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Genetics

Targeted homologous recombination at the endogenous adenine phosphoribosyltransferase locus in Chinese hamster cells

(gene targeting/transfection/targeted integration/gene conversion)

GERALD M. ADAIR†‡, RODNEY S. NAIRN†, JOHN H. WILSON§, MICHAEL M. SEIDMAN¶, KATHERINE ANN BROTHERMAN†, CHRISTY MACKINNON†, AND JULIA B. SCHEERER†

†The University of Texas M. D. Anderson Cancer Center, Science Park-Research Division, Smithville, TX 78957; §Marrs McLean Department of Biochemistry, Baylor College of Medicine, Houston, TX 77030; and ¶Otsuka Pharmaceutical Co., Ltd., Maryland Research Laboratories, 9900 Medical Center Drive, Rockville, MD 20850

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ABSTRACT **We have developed a system that permits analysis of targeted homologous recombination at an endogenous, chromosomal gene locus in cultured mammalian cells. Using a hemizygous, adenine phosphoribosyltransferase (APRT)-deficient, Chinese hamster ovary (CHO) cell mutant as a transfection recipient, we have demonstrated correction of a nonrevertible deletion mutation by targeted homologous recombination. Transfection with a plasmid carrying a fragment of the *APRT* gene yielded APRT$^+$ recombinants at a frequency of $\approx 4.1 \times 10^{-7}$. The ratio of targeted recombination to nontargeted integrations of plasmid sequences was ≈1:4000. Analysis of 31 independent APRT$^+$ recombinants revealed conversions of the endogenous *APRT* gene, targeted integration at the *APRT* locus, and a third class of events in which the plasmid donor *APRT* fragment was converted to a full-length, functional gene.**

Targeted homologous recombination is a powerful tool for genetic manipulation in yeast, permitting precise gene correction, site-specific gene modification, or targeted gene disruption at any chromosomal locus for which a cloned sequence is available (1–4). Potential applications of such techniques in mammalian cells include (*i*) site-specific gene modification or precise targeting of engineered gene sequences into their normal chromosomal environments for studying expression and structure–function relationships; (*ii*) generation of animal models for the study of heritable human diseases by targeted gene disruption or insertional mutagenesis of gene loci in mouse embryo cells; and (*iii*) precise replacement or correction of defective human genes as an approach to gene therapy. However, the paucity of good assay systems and selectable markers for studying homologous recombination at endogenous gene loci and the propensity of mammalian cells for integration of foreign DNA by illegitimate recombination events requiring little or no homology (5–7) have been major impediments to the practical application of gene targeting approaches in mammalian cells.

Most studies of targeted homologous recombination in mammalian cells have used one of two basic approaches: analysis of the production of viable viruses by recombination between defective exogenous and chromosomally integrated viral sequences (8–11) or introduction and stable integration of a defective viral or bacterial gene sequence into a mammalian cell genome to serve as an artificial chromosomal target sequence for subsequent homologous recombination events (12–16). Very few studies have used mammalian genes in their normal chromosomal environments as targets for homologous recombination (17–19).

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We have developed a system that permits analysis of targeted homologous recombination at an endogenous, chromosomal gene locus in cultured mammalian cells. This system utilizes a hemizygous adenine phosphoribosyltransferase (*APRT*) gene (20–24) as a target for homologous recombination. In this paper we (*i*) demonstrate targeted correction of a nonrevertible *APRT* deletion mutation by homologous recombination of a transfection-introduced plasmid *APRT* sequence with the defective chromosomal locus; (*ii*) determine the relative frequencies of targeted homologous recombination and nontargeted integration of plasmid sequences; and (*iii*) analyze 31 independent APRT$^+$ recombinants to determine the nature of the targeted recombination events.

