3304–3310 *Nucleic Acids Research, 2001, Vol. 29, No. 16*

Suppression of gene amplification and chromosomal DNA integration by the DNA mismatch repair system

Ching-Tai Lin*, Yi Lisa Lyu[1], Hai Xiao[2], Wei-Hsin Lin and Jacqueline Whang-Peng

National Health Research Institute, Cancer Research Division, Cooperative Laboratory, Veterans General Hospital, 201 Shih-Pai Road, Sec. 2, Taipei 112, Taiwan, Republic of China, [1]MCB, Harvard University, 7 Divinity Avenue, Cambridge, MA 02138, USA and [2]Department of Pharmacology, Robert Wood Johnson Medical School, University of Medicine and Dentistry of New Jersey, Piscataway, NJ 08854, USA

Received May 24, 2001; Revised and Accepted July 4, 2001

ABSTRACT

Mismatch repair (MMR)-deficient cells are shown to produce >15-fold more methotrexate-resistant colonies than MMR normal cells. The increased resistance to methotrexate is primarily due to gene amplification since all the resistant clones contain double-minute chromosomes and increased copy numbers of the *DHFR* gene. In addition, integration of linearized or retroviral DNAs into chromosomes is also significantly elevated in MMR-deficient cells. These results suggest that in addition to microsatellite instability and homeologous recombination, MMR is also involved in suppression of other genome instabilities such as gene amplification and chromosomal DNA integration.

INTRODUCTION

Mismatch repair (MMR) has been shown to suppress both microsatellite instability and homeologous recombination (reviewed in 1–3). In the current study, we have investigated whether MMR is also involved in suppression of other genomic instabilities such as gene amplification and chromosomal DNA integration.

Gene amplification occurs during the development of many organisms (4), emergence of drug resistance (5) and progression of cancer (5–7). Amplified genes are often found to organize as giant inverted repeats in the forms of either circular inverted dimers (e.g. double-minute chromosomes) (8–10) or head-to-head linear arrays (e.g. homogeneous staining region) (11). It was proposed that generation of DNA fragments with free ends (generated through over-replication or during excision of the replication bubble) could be the initiation event during gene amplification process(es) (5,12). Subsequent processing of these linear DNA fragments can lead to the formation of extrachromosomal circular DNA as the gene amplification product. There are different ways that the linear DNA with free ends can be processed. It is generally accepted that non-homologous end joining (NHEJ) is the major mechanism responsible for the repair of DNA double-stranded breaks (13). DNA replication prior to double-strand breaks (excision of the replication bubble) or after the excision of the linear DNA fragment from chromosome can produce a pair of identical linear DNAs with their quasi-identical ends located in close proximity spatially. Joining of these ends through NHEJ will lead to the production of an extrachromosomal circular DNA with giant inverted duplications (inverted dimer) separated by two unique junctions. NHEJ is the essential step of breakage-fusion-bridge cycles proposed to explain the formation of homogeneous staining region (11). This mechanism is also used to explain the formation of the homogeneous staining region (HSR) during gene amplification in hamster cells (14,15).

We show that MMR-deficient cells exhibit increased drug resistance frequencies toward methotrexate (MTX). Analysis of MTX-resistant colonies has indicated the presence of double-minute chromosomes and amplification of the *DHFR* gene. The frequencies of chromosomal DNA integration as assayed by both transfection of linear DNAs and retroviral integration were also increased significantly in MMR-deficient cells. These results suggest that the MMR system can suppress both gene amplification and chromosomal DNA integration.

MATERIALS AND METHODS

Cell lines

HCT116+Ch2 (MMR$^-$) and HCT116+Ch3 (MMR$^+$) cell lines, which were derived from HCT116 (human colorectal carcinoma with *hMLH1*$^{-/-}$) cells, were obtained from Dr C. R. Boland (University of California at San Diego, CA) (16). Plasmid pC9MLHWT (gift from Dr B. Vogelstein, Johns Hopkins Oncology Center, MD), which harbors the wild-type *hMLH1* cDNA (under the control of CMV promoter), was used to transfect HCT116 cells by electroporation and the resulting G418-resistant clone which expressed the hMLH1 protein was termed HCT116+hMLH1. DLD-1+Ch2 (MMR$^+$) and HEC59+Ch2 (MMR$^+$) cell lines and their respective MMR-deficient mutant cell lines, DLD-1 (hMSH6$^-$) and HEC59 (hMSH2$^-$), were provided by Dr Thomas A. Kunkel (National Institute of Environmental Health Sciences, NC) (17,18). Cells were cultured in complete RPMI medium supplemented with 10% fetal bovine serum. DLD-1+Ch2, HEC59+Ch2, HCT116+Ch2 and HCT116+Ch3 were cultured in the presence of 400 μg/ml of G418. HCT116+hMLH1 cells were cultured in the presence of 100 μg/ml of G418.

*To whom correspondence should be addressed. Tel: +886 2 28712121; Fax: +886 2 28733664; Email: linct@nhri.org.tw

Single step gene amplification test

Subconfluent cells (2.5×10^6) were seeded in 100×20 mm culture dishes with complete RPMI-1640 medium supplemented with 10% commercially dialyzed fetal bovine serum (Gibco) 24 h prior to drug selection. Following medium change, MTX was added ($14 \times IC_{50}$) to each plate and the plates were incubated in a 37°C incubator supplied with 5% CO_2. Depending on the growth of the cells, as judged by the color of the medium, cells were replenished with fresh medium in the presence of continuous MTX selection. Colonies were either picked or stained with methylene blue after 1 month of growth. The plating efficiency of cell lines was determined by seeding 100 cells in 100×20 mm culture dishes and counting the colonies after 10 days of growth.

