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Cell Cycle Regulation of the Human DNA Mismatch Repair Genes *hMSH2*, *hMLH1*, and *hPMS2*[1]

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Abstract

Hereditary nonpolyposis colorectal cancer is a cancer susceptibility syndrome that has been found to be caused by mutations in any of several genes involved in DNA mismatch repair, including *hMSH2*, *hMLH1*, or *hPMS2*. Recent reports have suggested that *hMSH2* and *hMLH1* have a role in the regulation of the cell cycle. To determine if these genes are cell cycle regulated, we examined their mRNA and protein levels throughout the cell cycle in IMR-90 normal human lung fibroblasts. We demonstrate that the levels of hMSH2 mRNA and protein do not change appreciably throughout the cell cycle. Although hMLH1 mRNA levels remained constant, there was a modest (approximately 50%) increase in its protein levels during late G_1 and S phase. The levels of hPMS2 mRNA fluctuated (decreasing 50% in G_1 and increasing 50% in S phase), whereas hPMS2 protein levels increased 50% in late G_1 and S phase. Our data indicate that, at least in normal cells, the machinery responsible for the detection and repair of mismatched DNA bases is present throughout the cell cycle.

Introduction

HNPCC[3] is a familial cancer syndrome accounting for approximately 1–5% of the overall colon cancer incidence in the Western world (1). Defects in the *hMSH2*, *hMLH1*, and *hPMS2* genes, which are involved in MMR, have been found to co-segregate with the disease (2–6). These genes are homologues of members of the *MutHLS* DNA repair pathway in bacteria, primarily responsible for correcting mismatched DNA base pairs that arise as a result of misincorporation errors during DNA replication (7). *hMSH2* encodes the human homologue of the bacterial MutS protein, which is responsible for recognizing mispaired bases in DNA (8). It was found mutated in 31% of 48 HNPCC kindreds examined (9). Two additional MutS homologues, hMSH3 and hMSH6 (GTBP/p160), have been identified (10), which appear to interact with hMSH2 and alter its binding specificity for single base pair and insertion/deletion mismatches. (11). The *hMLH1* gene encodes the homologue of the MutL protein, accounting for 33% of HNPCC (9). The function of hMLH1 is not yet known, although it is likely to interact with hMSH2 (12). *hPMS2*, which appears to be mutated in only 4% of these HNPCC kindreds (9), also encodes a homologue of MutL and is thought to interact with hMLH1 (12).

It has recently been suggested that the MMR system influences the cell cycle (13–15). The human colon tumor cell line HCT116 has no normal *hMLH1* gene product. When a normal copy of *hMLH1* on chromosome 3 was introduced via microcell chromosome transfer into HCT116, an enhanced G_2 arrest was noted upon exposure to 6-thioguanine, a DNA base analogue that mimics a mismatch upon incorporation into DNA. In addition, we recently demonstrated that hMLH1 plays a regulatory role in the G_2-M arrest following treatment with ionizing radiation.[4] Furthermore, both the human colon adenocarcinoma cell line LoVo and the human ovarian tumor cell line 2774, which are deficient in wild-type hMSH2, exhibited an attenuated G_2 arrest following treatment with the DNA alkylating agent *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine compared to the human colon adenocarcinoma line SW480, which contains wild-type hMSH2 and hMLH1 (15). Finally, in yeast, both MSH2 and MLH1 were found to interact with proliferating cell nuclear antigen, a multifunctional protein involved in the cell cycle which interacts with DNA polymerases δ and ϵ as well as cyclin/cyclin-dependent kinase complexes (16). In this study, we wished to determine if hMSH2, hMLH1, and hPMS2 mRNA and protein levels were regulated throughout the cell cycle.

Materials and Methods

Cell Culture and Synchrony. IMR-90 fibroblasts (derived from normal human fetal lung) were obtained from the American Type Culture Collection (Rockville, MD) and maintained in DMEM (Life Technologies, Inc., Gaithersburg, MD) supplemented with 10% FBS (Hyclone, Logan, UT), 100 units/ml penicillin, and 100 μg/ml streptomycin and grown in a 90% air-10% CO_2 atmosphere at 37°C. Cells were synchronized using common methods (17, 18), and cultures were grown no more than six passages when used. IMR-90 cells were allowed to grow to confluence and then maintained for approximately 60 h in DMEM with 0.1% FBS to enhance G_0 arrest. To induce proliferation, cell monolayers were dissociated (using 0.05% trypsin with 0.53 mM EDTA), and cells were replated at a 1:3 dilution in DMEM containing 20% FBS supplemented with additional vitamins and essential and nonessential amino acids (to twice their original concentrations).