MATERIALS AND METHODS

Cell Lines and Culture Conditions. CHO-ATS-49 is a spontaneous 8-azaadenine-resistant, APRT-deficient mutant derived from CHO-AT3-2 (20–22). ATS-49 cells are hemizygous for an *Mbo* II *APRT* restriction fragment length polymorphism that reflects loss of the exon V *Mbo* II restriction site (Fig. 1*a*). A spontaneous 6-thioguanine-resistant, hypoxanthine (guanine) phosphoribosyltransferase (HPRT)-deficient subline, ATS-49tg, was used for targeting experiments. Cells were maintained as exponentially growing monolayer cultures in alpha modified Eagle's minimal essential medium (α-MEM) supplemented with 10% fetal bovine serum, penicillin, and streptomycin (20, 21).

Plasmid DNAs. Plasmid pAG-7 (Fig. 1*b*) was constructed by replacing the 0.7-kb *Eco*RI–*Bam*HI region of pSV2gpt (25, 26) with a 2.6-kb *Eco*RI–*Bam*HI fragment derived from pHaprt-1 (23). This fragment includes only the 3′ portion of the Chinese hamster *APRT* gene (22). During the construction of pAG-7, a *Pst* I site in the bacterial ampicillin-resistance gene was removed in such a way as to leave the gene functional, making the *Pst* I site in the *APRT* gene fragment unique.

Transfections. Plasmid DNAs were introduced into ATS-49tg cells by calcium phosphate transfection (27, 28). Each 100-mm dish received 8 μg of plasmid DNA. No carrier DNA was used. After 4 hr of exposure to calcium phosphate/DNA precipitates, cells were treated for 25 min with 10% (vol/vol) dimethyl sulfoxide and maintained in α-MEM for ≈40 hr before exposure to selective media.

Selections. APRT$^+$ recombinants were selected in ALASA (25 μM alanosine/50 μM azaserine/100 μM adenine) medium (21, 28). The frequency of GPT$^+$ transformants was monitored

Abbreviations: APRT, adenine phosphoribosyltransferase; HPRT, hypoxanthine (guanine) phosphoribosyltransferase; GPT, guanine phosphoribosyltransferase; WT, wild type; ALASA, alanosine/azaserine/adenine; HAT, hypoxanthine/amethopterin/thymidine.

‡To whom reprint requests should be addressed.

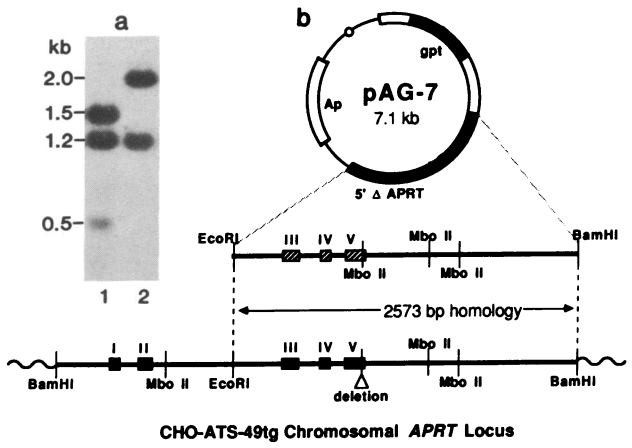

FIG. 1. Target gene and targeting vector. (*a*) *Mbo* II-digested DNAs from CHO-AT3-2 (lane 1) and CHO-ATS-49tg (lane 2) after hybridization with the 3.9-kilobase (kb) *APRT* probe, showing loss of the exon-V *Mbo* II site in ATS-49tg. Internal 1.5- and 0.5-kb fragments are generated by *Mbo* II sites in intron 2, exon V, and just downstream of the *APRT* gene; the upstream and downstream genomic flanking sequences are present on overlapping 1.2-kb *Mbo* II fragments. (*b*) Diagram showing the shared *APRT* sequence homology [2573 base pairs (bp)] between pAG-7 and the targeted ATS-49tg chromosomal *APRT* locus. The location of the ATS-49tg *APRT* deletion site is indicated. Ap, ampicillin-resistance gene; *gpt*, guanine phosphoribosyltransferase gene.

by plating a small aliquot of cells from each culture into HAT (100 μM hypoxanthine/2 μM amethopterin/50 μM thymidine) medium. One APRT$^+$ recombinant was picked from each independent transfection population for molecular analysis. Alanosine (NSC-529469) was obtained from the Drug Synthesis and Chemistry Branch of the National Cancer Institute.

