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Targeted gene modification in mismatch-repair-deficient embryonic stem cells by single-stranded DNA oligonucleotides

Marleen Dekker, Conny Brouwers and Hein te Riele*

Division of Molecular Biology, The Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX Amsterdam, The Netherlands

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ABSTRACT

Gene targeting through homologous recombination in murine embryonic stem (ES) cells is already strongly suppressed by DNA mismatch-repair (MMR)-dependent anti-recombination when targeting construct and target locus differ at <1% of the nucleotide positions. We demonstrate that MMR activity also raises a strong impediment to gene modification mediated by small synthetic DNA oligonucleotide sequences. In the absence of the DNA MMR gene *MSH2*, synthetic single-stranded deoxyribo-oligonucleotides can be used to site-specifically modify the ES cell genome. We show that PCR-based procedures can be used to identify and clone modified cells. By this method we have substituted a single codon in the retinoblastoma gene.

INTRODUCTION

Gene targeting in embryonic stem (ES) cells is widely used to introduce specific genetic modifications into the mouse germline (1). In its simplest form, the technology involves the generation of a so-called targeting construct in which a selectable marker gene (e.g. *neo*, *hyg*, *pur*) is flanked by DNA sequences that are largely identical to the specific chromosomal locus to be modified. On entry of the targeting construct into the cell, homologous recombination between the flanking sequences and their chromosomal counterparts will result in the integration of the marker gene into the chromosome, thereby disrupting the gene of interest (2). In addition, protocols have been developed to introduce subtle modifications as small as the insertion, deletion or substitution of a single base pair. For example, in a two-step protocol, homologous recombination is used to introduce the subtle mutation into the gene of interest concomitantly with a dominantly/negatively selectable marker gene, the latter subsequently being removed via intra-chromosomal homologous or Cre/*lox*-mediated site-specific recombination (3,4). An alternative approach to introduce subtle gene modifications may be the use of synthetic single-stranded DNA oligonucleotides, as has been proven successful in the yeast *Saccharomyces cerevisiae* (5). Single-stranded oligonucleotides have also been used in human cells to modify an episomally-located gene; however, this approach has not been followed up by targeting of chromosomally-located genes (6). Instead, oligonucleotide-directed gene modification in mammalian cells has made use of chemically-modified single-stranded oligonucleotides, chimeric RNA/DNA oligonucleotides or triple-helix-forming oligonucleotides (7–10), all containing phosphorothioate linkages or 2′-*O*-methyl-RNA residues. While the enhanced resistance of these oligonucleotides to intracellular nucleolytic degradation has been considered critical to the success of these approaches (11), the mechanism of transfer of genetic information from the oligonucleotide to the target remains largely elusive. Somewhat surprisingly, no reports have appeared describing the successful use of chemically-modified oligonucleotides in ES cells.

We have previously demonstrated that the efficiency of gene targeting in ES cells is strongly suppressed by DNA mismatch-repair (MMR)-dependent anti-recombination, which is already activated by sequence dissimilarities between targeting construct and target locus as small as 0.6% (12,13). We reasoned that this phenomenon could also raise a strong impediment to gene modification mediated by small synthetic DNA oligonucleotide sequences which differ from the target locus by one or a few nucleotides. To test this hypothesis, we have introduced into MMR-proficient and -deficient ES cells [the latter carrying a homozygous disruption in the central MMR gene *Msh2* (13)] a recombination reporter construct consisting of a *neo* gene that was rendered inactive by either a two base pair insertion disrupting the open reading frame or a base pair substitution in the start codon. We then investigated whether these mutations could be restored by sequence-specific single-stranded deoxyribo-oligonucleotides.