Cell Cycle Analyses. Flow cytometric analyses were performed using common techniques (19). After trypsinization, cells were washed in PBS, fixed in 70% ethanol, and then stained in PBS containing 1% NP-40, 1 mg/ml RNase A, and 0.1 mg/ml propidium iodide for at least 30 min. Stained nuclei were analyzed for DNA-propidium iodide fluorescence using a Becton Dickinson FACScan (San Jose, CA) at a laser setting of 15.2 mW and an excitation wavelength of 488 nm. Data were analyzed using ModFit LT, version 1.01 (Verity Software House, Topsham, ME).

RNA Isolation and Slot-Blotting. Total RNA was purified using RNAzol B (Tel-Test, Friendswood, TX) following the manufacturer's instructions. Two μg of total RNA were transferred to a Zeta-Probe membrane (Bio-Rad, Richmond, CA) using the Minifold II slot-blot system (Schleicher & Schuell, Keene, NH) and probed with the appropriate randomly primed cDNA following the manufacturer's instructions (Boehringer Mannheim, Indianapolis, IN). 36B4 (human acidic ribophosphoprotein P0) was acquired from Dr. Pierre

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[3] The abbreviations used are: HNPCC, hereditary nonpolyposis colorectal cancer; MMR, mismatch repair; hMSH, human MutS homologue; hMLH, human MutL homologue; hPMS, human postmeiotic segregation homologue; FBS, fetal bovine serum.

[4] T. W. Davis, C. Wilson-Van Patten, M. Meyers, T. Wilson, K. A. Kunugi, S. Acharya, M. Koi, C. R. Boland, T. J. Kinsella, R. Fishel, and D. A. Boothman. Defects in the DNA mismatch repair protein hMLH1 alter G_2-M arrest following DNA-damaging agents, submitted for publication.

Chambon (INSERM, Strasbourg Cedex, Paris) (20). Human MSH2, MLH1, and PMS2 cDNAs have been described (2, 4, 6). RNA levels were quantified using the ImageQuant software, version 3.3, of the Molecular Dynamics PhosphorImager (Sunnyvale, CA).

Preparation of Crude Cell Lysates and Western Analyses. Cells were scraped into ice cold PBS, pelleted by centrifugation, resuspended in five volumes of ice-cold suspension buffer [100 mM NaCl, 10 mM Tris (pH 7.6), 1 mM EDTA, 1 μg/ml aprotinin, and 100 μg/ml phenylmethylsulfonyl fluoride] followed by five volumes of gel-loading buffer [100 mM Tris (pH 6.8), 200 mM DTT, 4% SDS, and 20% glycerol], and placed in a boiling water bath for 10 min (21). Thirty μg of each extract were separated on 8% SDS-PAGE gels using the Mini-PROTEAN II electrophoresis system (Bio-Rad) and transferred overnight at 40 V onto polyvinylidene difluoride membranes (Immobilon-P; Millipore, Danvers, PA) in transfer buffer (25 mM Tris base, 192 mM glycine, and 15% methanol) using the Mini Trans-Blot electrophoretic transfer cell (Bio-Rad) following the manufacturer's instructions. Blots were blocked for at least 1 h in PBST (PBS with 0.05% Tween 20) containing 10% nonfat dry milk. They were then incubated with a primary antibody at a dilution of 1:2500 (for anti-hMSH2, hMLH1, topoisomerase IIα, or α-tubulin) or 1:500 (for anti-hPMS2) at room temperature in PBST for 1.5 h and then washed three times (10 min each) in a large volume of PBST. Polyclonal hMSH2 and monoclonal hMLH1 and hPMS2 antibodies were generated by Oncogene Science (Cambridge, MA) using overproduced and purified proteins and characterized for specificity in total cell extracts. Polyclonal topoisomerase IIα antibody was obtained from Genosys Biotechnologies (The Woodlands, TX), and monoclonal α-tubulin antibody was acquired from Oncogene Science. Blots were then incubated in PBST for 1 h with a 1:7500 dilution of a horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA), either anti-mouse IgG (for anti-hMLH1, hPMS2, or α-tubulin) or anti-rabbit IgG (for anti-hMSH2 or topoisomerase IIα), and washed as above. Bound antibodies were detected using the SuperSignal chemiluminescence substrate (Pierce, Rockford, IL) on Fuji RX medical X-ray film (Fuji Photo Film, Tokyo, Japan). Quantitation was performed using the Molecular Analyst image analysis software (version 1.3) of the Gel Doc 1000 gel documentation system (Bio-Rad). The values given in the text represent the means and SEs of the levels of the indicated proteins (after correcting for loading variations against α-tubulin levels) from at least two independently done Western blots.