DNA Isolation and Molecular Analysis. DNA was isolated either by the NaDodSO$_4$/proteinase K method (21, 27) or by a simple salting-out procedure (29). DNA samples (10–15 μg) were digested by overnight incubation with restriction enzymes in the buffers recommended by the suppliers (Boehringer Mannheim and New England Biolabs), electrophoresed for 560 V·hr in 0.8% agarose gels, and transferred to nitrocellulose membranes (21). [α-^{32}P]dCTP-labeled probes were prepared by nick-translation (21, 27) or random oligonucleotide-primed synthesis (30), using either the 3.9-kb *Bam*HI fragment derived from pHaprt-1 or a 1.3-kb *Bam*HI–*Eco*RI fragment that includes only the 5′ portion of the *APRT* gene. Hybridizations and autoradiography were carried out as described (21, 27).

RESULTS

Correction of a Nonrevertible Mutation at the Endogenous *APRT* Locus by Targeted Homologous Recombination. CHO-ATS-49tg cells are hemizygous for the *APRT* locus; they contain a single, mutationally altered copy of the *APRT* gene. A small (<20-base-pair) deletion has resulted in loss of the exon-V *Mbo* II restriction site (Fig. 1*a*). No APRT$^+$ revertants of this cell line have been obtained after screening >10^9 cells. However, targeted homologous recombination between transfection-introduced plasmid-derived *APRT* sequences and the defective chromosomal *APRT* gene in ATS-49tg should yield APRT$^+$ (ALASA^r) recombinants. To search for such recombinants, ATS-49tg cells were transfected with *Pst* I-linearized pAG-7 DNA, which shares ≈2.6 kb of *APRT* sequence homology with the target gene (Fig. 1*b*), or with *Bam*HI-linearized pSV2gpt DNA, which lacks any *APRT* gene sequences.

Results of these experiments are summarized in Table 1. The two plasmid DNAs yielded comparable frequencies of GPT$^+$ (HAT^r) transformants, reflecting nontargeted integration and expression of plasmid *gpt* sequences. No APRT$^+$ (ALASA^r) clones were obtained after transfection of ≈10^8 ATS-49tg cells with *Bam*HI-linearized pSV2gpt DNA. However, transfection of ATS-49tg cells with *Pst* I-linearized pAG-7 DNA yielded APRT$^+$ colonies (presumptive targeted recombinants) at a frequency of ≈4 × 10^{-7}. The ratio of the APRT$^+$ and GPT$^+$ frequencies (≈1:4000) provides a measure of the relative frequency of targeted homologous recombination versus nontargeted integration of plasmid sequences.

Southern Blot Hybridization Analysis of APRT$^+$ Recombinant Clones. To determine the nature of the targeted homologous recombination events, DNA samples from 31 independent APRT$^+$ clones were digested with *Mbo* II and seven other restriction enzymes and subjected to Southern blot hybridization analysis using full-length (3.9-kb) and 5′-end-specific (1.3-kb) *APRT* probes. APRT$^+$ clones with multiple nontargeted integrations of plasmid sequences show complex restriction fragment patterns with the full-length probe, because it detects plasmid-derived *APRT* sequences as well as the targeted chromosomal gene. Hybridization with the 1.3-kb (5′-end-specific) *APRT* probe allows selective identification of fragments containing *APRT* sequences unique to the target gene, greatly facilitating interpretation of restriction patterns and deduction of the nature of the recombinational events. Throughout this paper, restriction fragments that are hybridized by the 5′-end-specific *APRT* probe will be identified by an asterisk (*).