MATERIALS AND METHODS

The *neo* gene was essentially derived from plasmid pMC1neo (14) in which the sequence around the start codon (between the *Pst*I site and the fifth codon) was replaced by sequences depicted in Figure 1b and c (target 1 and 2, respectively). The *Rosa26*-targeting construct consisted of a 13 kb *Rosa26* fragment (15) (J.-H.Dannenberg, personal communication) carrying a promoterless *histidinol*-resistance gene with an upstream splice acceptor site inserted into the first intron. Defective *neo* genes were placed immediately downstream of

*To whom correspondence should be addressed. Tel: +31 20 512 20 84; Fax: +31 20 669 13 83; Email: h.t.riele@nki.nl

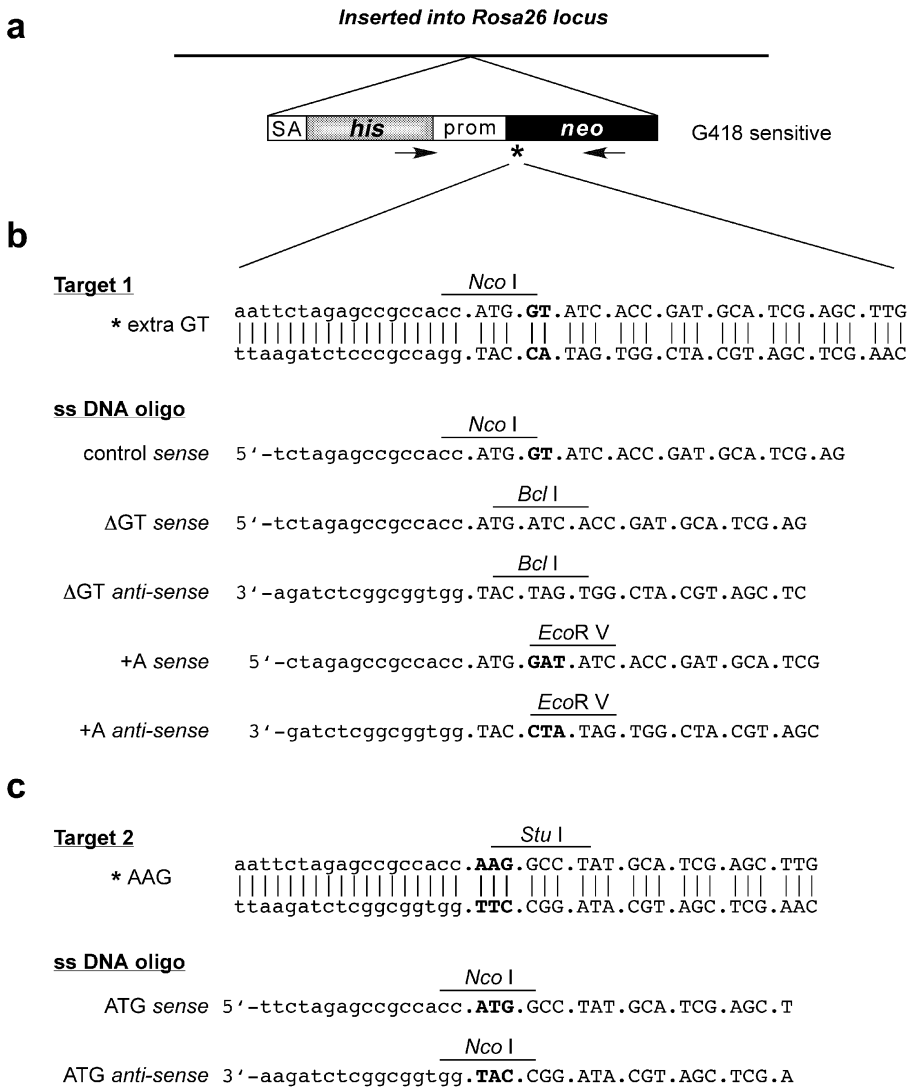

Figure 1. A selectable target for oligonucleotide-directed gene modification. (**a**) Single-copy integration of a defective *neo* gene into the *Rosa26* locus. The targeting vector contains a promoterless *histidinol*-resistance gene (*his*) preceded by a splice acceptor site (SA); the *neo* gene is driven by the MC1 promoter (prom). The asterisk indicates the position of an inactivating mutation in *neo*. The arrows represent PCR primers used to amplify a diagnostic 1200 bp fragment. (**b**) Target 1 contains a GT frameshift mutation immediately downstream of the start codon. This mutation was restored by deletion of GT using single-stranded DNA oligonucleotides ΔGT *sense* and ΔGT *anti-sense*, or insertion of A using oligonucleotides +A *sense* and +A *anti-sense*. Restoration of *neo* leads to loss of *Nco*I and gain of *Bcl*I (ΔGT) or to gain of *Eco*RV (+A). Lower-case letters represent non-coding bases. (**c**) Target 2 contains a T to A point mutation in the start codon. AAG was restored to ATG using oligonucleotides ATG *sense* and ATG *anti-sense* leading to loss of *Stu*I and gain of *Nco*I.

his. Targeting vectors were introduced into ES cells by electroporation as described (12). Over 90% of histidinol-resistant clones (selected in 2.0 mM histidinol) were the result of homologous recombination at *Rosa26* without additional random integrations as verified by Southern blotting.

