Gene targeting by single-stranded DNA oligonucleotides in mouse embryonic stem cells

Aarts, M.

2010

document version
Publisher's PDF, also known as Version of record

Chapter

3

Transient suppression of MLH1 allows effective single-nucleotide substitution by single-stranded DNA oligonucleotides

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Short synthetic single-stranded oligodeoxyribonucleotides (ssODNs) can be used to introduce subtle modifications into the genome of mouse embryonic stem cells. We have previously shown that the DNA mismatch repair (MMR) system imposes a strong barrier to effective application of ssODN-mediated gene targeting. Transient down-regulation of the mismatch recognition protein MSH2 to ten percent of the normal level allowed substitution of 3 or 4 nucleotides, while 1 or 2 nucleotide substitutions were still suppressed. Here, we demonstrate that single- or dinucleotide substitution can be achieved by transient down-regulation of the downstream MMR protein MLH1. Although MLH1 knockdown increased the level of spontaneous mutagenesis, modified alleles could be introduced into the germ line of mice. Thus, transient MLH1 suppression provides a valuable extension of the MSH2 knockdown strategy, allowing ssODN-directed substitution of single nucleotides.

INTRODUCTION

Gene targeting by single-stranded oligodeoxyribonucleotides (ssODNs) is an alternative technique for the introduction of site-specific sequence alterations into the mouse genome. The procedure only requires short synthetic ssODNs that are homologous to the target locus, except for a single or a few centrally located bases that comprise the desired genetic alteration [1,2]. Incorporation of the ssODN during DNA replication appears to be the predominant mechanism underlying ssODN-mediated gene targeting [3-6]. According to this model, the ssODN anneals to its complementary genomic target sequence, when this is present as a region of single-stranded DNA near the replication fork. Then, the ssODN serves as a primer for DNA synthesis to become extended by the endogenous replication machinery, resulting in physical incorporation of the ssODN into the newly synthesized DNA strand [7]. Following annealing, DNA mismatches are formed between the ssODN and its chromosomal complement. These mismatches are recognized and removed by the DNA mismatch repair (MMR) system, thereby preventing introduction of the desired genetic alteration. Thus, MMR raises a strong barrier to ssODN-directed gene modification as has been demonstrated in mouse embryonic stem (ES) cells [8] and in *E. coli* [9-11].

In eukaryotes, DNA mismatches are recognized by either one of two heterodimeric protein complexes: MSH2/MSH6 (MutSα) and MSH2/MSH3 (MutSβ) that have specific and partially redundant mismatch recognition capacities (reviewed in [12]). The MSH2/MSH6 heterodimer mainly recognizes single base-base mismatches and small loops of one or two nucleotides, while the MSH2/MSH3 complex has more affinity for larger loops of two to five unpaired nucleotides. Upon mismatch binding, the MSH2/MSH3 and MSH2/MSH6 complexes activate the MLH1/PMS2 (MutLα) complex, which triggers a downstream repair cascade, resulting in removal of the DNA strand carrying the erroneous nucleotide(s). One manifestation of defects in the MMR machinery is microsatellite instability (MSI), *i.e.*, alterations in the length of simple repetitive DNA tracts such as $(A)_n$ or $(CA)_n$. In the absence of MMR, misalignment of the nascent and template DNA strands due to DNA polymerase slippage errors remains unnoticed and this leads to expansion or contraction of the repetitive tract. Mutations in the

Msh2, *Msh6*, *Mlh1* and *Pms2* genes caused elevated spontaneous mutation rates and MSI in *S. cerevisiae* [13-18] and led to MSI and cancer predisposition in mice [19-25]. Moreover, inherited defects in these MMR genes underlie the cancer syndrome hereditary nonpolyposis colorectal cancer (HNPCC or Lynch syndrome) in humans, which is characterized by development of MSI tumors at early age [26-30]. Mutations in the *Msh3* gene did not induce tumorigenesis in mice [31,32], and only moderate MSI in *S. cerevisiae* [33,34], whereas *Msh6*-deficient mice developed tumors with low level MSI [23]. These data suggest that the MSH2/MSH3 and MSH2/MSH6 complexes have overlapping roles in maintaining genetic stability at simple sequence repeats.

We have previously shown that the recognition specificities of the MSH2/MSH6 and MSH2/MSH3 protein complexes could be exploited to allow the introduction of specific types of sequence alterations by ssODNs. Ablation of *Msh6* allowed effective introduction of 1-, 2-, 3- and 4-nt substitutions, while nucleotide insertions were still suppressed by residual MSH2/MSH3 activity [35]. On the other hand, *Msh3*-deficient ES cells were permissive for nucleotide insertions [36], whereas nucleotide substitutions were still suppressed by the remaining MSH2/MSH6 complexes [35]. Accordingly, *Msh2*-deficient ES cells were permissive for all types of nucleotide substitutions and insertions [36]. Although MMR deficiency strongly improved the efficacy of ssODN-mediated gene targeting, general application is hampered by the mutator phenotype associated with constitutive MMR deficiency (reviewed in [37]). This problem may be circumvented by transient rather than constitutive suppression of MMR. Indeed, we found that temporary down-regulation of *Msh2* by RNA interference generated a time window that allowed gene modification by ssODNs [35]. However, effective gene modification was restricted to substitutions of four, sometimes three, adjacent nucleotides that could be introduced with frequencies comparable to those in constitutive *Msh2*-deficient cells [36]. In contrast, simple nucleotide substitutions and 1- and 4-nt insertions were still suppressed by the residual level of MSH2, which was 10 percent of the normal level. Here, we have investigated whether transient knockdown of the downstream MMR gene *Mlh1* rendered cells permissive for ssODN-mediated gene targeting. Furthermore, the occurrence of frameshift mutations at simple sequence repeats was assessed to compare the level of spontaneous mutagenesis resulting from transient and constitutive MMR suppression.