Restoration of the exon-V *Mbo* II site at the ATS-49tg chromosomal *APRT* locus by targeted recombination should be accompanied by the reappearance of the *1.5-kb *Mbo* II fragment characteristic of the wild-type (WT) CHO *APRT* gene. Each of the 31 APRT$^+$ clones analyzed had indeed regained this fragment (as illustrated by six cell lines in Fig. 2*a*). The majority of the APRT$^+$ clones analyzed (19/31) showed simple restriction fragment patterns after with very few (<3) or no untargeted integrations. However, 12 clones showed more complex patterns reflecting multiple nontargeted integrations of single and/or tandemly arrayed pAG-7 plasmid sequences.

Table 1. Targeted correction of the *APRT* gene in CHO-ATS-49tg cells

Plasmid vector*	Total cells transfected, no. × 10^{-8}	APRT$^+$ colonies			GPT$^+$ colonies		APRT$^+$/GPT$^+$ frequency ratio
		No.	Frequency† × 10^7	Yield‡	Frequency† × 10^3	Yield‡	
pSV2gpt	0.96	0	0	0	1.3	164	—
pAG-7	1.20	33	4.1	0.05	1.6	201	1:3900

*Eight micrograms of *Bam*HI-linearized pSV2gpt DNA was added to each of 80 dishes (≈1.2 × 10^6 cells per dish); 8 μg of *Pst* I-linearized pAG-7 DNA was added to each of 100 dishes (≈1.2 × 10^6 cells per dish).

†Forty hours after treatment with calcium phosphate-precipitated DNAs, two-thirds of each transfected cell population was plated into ALASA selection medium; one-fourth of each transfected cell population was simultaneously plated into HAT selection medium. Data are expressed as the frequency of ALASA^r (APRT$^+$) or HAT^r (GPT$^+$) colonies per transfected cell.

‡Colonies per microgram of DNA per 10^6 transfected cells.

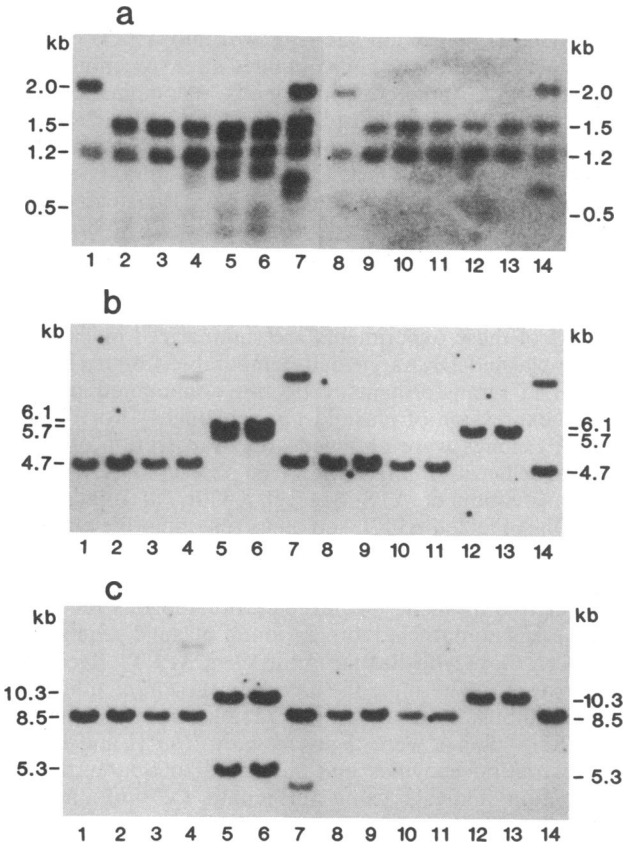

FIG. 2. Southern blot hybridization analysis of CHO-ATS-49tg and APRT$^+$ recombinant DNAs, following digestion with *Mbo* II (*a*), *Bgl* II (*b*), or *Hin*dIII (*c*). Lanes: 1 and 8, CHO-ATS-49tg; 2 and 9, clone 8-44; 3 and 10, clone 8-77; 4 and 11, clone 8-11; 5 and 12, clone 8-35; 6 and 13, clone 8-57; 7 and 14, clone 8-39. Lanes 1–7 were hybridized with the full-length 3.9-kb Chinese hamster *APRT* probe; lanes 8–14 were hybridized with a 1.3-kb probe that is specific for 5′-end, chromosomal *APRT* gene sequences.