Deoxyribo-oligonucleotides were obtained from Sigma-Genosys Ltd. For oligo-targeting experiments, cells were seeded in 6-well plates at a density of 7×10^5 per well. The next day, cells were exposed for 1 h to 1.4 ml of serum-free medium containing 3–7 μg of oligonucleotide plus Tfx™-50 (31.5 μl) or TransFast™ (63 μl) lipofection reagent (Promega Corporation). After addition of 4 ml of medium plus serum, cells were incubated overnight. The next day, cells were counted and replated in selective medium containing 400 μg (target 1 cells) or 600 μg (target 2 cells) of G418. After 8–10 days, G418-resistant colonies were counted. For DNA extraction, colonies were picked and expanded in 96-well plates. DNA was analyzed by PCR as described in the legend to Figure 2 (primer sequences are available upon request).

Rb-1 mRNA was amplified by reverse transcription using a primer in exon 22, followed by PCR using primers in exons 19 and 20. The exon 19 primer was used for sequencing (primer sequences are available upon request).

RESULTS

Oligonucleotide-mediated deletion of two base pairs

To generate a selectable target for oligonucleotide-directed gene modification ('oligo targeting'), we constructed a defective *neo* gene (*neo*^{s}) by inserting a GT frameshift mutation immediately downstream of the start codon (target 1,

Table 1. Oligonucleotide-mediated correction of a GT frameshift mutation in *Msh2*$^{-/-}$ cells

Oligonucleotide	No. of G418-resistant colonies			
	Experiment 1	Experiment 2	Experiment 3	Experiment 4
ΔGT *sense*	22	36	37	0
ΔGT *anti-sense*	60	24	39	ND
ΔGT *ds*	1	1	ND	ND
Control *sense*	ND[a]	0	0	ND

ES cells were plated at 7×10^5 cells/10 cm^2. The next day cells were exposed for 1 h to lipofection medium consisting of 1.4 ml of medium (without serum), 7 μg of DNA plus 31.5 μl Tfx-50 (Experiment 1); 21 μl Transfast (Experiment 2); 63 μl Transfast (Experiment 3); without lipofection reagent (Experiment 4). After addition of 4 ml of serum-containing medium, cells were incubated overnight. The next day, cells were trypsinized, plated on 10 cm plates and grown in the presence of G418 (400 μg/ml). Colonies were counted after 8–10 days.
[a]ND, not determined.

Table 2. Oligonucleotide-mediated correction of a GT frameshift mutation in wild-type cells

Oligonucleotide	No. of G418-resistant colonies			
	Experiment 1	Experiment 2	Experiment 3	Experiment 4
ΔGT *sense*	0	0	0	0
ΔGT *anti-sense*	ND[a]	0	0	ND

See footnote to Table 1.

Fig. 1b). The *neo*^{s} allele was placed adjacent to a *histidinol*-resistance gene into a gene-targeting vector that allowed single-copy integration at the same chromosomal locus (*Rosa26*) in MMR-proficient and -deficient (*Msh2*$^{-/-}$) (13) ES cells (Fig. 1a). To restore the *neo* open-reading frame in target 1, a single-stranded oligonucleotide sequence was used, consisting of 35 (non-chemically-modified) deoxyribonucleotides and differing from the target locus by the absence of the GT dinucleotide (ΔGT *sense*, Fig. 1b). Oligonucleotides were introduced into ES cells by liposome-mediated DNA transfer and cells were selected for restoration of *neo* in G418-containing medium. Tables 1 and 2 show the results of several experiments demonstrating that G418-resistant colonies were readily obtained with ΔGT *sense* but not with a control oligonucleotide carrying the GT frameshift mutation (control *sense*, Fig. 1b). Strikingly, G418-resistant colonies were only obtained in *Msh2*-deficient ES cells but not in wild-type cells, indicating that MMR imposes a strong barrier to oligonucleotide-mediated gene modification. To verify whether restoration of *neo* activity was indeed the result of deletion of the GT frameshift mutation, DNA from target 1 cells and G418-resistant clones was amplified by PCR using the primer pair indicated in Figure 1a. The resulting 1200 bp DNA fragments were analyzed for the presence of *Nco*I (indicative of the *neo*^{s} allele) and *Bcl*I (indicative of reactivation of *neo* by deletion of GT) restriction sites. G418 resistance corresponded in all cases with loss of *Nco*I and gain of *Bcl*I (Fig. 2a).