RESULTS

Reporter systems

We have previously developed two selectable reporter systems consisting of mutant neomycin (*neo*) resistance genes to monitor the efficacy of ssODN-mediated gene modification [8]. The *neo* cassette was mutated by either a single base substitution (ATG to AAG) in the start codon (Target 2, Figure 1A), or a 2 base pair insertion (extra GT) immediately after the start codon, causing a frameshift mutation (Target 1, Figure 2A). A single copy of these mutant *neo* genes was stably integrated into the *Rosa26* locus of wild-type mouse ES cells. ssODNs were designed to correct the mutant *neo* sequence by introducing an in-frame ATG start codon (Figure 1B) or by introducing extra base pairs (+1 or +4 nt) to restore the *neo* open reading frame (Figure 2B) resulting in G418 resistance. MMR activity imposes a strong barrier to ssODN-mediated gene targeting in mouse ES cells [8,36]. Previously, we have demonstrated that transient down-regulation of the central MMR gene *Msh2*

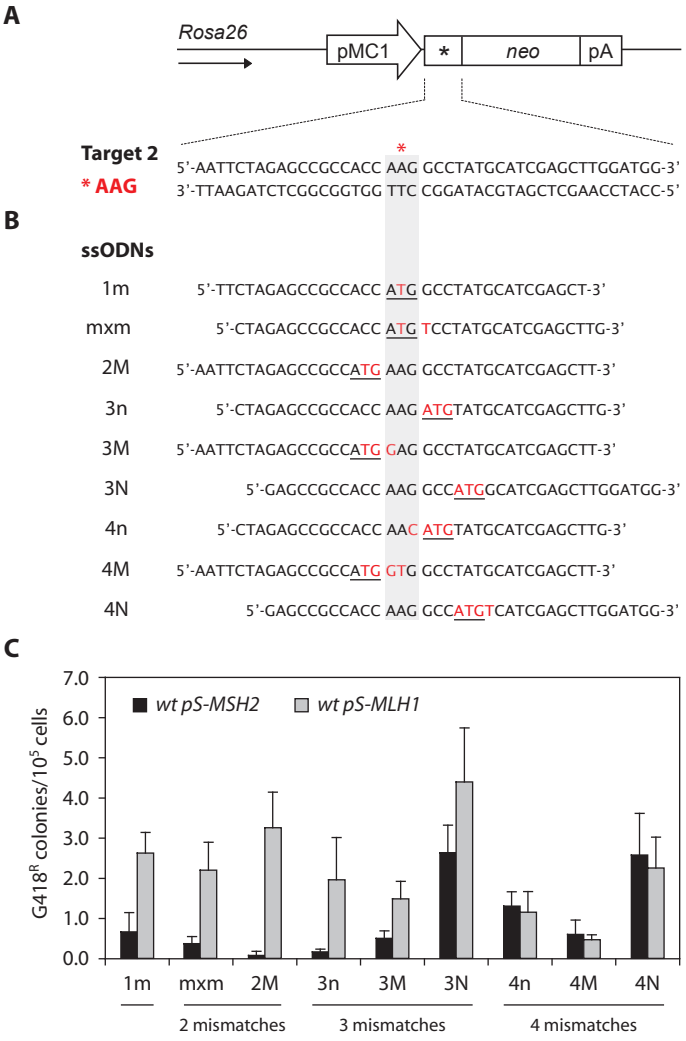

Figure 1 ssODN-mediated nucleotide substitutions.
*(**A**) A single copy of a defective neomycin gene (neo) in which the start codon (ATG) is replaced for AAG (*) (Target 2) was inserted into the Rosa26 locus of wild-type ES cells. (**B**) Activity of the neo gene can be restored by single-stranded oligodeoxyribonucleotides (ssODNs) that substitute one to four nucleotides (indicated in red) to create a new ATG start codon (underlined). (**C**) Correction of the neo reporter by the substitution ssODNs in wild-type ES cells transfected with pS-MSH2 (black bars) or pS-MLH1 (grey bars). The targeting efficiency is the number of G418-resistant colonies per 10^5 cells that were plated after ssODN exposure. Data of pS-MSH2 transfected wild-type ES cells (black bars) are taken from Ref. [35] and shown as controls. Error bars represent the standard deviation (s.d.) of at least four independent experiments.*

by RNA interference was sufficient to allow effective substitution of 3 to 4 adjacent nucleotides in wild-type ES cells [35]. However, simple nucleotide substitutions (1-2 nt) and nucleotide insertions were still suppressed by the activity of residual MMR complexes.

ssODN-mediated gene targeting in MLH1 knockdown ES cells

We investigated whether transient down-regulation of MLH1 could also render cells permissive for ssODN-mediated gene targeting. Wild-type ES cells containing the mutant *neo* reporter gene were transfected with a pSUPER vector expressing a short RNA hairpin directed against *Mlh1* (pS-MLH1), followed by puromycin selection for 2 days to eliminate untransfected cells. Western blot analysis revealed that the MLH1 protein levels were >10-fold reduced in pS-MLH1 transfected ES cells (Figure 3), while the *Mlh1* mRNA levels were reduced to 28% (± 4) of the wild-type levels as determined by quantitative RT-PCR (data not shown). MLH1 protein levels remained strongly reduced for 3 days and then gradually increased to