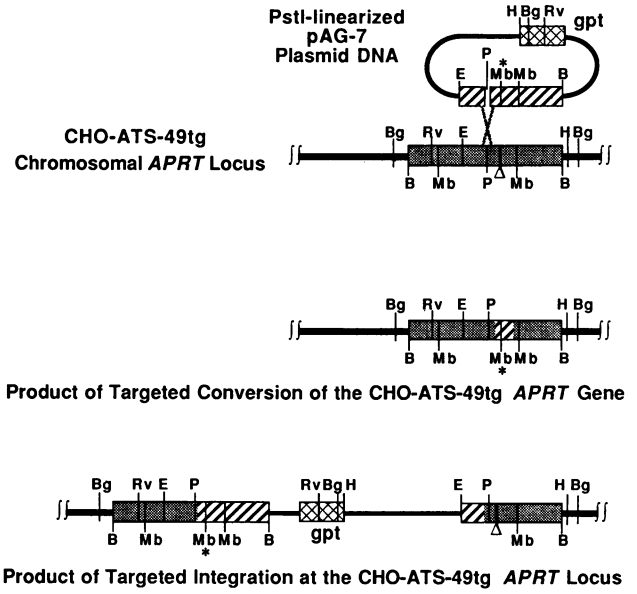

FIG. 3. Targeted homologous recombination of pAG-7 *APRT* sequences with the CHO-ATS-49tg chromosomal *APRT* locus, showing the predicted targeted conversion and integration products. Crossed lines indicate the region where recombination is initiated. Conversion and integration, as drawn, represent potential outcomes of recombination at this site. Restriction enzyme recognition sites: B, *Bam*HI; Bg, *Bgl* II; E, *Eco*RI; Rv, *Eco*RV; H, *Hin*dIII; Mb, *Mbo* II; P, *Pst* I.

We anticipated that APRT$^+$ recombinants might arise by at least two types of recombination events: targeted conversion of the chromosomal *APRT* gene and targeted integration into the chromosomal *APRT* locus (Fig. 3). These two classes of recombinants can be distinguished by their diagnostic restriction fragment patterns. APRT$^+$ recombinants arising by targeted conversion will have normal, WT restriction patterns for all restriction enzymes. APRT$^+$ recombinants arising by targeted integration will have diagnostic restriction fragment patterns for *Bgl* II (*6.1 and 5.7 kb), *Hin*dIII (*10.3 and 5.3 kb), *Eco*RV (*2.2, *4.6 and 15 kb), and *Sac* I (≈*21 kb). For example, since there are no *Sac* I restriction sites in pAG-7 and the genomic sites lie well outside of the *APRT* gene sequence, a targeted integration would increase the size of the WT *14-kb *Sac* I fragment to ≈21-kb, while a conversion event would leave this fragment unchanged. Both types of APRT$^+$ recombinants have been observed.

Given the nature of the pAG-7 *APRT* donor sequence (which lacks the entire 5′ portion of the *APRT* gene, including the promoter region and first two exons), we did not anticipate that it would be possible to recover recombination products in which the incoming plasmid sequence had been corrected by recombination with the targeted chromosomal gene and then integrated elsewhere in the genome. Surprisingly, this class of recombinants accounted for nearly one-third of the APRT$^+$ clones analyzed.