Tables 1 and 2 also show that ΔGT *sense* and the complementary sequence ΔGT *anti-sense* (Fig. 1b) performed equally well in generating G418-resistant colonies. However, when both sequences were allowed to anneal prior to introduction into cells (ΔGT *ds*), only a few G418-resistant

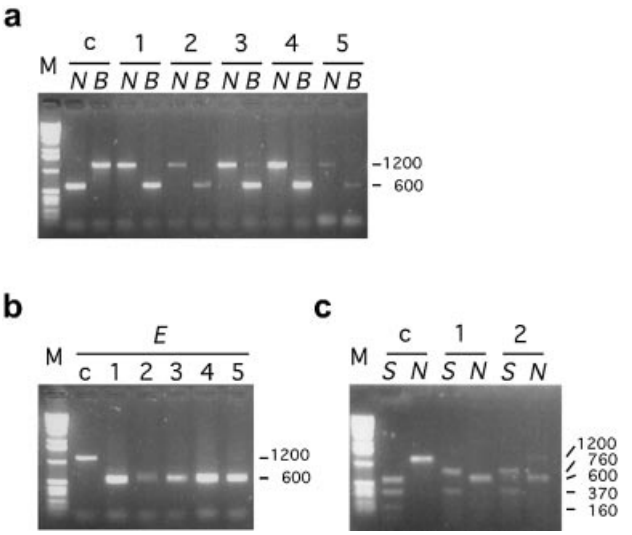

Figure 2. Confirmation of oligonucleotide-mediated restoration of *neo* by restriction site analyses. DNA from target 1 or target 2 cells and G418-resistant clones obtained by exposure to DNA oligonucleotides was amplified using the primer pair indicated in Figure 1a. The resulting 1200-bp fragments were digested with diagnostic restriction enzymes. (**a**) The 1200 bp fragment of target 1 cells (lane c) contains *Nco*I (giving two 600-bp fragments) and lacks *Bcl*I. G418-resistant clones resulting from exposure to ΔGT *sense* (lanes 1–5) have lost *Nco*I and gained *Bcl*I. (**b**) The 1200 bp fragment of target 1 cells (lane c) lacks an *Eco*RV site. G418-resistant clones resulting from exposure to +A *sense* (lanes 1–5) have gained an *Eco*RV site. (**c**) The 1200 bp fragment of target 2 cells (lane c) contains a *Stu*I site at the AAG mutation and several others upstream (giving a 600 bp fragment and several smaller fragments) and lacks *Nco*I. G418-resistant clones resulting from exposure to ATG *sense* (lanes 1 and 2) have lost the diagnostic 600 bp *Stu*I fragment and gained a central *Nco*I site. *B*, *Bcl*I; *E*, *Eco*RV; *N*, *Nco*I; *S*, *Stu*I.

Table 3. Optimization of oligo-targeting protocol using target 1: effect of oligonucleotide length

Oligonucleotide		No. of cells plated[a]	No. of G418-resistant colonies	Efficiency[b] (/10^5)
ΔGT *sense*	20 nt	2.3×10^6	24	1.0
ΔGT *sense*	35 nt	2.2×10^6	42	1.9
ΔGT *sense*	47 nt	2.3×10^6	45	2.0
ΔGT *sense*	60 nt	2.4×10^6	40	1.7

7×10^5 *Msh2*$^{-/-}$ ES cells/10 cm^2 were exposed for 1 h to 7 μg of oligonucleotide.
[a]Cells were counted prior to reseeding in selective medium.
[b]No. of G418-resistant cells per no. of cells plated.

colonies were obtained, indicating that gene modification can be mediated by single- but not double-stranded DNA oligonucleotides.