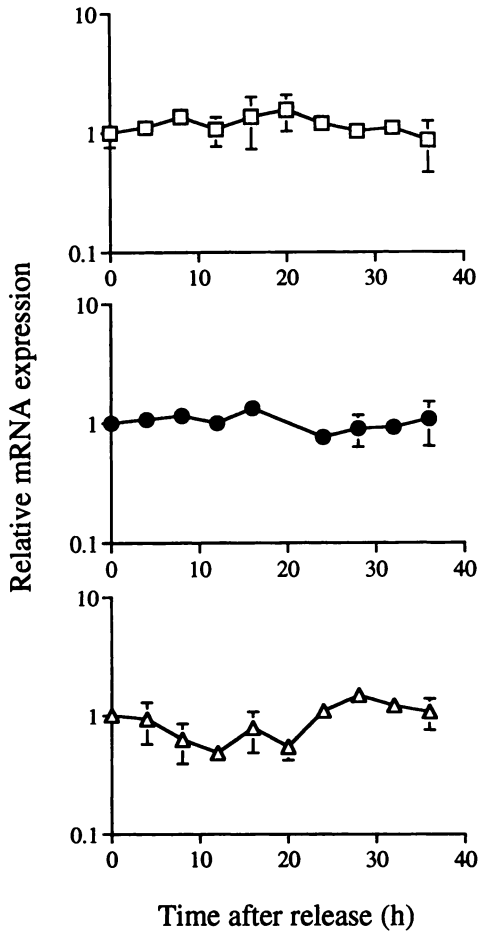

Fig. 2. Relative mRNA levels of hMSH2 (□), hMLH1 (●), and hPMS2 (Δ) after subconfluent release of IMR-90 cells into medium containing high serum. Data are the means from PhosphorImager scans of slot blots done in duplicate. All levels were corrected for loading variations against the levels of constitutively expressed 36B4 mRNA. *Bars*, SE.

Results and Discussion

Early passage IMR-90 cells were growth-arrested both by allowing them to reach confluence and then by growing them for 60 h in medium containing low serum. The cells were then induced to reenter the cell cycle by replating them at low density into medium containing a high concentration of serum. This is a typical synchronization treatment procedure for normal cells (17, 18). IMR-90 cells, which were derived from normal human fetal lung, were chosen instead of colorectal cancer cells because tumor cells often show cell cycle dysregulation (22). Samples were taken at regular intervals and analyzed by flow cytometry (Fig. 1). Initially, 76% of the cells were in G_0-G_{G_1}. At 28 h, 60% of IMR-90 cells were in S, and a subsequent G_2 peak was seen at 35 to 40 h (approximately 35%). The 31-h time point represents an 8-fold enrichment of IMR-90 cells in S phase cells compared to time zero, confluent cultures.

