Targeted recombination with single-stranded DNA vectors in mammalian cells

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Received November 18, 1992; Accepted January 4, 1993

ABSTRACT

We studied the ability of single-stranded DNA (ssDNA) to participate in targeted recombination in mammalian cells. A 5' end-deleted adenine phosphoribosyltransferase (*aprt*) gene was subcloned into M13 vector, and the resulting ssDNA and its double-stranded DNA (dsDNA) were transfected to APRT[−] Chinese hamster ovary cells with a deleted *aprt* gene. APRT⁺ recombinants with the ssDNA was obtained at a frequency of 3×10^{-7} per survivor, which was almost equal to that with the double-stranded equivalent. Analysis of the genome in recombinant clones produced by ssDNA revealed that 12 of 14 clones resulted from correction of the deletion in the *aprt* locus. On the other hand, the locus of the remaining 2 was not corrected; instead, the 5' deletion of the vector was corrected by end extension, followed by integration into random sites of the genome. To exclude the possibility that input ssDNA was converted into its duplex form before participating in a recombination reaction, we compared the frequency of extrachromosomal recombination between non-complementary ssDNAs, and between one ssDNA and one dsDNA, of two phage vectors. The frequency with the ssDNAs was 0.4×10^{-5} , being 10-fold lower than that observed with the ssDNA and the dsDNA, suggesting that as little as 10% of the transfected ssDNA was converted into duplex forms before the recombination event, hence 90% remained unchanged as single-stranded molecules. Nevertheless, the above finding that ssDNA was as efficient as dsDNA in targeted recombination suggests that ssDNA itself is able to participate directly in targeted recombination reactions in mammalian cells.

INTRODUCTION

Gene targeting by homologous recombination in mammalian cells is a powerful, new technology to manipulate the chromosomal gene in a planned manner (1). It has been used to study the

function of specific genes in cultured mammalian cells or transgenic mice (2, 3). However, targeted homologous recombination is an extremely rare event (10^{-6} – 10^{-8} per survivor after transfection), while nonhomologous recombination (random integration) occurs at three to four orders of magnitude higher rates (4–6). This makes it very inefficient, laborious and time-consuming to identify and isolate rare targeted cells within a large population of cells transfected with vectors.

Studies on the mechanism of targeted recombination in mammalian cells may allow the development of more improved technology for manipulating easily and precisely the mammalian genome, and provide insights into mechanisms of genetic rearrangements. In all currently accepted models for homologous recombination (7–9), ssDNA is essential in the recombination process. The double-strand break repair model (8) assumes that homologous recombination is initiated by invasion of a single-stranded 3' end of a gapped dsDNA into the homologous region of the partner duplex followed by strand exchange to form a D-loop. Subsequent gap repair and branch migration result in the formation of two Holliday junctions, which are resolved by an endonuclease(s) to yield two recombination products. Similarly, in the single-strand annealing model (9), the creation of single-stranded ends from recombining DNA duplexes is the first key process; these ends pair with each other at their complementary regions and form a heteroduplex joint to give rise to one recombination product.

A central role of ssDNA in an early step of recombination processes is also substantiated by *in vitro* studies on the molecular mechanism of the *Escherichia coli* RecA protein (10–13) and the *Ustilago maydis* Rec1 protein (14). These proteins bind preferentially to a single-stranded region of one duplex, promote its homologous pairing with the other duplex, and mediate strand exchange between the two sequences. Similar proteins with such activities have recently been identified in mammalian cells (15–18).

In *Saccharomyces cerevisiae* (19), *Streptomyces* (20) and *Ascombolus immersus* (21), ssDNA can participate directly in homologous recombination with chromosomal sequences. Efficient extrachromosomal homologous recombinations between

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ssDNA and dsDNA have been reported in mammalian (22) and plant cells (23). However, participation of ssDNA in targeted recombination with chromosomal genes remains unexplored in mammalian cells.

In this report, with single-stranded targeting vectors, we have investigated gene correction events at the deleted *aprt* locus in a mutant Chinese hamster ovary (CHO) cell line. We indicate that single-stranded vectors undergo homologous recombination with the target locus as efficiently as the equivalent double-stranded form, and suggest that ssDNA is able to participate directly in targeted recombination reactions.