To optimize the efficiency of oligo targeting, we varied the length of the oligonucleotide sequence (Table 3), the amount of oligonucleotide (Table 4), the number of lipofected cells, the duration of exposure to DNA/lipofection reagent and the effect of chemical modifications in the oligonucleotide (Table 5). In order to better compare the efficiency of oligo targeting for different protocols, we counted the number of cells that had survived the lipofection procedure and were reseeded into selective medium. The length of the oligonucleotide did not strongly affect the frequency of gene modification: Table 3 shows that ΔGT *sense* oligonucleotides

Table 4. Optimization of oligo-targeting protocol using target 1: effect of amount of oligonucleotide

Oligonucleotide	μg	No. of cells plated[a]	No. of G418-resistant colonies	Efficiency[b] (/10^5)
ΔGT *anti-sense*	3	1.7×10^6	44	2.6
ΔGT *anti-sense*	7	2.2×10^6	39	1.8
ΔGT *anti-sense*	14	2.5×10^6	36	1.4
ΔGT *anti-sense*	28	1.8×10^6	27	1.5

7×10^5 *Msh2*$^{-/-}$ ES cells/10 cm^2 were exposed for 1 h to 3, 7, 14 or 28 μg of oligonucleotide plus Transfast (27, 63, 128 or 256 μl, respectively).
[a]Cells were counted prior to reseeding in selective medium.
[b]No. of G418-resistant cells per no. of cells plated.

Table 5. Optimization of oligo-targeting protocol using target 1: effect of chemical modifications

Oligonucleotide	No. of cells plated[a]	No. of G418-resistant colonies	Efficiency[b] (/10^5)
ΔGT *sense*	2.8×10^6	72	2.6
ΔGT *sense*-U	2.9×10^6	34	1.2
ΔGT *sense-S*	2.8×10^6	21	0.75
ΔGT *sense-SS*	2.7×10^6	10	0.37

7×10^5 *Msh2*$^{-/-}$ ES cells/10 cm^2 were exposed for 1 h to 3 μg of oligonucleotide plus 27 μl of Transfast. ΔGT *sense*-U contains three 2′-*O*-methyl-uracil residues added to its 5′ end; ΔGT *sense-S* contains three phosphorothioate linkages at its 5′ end; ΔGT *sense-SS* contains three phosphorothioate linkages at its 5′ end and three at its 3′ end.
[a]Cells were counted prior to reseeding in selective medium.
[b]No. of G418-resistant cells per no. of cells plated.

Table 6. Oligonucleotide-mediated GT→GAT using target 1

Oligonucleotide	No. of cells plated[a]	No. of G418-resistant colonies	Efficiency[b] (/10^5)
+A *sense*	2.5×10^6	27	1.1
+A *anti-sense*	2.3×10^6	26	1.1

7×10^5 *Msh2*$^{-/-}$ ES cells/10 cm^2 were exposed for 1 h to 7 μg of oligonucleotide plus Transfast (63 μl).
[a]Cells were counted prior to reseeding in selective medium.
[b]No. of G418-resistant cells per no. of cells plated.

Table 7. Oligonucleotide-mediated GT correction in wild-type cells

Oligonucleotide	No. of cells lipofected	No. of cells plated[a]	No. of G418-resistant colonies	Efficiency[b] (/10^7)
ΔGT *sense*	4.2×10^6	2.0×10^7	1	0.5
+A *sense*	4.2×10^6	2.2×10^7	3	1.4

4.2×10^6 *wild-type* ES cells on a 6 well plate (6×10 cm^2) were exposed for 1 h to 7 μg of DNA plus Transfast (63 μl) per well.
[a]Cells were counted prior to reseeding in selective medium.
[b]No. of G418-resistant cells per no. of cells plated.

of 35, 47 or 60 residues were almost equally effective, while a sequence of 20 residues was slightly less effective. Taken together these experiments revealed that a 1 h exposure of 7×10^5 cells to 3 μg of a 35 nt sequence gave an optimal targeting frequency of 2.6 per 10^5 cells (Tables 4 and 5, and results not shown). The actual oligo-targeting frequency may be somewhat higher as the plating efficiency of ES cells in these experiments was ~20–50% (not shown). Strikingly, the presence of phosphorothioate linkages or 2′-*O*-methyl-RNA residues decreased the efficiency of oligo targeting (Table 5).