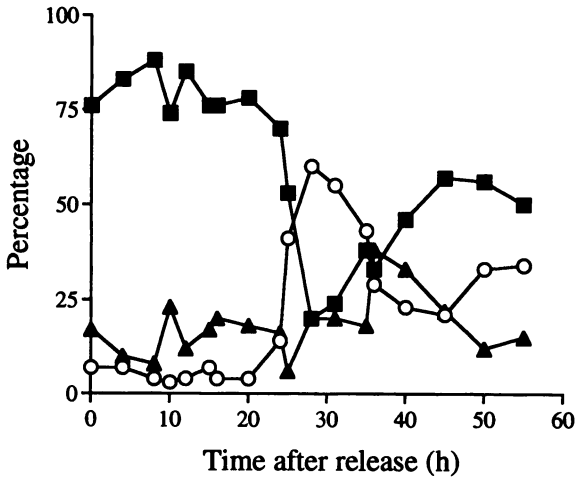

Fig. 1. Percentage of IMR-90 cells in each phase of the cell cycle following subconfluent release into medium containing a high concentration of serum. The symbols denote cells in G_0-G_1 (■), S (○), and G_2-M (▲) phases.

Drug treatments are often used to synchronize cells (23); however, treatment with drugs may additionally result in DNA damage. We were concerned about using agents that might introduce DNA damage because the cell cycle regulation of DNA repair genes was being examined. For example, hydroxyurea, which disturbs the deoxynucleotide triphosphate pool and thus inhibits DNA synthesis, has been reported to increase the frequency of chromosomal aberrations (24). Thymidine, which inhibits DNA synthesis and thus cell cycle progression when present at high concentrations, also may induce chromosomal aberrations (25). Aphidicolin, an inhibitor of DNA polymerase α which causes cells to accumulate at the G_1-S border, even when used at low doses, also damages DNA (26). Thus, we opted for the more physiological mechanism of growth arrest and cell synchrony that is inherent in confluent IMR-90 cells.

Total RNA was isolated at selected time points during the synchronization of IMR-90 cells described above (Fig. 2). mRNA levels were normalized to 36B4 mRNA levels to correct for loading variations;

Fig. 3. Western analyses of IMR-90 whole-cell extracts after release into medium containing high serum. Shown are topoisomerase IIα (170 kDa), hMSH2 (100 kDa), hMLH1 (83 kDa), hPMS2 (115 kDa), and α-tubulin (55 kDa). The *arrow* denotes the hPMS2 protein band.

this transcript is known not to be regulated by cell growth or DNA damage (27). The levels of hMSH2 and hMLH1 did not change significantly throughout the cell cycle. However, hPMS2 mRNA levels dropped approximately 50% in mid- to late-G_1 and rose 50% in S and 25% in G_2-M relative to G_0-G_1 levels. It should be noted that hPMS2 mRNA levels were very low and were difficult to detect in normal cells by Northern blot hybridization analyses.

Whole-cell extracts were also examined for alterations in MMR protein expression (Fig. 3). As a positive control for cell cycle synchronization, we examined the levels of topoisomerase IIα, a protein expressed only during S and G_2 phases of the cell cycle and which is required for DNA replication and chromosome disjunction at mitosis due to its ability to alter DNA supercoiling (28). Topoisomerase IIα protein levels began to rise in S phase at 25 h as expected (28). hMSH2 protein remained at constant, high levels throughout the experiment. hMLH1 protein levels were also high and expressed in all phases of the cell cycle, but there appeared to be a modest increase in late G_1 and in S phase; hMLH1 protein levels increased 43 ± 4% at 15 h, 63 ± 2% at 20 h, and 37 ± 1% at 25 h. hPMS2 was expressed at very low levels, consistent with the low level of hPMS2 mRNA. It also increased late G_1 and in S phase; hPMS2 protein levels rose 33 ± 14% at 15 h, 54 ± 9% at 20 h, 66 ± 8% at 25 h, and 29 ± 0% at 31 h. The levels of α-tubulin served as a gel loading control. These data strongly suggest that hMSH2, hMLH1, and hPMS2 are not strongly cell cycle regulated.

MMR Functions as a Mutation-Avoidance System. Mismatches can arise in DNA by DNA polymerase replication errors, as well as through direct physical damage to nucleotides (29). Our data using synchronized, normal IMR-90 fibroblasts indicate that the levels of MMR proteins remained relatively constant throughout the cell cycle. We conclude that, in general, MMR proteins have an important, constitutive role in protecting the cell from the effects of replication misincorporation and perhaps DNA damage. We are currently examining the cell cycle regulation of these MMR proteins in various colon carcinoma and other tumor cells to investigate whether MMR transcript and protein expression is dysregulated in neoplastic or preneoplastic cells compared to normal cells.

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