DHFR gene copy number analysis

Genomic DNAs were isolated from 2×10^6 MTX^r or parental cells. Cells were pelleted and lysed in 1 ml of lysis buffer (25 mM Tris–HCl pH 8.0, 25 mM EDTA, 1% SDS, 0.5 mg/ml proteinase K). Cell lysates were incubated at 50°C overnight. Following ethanol precipitation and resuspension, RNaseA was added to 100 µg/ml and the mixture was kept at 37°C for 30 min. After phenol extraction (twice), DNAs were precipitated with 0.1 vol 3 M NaOAc and 2 vol ethanol. DNA pellets were washed once with 70% ethanol, air-dried and resuspended in 50 µl of TE buffer. Upon digestion with *Eco*RI restriction enzyme, the 1.8 kb genomic DNA fragment containing part of the *DHFR* gene was detected by Southern hybridization analysis using the ^{32}P-labeled *DHFR* genomic DNA probe.

Analysis of metaphase chromosomes

Cells (2×10^4) were seeded on 100×20 mm dishes. After 48 h, cells were replenished with fresh medium followed by addition of colcemid (Sigma) (400 µg/ml). Following incubation at 37°C for 10–15 h, cells were dislodged from dishes by trypsin–EDTA. Cells were collected by centrifugation at 100 *g* for 5 min and then resuspended in 5 ml of a hypotonic solution (0.04 M KCl and 0.025 M sodium citrate) and incubated at 37°C for 20 min. An equal volume of ice-cold acetic acid/methanol (1:3) was added to the cell mixture. Cells were pelleted and resuspended in 5 ml of acetic acid/methanol and incubated on ice for 10 min. Cell pellets were collected again after centrifugation for 2 min at 100 *g* and resuspended in 200 µl of ice-cold acetic methanol. The cell suspension (3 µl) was dropped onto a cold slide and the slide was quickly dried off on top of flame. The chromosome spread was mounted with mounting medium [0.1 M Tris, pH 9.2, 50% glycerol, 1 µg/ml *p*-phenylenediamine (Sigma), 1 µg/ml DAPI] and viewed under a fluorescence microscope.

Cytological immunostaining of hMLH1

Cells were grown on glass coverslips overnight. The cells were then fixed with the 50% acetone/50% methanol solution after washing with PBS. Immunostaining was performed following the manufacturer's instructions (Dako, CA). The fixed cells were incubated for 30 min at room temperature with antibodies against human hMLH1 (mAb G168-15, PharMingen, USA), dilution 1:50. Immuno-reactivity was detected using a two-step peroxidase detection system (Dako). Staining was evaluated under microscope after hematoxylin counterstain.

DNA transfection into mammalian cells

The linear DNA substrate used for transfection was generated by linearization of 100 µg of pTK-hygro (Clontech) with *Hin*dIII, followed by phenol/$CHCl_3$ extraction and ethanol precipitation. The linear DNA was purified from agarose gel after electrophoresis in Tris–acetate–EDTA buffer (TAE). Gel-purified linear DNA substrate (5 µg) was electroporated (500 µF and 400 V) into MMR-deficient cells (HCT116+Ch2, DLD-1 and HEC59) and MMR-proficient cells (HCT116+Ch3, DLD-1+Ch2 and HEC59+Ch2) using the Gene Pulser apparatus from Bio-Rad (Hercules, CA). Transfected cells were resuspended in RPMI-1640 plus 10% FBS and 400 µg/ml G418 after placing on ice for 10 min. Various concentrations of hygromycin B were added to transfected cells after 24–36 h. Cells were replenished with medium containing the selecting drugs (G418 and/or hygromycin B) every 3–4 days. The resistant colonies appeared after 3–4 weeks.

Integration assay with the infection of retroviruses

The packaging cell line GPGC/GIP that contains replication defective pseudo-type MMLV retroviruses was kindly provided by Dr Joseph P. Dougherty (Robert Wood Johnson Medical School, University of Medicine and Dentistry of New Jersey, NJ). The replication defective pseudo-type MMLV retrovirus contains the puromycin-resistant marker gene (for integration selection). Viruses were collected from the medium of the packaging cells without any selection drugs. The retrovirus infection was performed in 50 µg/ml ploybrene (Sigma). Host cells (5×10^5) were infected with viruses at 37°C with 5% CO_2 for 40 min by rocking the dishes gently to redistribute the virus every 10 min. The polybrene solutions were then removed and replaced with fresh growth medium. Puromycin (10 or 50 µM; Sigma) was added after 24 h and cells were grown in puromycin for 10 days.

RESULTS

Mismatch repair (MMR)-deficient cells exhibit increased frequencies of gene amplification

The pathways (see Introduction) just described for gene amplification point out the requirement of homeologous recombination processes. MMR has been shown to suppress homeologous recombination in prokaryotic and eukaryotic cells (19,20). Thus only mutant cells defective in MMR are expected to be competent in these processes. We have tested whether MMR-deficient cells exhibit higher resistance frequencies toward MTX. Isogenic pairs of MMR^+ and MMR^- human cell lines were used. The HCT116 (human colorectal carcinoma) cell line is defective in MMR and has known microsatellite instability due to mutation in the *hMLH1* locus. The same cell line has been transduced with chromosome 3 (carrying the wild-type *hMLH1* gene), and the resulting 'revertant' cell line exhibits the MMR^+ phenotype (21). The cell line (HCT116+Ch2) in which chromosome 2 has been transferred is a control and has the same phenotype as HCT116. As shown in Figure 1A and B, HCT116+Ch2 (MMR^-) cells gave rise to