Table 8. Oligonucleotide-mediated AAG→ATG using target 2

Oligonucleotide	No. of cells plated[a]	No. of G418-resistant colonies	Efficiency[b] (/10^5)
ATG *sense*	4.3×10^6	53	1.2
ATG *anti-sense*	4.4×10^6	61	1.4
PBS	4.5×10^6	0	0

See footnote to Table 6.

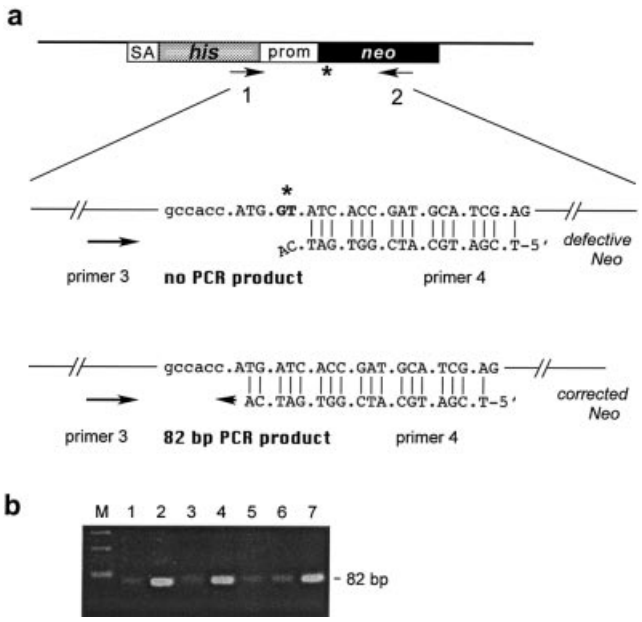

Figure 3. Procedure to identify oligo-targeted cells without selection. (**a**) Primers 1 and 2 were used to amplify 1200-bp fragments from pools of cells, which were subsequently used as templates in a second PCR round using the nested primers 3 and 4. Primer 4 is specific for the planned modification giving an 82 bp fragment. (**b**) Efficient amplification of the 82 bp fragment was observed in pools 2, 4 and 7, indicating the presence of modified cells.

Table 9. Oligonucleotide-mediated GT correction in *Msh2*$^{-/-}$ cells

Oligonucleotide	No. of cells plated	No. of wells with G418-resistant cells	Efficiency[a] (/10^5)
ΔGT *sense*	2 × 96-well plates 1000 cells/well	8	4.2

7×10^5 *Msh2*$^{-/-}$ ES cells were exposed for 1 h to 7 μg of DNA plus Transfast (63 μl). Cells were seeded into two 96-well plates at a density of 1000 cells/well. After 2 days, cells in each well were split into two new wells: one was used for DNA isolation and one for growth in selective medium.
[a]No. of G418-resistant cells per no. of cells plated.

Insertion and substitution of a single base pair

Instead of deleting the GT dinucleotide, the *neo* open-reading frame in target 1 could also be restored by inserting a single A between G and T using +A *sense* and *anti-sense* oligonucleotides (Fig. 1b). The two oligonucleotides performed equally