MATERIALS AND METHODS

Cells and culture conditions

All CHO cell lines used in this study were provided by M. Meuth (24). D422 is the wild-type cell line which is hemizygous for the *aprt* gene and proficient in APRT activity. S118 is a spontaneous APRT⁻ mutant line having a 422-base pair (bp) deletion in the *aprt* locus; S10 lacks the whole *aprt* gene and possesses no APRT activity. Cells were routinely maintained as monolayers in ES medium supplemented with 5% calf serum in a 5% CO₂ incubator as described previously (25). After transfection, selection of APRT⁺ colonies were carried out in adenine/azaserine-containing medium (AA medium) as described (25). In this selective medium, APRT⁺ cells are able to grow (AA-resistant; AA^r) but APRT⁻ are not (AA-sensitive; AA^s).

Vector construction

The M13 phage vectors, mAYS and mAYR, were constructed from plasmid pAY1 (25) carrying a 3.3-kilobase (kb) *EcoRV*-*Bam*HI insert of the wild-type *aprt* gene cloned into pUC8 (Figure 1A). mAYS was made by subcloning a 3.3-kb *EcoRV*-*Hind*III fragment of pAY1 into M13mp18 at the *Hinc*II-*Hind*III site (Figure 1B). To construct mAYR, a 3.3-kb *EcoRV*-*Bam*HI fragment of pAY1 was ligated with the M13mp18 digested with *Hinc*II and *Bam*HI (Figure 1B). Therefore, the *aprt* insert of mAYS and mAYR is in an identical and opposite direction, respectively, to the *lacZ* gene in the M13 vector. mS118R was constructed from plasmid pS118 carrying a 3.5-kb *Bam*HI fragment of the deleted *aprt* gene cloned from S118 cells (24); this fragment was subcloned into the *Bam*HI site of M13mp18 in the opposite direction to the *lacZ* gene (Figure 1B). Their ssDNAs and double-stranded, replicative forms were prepared by the standard procedures (26).

DNA transfection

For targeting experiments, S118 cells (4×10^6) were transfected with 8 μ g of targeting vector by electroporation, AA^r (APRT⁺) recombinant colonies were selected in AA medium, and their frequency was calculated and expressed as the number of the colonies per survivor as described previously (25). For experiments of extrachromosomal recombination, S10 cells (4×10^6) were cotransfected with 1 μ g each of two DNA vectors, and the frequency of AA^r recombinant colonies was calculated as above.

Southern blot and PCR analysis

Total genomic DNA was isolated from each recombinant clone, digested with *Bam*HI, and subjected to Southern blot analysis as described previously (25), using the 3.9-kb *Bam*HI fragment containing the wild-type *aprt* gene as a hybridization probe

(Figure 1A). Conditions for PCR analysis were also described previously (25). For this analysis, primers I (5'-CAGGGGC-TGCACCAAAGTGT-3') and II (5'-TCGCTTCCGGATGAGC-ACAC-3') were made to sequences flanking the region deleted in S118 cells. Primer III (5'-TCTCCTCGTGCTGGATCGCT-3') was made to the sequence 120-bp upstream of the transcription initiation site of the gene absent in both mAYS and mAYR, while primer IV (5'-GCCAGGAGGAACCACTAGGA-3') was made to the sequence which is deleted in S118 cells.

RESULTS

Targeted correction with ssDNA

Recipient S118 cells are hemizygous for the *aprt* locus and totally deficient in APRT activity (AA^s), because the cells have a 422-bp deletion ranging from intron 2 to exon 3 of the gene (Figure 1A) (24). For targeting experiments, we constructed two phage vectors, mAYS and mAYR (Figure 1B), carrying the 5'-end deleted *aprt* gene present in the pAY1 plasmid (Figure 1A), thereby completely lacking any transforming activity (data not shown). We used these vectors in a single-stranded form, ssmAYS and ssmAYR, carrying antisense and sense strands, respectively, of the *aprt* gene, or in a corresponding double-stranded replicative form (dsmAYS, dsmAYR). In order to test the ability of ssDNA to participate in targeted recombination, each of these DNAs was electroporated to S118 cells and AA^r (APRT⁺) colonies were selected in AA medium, as described in Materials and Methods.