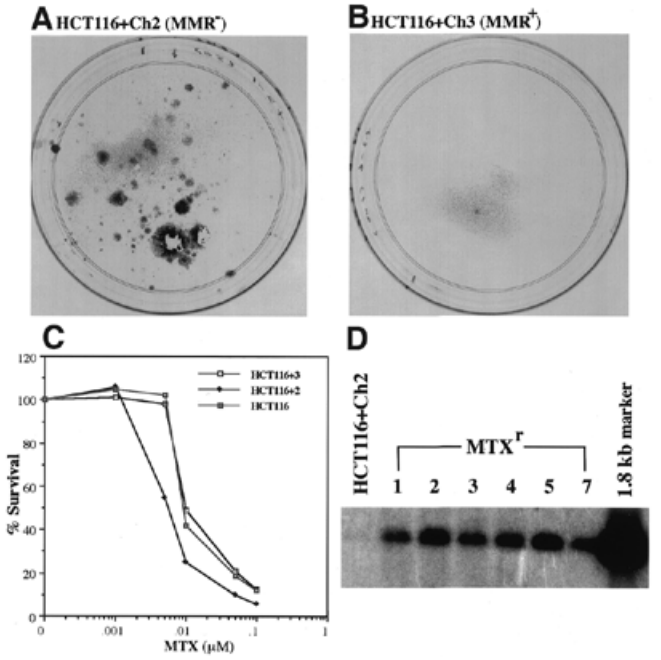

Figure 1. Suppression of gene amplification in HCT116 (*hMLH1*$^{-/-}$) by transfected chromosome 3 carrying the wild-type *hMLH1* gene. (**A** and **B**) Selection of MTX resistance in HCT116+Ch2 (hMLH1^{-}; MMR^{-}) and HCT116+Ch3 (hMLH1^{+}; MMR^{+}) cells. Cells (2×10^6) were plated in 100×20 mm plates in the presence of MTX ($14 \times IC_{50}$). The plates were incubated at 37°C for 28 days. Colonies were then fixed and stained with methylene blue. This experiment has been repeated four times and essentially the same results were obtained. (**C**) IC_{50} determination using the 4-day MTT assay (55). (**D**) Determination of the *DHFR* gene copy number in MTX^{r} HCT116+Ch2 clones. Genomic DNAs were isolated and the same amount of DNAs was digested with *Eco*RI and analyzed by agarose gel electrophoresis. The amplified *DHFR* gene was detected by the ^{32}P-labeled *DHFR* cDNA probe. Genomic DNA isolated from HCT116+Ch2 was used as the control. All six MTX^{r} of HCT116+Ch2 clones contained the amplified 1.8 kb DNA fragment.

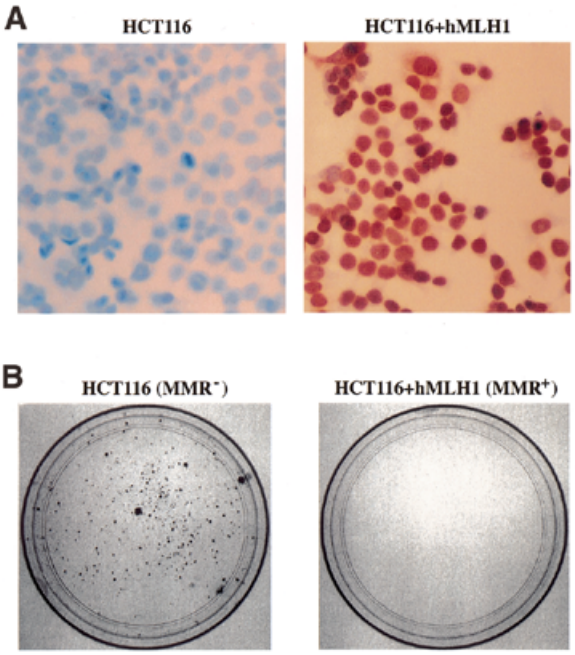

Figure 2. Suppression of gene amplification in HCT116 (*hMLH1*$^{-/-}$) by transfected cDNA carrying the wild-type *hMLH1* gene. (**A**) Nuclear expression of hMLH1 in HCT116 cells transfected with wild-type *hMLH1* cDNA. Immunostaining analysis of HCT116 and HCT116+hMLH1 cells was performed with monoclonal anti-hMLH1 antibody as described in Materials and Methods. The positive staining of hMLH1 was observed as red–brown color only in the nuclei of HCT116+hMLH1 cells under regular microscopy. (**B**) Suppression of gene amplification in HCT116 (*hMLH1*$^{-/-}$) cells transfected with the wild-type *hMLH1* cDNA. Selection of MTX resistance was performed in HCT116 (MMR^{-}) and HCT116+hMLH1 (MMR^{+}) cells. Cells (2×10^6) were plated in 100×20 mm plates in the presence of MTX ($14 \times IC_{50}$). The plates were incubated at 37°C for 28 days. Colonies were then fixed and stained with methylene blue. This experiment has been repeated three times and essentially the same results were obtained.

>40-fold more MTX^{r} colonies than HCT116+Ch3 (MMR^{+}) cells (Fig. 1A and B). The parental cell line HCT116 also gave rise to ~40-fold more MTX^{r} colonies than HCT116+Ch3 cell line (Fig. 2B). The IC_{50} of MTX (Fig. 1C) for HCT116+Ch2 (MMR^{-}) cells is ~0.006 μM that is slightly lower than the IC_{50} of MTX for HCT116 MMR^{-} (0.009 μM) cells and HCT116+Ch3 MMR^{+} (0.01 μM) cells, suggesting that the higher drug resistance frequency in hMLH1-deficient cells is not due to the difference in MTX sensitivity.

Amplification of the *DHFR* gene is known to be one major resistance mechanism to MTX (22–26). In order to verify that MTX resistance in our system was indeed due to amplification of the *DHFR* gene, we determined the copy number of the *DHFR* gene in MTX^{r} clones. The same amount of genomic DNAs isolated from parental and various MTX^{r} cells was digested with *Eco*RI, and the digested DNA fragments were separated by gel electrophoresis. Southern hybridization was performed to determine the copy number of the *DHFR* gene using a ^{32}P-labeled 1.8 kb fragment of *Eco*RI-restricted *DHFR* genomic DNA as a probe. As shown in Figure 1D, the *DHFR* gene in every clone isolated from MTX^{r} cells of HCT116+Ch2 was amplified (>4-fold). Microscopic examination of the resistant cells from several clones has also revealed the presence of multiple double-minute chromosomes (data not shown).

To further verify the role of *hMLH1* and MMR system in suppression of gene amplification

In order to rule out the possibility that some unknown factors other than *hMLH1* gene on chromosome 3 are responsible for the suppression of *DHFR* gene amplification in HCT116+Ch3 cells, we introduced the wild-type *hMLH1* cDNA into cell line HCT116 (hMLH1$^{-/-}$). The expression of the *hMLH1* cDNA was confirmed by immunostaining with monoclonal anti-hMLH1 antibody (Fig. 2A). Mutant *hMLH1* (homozygous mutation at codon 252) if expressed in HCT116 cannot be recognized by the same antibody (Fig. 2A). Again, HCT116 (MMR^{-}) cells gave rise to ~40-fold more MTX^{r} colonies than HCT116+hMLH1 (MMR^{+}) cells (Fig. 2B) as that shown in Figure 1A and 1B.