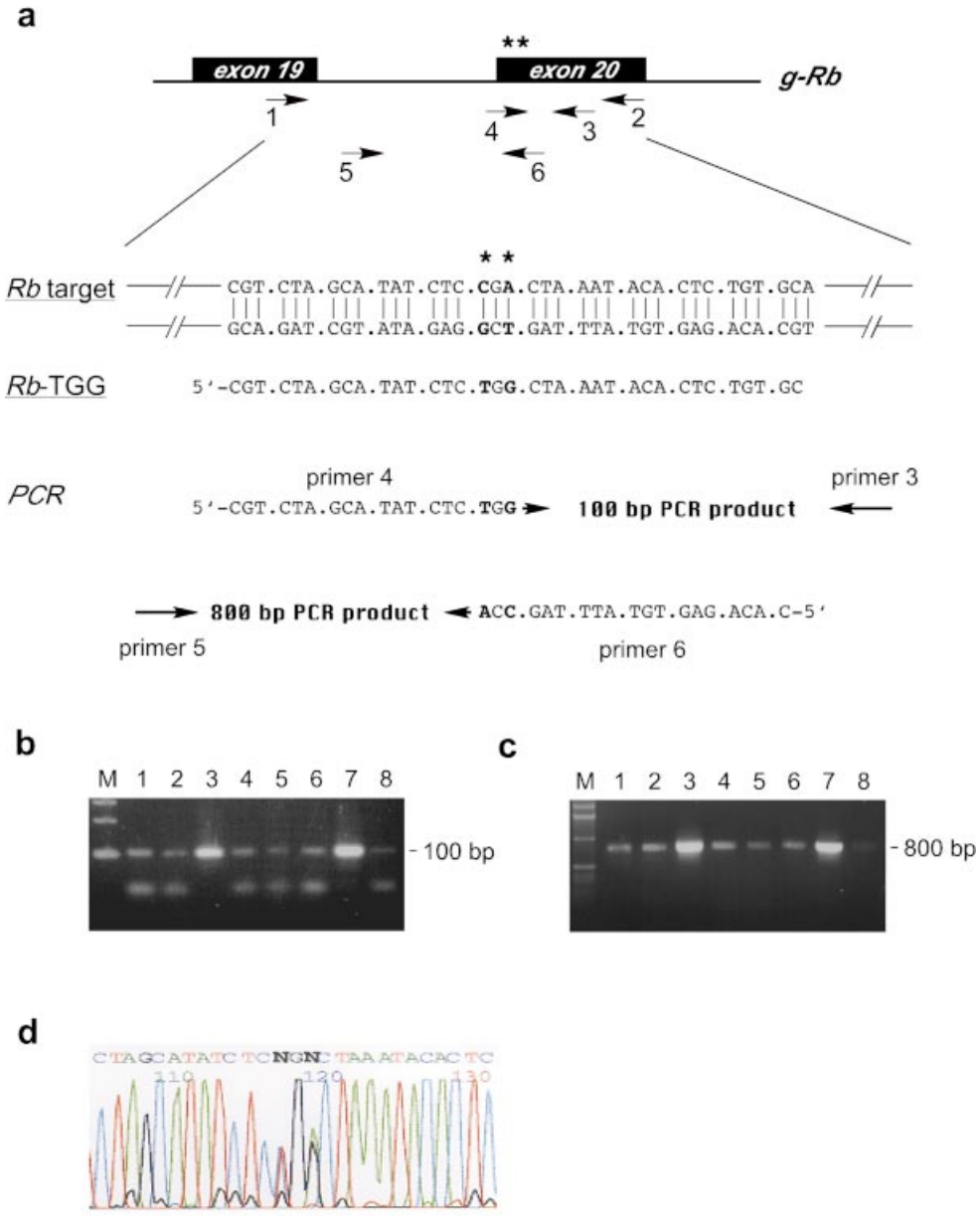

Figure 4. Oligonucleotide-mediated substitution of a single codon CGA for TGG in the retinoblastoma gene. (**a**) Base pairs to be substituted are located in exon 20 of *Rb-1* and are indicated by asterisks. Primers 1 and 2 were used to amplify 1500 bp fragments from pools of cells, which were subsequently used as templates in a second PCR round using the nested primers 3 and 4 or 5 and 6; primers 4 and 6 are specific for the planned modification. After exposure to the single-stranded DNA oligonucleotide *Rb*-TGG, cells were seeded in 96-well plates at a density of 1000 cells per well. Cells from wells giving a strong PCR signal were reseeded in 96-well plates at a density of 100 cells per well. (**b**) Positive wells (lanes 3 and 7) after the second round of enrichment identified with primer pair 3,4 giving a 100 bp PCR product or (**c**) primer pair 5,6 giving an 800 bp PCR product. Cells from positive wells were clonally expanded giving pure cultures of modified cells. (**d**) Sequencing reveals the presence of wild-type and modified nucleotides in *Rb-1* mRNA in purified clones.

well, giving G418-resistant colonies at a frequency of $1/10^5$ (Table 6). In all cases tested, G418 resistance was associated with the gain of an *Eco*RV restriction site in the diagnostic 1200 bp PCR fragment (Fig. 2b). Correction of target 1 by +A *sense* was, similar to ΔGT *sense*, extremely inefficient in wild-type cells (Table 7). Thus, MSH2 activity suppressed the efficiency of oligo targeting ~100-fold.

