aid in restriction enzyme digestion of the final PCR product. The final PCR product was digested with NheI/HindIII, gel-isolated and sub-cloned into the multiple cloning site of the pGL3-Basic Vector (Promega, Madison, WI) immediately upstream of the luciferase reporter gene to produce the −1781 hMLH1 pGL3 construct. The −1781 hMLH1 pGL3 construct was sequenced on an ABI 3700 capillary sequencer.

hMLH1 Deletion Constructs

Serial deletions of the 5′ end of the −1781-bp hMLH1 promoter region were generated in separate reactions using the following restriction enzymes to shorten the 5′-end of the promoter: EcoRV (−914 bp); ApaI (−577 bp); SacI (−294 bp); and BsaI (−195 bp). Following restriction enzyme digestion of the −1781-bp hMLH1-pGL3 vector with the above enzymes, the ends were polished blunt using T4 polymerase, and the fragments (sizes shown in brackets next to the restriction enzymes; above) excised by digestion of their 3′ ends with HindIII. The 5′-shortened fragments were subsequently gel-isolated, and re-ligated into the SmaI/HindIII sites of the pGL3 Basic Vector. When restriction enzymes sites were not available, deletions were made by PCR as described above utilizing the following 5′ PCR primers: (−250 bp; 5′-CGCAAGCGCATATCTTCTAGG-3′); (−150 bp; 5′-GTGCAACATCAAAATGC-3′); and (−100 bp; 5′-TACAGCTGAAAGAGAACCAGTGAG-3′). All primers had the ATAT[NheI] sequence added to their 5′ ends to facilitate cloning into the pGL3 Basic Vector as described above. The 3′ PCR primer was the same as described for the −1781-bp fragment above, with the −1781-bp hMLH1 promoter fragment acting as the template. All deletion constructs were sequenced to ensure authenticity before their use in transfection assays.

hMLH1 Linker Mutations

A series of contiguous mutation of the hMLH1 proximal promoter region between −294 and −195 was accomplished using the Quick Change Mutagenesis Kit (Stratagene, La Jolla, CA). Complementary 45-mer primers were synthesized, containing a 15-bp EcoRI linker “mutant” sequence flanked by 15 bp of wt sequence derived from either side of the sequence within the hMLH1 upstream/promoter region to be replaced. The 15-bp EcoRI linker sequence 5′-ATAA-GAATTCATATA-3′ was chosen as a mutating replacement as we have previously used it as a replacement sequence in A2780 transfection assays, and have shown that it has no inherent transcriptional enhancer activity (40).

Transient Transfection Assays

DNA constructs were transiently transfected into cells using LipofectAMINE Plus Reagent (Life Technologies). Twenty-four hours before transfection, cells were sub-cultured onto 60-mm plates so that they would be at 50–60% confluence the following day. Transfections were optimized for the amounts of DNA, PLUS reagent, and LipofectAMINE per 60-mm culture dish as follows: 0.6 μg hMLH1-pGL3 construct; 0.1 μg pRL-TK vector (Promega); 2.4 μl PLUS Reagent; and 3.6 μl of LipofectAMINE Reagent. The above reagents and DNA constructs were mixed and incubated with the cells overnight (18 h) according to the manufacturer’s protocol. The following day, the culture medium was replaced and the cells placed back into a 37°C, 95% O2, 5% CO2 incubator for an additional 24 h.

Luciferase Assay

Cells were harvested from the plates following a single wash of PBS to remove residual media in 400 μl/plate of Passive Lysis Buffer (Promega) by scraping. Cell lysates were frozen at −20°C to ensure complete lysis of the cells. Luciferase activity in the cell lysates was determined using the Dual-Luciferase Reporter Assay System (Promega) to allow sequential determination in the same sample of both the firefly luciferase activity from the hMLH1-pGL3 constructs and the transfection efficiency from the Renilla-luciferase activity of the pRL-TK vector. All assays were carried out in a single-sample luminometer (DIGENE Diagnostics, Gaithersberg, MD) model DRC-1. All reported firefly luciferase values were normalized for transfection efficiency using the pRL-TK, Renilla-luciferase value. Statistically significant differences in promoter activity of the various hMLH1-pGL3 constructs were determined by ANOVA.

DNA Methylation Assay

The methylation status of CpG sites within the 55-bp region (−250 to −196) were determined using the CpGenome Kit (Intergen, Purchase, NY), bisulfite modification coupled methylation specific PCR (13). Briefly, genomic DNA was isolated from cell cultures using the Wizard Genomic DNA Purification Kit (Promega). One microgram of genomic DNA was incubated with DNA modification Reagent (CpGenome Kit, Intergen) for 16–20 h at 50°C following the manufacturer’s protocol. The following day, the DNA was cleaned up and purified using Wizard DNA Clean Up System (Promega). The purified DNA was then subjected to PCR using primers adjacent to the −250 to −195-bp region of interest. The PCR product was run on 4% Neusieve, isolated, and sequenced on an ABI 3700 capillary sequencer. Because methylation of cytosine residues prevents their conversion to uridine, the ratio of C/C+T for each cytosine residue was calculated and used to determine the percentage of methylation.

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References

Multiple Elements Regulate hMLH1 Activity


Molecular Cancer Research

Cell Type-Dependent Regulation of hMLH1 Promoter Activity Is Influenced by the Presence of Multiple Redundant Elements\(^1\) 1 Feature Films for Families (CEO: Forrest S. Baker III); The Smith Cancer Institute; The Joni Spafford Whitney Endowment; Warren and Kay Forsythe; The Deseret Foundation; and The Annette Montgomery Bourne Endowment.

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