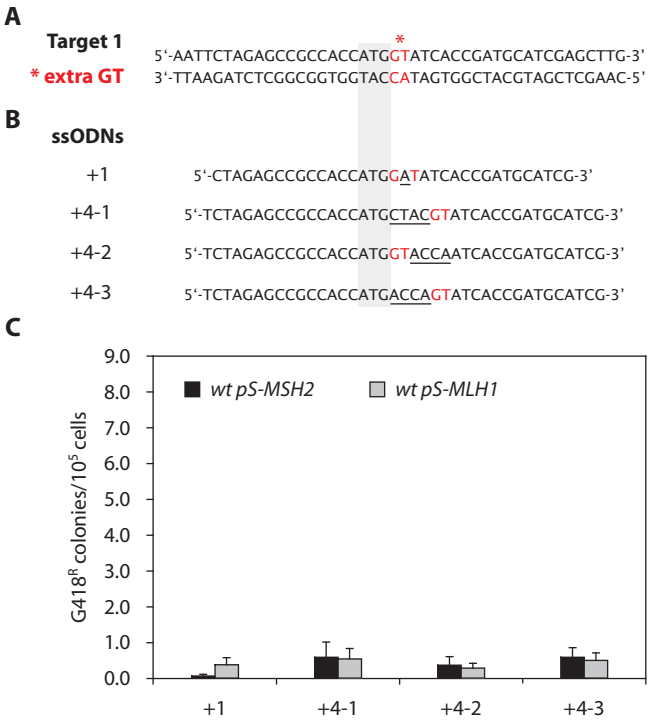

Figure 2 ssODN-mediated nucleotide insertions.
*(**A**) A single copy of a defective neomycin gene (neo) carrying a GT insertion (*, indicated in red) after the ATG start codon (Target 1) was inserted into the Rosa26 locus of wild-type ES cells. (**B**) Activity of the neo gene can be restored by single-stranded oligodeoxyribonucleotides (ssODNs) inserting either one (+1) or four (+4) nucleotides (underlined) to regenerate the open reading frame. (**C**) Correction of the neo reporter by the insertion ssODNs in wild-type ES cells transfected with pS-MSH2 (black bars) or pS-MLH1 (grey bars). The targeting efficiency is the number of G418-resistant colonies per 10^5 cells that were plated after ssODN exposure. Data of pS-MSH2 transfected wild-type ES cells (black bars) are taken from Ref. [35] and shown as controls. Error bars represent the s.d. of at least four independent experiments.*

approximately 50% of the wild-type levels on day 4 after puromycin selection. During the brief period of MLH1 knockdown (day 0 or 1; Figure 3), cells were transfected with different substitution ssODNs to correct the AAG-*neo* reporter gene (Target 2, Figure 1). In contrast to transient down-regulation of MSH2, transient down-regulation of MLH1 allowed effective introduction of all tested nucleotide substitutions, regardless of the number of mismatches that were generated by the ssODNs (Figure 1C; data of pS-MSH2 transfected wild-type cells were taken from Ref. [35]). In particular, simple nucleotide substitutions (ssODNs 1m, mxm, 2M, 3n and 3M) could now efficiently be introduced, reaching frequencies of ~2.3 x 10^{-5}, which was 3- to 44-fold higher than in MSH2 knockdown cells and 50-100% of the frequencies obtained in *Msh2*$^{-/-}$ cells [36]. The targeting efficiencies of ssODNs generating more complex mismatches (ssODNs 3N, 4n, 4M, and 4N) were not significantly altered in MLH1 knockdown cells compared to MSH2 knockdown cells.

Wild-type ES cells carrying the GT-*neo* reporter were used to investigate the effect of MLH1 knockdown on the efficacy of insertion ssODNs (Target 1, Figure 2). Remarkably, transient down-regulation of MLH1 did not improve the efficacy of 4 nt insertion ssODNs, reaching frequencies of only ~0.4 x 10^{-5} (Figure 2C). Only insertion of a single nucleotide (ssODN +1) was 6-fold enhanced after MLH1 knockdown compared to MSH2 knockdown. In sum, transient suppression of MLH1 activity rendered cells permissive for nucleotide substitutions ranging from 1- to 4-nt, while nucleotide insertions were still significantly suppressed. These results indicate that MLH1 knockdown leads to a more rigorous abrogation of MMR, as opposed to MSH2 knockdown, which only allowed the introduction of complex nucleotide substitutions [35].

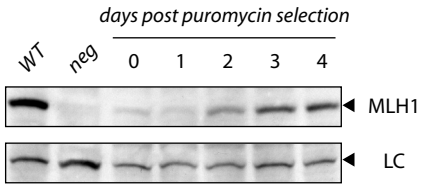

Figure 3 Protein levels after MLH1 suppression. *Western blot analysis of wild-type mouse ES cells transfected with pS-MLH1 followed by puromycin selection for two days. Whole-cell extracts were analyzed for MLH1 expression for five consecutive days. WT, wild-type ES cells not treated with pS-MLH1; neg, Mlh1-deficient human HCT116 cells; LC, non-specific band as loading control.*

Effects of transient MLH1 suppression

To obtain an indication of the level of accumulation of spontaneous mutations as a result of transient MLH1 suppression, we assessed the occurrence of frameshift mutations at simple sequence repeats. For this purpose, we have used two slippage reporter constructs consisting of a *neo* gene in which the open reading frame was disrupted by either a $(G)_{10}$ or a $(CA)_{15}C$ repeat. Polymerase slippage errors that remain unrepaired in the absence of MMR activity may lead to *e.g.* deletion of a single G or addition of a CA restoring the open reading frame and resulting in G418 resistance.