To further verify the roles of MMR systems in the suppression of gene amplification, cell lines have mutations in other MMR genes and their chromosome complemented cell lines were chosen to perform MTX drug resistance test. The human endometrial tumor cell lines HEC59 (*hMSH2*$^{-}$) and HEC59+Ch2 (*hMSH2*$^{+}$) and colon cancer cell lines DLD-1 (*hMSH6*$^{-}$) and DLD-1+Ch2 (*hMSH6*$^{+}$) were used to test the effect of hMSH2 or hMSH6 mutation on *DHFR* gene amplification. Again, HEC59 and DLD-1 (MMR^{-}) cells gave rise to

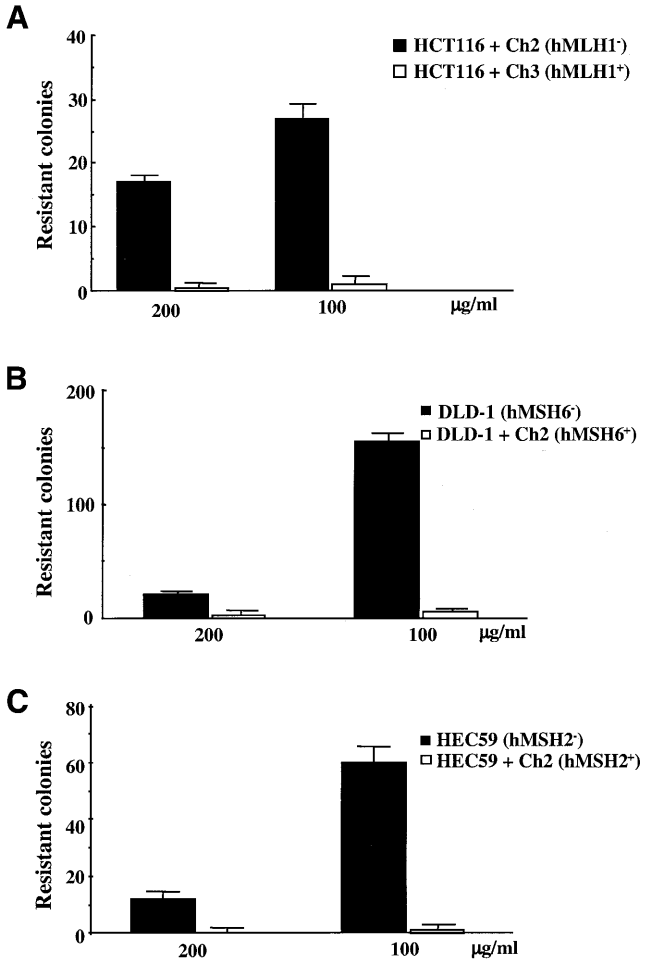

Figure 3. MMR deficiency enhances chromosomal integration of transfected linear DNA. Three pairs of MMR-deficient and MMR-proficient cells were transfected with linearized pTK-hyg DNAs (see Materials and Methods) and then selected with indicated concentrations of hygromycin B. (**A**) hMLH1$^-$ (HCT116+Ch2) and hMLH1$^+$ (HCT116+Ch3) cells. (**B**) hMSH6$^-$ (DLD-1) and hMSH6$^+$ (DLD-1+Ch2) cells. (**C**) hMSH2$^-$ (HEC59) and hMSH2$^+$ (HEC59+Ch2) cells. The results presented are the average of at least three independent experiments.

more MTX^r colonies than HEC59+Ch2 and DLD-1+Ch2 (MMR$^+$) cells by a factor of >15 (data not shown).

MMR-deficient cells exhibit elevated frequencies of chromosomal DNA integration

Our MTX results suggest that MMR may be involved in suppression of gene amplification in mammalian cells. To determine whether MMR may also be involved in suppressing NHEJ that was proposed as a possible pathway for gene amplification (see Introduction), we have tested chromosomal DNA integration in MMR-deficient and MMR-proficient cells. Linear DNA (pTK-hyg restricted with *Hin*dIII) was transfected into MMR wild-type (HCT116+Ch3, DLD-1+Ch2 or HEC59+Ch2) and their respective mutant (HCT116+Ch2, DLD-1 or HEC59) cells. Results show an average of ~15-fold higher transfection efficiency with MMR mutant cells (Fig. 3). The chromosomal integration of linear DNA was determined by Southern analysis with ^{32}P-labeled pTK-hyg (data not shown).

MMR-deficient cells exhibit elevated frequency of retroviral integration

Integration of retroviral DNA into the host genome is part of the replication cycle of retroviruses (27). The proposed pre-integration structure, which is generated by the reaction of integrase, contains a 4–6-base gap and a 2-base mismatch at each end (28). Thus, retroviral integration can also be considered as a type of non-homologous recombination. To test whether MMR also suppresses the integration of retroviral DNA, the amphotropic retroviruses were used to infect MMR$^-$ (HCT116+Ch2, DLD-1 or HEC59) and MMR$^+$ (HCT116+Ch3, DLD-1+Ch2 or HEC59+Ch2) human cells. Puromycin (10 or 50 μM) was used to select for integrants. As shown in Figure 4, MMR-deficient mutant cells exhibited higher (average 4.2 ± 1.0-fold) integration frequency from four independent infection experiments (see Materials and Methods).

DISCUSSION

In this study, our results have clearly demonstrated that MMR suppresses *DHFR* gene amplification (Figs 1 and 2) and chromosomal DNA integration of the linear DNA in human cells (Fig. 3). The mechanisms by which the *DHFR* gene is amplified and the linear DNA is integrated into the chromosome are not clear but may involve NHEJ processes (29,30).