Targeted Conversion of the Endogenous CHO-ATS-49tg *APRT* Gene. The majority of APRT$^+$ recombinants with simple restriction patterns (15/19) appeared to have arisen by targeted conversion of the ATS-49tg *APRT* locus. Thirteen of these clones showed no evidence of nontargeted integration of pAG-7 plasmid sequences. Two such recombinants, which appeared to contain only a single, WT copy of the *APRT* gene, are shown in Fig. 2 (clone 8-44 in lanes 2 and 9 and clone 8-77 in lanes 3 and 10). Both clones have a GPT$^-$ (HAT^s) phenotype. Both showed loss of the *2.0-kb ATS-49tg *Mbo* II fragment and reappearance of a WT *1.5-kb fragment (Fig. 2*a*); all other restriction fragment patterns are unchanged. Clone 8-11 (Fig. 2, lanes 4 and 11) is an example of a recombinant with a targeted conversion of the ATS-49tg *APRT* locus plus a single, nontargeted integration of pAG-7 elsewhere in its genome. The additional bands present in lane 4 in Fig. 2 *b* and *c* represent novel junction fragments produced by the nontargeted integration. Novel junction fragments were also observed after digestion with other enzymes; such fragments can be detected only with the 3.9-kb probe. Clone 8-66 (data not shown) reflected two nontargeted integrations. Both of these clones have a GPT$^+$ (HAT^r) phenotype. Curiously, of the 12 APRT$^+$ recombinants that showed multiple nontargeted integrations, only 1 (8-33) appears to have arisen by targeted conversion.

Targeted Integration at the CHO-ATS-49tg *APRT* Locus. Three APRT$^+$ clones with simple restriction fragment patterns arose by targeted integration at the *APRT* locus. Each displayed a GPT$^+$ phenotype. Two of these recombinants (clones 8-35 and 8-57; Fig. 2, lanes 5 and 12 and lanes 6 and 13, respectively) showed no evidence of untargeted integration of pAG-7 plasmid sequences. The third clone (8-52; data not shown) showed a targeted integration plus a nontargeted integration of two tandem copies of pAG-7 elsewhere in its genome. As shown in Fig. 2*b*, digestion with *Bgl* II (which does not cut within the *APRT* gene but cuts once within the pAG-7 *gpt* gene) yields a single *4.7-kb fragment for ATS-49tg or APRT$^+$ recombinants that have arisen by conversion, whereas targeted integrations generate diagnostic *6.1- and 5.7-kb recombinant fragments. Digestion with *Hin*dIII (which likewise does not cut within the *APRT* gene but cuts once within *gpt*) yields an *8.5-kb fragment for ATS-49tg or

APRT$^+$ convertants, whereas targeted integrations generate diagnostic *10.3- and 5.3-kb recombinant fragments (Fig. 2*c*). The extra bands in lanes 5 and 6 of Fig. 2*a* represent recombinant fragments defined by *Mbo* II sites within the targeted *APRT* locus together with sites in the integrated pAG-7 sequences. Addtional evidence of targeted integration in clones 8-35, 8-57, and 8-52 was provided by the observation of diagnostic *Eco*RV (*2.2-, *4.6-, and 15-kb) and *Sac* I (*21-kb) restriction fragments (data not shown). *Bgl* II, *Hin*dIII, *Eco*RV, and *Sac* I restriction fragments indicative of targeted integration were also detected in 3 of the 12 APRT$^+$ clones that showed multiple nontargeted integrations.