Results of these experiments demonstrate that APRT⁺ recombinant clones were indeed recovered when the ssDNA vectors were transfected as summarized in Table 1. The frequency of targeted recombination with ssmAYR was 3.1×10^{-7} per survivor after transfection; this was almost equal to the frequency (3.5×10^{-7}) with dsmAYR, its double-stranded equivalent. A similar ability of ssmAYS was also observed; the targeting frequency with this ssDNA was 3.2×10^{-7} per survivor, although the corresponding dsDNA (dsmAYS) gave a 2-fold higher frequency of 6.3×10^{-7} . These results demonstrate that ssDNA has the same ability of targeted recombination as dsDNA. Furthermore, the same frequencies observed with ssmAYR and ssmAYS prove that the sense and antisense DNAs of the *aprt* gene are able to participate equally in the recombination reaction.

Characterization of recombination events

To know the nature of the targeted recombination events, we analyzed the structure of the *aprt* gene in each of 7 independent clones obtained with ssmAYR (Figure 2, lanes 3–9) and 7 clones

Table 1. Frequencies of targeted recombination with ssDNA and dsDNA vectors.

Vectors	No. of experiments	Frequency* of APRT ⁺ clones ($\times 10^7$)
ssmAYR	6	3.1 ± 2.0
dsmAYR	3	3.5 ± 0.6
ssmAYS	8	3.2 ± 1.7
dsmAYS	2	6.3 ± 3.5

* Recipient S118 cells were transfected with each of the indicated vectors, and APRT⁺ recombinant colonies were selected in adenine/azaserine (AA) medium as described in Materials and Methods. The frequency of the recombinant colonies is expressed as a function of surviving cells after transfection. Data are given as the mean $\pm$ standard error of the frequencies obtained in the indicated number of experiments.

with ssmAYS (lanes 12–18) by Southern hybridization. Genomic DNAs of wild-type D422, recipient S118, and each recombinant clone were digested with *Bam*HI, electrophoresed in agarose gels and probed with the 3.9-kb *Bam*HI fragment containing the whole *aprt* gene (see the top of Figure 1A). The D422 cells gave a single 3.9-kb band (lanes 2 and 11), which contained the wild-type *aprt* sequence as shown in Figure 1A (24), while a deleted, 3.5-kb fragment was seen in recipient S118 cells (lanes 1 and 10). Among the 14 recombinant clones analyzed, recombinants 1 to 6 (lanes 3 to 8), 8 (lane 12), and 10 to 14 (lanes 14 to 18) showed only the 3.9-kb restriction fragment like the D422 line without any other band. These results indicate that the 422-bp deletion of the *aprt* locus in S118 cells was corrected with the

corresponding sequence of the introduced ssDNA vectors. In contrast, recombinant 7 (lane 9) had 3.5- and 8.0-kb bands, while recombinant 9 (lane 13), 3.5- and 3.9-kb bands. Like S118 cells, the existence of the common 3.5-kb band in both recombinant clones suggested that the deleted *aprt* gene in the recipient cells remained uncorrected.

The genome of each clone was further analyzed by PCR (Figure 3). PCR primers, I and II, made to sequences flanking the region deleted in S118 cells, were used to amplify predicted bands (Figure 3A). In wild-type D422 cells, a 596-bp band was amplified (Figure 3B, lanes 2 and 11), while in recipient S118 cells, a 174-bp band was amplified (lanes 1 and 10). Recombinants which gave the 3.9-kb band in the Southern hybridization shown in Figure 2 (recombinants 1–6, 8, and 10–14) only amplified the 596-bp band (Figure 3B, lanes 3 to 8, 12, and 14 to 18), confirming that the 422-bp deletion in the recipient cells was corrected. In recombinants 7 and 9 (lanes 9 and 13), both 596- and 174-bp bands were amplified. The presence of this smaller band indicates that the target locus was not corrected and the greater band appears to have been derived from the targeting vector itself integrated randomly into the genome. These data are all consistent with the results obtained by the Southern blot analysis described above (Figure 2).