At the $(G)_{10}$ repeat, pS-MLH1 transfected wild-type cells showed a slippage frequency that was ~1400-fold higher than in control wild-type cells (Table 1). However, at the $(CA)_{15}C$ repeat, transient MLH1 suppression resulted in only a 6-fold increase in slippage frequency compared to wild-type control cells. After transient MLH1 knockdown, the slippage frequencies at both the $(G)_{10}$ repeat and $(CA)_{15}C$ repeat were approximately 3-fold higher than after transient MSH2 knockdown [35], but still 4- to 11-fold lower than in constitutive *Msh2*-deficient ES cells (Table 1).

In addition, we have determined the mutation frequency at the *Hprt* locus, assayed by the appearance of 6-thioguanine (6TG)-resistant cells. Upon transient down-regulation of MLH1, the number of 6TG-resistant colonies per 10^6 cells varied between 1 and 6, whereas only a few *Hprt* mutants were found in untreated wild-type cells (Table 2). However, the number of *Hprt* mutants in the pS-MLH1 transfected wild-type cells was approximately 3-fold lower compared to *Msh2*$^{-/-}$ control ES cells. Transient MLH1 suppression resulted in approximately 2-fold higher levels of *Hprt* mutants than transient MSH2 suppression [35], again indicating that MLH1 knockdown more severely compromised MMR activity.

Frameshift mutation rates in constitutive MMR-deficient ES cell lines

Remarkably, transient disabling of the MMR system differentially affected the occurrence of frameshift mutations at the two simple sequence repeats: the slippage frequency at the $(G)_{10}$ repeat increased dramatically (>1000-fold), while the slippage frequency at the $(CA)_{15}C$ repeat was only mildly elevated (<10-fold). To gain more insight into the suitability of the $(G)_{10}$ and $(CA)_{15}C$ reporters as indicators of a mutator phenotype, we studied their behaviour in ES cell lines with constitutive MMR gene defects. To this aim, single copies of the *neo* slippage reporter constructs were inserted into the *Rosa26* or *Rb* locus of wild-type, *Msh3*$^{-/-}$, *Msh6*$^{-/-}$,

Table 1 ***Frameshift mutations at simple sequence repeats.***

Genotype	G418-resistant colonies / 10^6 cells	
	$(G)_{10}$ repeat[a]	$(CA)_{15}C$ repeat[b]
WT pS	0.75 (±0.74)	78 (±45)
WT pS-MLH1	1052 (±132)	496 (±128)
***Msh2*$^{-/-}$ pS**	4120 (±562)	5433 (±1723)

[a] *$(G)_{10}$ repeat in Rb locus.*
[b] *$(CA)_{15}C$ repeat in Rosa26 locus.*
Results (± s.d.) are shown from 4 independent experiments.

Table 2 ***Spontaneous mutation frequency at the Hprt locus.***

Genotype	Number of HPRT mutants / 10^6 cells			
	Exp. 1	Exp. 2	Exp. 3	Exp. 4
WT pS	0	1	1	1
WT pS-MLH1	2	2	1	6
***Msh2*$^{-/-}$ pS**	6	6	ND	9

ND*, not determined*

Table 3 ***Frameshift mutation rate at $(G)_{10}$ repeat in MMR-deficient ES cell lines.***

Genotype	Mutation rate (x 10^{-5})[a]	
	***Rosa26* locus**	***Rb* locus**
WT	0.05 (±0.03)	0.03 (±0.01)
***Msh3*$^{-/-}$**	1.5 (±2.6)	0.3 (±0.03)
***Msh6*$^{-/-}$**	14 (±0.6)	42 (±11)
***Msh2*$^{-/-}$**	145 (±18)	137 (±20)
***Msh3*$^{-/-}$*Msh6*$^{-/-}$**	108 (±48)	ND

[a] *Number of mutants per cell division.*
Results (± s.d.) are shown from 2-8 independent subclones.

Table 4 ***Type of $(G)_{10}$ repeat alterations.***

Genotype	–4 bp	–1 bp	+2 bp	Total
WT	10			10
***Msh3*$^{-/-}$**	22			22
***Msh2*$^{-/-}$**	2	35		37

Number of colonies with repeat alterations (deletion of 4 bp, deletion of 1 bp, addition of 2 bp) is indicated.

Msh2$^{-/-}$ and *Msh3*$^{-/-}$*Msh6*$^{-/-}$ ES cells [19,31]. As shown in Table 3, the $(G)_{10}$ repeat was very stable in wild-type ES cells (3×10^{-7} to 5×10^{-7} repeat alterations/cell division). However, the stability of this repeat was strongly affected by disruption of MMR genes: in *Msh2*$^{-/-}$ cells the mutation rates increased up to 4500-fold to 1.4×10^{-3} repeat alterations/cell division. *Msh6*-deficiency resulted in a 300- to 1400-fold increase, while *Msh3*-deficiency moderately affected the slippage rate at the $(G)_{10}$ repeat (10- to 30-fold increase). *Msh3*$^{-/-}$*Msh6*$^{-/-}$ cells showed the same level of instability as the *Msh2*$^{-/-}$ cell line. The occurrence of slippage errors appeared to be largely independent of the chromosomal location of the repeat (*Rosa26* or *Rb* locus).

The type of alterations at the $(G)_{10}$ repeat was determined in a series of G418-resistant colonies (Table 4). *Msh2*$^{-/-}$ colonies predominantly resulted from 1 bp deletions (35/37 = 95%) and occasionally from 4 bp deletions (2/37 = 5%). In wild-type and *Msh3*$^{-/-}$ colonies only 4 bp deletions were found, indicating that 1 bp deletions were efficiently suppressed by MSH2/MSH6 activity. Remarkably, none of the investigated colonies showed 2 bp additions, suggesting that this type of replication error did not occur at the $(G)_{10}$ repeat.