We have also demonstrated that MMR suppresses retroviral integration (Fig. 4). This result is quite unexpected since retroviral integration is primarily carried out by the integrase (IN) (31,32). The pre-integration complex contains two 4–6-base gaps with two 2-base flaps (28). The cellular enzymes involved in processing this pre-integration complex are not known. However, it has been reported that retroviral integration is reduced in cells defective in repair of double-strand breaks (e.g. DNA-PK mutant cells) (33). DNA-PK is known to be a key component of NHEJ (reviewed in 34–36). The idea that DNA-PK is directly involved in integration processes and/or protects the cells from DNA breaks (a side-effect of integration) was proposed (28,33). Moreover, our results also suggest that retroviral integration could have overlapping steps with NHEJ that is suppressed by MMR as the assay of chromosomal integration of the linear DNA (Fig. 3).

The detailed mechanism(s) for NHEJ remains elusive. Factors such as DNA ligase IV, Xrcc4, Ku70, Ku80(86) and DNA-PKcs have been shown to be required for NHEJ *in vivo* (37–44) and *in vitro* (45). End-joining appears to involve at least two pathways: the blunt end ligation that does not require pairing of bases and the joining of ends with single-stranded extensions that depends on short sequence homologies (29,46). Therefore, end-joining through pairing of short homologies could potentially generate an intermediate containing mismatches (i.e. a heteroduplex intermediate or DNA with protruding ends). Indeed, studies in yeast have suggested that Msh2p and Msh3p are involved in double-strand break-induced recombination (47). It was suggested that Msh2p and Msh3p are required to remove 3′ non-homologous DNA tails during genetic recombination initiated on non-homologous ends of a double-strand break (48). It seems possible that some MMR proteins may be involved in certain aspects of NHEJ in mammalian cells as well. However, our results indicate that

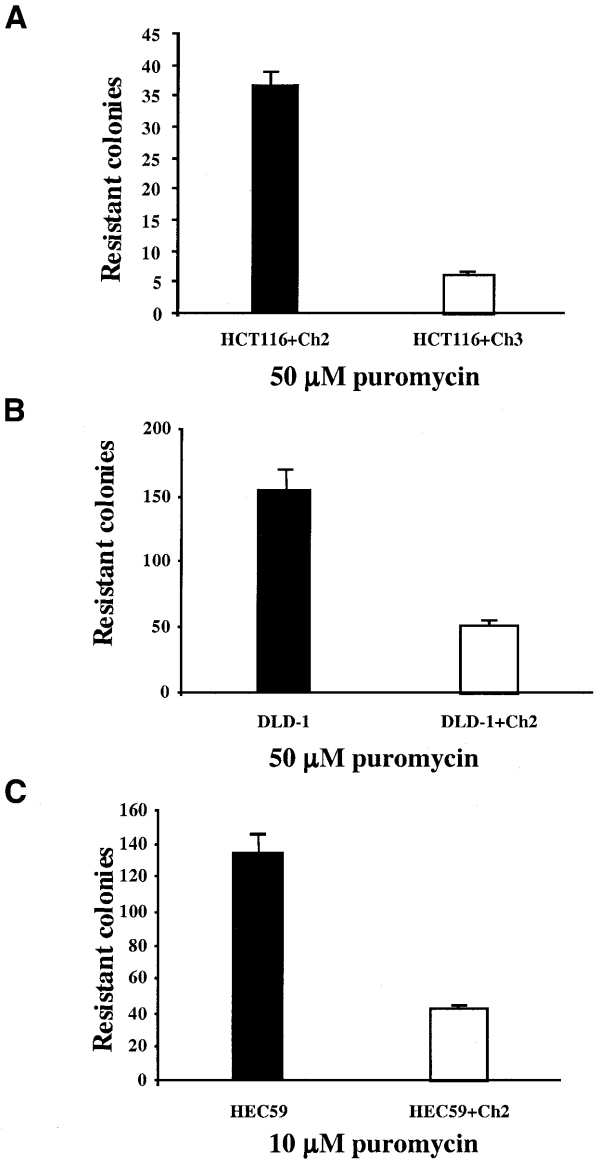

Figure 4. MMR deficiency increases the frequency of chromosomal integration of retrovirus. Three pairs of MMR-deficient and MMR-proficient cells were infected with amphotropic retrovirus (see Materials and Methods) and then selected with indicated concentrations of puromycin. (**A**) hMLH1$^-$ (HCT116+Ch2) and hMLH1$^+$ (HCT116+Ch3) cells. (**B**) hMSH6$^-$ (DLD-1) and hMSH6$^+$ (DLD-1+Ch2) cells. (**C**) hMSH2$^-$ (HEC59) and hMSH2$^+$ (HEC59+Ch2) cells. The results are the average of four independent experiments.

these MMR proteins could be involved in suppression rather than promotion of NHEJ.

MMR is known to suppress homeologous recombination in both prokaryotic and eukaryotic cells (19,20). Our previous studies have demonstrated that recombinational rearrangements mediated by imperfect inverted repeats, which do not involve the formation of a Holliday junction, are also suppressed by MMR (49). It has been suggested that MMR suppresses homeologous recombination by aborting a heteroduplex recombinational intermediate (49). One possible explanation for our results is that NHEJ may also involve the formation of a heteroduplex intermediate that is aborted/destroyed by MMR. Consequently, MMR may suppress both gene amplification and chromosomal DNA integration by the same mechanism, i.e. aborting the putative heteroduplex intermediates.

Previous studies in the *Escherichia coli* system have shown that the giant circular inverted dimers, resembling the structure of some gene amplification products, can be generated via recombination/rearrangement mediated by small inverted repeats (49–51). In this case, circular plasmid DNAs containing a pair of small inverted repeats were shown to mediate efficient recombination/rearrangement to produce circular inverted dimers. It remains unknown whether circular DNAs containing a pair of small inverted repeats could be the precursors of inverted duplications for gene amplification as we observed in *E.coli*. One argument is what the driving force will be to generate the inverted dimer from the circular monomer. Alternatively, and very likely, the circular inverted dimers are directly formed from linear chromosomes. Since there are abundant repetitive DNA sequences (e.g. human *Alu* sequences) in mammalian cells, the presence of one or even two pairs of inverted repeats in the region to be amplified should not present a problem. Indeed, studies of the amplified H-circles from drug-resistant *Leishmania* have demonstrated that the unamplified H-locus is bracketed by two pairs of inverted repeats of 198 and 1241 bp, respectively, one at each end of the H-locus (52,53). These two pairs of inverted repeats have been suggested to be crucial for the amplification of the H-circles (52,53). Our current studies further demonstrate that linear DNA constructs containing inverted repeats at each end can also give rise to circular inverted dimers in both mammalian cells and *E.coli* (54). In addition, our results point to a model in which a dumbbell-like DNA intermediate, generated through nuclease/helicase processing of the linear DNA substrate, is responsible for the formation of the circular inverted dimer (54).