As the *aprt* locus of recombinants 7 and 9 remained uncorrected, they were expected to have restored APRT activity by extending the 5'-deleted sequence of the targeting vector. To examine this directly, we analyzed the 5' region of the *aprt* gene in those clones by PCR (Figure 3C). Primer III was made to the sequence 120-bp upstream of the transcription start site of the *aprt* gene that is lost in mAYS and mAYR, while primer IV made to the sequence that is deleted in recipient S118 cells (Figure 3A). Amplification of the predicted 1114-bp sequence between the two primers occurred with the D422 cell DNA (Figure 3C, lane 2) but not with the S118 cell DNA (lane 1). As expected, both recombinants 7 (lane 3) and 9 (lane 4) exhibited the amplified band with the same size as that seen in wild-type D422 cells. These results demonstrate that the *aprt* sequence in the incoming ssDNA vector was corrected by extending its 5' truncated end at least up to the region corresponding to the primer III. We have already reported a similar gene correction event,

Figure 1. Maps of the *aprt* locus in the CHO cell lines, and structure of targeting vectors constructed. (A) The wild-type D422 line carries one copy of the functional *aprt* gene which resides between two *Bam*HI sites [3.9-kilobase (kb) long]. As indicated by open arrowheads, mutant line S118 has a 422-base pair (bp) deletion from intron 2 to exon 3 in the *aprt* gene, while S10 entirely lacks the *aprt* gene. Plasmid pAY1 (25) loses the sequence from the *Bam*HI site upstream of the promoter region to the unique *Eco*RV site in exon 2. pS118 (provided by M. Meuth) is the plasmid in which the 3.5-kb *Bam*HI fragment of the *aprt* gene in S118 cells was cloned into the *Bam*HI site of the pUC8. Shaded boxes indicate exons numbered according to the map by Nalbantoglu *et al.* (24), and thick open bars, introns. Thick heavy lines indicate the chromosomal sequence flanking the *aprt* gene. Broken lines indicate the pUC8 plasmid sequence depicted linearly. Open arrowheads indicate the termini of the deleted region in the *aprt* and flanking sequences. (B) Targeting vectors were constructed by subcloning the *aprt* insert of pAY1 or pS118, into the M13mp18 vector as described in Materials and Methods. mAYS contains the *aprt* insert of pAY1 in the same direction as the *lacZ* gene of the phage vector; mAYR does in its opposite direction. m118R possesses the 3.5-kb *aprt* insert of pS118 in the opposite direction to the *lacZ* gene. The *aprt* insert of pAY1 or pS118 is shown by striped or crosshatched boxes, respectively. Arrows indicate the direction of the *aprt* inserts. Figures are not drawn to scale. B, *Bam*HI; E, *Eco*RV; H, *Hind*III; Hc, *Hinc*II.

Figure 2. Southern blot analysis of genomic DNA from recombinant clones produced by transfection with ssDNA vectors. Genomic DNA (5 μ g) purified from individual clones was restricted with *Bam*HI. Southern hybridization was performed using the 32 P-labeled, 3.9-kb *Bam*HI probe (shown at the top of Figure 1A). As controls, *Bam*HI-cut genomic DNA (5 μ g) of wild-type D422 (lanes 2 and 11) and recipient S118 (lanes 1 and 10) cells was also run. Recombinants 1 to 7 (lanes 3 to 9) and 8 to 14 (lanes 12 to 18) were yielded by transfection with ssmAYR and ssmAYS, respectively.

Figure 3. PCR analysis of genomic DNA from recombinant clones. Genomic DNA (50 ng) prepared for Southern hybridization in Figure 2 was used for amplification by PCR and the product was resolved in a 1.5% agarose gel as described in Materials and Methods. (A) Locations of four synthetic primers used. Primers I, II, III, and IV are indicated by thick arrows. Symbols are the same as those in Figure 1. (B) Primers I and II were configured to amplify a 596-bp sequence of the *aprt* gene present in wild-type D422 cells (lanes 2 and 11), and a 174-bp sequence in recipient S118 cells (lanes 1 and 10). The numbers at the top of the gel are the same as those in Figure 2. (C) Primers III and IV were configured to amplify a 1114-bp sequence between them. mAYS and mAYR lack the sequence corresponding to primer III, and S118 cells lack the sequence corresponding to primer IV. Thus, it was expected that only correction of incoming targeting vectors results in the positive amplification of this sequence in recombinant clones. Lanes 1, S118; 2, D422; 3, recombinant 7; 4, recombinant 9.

termed end extension repair, of dsDNA targeting vectors mediated by homologous recombination (25).