To study oligonucleotide-mediated base substitution, we generated target 2 carrying a point mutation in the start codon: AAG (Fig. 1c). ATG *sense* and *anti-sense* oligonucleotides (Fig. 1c) performed equally well in restoring *neo* activity (Table 8), which in all cases tested was accompanied by loss of *Stu*I and gain of *Nco*I restriction sites (Fig. 2c).

Oligo targeting without selection

We next tested whether the efficiency of oligo targeting was sufficiently high to identify cells that had undergone oligonucleotide-directed gene modification without applying positive selection. *Msh2*$^{-/-}$/target 1 ES cells exposed to ΔGT *sense* were seeded into two 96-well plates at a density

of 1000 cells per well. Cells were cultured in non-selective medium and then split into new wells for further growth in non-selective or G418-containing medium. DNA was extracted from cell pools grown in non-selective medium and subjected to the PCR protocol depicted in Figure 3a. Primers 1 and 2 were used to amplify a 1200 bp fragment that was subjected to a second PCR amplification round using the nested primers 3 and 4. Primer 4 is specific for the planned modification giving an 82 bp PCR product, while non-modified DNA would not give a product due to improper annealing of the two 3′ nucleotides of primer 4. Efficient amplification of an 82 bp fragment was found in 8 out of 192 wells (Fig. 3b). In each case, efficient amplification corresponded to the presence of G418-resistant cells in parallel pools grown under selective conditions (Table 9), thereby validating this procedure to identify pools in which part of the cells contained the planned modification. Repeated rounds of this procedure will readily lead to enrichment and finally clonal purification of the oligonucleotide-mediated modification, alleviating the need for a selectable phenotype.

We used this procedure to substitute a single codon CGA for TGG in the retinoblastoma gene *Rb-1* (Fig. 4a). *Msh2*$^{-/-}$ ES cells were exposed to the single-stranded deoxyribo-oligonucleotide *Rb*-TGG and seeded into 96-well plates at a density of 1000 cells per well. Cells were cultured and each well was split in two. With PCR primers specific for the planned modification (Fig. 4a), we identified two wells giving a strong PCR product and eight giving a weaker signal out of 192 wells. Cells from the strongly positive wells were reseeded in a 96 well plate at a density of 100 cells per well. After culturing, two positive wells were identified (Fig. 4b and c). From these wells, single cell clones were prepared yielding cell cultures that were clonal for the planned oligonucleotide-mediated modification. Finally, from these clones, *Rb-1* mRNA was prepared and sequenced. From each clone the sequence showed a superposition of the wild-type and modified message without any additional alteration (Fig. 4d).

DISCUSSION

Our results demonstrate that small non-chemically-modified single-stranded deoxyribonucleotide sequences can be used to site-specifically introduce subtle alterations into the ES cell genome. However, this procedure appeared only effective in the absence of the central MMR protein MSH2. We speculate that the single-stranded oligonucleotide upon annealing to its chromosomal complement can serve as a primer for DNA synthesis. However, the presence of the genetic alteration leads to a mismatch in the primer-template heteroduplex which, in wild-type cells, activates the MMR machinery to remove the newly synthesized strand including the oligonucleotide thereby preventing the modification to become fixed into the genome. At present it is unclear whether annealing of the single-stranded oligonucleotide to its chromosomal complement occurs in the context of a replication fork or requires the assistance of proteins involved in homologous recombination. Further insight into the mechanism of oligo targeting requires its frequency to be determined in cells with defects in other MMR genes and in recombination-defective backgrounds.

Oligo targeting may provide a valuable addendum to current gene-targeting procedures basically only requiring sequence information, and find an application in the modification of industrially and medically important cell types and organisms. However, we do realize that its general applicability may be hampered by the mutator phenotype associated with MSH2 deficiency. This may lead to inadvertent genetic alterations on top of the desired oligonucleotide-mediated modification. One approach to circumvent this problem may be transient inactivation of MSH2. Interestingly, several small molecule inhibitors of MMR activity *in vitro* have been identified such as adriamycin (16) and vanadate (17). While at the effective concentrations these compounds are highly toxic to cells, they may serve as lead compounds for the development of reversable inhibitors of MMR *in vivo*. Under such conditions, oligo targeting may occur sufficiently effective while the accumulation of spontaneous mutations remains below an acceptable level.

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