Based on these studies, we propose the homeologous recombination processes including the NHEJ pathway (see Introduction) as well as a dumbbell model for gene amplification as shown in Figure 5. The dumbbell model involves the excision of a linear DNA substrate containing two pairs of small inverted repeats (imperfect) near each end of the DNA, the processing of the linear DNA by nuclease/helicase to expose the single-stranded inverted repeats, and the formation of dumbbell-like DNA intermediate by self-annealing the single-stranded inverted repeats. Therefore, both the NHEJ pathway and the dumbbell model for gene amplification may involve the formation of a heteroduplex intermediate that is aborted/destroyed by MMR. It remains to be established if gene amplification in tumor cells indeed involves such homeologous recombination processes.

ACKNOWLEDGEMENTS

We would like to thank Drs C. R. Boland and Thomas A. Kunkel for providing the MMR-deficient human cell lines. We also thank Dr B. Vogelstein for the cDNA of hMLH1 and Dr Joseph P. Dougherty for providing the retroviruses and Dr Leroy F. Liu for helpful discussions. This work was supported by NHRI grants in Taiwan.

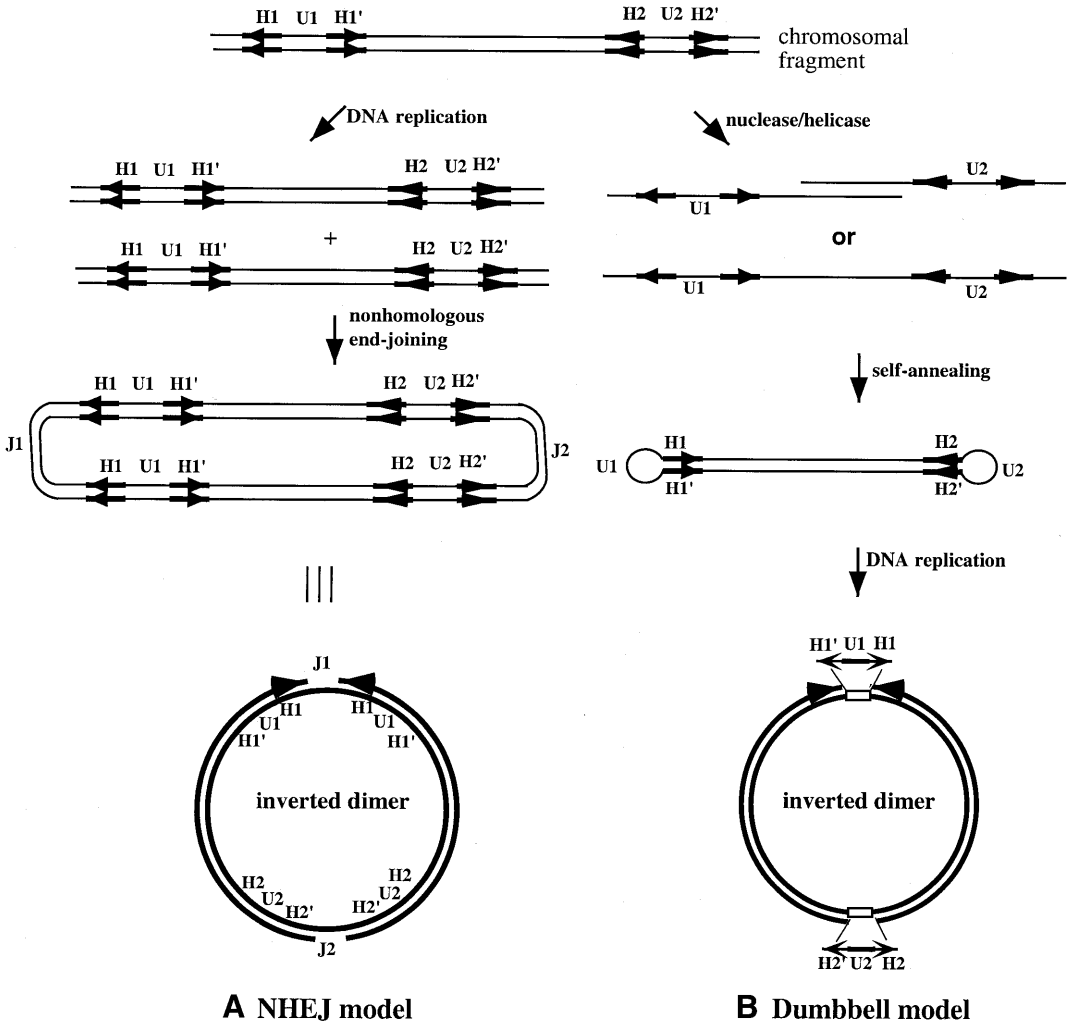

Figure 5. Homeologous (non-homologous) recombination processes for gene amplification. (**A**) NHEJ pathway for inverted duplications. (**B**) A dumbbell model for inverted dimer formation. Two pairs of inverted repeats are imperfect and form homeologous pairing to generate dumbbell-like DNA intermediate. For details see Introduction and Discussion.