The extent of ssDNA converting into the duplex form

Although transfected ssmAYS and ssmAYR were able to correct the deleted *aprt* locus as described above, there remained the possibility that these input ssDNAs would be converted into duplex forms following transfection so that they could take part in targeted recombination reactions. To know the extent of ssDNA converting into its double-stranded form after transfection, we examined the frequency of extrachromosomal recombination between two noncomplementary ssDNAs with nonoverlapping deletions. Since at least one of the two DNAs must be altered to the duplex DNA to undergo recombination reactions, we will be able to presume the extent of this alteration by comparing the above frequency with that of extrachromosomal recombination between one ssDNA and one dsDNA (replicative form). Two single-stranded vectors, ssmAYR and ssm118R, which lack the different regions of the *aprt* gene (Figure 1B),

Table 2. Frequencies of extrachromosomal recombination between ssDNA vectors and between ssDNA and dsDNA vectors

Vectors*	No. of experiments	Frequency** of APRT ⁺ clones ($\times 10^5$)
ssmAYR + ssm118R	6	0.39 $\pm$ 0.20
ssmAYR + dsm118R	5	4.2 $\pm$ 1.7

* Recipient S10 cells lacking the whole *aprt* locus were cotransfected with each of vectors and APRT⁺ recombinant colonies were selected in adenine/azaserine (AA) medium as described in Materials and Methods. The structure of the vectors used is shown in Figure 1B. The frequency of APRT⁺ recombinant colonies is expressed as described in Table 1.

** Data represent the mean $\pm$ standard error in the indicated number of experiments.

Figure 4. Possible reaction pathways to explain targeted recombination between ssDNA vector and chromosomal gene. In A, strand exchange between the ssDNA and the chromosomal duplex DNA would create a heteroduplex with a mismatch region. In B, a similar heteroduplex would be created by pairing of the ssDNA with the complementary, overhanging ssDNA tails of the gap formed on one sister chromatid in an eye form during the chromosomal DNA replication. Subsequent mismatch repair of the heteroduplex would give rise to the corrected wild-type locus. Thin circle lines indicate a single-stranded targeting vector; thick lines, the DNA duplex of the chromosome including the *aprt* locus. Arrows indicate the 3'-ends of the duplexes. Open triangles represent the deleted region of the *aprt* genes on the chromosome and targeting vector. Broken lines represent a newly synthesized DNA.

were co-transfected to S10 cells (which delete the entire *aprt* locus) as described in Materials and Methods. Homologous recombination between the two noncomplementary ssDNAs occurred at a very low frequency of 0.39×10^{-5} per survivor (Table 2), proving that some amount of these input ssDNAs was converted to double-stranded forms by synthesising of their opposite strand. However, this frequency was 10 times lower than the frequency (4.2×10^{-5}) of recombination between

ssmAYR and dsm118R, the double-stranded equivalent of ssm118R. These results suggest that as little as 10% of the introduced ssDNA was converted into the duplex form before participating in the recombination reactions. If targeted recombination requires dsDNA as incoming vector, the targeting frequency with ssDNA would be proportional to the extent of conversion of the ssDNA into its double-stranded form. However, as shown in Table 1, the clear similarity in targeted recombination frequencies observed with single-stranded and double-stranded DNA vectors suggests that ssDNA itself is able to participate directly in targeted recombination without converting into the duplex form in mammalian cells.