Together, these results indicate that slippage errors at the $(G)_{10}$ repeat are very efficiently repaired by the MMR system.

Remarkably, the $(CA)_{15}C$ repeat was less stable in wild-type ES cells than the $(G)_{10}$ repeat, showing mutation rates of 9 x 10^{-6} to 4 x 10^{-5} repeat alterations/cell division (Table 5). In *Msh2*$^{-/-}$ cells, the mutation rate increased 110-fold at the *Rosa26* locus (9.9 x 10^{-6} repeat alterations/cell division), but only 9-fold at the *Rb* locus (3.2 x 10^{-6} repeat alterations/cell division), indicating that the occurrence of replication errors and their repair were affected by differences in sequence context. The relatively high mutation rate of the $(CA)_{15}C$ repeat in wild-type cells suggests that many dinucleotide slippage errors escaped detection by the MMR system and this was particularly the case when the $(CA)_{15}C$ repeat was inserted at the *Rb* locus. The mutation rate was not strongly affected by *Msh3*-deficiency, indicating a dominant role for MSH2/MSH6-directed repair. Nevertheless, *Msh6*-deficiency elevated the mutation rate only 3- to 6-fold, indicating that also MSH2/MSH3 activity was capable of suppressing dinucleotide slippage errors.

At the $(CA)_{15}C$ repeat, restoration of the *neo* open reading frame can only be achieved by slippage errors leading to addition of 2 or 8 bp or deletion of 4 or 10 bp. By analyzing the type of repeat alterations that were present

Table 5 ***Frameshift mutation rate at $(CA)_{15}C$ repeat in MMR-deficient ES cell lines.***

Genotype	Mutation rate (x 10^{-5})[a]	
	***Rosa26* locus**	***Rb* locus**
WT	0.9 (±0.2)	3.7 (±1.8)
***Msh3*$^{-/-}$**	1.3 (±0.7)	1.0 (±0.4)
***Msh6*$^{-/-}$**	5.0 (±0.5)	10 (±2.0)
***Msh2*$^{-/-}$**	99 (±20)	32 (±14)
***Msh3*$^{-/-}$*Msh6*$^{-/-}$**	73 (±34)	ND

[a] *Number of mutants per cell division.*
Results (± s.d.) are shown from 2-14 independent subclones.

Table 6 ***Type of $(CA)_{15}C$ repeat alterations.***

Genotype	–10 bp	–4 bp	+2 bp	Total
WT			51	51
***Msh3*$^{-/-}$**	6	13	37	56
***Msh6*$^{-/-}$**			68	68
***Msh2*$^{-/-}$**	1	18	77	96
***Msh3*$^{-/-}$*Msh6*$^{-/-}$**		9	60	69

Number of colonies with repeat alterations (deletion of 10 bp, deletion of 4 bp, addition of 2 bp) is indicated.

in the G418-resistant colonies, we found in wild-type and *Msh6*$^{-/-}$ colonies only 2 bp additions (Table 6), indicating that larger additions (8 bp) or deletions (4 or 10 bp) were strongly suppressed by MSH2/MSH3 activity. Two-base pair additions were also the main type of slippage errors in the *Msh2*$^{-/-}$ and *Msh3*$^{-/-}$*Msh6*$^{-/-}$ colonies, although 4 bp deletions were observed in 13-19% of the colonies (18/96 and 9/69, respectively). A substantial percentage (6/56 = 11%) of the *Msh3*$^{-/-}$ colonies showed 10 bp deletions, confirming strong MSH2/MSH3 activity towards large slippage errors.

In summary, these results show that MSH2/MSH6 was the main suppressor of single nucleotide deletions and MSH2/MSH3 predominantly suppressed large insertions or deletions, which is consistent with previous reports [12]. More strikingly, our results show that 1-nt slippage at the $(G)_{10}$ repeat is far more efficiently repaired than 2-nt slippage at the $(CA)_{15}C$ repeat. Somewhat counter-intuitively, transient disabling of the MMR system by MLH1 knockdown increased the slippage frequency at the $(G)_{10}$ mononucleotide repeat to roughly the same level as that of the $(CA)_{15}C$ dinucleotide repeat. Moreover, the slippage frequencies in the MLH1 knockdown cells were most similar to the mutation rates obtained in the constitutive *Msh6*-deficient cell line, suggesting that MLH1 suppression may have mainly abrogated MMR activity initiated by the MSH2/MSH6 complex. This notion was supported by the increased targeting efficiencies of the substitution ssODNs in the MLH1 knockdown cells (Figure 1C), whereas the insertion ssODNs were still suppressed most likely by MSH2/MSH3-directed repair (Figure 2C).

DISCUSSION

Previously, we have established a generally applicable procedure for the generation of subtle gene modifications in mouse ES cells using ssODN-mediated gene targeting in combination with a temporary knockdown of the *Msh2* gene [35]. This procedure allowed effective substitution of 4 nt, but the introduction of simple nucleotide (1-, 2- and some 3-nt) substitutions was still suppressed by the activity of residual mismatch recognition complexes. Here, we demonstrate that transient down-regulation of the downstream MMR gene *Mlh1* rendered cells permissive for all tested nucleotide substitutions, ranging from 1- to 4-nt (Figure 1C). Since MLH1 functions downstream in MMR, its suppression should leave the recognition of all types of mismatches by MSH2/MSH3 and MSH2/MSH6 intact. Thus, in MLH1 knockdown cells, activation of the downstream MMR response, including degradation and resynthesis of the DNA strand carrying the mismatch, has become the rate-limiting step in the MMR reaction irrespective of the type of substitution.