REFERENCES

1. Lengauer,C., Kinzler,K.W. and Vogelstein,B. (1997) Genetic instability in colorectal cancers. *Nature*, **386**, 623–627.
2. Prolla,T.A. (1998) DNA mismatch repair and cancer. *Curr. Opin. Cell Biol.*, **10**, 311–316.
3. Harfe,B.D. and Jinks-Robertson,S. (2000) DNA mismatch repair and genetic instability. *Annu. Rev. Genet.*, **34**, 359–399.
4. Kafatos,F.C., Mitsialis,S.A., Spoerel,N., Mariani,B., Lingappa,J.R. and Delidakis,C. (1985) Studies on the developmentally regulated expression and amplification of insect chorion genes. *Cold Spring Harb. Symp. Quant. Biol.*, **50**, 537–547.
5. Stark,G.R., Debatisse,M., Giulotto,E. and Wahl,G.M. (1989) Recent progress in understanding mechanisms of mammalian DNA amplification. *Cell*, **57**, 901–908.
6. Bishop,J.M. (1991) Molecular themes in oncogenesis. *Cell*, **64**, 235–248.
7. Brison,O. (1993) Gene amplification and tumor progression. *Biochim. Biophys. Acta*, **1155**, 25–41.
8. Ford,M. and Fried,M. (1986) Large inverted duplications are associated with gene amplification. *Cell*, **45**, 425–430.
9. Fried,M., Feo,S. and Heard,E. (1991) The role of inverted duplication in the generation of gene amplification in mammalian cells. *Biochim. Biophys. Acta*, **1090**, 143–155.
10. Nonet,G.H., Carroll,S.M., DeRose,M.L. and Wahl,G.M. (1993) Molecular dissection of an extrachromosomal amplicon reveals a circular structure consisting of an imperfect inverted duplication. *Genomics*, **15**, 543–558.
11. Coquelle,A., Pipiras,E., Toledo,F., Buttin,G. and Debatisse,M. (1997) Expression of fragile sites triggers intrachromosomal mammalian gene amplification and sets boundaries to early amplicons. *Cell*, **89**, 215–225.
12. Schimke,R.T. (1988) Gene amplification in cultured cells. *J. Biol. Chem.*, **263**, 5989-5992.
13. Jeggo,P.A. (1998) Identification of genes involved in repair of DNA double-strand breaks in mammalian cells. *Radiat. Res.*, 580–591.
14. Ma,C., Martin,S., Trask,B. and Hamlin,J.L. (1993) Sister chromatid fusion initiates amplification of the dihydrofolate reductase gene in Chinese hamster cells. *Genes Dev.*, **7**, 605–620.
15. Smith,K.A., Stark,M.B., Gorman,P.A. and Stark,G.R. (1992) Fusions near telomeres occur very early in the amplification of CAD genes in Syrian hamster cells. *Proc. Natl Acad. Sci. USA*, **89**, 5427–5431.
16. Koi,M., Umar,A., Chauhan,D.P., Cherian,S.P., Carethers,J.M., Kunkel,T.A. and Boland,C.R. (1994) Human chromosome 3 corrects mismatch repair deficiency and microsatellite instability and reduces N-methyl-N′-nitro-N-nitrosoguanidine tolerance in colon tumor cells with homozygous hMLH1 mutation [published erratum appears in *Cancer Res.* (1995) **55**, 201]. *Cancer Res.*, **54**, 4308–4312.
17. Umar,A., Koi,M., Risinger,J.I., Glaab,W.E., Tindall,K.R., Kolodner,R.D., Boland,C.R., Barrett,J.C. and Kunkel,T.A. (1997) Correction of hypermutability, N-methyl-N′-nitro-N-nitrosoguanidine resistance, and defective DNA mismatch repair by introducing chromosome 2 into human