DISCUSSION

We have studied the ability of ssDNA as a vector to undergo targeted recombination. Using a deleted *aprt* locus in a CHO mutant line as a target, transfection with ssDNA vectors indeed gave APRT⁺ recombinants (Table 1). To our knowledge, this is the first demonstration of successful targeted recombination with ssDNA in mammalian cells. Also, it should be noted that the targeting frequency observed with ssDNA was the same as that with the equivalent dsDNA (Table 1). Furthermore, using M13 phage constructs (ssmAYS and ssmAYR) with *aprt* inserts of opposite orientation from each other, we found both sense and antisense strands to be equally effective for targeted recombination reactions (Table 1). The possibility that ssDNA was converted to its double-stranded form to act as a recombination substrate prior to participating into homologous recombination was excluded by the following reasons. First, the M13 constructs used are unable to replicate because they do not contain any mammalian replication origin; Rauth *et al.* have observed no extensive conversion of single-stranded M13 constructs introduced into both human and monkey cells (22). Second, by comparing the frequencies of extrachromosomal recombination between noncomplementary ssDNAs and between ssDNA and dsDNA, of two phage vectors (Table 2), we could presume the extent of introduced ssDNA converted into the double-stranded form to be as little as 10% before its participation in recombination reactions. In spite of such a low level of conversion of ssDNA into the corresponding dsDNA, the targeting frequency with ssDNA was found to be almost equal to that with the dsDNA equivalent (Table 1). These results suggest that ssDNA is able to participate directly in the targeted recombination in mammalian cells and are consistent with those shown in *S.cerevisiae* (19).

Of the 14 recombinant clones produced with ssDNA vectors (ssmAYS and ssmAYR), 12 had the *aprt* locus ideally corrected without carrying additional sequences (Figures 2 and 3B, lanes 3–8, 12, and 14–18), indicating that ssDNA could act as a recombination donor. On the other hand, in the remaining 2 clones (recombinants 7 and 9), the target locus remained uncorrected (Figures 2 and 3B, lanes 9 and 13) but the 5'-deleted *aprt* sequence of the vectors was corrected by extension of the end (Figure 3C, lanes 3 and 4). These data also prove the ability of ssDNA to act as a recombination acceptor. However, we infer from the results shown in Table 2 that about 10% of the input ssDNA would be converted to its double-stranded form and participate in targeted recombination events. Correction events of mutated targeting vectors in a double-stranded form have been described (27, 28). We have also observed that, when the circular double-stranded pAY1 vector (see Figure 1A) was transfected to S118 cells, the majority of recombinants (18 out of 24

APRT⁺ clones) were derived by correction of the vector itself. We designated this phenomenon as end extension repair of targeting vector and discussed the mechanism of this reaction (25). Therefore, recombinants 7 and 9 (in Figure 2) would arise by correcting the 5'-deleted *aprt* sequence of the double-stranded form to which the introduced ssDNA has been converted.

We may explain the targeted recombination events observed with ssDNA by two possible reaction pathways illustrated in Figure 4. One pathway (A) is mediated by a strand transfer reaction between ssDNA targeting vector and the chromosomal DNA, similarly to the mechanism of extrachromosomal recombination between single-stranded and double-stranded DNAs in mammalian cells (22), or recombination between ssDNA and chromosomal genes in *S.cerevisiae* (19). First, the ssDNA vector would invade the homologous region of the *aprt* target locus and form a D-loop structure; resolution of this structure by an endonuclease(s) would result in the formation of a heteroduplex with a mismatched region. Repair of this mismatched region (29) would produce the wild-type *aprt* locus. The other pathway (B) is principally based on the single-strand annealing model (9). We assume DNA replication at S phase of the chromosome including the *aprt* locus. Both endonuclease and exonuclease cuts of this newly replicated DNA in one sister chromatid in an eye form would give rise to a double-stranded gap with overhanging single-stranded tails, which would occasionally be able to pair with an incoming ssDNA molecule. The regions of the ssDNA not annealed to the chromosomal *aprt* sequence would be digested followed by ligation. As a result, an heteroduplex similar to that shown in A would be formed and its single-stranded gap would be repaired during or after replication and segregation of the chromosome. In *S.cerevisiae* cells (19), many recombinants after transfection with ssDNA result from gene insertion (30). However, as far as we studied, we have not yet obtained such a type of recombinants. This may support the above pathways shown in Figure 4.

ACKNOWLEDGEMENTS

We thank Miss H.Kojima for her technical assistance. This work was supported in part by grants from the Ministry of Education, Science, and Culture of Japan and from Kanagawa Nanbyo Foundation of Japan.

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