Surprisingly, nucleotide insertions were not effective in the MLH1 knockdown cells (Figure 2C): targeting efficiencies remained at only 5-14% of the levels found in *Msh2*$^{-/-}$ ES cells [36], which was comparable to the levels in the MSH2 knockdown cells [35]. Perhaps the suppression of nucleotide insertions required only very little MMR activity. Alternatively, nucleotide insertions might be suppressed via an MLH1-independent mechanism, requiring only the activity of the MSH2/MSH3 complex for their removal [36]. Testing the efficacy of the insertion ssODNs in *Mlh1*-deficient ES cells may solve this question.

To obtain an indication for the accumulation of undesired mutations due to transient suppression of MLH1 activity, we used the mutation frequency at two simple sequence repeats disrupting the open reading frame of a *neo* reporter gene as readout (Table 1). As expected, transient MLH1 suppression increased the mutation frequency at both the mononucleotide and dinucleotide repeat. Strikingly, the slippage frequency at the $(G)_{10}$ repeat was more than 1000-fold increased, while the slippage frequency at the $(CA)_{15}C$ repeat was only 6-fold increased upon transient MLH1 knockdown.

To assess the suitability of the simple sequence repeat reporters as a readout for MMR activity, we determined the mutation rates at both repeats in constitutively MMR-deficient ES cell lines. From the mutation frequencies at the *Rosa26* locus given in Tables 3 and 5 and the types of sequence alterations shown in Tables 4 and 6, we estimated the repair capacities of MSH2/MSH6 and MSH2/MSH3. As shown in Table 7, MSH2/MSH6 was nearly 100% effective in repairing -1 bp slippage errors at the $(G)_{10}$ repeat since the G418-resistant colonies appearing in *Msh3*-deficient ES cells were all the result of -4 bp slippage errors (Table 4). In turn, MSH2/MSH3 was very effective in repairing large slippage errors as deduced from the absence of 10 and 4 bp deletions at the $(CA)_{15}C$ repeat in *Msh6*-deficient cells (Table 6). Although not directly tested, it is likely that MSH2/MSH3 also strongly suppressed 4 bp deletions at the $(G)_{10}$ repeat. Thus, the high activity of MSH2/MSH6 towards -1 bp slippage errors and of MSH2/MSH3 towards -4 bp slippage errors explains the very low mutation frequency at the $(G)_{10}$ repeat in wild-type cells. It is of note that restoration of the *neo* open reading frame by 2 bp insertion did not occur at the $(G)_{10}$ repeat. This is somewhat puzzling as 2 bp insertions at the $(CA)_{15}C$ repeat were frequently observed in *Msh2*-deficient cells (Tables 5 and 6). Perhaps an extrahelical GG is structurally less-well tolerated than an extrahelical CA. Of the +2 bp slippage errors at the $(CA)_{15}C$ repeat, MSH2/MSH6 could only repair ±99% and MSH2/MSH3 ±95%. We conclude from this analysis that MMR is more effective at -1 bp errors than at +2 bp errors. These observations are in agreement with the high slippage rates of poly(G) tracts in *msh2* mutants in *S. cerevisiae* [16-18], whereas the slippage rates at $(GT)_{14}G$ and $(GT)_{16}G$ dinucleotide repeats increased only several 100-fold in *msh2* mutants [13,15,16,34]. Moreover, the mutation rates at dinucleotide repeats were on average several 100- to 1000-fold higher than at mononucleotide repeats in wild-type yeast strains supporting our observation that dinucleotide slippage errors were less efficiently repaired by the MMR system.

Table 7 ***Repair capacities of MSH2/MSH6 and MSH2/MSH3.***

Slippage error	MSH2/6	MSH2/3
–1 bp (in G repeat)	±100%	90%
+2 bp (in CA repeat)	99%	95%
–4 bp (in G or CA repeat)	98%	±100%

The repair efficiencies are estimated from the slippage frequencies at the Rosa26 locus in Tables 3 and 5 and the type of repeat alterations in Tables 4 and 6.

Although the $(G)_{10}$ repeat was much better stabilized by MMR than the $(CA)_{15}C$ repeat, both generated roughly the same level of G418-resistant colonies upon reduction of MMR protein levels. This shows that the very effective repair at the $(G)_{10}$ repeat was highly dependent on wild-type MMR protein levels. Moreover, at both repeats, MLH1 knockdown generated about 3-fold more G418-resistant colonies than MSH2 knockdown. Strikingly, the efficacy of ssODN-directed +1 or +4 nt insertion was still 6- to 120-fold suppressed in MLH1 and MSH2 knockdown cells, compared to *Msh2*$^{-/-}$ ES cells (Figure 2C). This observation suggested to us that small spontaneously occurring slippage errors may still effectively be repaired by reduced levels of MMR activity. This view is now challenged by the high mutation frequencies at the $(G)_{10}$ and $(CA)_{15}C$ repeats upon transient MMR protein knockdown. Apparently, frameshift mutagenesis at microsatellite sequences is more sensitive to reduction of MMR protein levels than ssODN-directed frameshift mutagenesis. Also the direct sequence context may play a role as in *S. cerevisiae*, it has been reported that besides the length and the primary sequence of the repeat, also the sequences flanking the repeat have a strong impact on the mutation rate [14,17,18,38].