tumor cells with mutations in MSH2 and MSH6. *Cancer Res.*, **57**, 3949–3955.
18. Malkhosyan,S., McCarty,A., Sawai,H. and Perucho,M. (1996) Differences in the spectrum of spontaneous mutations in the hprt gene between tumor cells of the microsatellite mutator phenotype. *Mutat. Res.*, **316**, 249–259.
19. Rayssiguier,C., Thaler,D.S. and Radman,M. (1989) The barrier to recombination between *Escherichia coli* and *Salmonella typhimurium* is disrupted in mismatch-repair mutants. *Nature*, **342**, 396–401.
20. de Wind,N., Dekker,M., Berns,A., Radman,M. and te Riele,H. (1995) Inactivation of the mouse Msh2 gene results in mismatch repair deficiency, methylation tolerance, hyperrecombination, and predisposition to cancer. *Cell*, **82**, 321–330.
21. Liu,L., Markowitz,S. and Gerson,S.L. (1996) Mismatch repair mutations override alkyltransferase in conferring resistance to temozolomide but not to 1,3-bis(2-chloroethyl)nitrosourea. *Cancer Res.*, **56**, 5375–5379.
22. Varshavsky,A. (1981) Phorbol ester dramatically increases incidence of methotrexate-resistant mouse cells: possible mechanisms and relevance to tumor promotion. *Cell*, **25**, 561–572.
23. Lee,T.C., Tanaka,N., Lamb,P.W., Gilmer,T.M. and Barrett,J.C. (1988) Induction of gene amplification by arsenic. *Science*, **241**, 79–81.
24. Mariani,B.D. and Schimke,R.T. (1984) Gene amplification in a single cell cycle in Chinese hamster ovary cells. *J. Biol. Chem.*, **259**, 1901–1910.
25. Tlsty,T.D. (1990) Normal diploid human and rodent cells lack a detectable frequency of gene amplification. *Proc. Natl Acad. Sci. USA*, **87**, 3132–3136.
26. Wright,J.A., Smith,H.S., Watt,F.M., Hancock,M.C., Hudson,D.L. and Stark,G.R. (1990) DNA amplification is rare in normal human cells. *Proc. Natl Acad. Sci. USA*, **87**, 1791–1795.
27. Brown,P.O. (1997) In Coffin,J.M., Hughes,S.H. and Varmus,H.E. (eds), *Retroviruses*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 161–203.
28. Coffin,J.M. and Rosenberg,N. (1999) Retroviruses. Closing the joint. *Nature*, **399**, 413, 415–416.
29. Roth,D.B. and Wilson,J.H. (1986) Nonhomologous recombination in mammalian cells: role for short sequence homologies in the joining reaction. *Mol. Cell. Biol.*, **6**, 4295–4304.
30. Merrihew,R.V., Marburger,K., Pennington,S.L., Roth,D.B. and Wilson,J.H. (1996) High-frequency illegitimate integration of transfected DNA at preintegrated target sites in a mammalian genome. *Mol. Cell. Biol.*, **16**, 10–18.
31. Craigie,R., Fujiwara,T. and Bushman,F. (1990) The IN protein of Moloney murine leukemia virus processes the viral DNA ends and accomplishes their integration *in vitro*. *Cell*, **62**, 829–837.
32. Katz,R.A., Merkel,G., Kulkosky,J., Leis,J. and Skalka,A.M. (1990) The avian retroviral IN protein is both necessary and sufficient for integrative recombination *in vitro*. *Cell*, **63**, 87–95.
33. Daniel,R., Katz,R.A. and Skalka,A.M. (1999) A role for DNA-PK in retroviral DNA integration. *Science*, **284**, 644–647.
34. Khanna,K.K. and Jackson,S.P. (2001) DNA double-strand breaks: signaling, repair and the cancer connection. *Nature Genet.*, **27**, 247–254.
35. Haber,J.E. (2000) Partners and pathways repairing a double-strand break. *Trends Genet.*, **16**, 259–264.
36. Karran,P. (2000) DNA double strand break repair in mammalian cells. *Curr. Opin. Genet. Dev.*, **10**, 144–150.
37. Gao,Y., Sun,Y., Frank,K.M., Dikkes,P., Fujiwara,Y., Seidl,K.J., Sekiguchi,J.M., Rathbun,G.A., Swat,W., Wang,J. *et al.* (1998) A critical role for DNA end-joining proteins in both lymphogenesis and neurogenesis. *Cell*, **95**, 891–902.
38. Grawunder,U., Zimmer,D., Fugmann,S., Schwarz,K. and Lieber,M.R. (1998) DNA ligase IV is essential for V(D)J recombination and DNA double- strand break repair in human precursor lymphocytes. *Mol. Cell*, **2**, 477–484.
39. Nussenzweig,A., Chen,C., da Costa Soares,V., Sanchez,M., Sokol,K., Nussenzweig,M.C. and Li,G.C. (1996) Requirement for Ku80 in growth and immunoglobulin V(D)J recombination. *Nature*, **382**, 551–555.
40. Zhu,C., Bogue,M.A., Lim,D.S., Hasty,P. and Roth,D.B. (1996) Ku86-deficient mice exhibit severe combined immunodeficiency and defective processing of V(D)J recombination intermediates. *Cell*, **86**, 379–389.
41. Ouyang,H., Nussenzweig,A., Kurimasa,A., Soares,V.C., Li,X., Cordon-Cardo,C., Li,W., Cheong,N., Nussenzweig,M., Iliakis,G. *et al.* (1997) Ku70 is required for DNA repair but not for T cell antigen receptor gene recombination *in vivo*. *J. Exp. Med.*, **186**, 921–929.
42. Gu,Y., Seidl,K.J., Rathbun,G.A., Zhu,C., Manis,J.P., van der Stoep,N., Davidson,L., Cheng,H.L., Sekiguchi,J.M., Frank,K. *et al.* (1997) Growth retardation and leaky SCID phenotype of Ku70-deficient mice. *Immunity*, **7**, 653–665.
43. Ramsden,D.A. and Gellert,M. (1998) Ku protein stimulates DNA end joining by mammalian DNA ligases: a direct role for Ku in repair of DNA double-strand breaks. *EMBO J.*, **17**, 609–614.
44. Liang,F., Romanienko,P.J., Weaver,D.T., Jeggo,P.A. and Jasin,M. (1996) Chromosomal double-strand break repair in Ku80-deficient cells. *Proc. Natl Acad. Sci. USA*, **93**, 8929–8933.
45. Baumann,P. and West,S.C. (1998) DNA end-joining catalyzed by human cell-free extracts. *Proc. Natl Acad. Sci. USA*, **95**, 14066–14070.
46. Kabotyanski,E.B., Gomelsky,L., Han,J.O., Stamato,T.D. and Roth,D.B. (1998) Double-strand break repair in Ku86- and XRCC4-deficient cells. *Nucleic Acids Res.*, **26**, 5333–5342.
47. Sugawara,N., Paques,F., Colaiacovo,M. and Haber,J.E. (1997) Role of *Saccharomyces cerevisiae* Msh2 and Msh3 repair proteins in double-strand break-induced recombination. *Proc. Natl Acad. Sci. USA*, **94**, 9214–9219.
48. Paques,F. and Haber,J.E. (1997) Two pathways for removal of nonhomologous DNA ends during double-strand break repair in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.*, **17**, 6765–6771.
49. Lyu,Y.L., Lin,C.T. and Liu,L.F. (1999) Inversion/dimerization of plasmids mediated by inverted repeats. *J. Mol. Biol.*, **285**, 1485–1501.
50. Bi,X. and Liu,L.F. (1996) DNA rearrangement mediated by inverted repeats. *Proc. Natl Acad. Sci. USA*, **93**, 819–823.
51. Lin,C.T., Lyu,Y.L. and Liu,L.F. (1997) A cruciform-dumbbell model for inverted dimer formation mediated by inverted repeats. *Nucleic Acids Res.*, **25**, 3009–3016.
52. Ouellette,M., Hettema,E., Wust,D., Fase-Fowler,F. and Borst,P. (1991) Direct and inverted DNA repeats associated with P-glycoprotein gene amplification in drug resistant *Leishmania*. *EMBO J.*, **10**, 1009–1016.
53. Ouellette,M. and Borst,P. (1991) Drug resistance and P-glycoprotein gene amplification in the protozoan parasite *Leishmania*. *Res. Microbiol.*, **142**, 737–746.
54. Lin,C.-T., Lin,W.-H., Lyu,Y.L. and Whang-Peng,J. (2001) Inverted repeats as genetic elements for promoting DNA inverted duplication: implications in gene amplification. *Nucleic Acids Res.*, **29**, in press.
55. Ausubel,F.M., Brent,R.E., Moore,D.D., Seidman,J.G., Smith,J.A. and Struhl,K. (eds) (1993) *Current Protocols in Molecular Biology*. Wiley, New York.