APRT$^+$ Recombinants That Retain an Unaltered Mutant *APRT* Gene at the Original Chromosomal Locus. Among the 19 APRT$^+$ clones with simple restriction patterns, 1 clone (8-39) did not appear to have arisen by either targeted conversion or targeted integration. As can be seen in Fig. 2*a* (lanes 7 and 14), while this clone has regained a *1.5-kb *Mbo* II fragment indicative of a reconstructed WT *APRT* gene, it has not lost the *2.0-kb *Mbo* II fragment characteristic of the ATS-49tg mutant allele. Based upon the restriction fragment patterns shown in Fig. 2, as well as information obtained from other restriction digests (not shown), it appears that clone 8-39 has retained an unaltered copy of the ATS-49tg *APRT* gene at the original chromosomal locus yet has somehow acquired a second, WT copy of the *APRT* gene, which has integrated elsewhere in the genome. Furthermore, of the 12 APRT$^+$ clones that showed complex restriction patterns with multiple nontargeted integrations of plasmid sequences, 8 resembled clone 8-39 in that they too had regained a *1.5-kb *Mbo* II fragment, characteristic of a reconstructed WT *APRT* gene, without having lost the original *2.0-kb *Mbo* II fragment. Each of these recombinants was found to contain at least two *Sac* I fragments that hybridized with the 1.3-kb (5′-end-specific) *APRT* probe; a *14-kb fragment corresponding to that of ATS-49tg and a second fragment of variable size that appeared to represent a junction fragment produced by random integration of the recombination-corrected input plasmid *APRT* sequence.

DISCUSSION

Most studies of targeted homologous recombination in mammalian cells have utilized "artificial" transfection- or electroporation-introduced, chromosomally integrated viral or bacterial gene sequences such as the herpes simplex virus thymidine kinase (*tk*) gene or the bacterial neomycin-resistance (*neo*) gene as recombinational targets (8-16). Many of the primary transfectants that have been employed in such targeting experiments have contained multiple single or tandem copies of the integrated target sequence (13, 14, 16), complicating analysis of recombinants and interpretation of the results. Such "artificial" target sequences may or may not be representative of a normal mammalian gene locus.

We have developed an experimentally adaptable system that permits analysis of targeted homologous recombination at an endogenous mammalian gene in its normal chromosomal context. Our system utilizes a hemizygous mutant CHO *APRT* gene (20–24) as a target locus. *APRT* is a constitutively expressed "housekeeping" gene that codes for a purine salvage-pathway enzyme (22). There are good forward and back selections for this locus (20–23). Hemizygous, nonrevertible *APRT* deletion mutants such as ATS-49tg are ideal for use in targeting experiments. The small size of the Chinese hamster *APRT* gene (22–24), its convenient distribution of restriction sites (22–24), and the absence of *APRT* pseudogenes in CHO cells (22) greatly facilitate molecular analysis of APRT$^+$ recombinants. Using ATS-49tg as a recipient cell line, we have demonstrated targeted correction of a nonrevertible *APRT* deletion mutation by homologous recombination of transfection-introduced plasmid *APRT* sequences with the defective chromosomal gene.

APRT$^+$ recombinants were obtained in our targeting experiments at a frequency of $\approx 4 \times 10^{-7}$, with a ratio of targeted recombination to nontargeted integration of $\approx$1:4000. Ratios ranging from about 1:100,000 to 1:100 have been reported in other targeting studies (12–14, 16, 19). The efficiency of targeted recombination varies at different locations in the genome and with different extents of homology in the input plasmid. Song *et al.* (16) detected targeted recombination in six out of eight transfectant cell lines with integrated *neo* target sequences. Targeted recombination frequencies varied over a 130-fold range (from 0.04 to 5.3 per microgram of plasmid DNA), with ratios of targeted recombination to nontargeted integration ranging from 1:500 to $\approx$1:75. In targeting experiments involving the *HPRT* locus in mouse embryo stem cells, Thomas and Capecchi (18) observed ratios of targeted recombination to nontargeted integration ranging from 1:40,000 to 1:950. They found the efficiency of targeted recombination to be strongly dependent upon the extent of shared homology between plasmid and target gene sequences, with targeting frequencies ranging from $\approx 4 \times 10^{-8}$ for a vector with 4.0 kb of target sequence homology to 4.1×10^{-7} for a vector with 9.1 kb of homology (18). In comparison, we have obtained targeted recombination at a frequency of 4.1×10^{-7} with a plasmid that carries only 2.6 kb of *APRT* sequence homology.