MLH1 knockdown cells also showed an increased spontaneous mutation frequency at the *Hprt* locus (Table 2). A possible source of these spontaneous mutations could be the incorporation of oxidized bases, such as 8-oxo-7,8-dihydroguanine (8-oxoG) that may lead to the formation of 8-oxoG:A mispairs [39]. Colussi *et al*. [40] demonstrated that the DNA of *Msh2*-deficient mouse embryonic fibroblasts (MEFs) contained more 8-oxoGs than wild-type MEFs, indicating that the MMR system is involved in excising incorporated 8-oxo-dGMP from the DNA. MTH1 provides a supplementary line of defence by eliminating oxidized purine nucleoside triphosphates (such as 8-oxo-dGTP) from the dNTP pool [41]. Overexpression of hMTH1 resulted in a 20-fold decrease in the *Hprt* mutation rate in *Msh2*-deficient MEFs [40], whereas the occurrence of frameshift mutations within mononucleotide repeats was decreased in some, but not all, MMR-defective human cancer cell lines [42]. We have investigated whether hMTH1 overexpression could diminish the mutator phenotype observed in the MLH1 knockdown cells, however this was not the case (data not shown). Nevertheless, oxidative damage may still contribute to the mutator phenotype in the MLH1 knockdown cells, since preliminary data suggest that culturing ES cells under low oxygen tension (~3% instead of ~20%) decreased the rate of spontaneous mutagenesis (data not shown). We are currently testing the feasibility of ssODN-directed gene modification under this condition.

In conclusion, transient down-regulation of MLH1 made wild-type ES cells permissive for the introduction of all types of nucleotide substitutions by ssODNs. This significantly improves the previously reported ssODN-mediated gene targeting procedure using transient MSH2 knockdown, by abolishing the suppression of simple substitutions of 1 and 2 nt. Nevertheless, transient knockdown of MSH2 or MLH1 strongly induced frameshift mutagenesis at two microsatellite repeat sequences. Although the level of frameshift mutagenesis in MSH2/MLH1 knockdown cells remained substantially below that in constitutatively MMR-defective cells, transient MSH2/MLH1 knockdown may not be harmless to cells. Full insight into the mutation load arising from transient MMR protein suppression may be obtained from whole genome sequencing of individual cell clones. This will also reveal whether the suppression of simple nucleotide sub-

stitutions in MSH2 knockdown cells can be taken as evidence for a low level of spontaneously occurring base-pair substitutions. However, more important is the question whether transiently low MMR protein levels lead to alterations that could interfere with subsequent phenotypic analyses of mutant cells or mice. In a previously generated *Msh2*$^{low/-}$ ES cell line that constitutively expressed 10% of wild-type MSH2 protein level, 2% of the tested dinucleotide microsatellites were instable as opposed to 32% in *Msh2*$^{-/-}$ ES cells and <1% in wild-type cells [43]. *Msh2*$^{low/-}$ cells were also fully capable of suppressing recombination between homologous but not identical DNA sequences, in contrast to *Msh2*$^{-/-}$ cells. Furthermore, although the mismatch correction capacity was somewhat compromised, the reduced MSH2 protein level was sufficient to prevent lymphomagenesis and development of intestinal tumors in *Msh2*$^{low/-}$ mice (unpublished observation). Thus, the level of spontaneously occurring mutations in *Msh2*$^{low/-}$ mice was not sufficient to induce development of tumor types that frequently arose in fully *Msh2*-deficient mice. The spontaneous frameshift mutation frequency in MLH1 knockdown cells was somewhat higher than in MSH2 knockdown cells, but we do not know yet how this would translate to tumor development in mice. However, we do know that in contrast to full ablation of MSH2, transient MLH1 knockdown and subsequent ssODN-mediated gene targeting allowed ES cell contribution to the germ line of chimeric mice and establishment of mutant mouse models. Whether or not transient suppression of MMR proteins is a serious concern may become clear from careful phenotypic assessment of such mouse lines.

ACKNOWLEDGEMENTS

We are grateful to Niek Wit for investigating the effect of hMTH1 overexpression on the mutator phenotype in MLH1 knockdown cells. We thank Dr. Margherita Bignami (Instituto Superiore di Sanità, Rome, Italy) for providing the hMTH1d construct. This work was supported by the Netherlands Genomics Initiative (Horizon project 050-71-051).

MATERIALS & METHODS

Cell lines and culture conditions

We developed two selectable targeting reporter systems consisting of mutant neomycin (*neo*) genes. The *neo* cassette was mutated by either a single base substitution (ATG > AAG) in the start codon (Target 2, Figure 1A), or a 2 bp insertion (extra GT) immediately after the start codon, causing a frameshift mutation (Target 1, Figure 2A). A single copy of these mutant *neo* genes was stably integrated into the *Rosa26* locus of wild-type ES cells as described previously [8]. 129/Ola-derived E14-IB10 ES cells [44] were cultured on MEF feeders in Glasgow minimal essential medium (GMEM) supplemented with 10% fetal calf serum (FCS), 1mM sodium pyruvate, 1x non-essential amino acids, 1 mM 2-mercaptoethanol and 1000 U per ml of leukemia inhibitory factor. For transfections and antibiotic selections, ES cells were cultured onto gelatin-coated plates in BRL (Buffalo rat liver cells)-conditioned medium [44].

Transfection

ssODNs and pSUPER plasmids were transfected following the TransFast-mediated transfection method described earlier [35]. Briefly, 7 x 10^5 ES cells were seeded onto a gelatin-coated 6-well in BRL-conditioned medium the day before transfection. For one well, 3 µg of pS-MLH1 and 27 µl of TransFast transfection agent (Promega) were diluted in 1.4 ml serum-free medium and incubated for 15 min at room temperature. After 75 min of exposure to the transfection mixture at 37 °C, 4 ml of BRL-conditioned medium with serum was added to the cells. The next day, puromycin selection (20 µg/ml; Sigma-Aldrich) was started for 2 days, after which cells were reseeded at a density of 7 x 10^5 cells per well for ssODN transfection as described above. The day after ssODN transfection, cells were counted and replated onto three gelatin-coated 100 mm dishes. G418 selection (750 µg/ml for Target 2 and 550 µg/ml for Target 1; GIBCO-Invitrogen) was started 48 hrs after transfection. After 10 days, G418-resistant colonies were stained with Leishman's eosin methylene blue solution (Merck) and counted. Transfection efficiencies were calculated by dividing the number of G418-resistant colonies by the number of cells that were plated 24 hrs after ssODN transfection.

ssODNs of 35-38 nt were designed to correct the mutant *neo* reporter genes by introducing an in-frame ATG start codon (Figure 1B) or by introducing extra base pairs (+1 or +4 nt) to restore the *neo* open reading frame (Figure 2B). Unmodified ssODNs, deprotected and desalted, were purchased from Sigma-Aldrich, Inc.

RNA interference

We used the pSUPER vector containing a puromycin resistance gene for expression of shRNAs in murine ES cells [45]. A 19 nt sequence corresponding to nucleotides 944-962 of the *Mlh1* gene (GenBank accession no. NM_026810.1), separated from the reverse complement of the same 19 nt sequence by a 9 nt non-complementary spacer (TTCAAGAGA), was inserted into the pSUPER vector (pS-MLH1). An empty vector with no gene-specific 19 nt sequence served as a non-silencing control (pS).

Western blot analysis

Western blot analysis was performed according to standard procedures. Protein extracts from 2 x 10^5 ES cells were separated by 7.5% SDS-PAGE and transferred to a nitrocellulose membrane. Rabbit polyclonal IgGs against MLH1 (1:1000; sc-581, Santa Cruz Biotechnology, Inc.) were used as primary antibodies and horseradish peroxidase-conjugated goat anti-rabbit IgG (Dako) was used as a secondary antibody. Signals were visualized using enhanced chemiluminescence (ECL).

Frameshift mutations at simple sequence repeats

We constructed two reporter cell lines consisting of a *neo* gene containing either a $(G)_{10}$ or a $(CA)_{15}C$ repeat disrupting the open reading frame. The *neo* gene was derived from plasmid pMC1neo [46] in which the sequence between the first and the fifth codon was replaced by either a $(G)_{10}$ or a $(CA)_{15}C$ repeat. The resulting *neo* slippage reporter genes were inserted into the *Rosa26-His* targeting vector

[8] and into the *129Rb*-his targeting vector which is based on 129*Rb*-hyg [47]. Single copies of the $(CA)_{15}C$ and $(G)_{10}$ *neo* reporters were stably integrated into either the *Rosa26* or *Rb* locus of *Msh2*$^{-/-}$, *Msh3*$^{-/-}$, *Msh6*$^{-/-}$, *Msh3*$^{-/-}$*Msh6*$^{-/-}$, and wild-type ES cell lines as described previously [19,31,47]. Single copy integration was confirmed by Southern blot analysis.

To calculate the frameshift mutation rate, single cells were expanded to approximately 8 x 10^6 cells (*Msh2*$^{-/-}$ and *Msh3*$^{-/-}$*Msh6*$^{-/-}$) or to approximately 2.0 x 10^8 cells (wild-type, *Msh3*$^{-/-}$ and *Msh6*$^{-/-}$). Then, 2 x 10^5 cells (*Msh2*$^{-/-}$ and *Msh3*$^{-/-}$*Msh6*$^{-/-}$) or 4 x 10^6 cells (wild-type, *Msh3*$^{-/-}$ and *Msh6*$^{-/-}$) were plated onto two 100 mm dishes in selective medium containing 400 µg/ml G418. Mutation rates were calculated according to the Luria and Delbrück method [48]: 0.6 x $G418^{total}$ = N x p x log (N x p), in which $G418^{total}$ is the total number of G418-resistant cells in a culture expanded to N cells, and p is the number of mutations per cell division. To determine the type of repeat alteration, DNA was isolated from G418-resistant colonies and the region encompassing the $(G)_{10}$ or $(CA)_{15}C$ repeat was amplified by PCR using forward primer *2-pmc1-5'* (GCATATTAAGGTGACGCGTGTGG) end-labelled with [γ-^{32}P]ATP and reverse primer *2-neo* (AAGCGGCCGGAGAACCTGCG). Amplified PCR products were separated on a denaturing polyacrylamide gel and autoradiographed.

To determine the slippage frequency after MLH1 down-regulation, wild-type ES cells containing the selectable slippage reporters were transfected with 3 µg of pS or pS-MLH1 followed by selection with puromycin for 2 days. After 7 days of cell culture, 4 x 10^6 cells were plated onto two 100 mm dishes in selective medium containing 600 µg/ml G418. As a control, 10^5 pS-transfected *Msh2*$^{-/-}$ cells were included in the experiment. After 10 days, G418-resistant colonies were stained and counted.

Spontaneous mutation frequency at Hprt locus

Wild-type ES cells were transfected with 3 µg of pS or pS-MLH1 followed by selection with puromycin for 2 days. pS-transfected *Msh2*$^{-/-}$ cells served as controls. After 7 days of cell culture, 4 x 10^6 cells were plated onto two 150 mm dishes in selective medium containing 10 µg/ml 6-thioguanine (6TG; Sigma-Aldrich). After 10 days, 6TG-resistant colonies were stained and counted.

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