In general, molecular analyses of recombinants obtained from mammalian targeting experiments have painted a rather confusing picture. In experiments utilizing a defective *neo* gene as a target sequence, Thomas *et al.* (14) obtained only gene conversion events. Song *et al.* (16) obtained gene conversion and single-crossover events at approximately equal frequencies. In targeting experiments with mouse embryo stem cells, Doetschman *et al.* (19) recovered two recombinants that appeared to have arisen by a simple crossover, while three others reflected crossover accompanied by gap repair or gene conversion. In "knockout" experiments employing sequence-replacement vectors, Thomas and Capecchi (18) observed only gene conversion events; when sequence-insertion vectors were used, approximately three-fourths of the recombinants arose by targeted integration of the vector sequences at the *HPRT* locus. A mechanistic association between gene conversion and crossing over is a common feature of recombination models derived from studies of meiotic recombination (31, 32).

We have observed three types of recombination events in the APRT$^+$ recombinants obtained from our targeting experiments. Most of these recombinants had simple restriction fragment patterns, with few or no untargeted integrations of pAG-7 plasmid sequences. Of the 31 independent APRT$^+$ clones analyzed, 16 arose by targeted correction (conversion) of the endogenous *APRT* gene by homologous recombination with plasmid donor *APRT* sequences. Six recombinants appeared to be the products of single crossovers (reciprocal exchanges) that resulted in targeted integration of the plasmid sequences at the *APRT* locus. Each of the 9 remaining APRT$^+$ clones retained an unaltered copy of the ATS-49tg *APRT* gene, at the original chromosomal locus, plus a second, WT copy of the *APRT* gene, which had integrated elsewhere in the genome.

We are particularly intrigued by the third class of recombination events. Although correction and subsequent integration of a defective plasmid gene with an internal deletion have been observed in other studies (14, 16), we did not anticipate such events in our experiments because the pAG-7 plasmid *APRT* sequence lacked the entire 5′ half of the *APRT* gene. Meiotic recombination models (31, 32) require homology on both sides of a double-strand break, gap, or mutation to effect correction by homologous recombination. Since the

donor plasmid in our experiments carried a truncated, 5'-end-deleted *APRT* gene fragment that was not flanked on its 5' side by any sequences homologous to the chromosomal *APRT* target sequences, it should not be a suitable substrate for correction by recombination. Nevertheless, in nine $APRT^+$ recombinants, this truncated plasmid *APRT* gene segment appears to have been converted to a complete, functional *APRT* gene, which would require the duplication and recombinational acquisition of ≈2 kb of chromosomal 5' *APRT* sequences.

We can envision at least two mechanisms by which such recombinants could have been generated: (*i*) one-armed, 3'-OH strand invasion into the chromosomal *APRT* gene duplex by the *Pst* I-linearized plasmid *APRT* DNA sequence, followed by elongation of the invading strand using a chromosomal *APRT* strand as a template, with branch migration and lagging-strand synthesis to generate a complete *APRT* gene on the plasmid duplex; or (*ii*) homologous recombination between input plasmid *APRT* sequences and an extrachromosomal, circular-DNA copy of the ATS-49tg *APRT* gene. The first mechanism would closely resemble the "join-copy" replication pathway that has been described in phage T4, in which replication forks are initiated from recombinational intermediates (33). The second mechanism is suggested by studies that have demonstrated the ubiquitous presence of heterogeneous populations of extrachromosomal, small polydisperse circular (spc) DNAs in the cells of higher eukaryotes (34, 35). These spcDNAs are derived from chromosomal DNAs and range in size from ≈0.15 to >250 kb (34, 35). Their heterogeneity and sequence complexity suggest that they are probably generated by multiple mechanisms (34–38) and that they may represent random samples of chromosomal DNA (35, 36). Wiberg *et al.* (38) observed a 5-fold increase in the levels of spcDNA in mammalian cells after calcium phosphate-mediated transfection; the new spcDNAs appeared to be derived from cellular DNA. Additional experiments will be required to distinguish between these alternative mechanisms.

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