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Chromosomal Double-Strand Breaks Induce Gene Conversion at High Frequency in Mammalian Cells

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Double-strand breaks (DSBs) stimulate chromosomal and extrachromosomal recombination and gene targeting. Transcription also stimulates spontaneous recombination by an unknown mechanism. We used *Saccharomyces cerevisiae* I-*Sce*I to stimulate recombination between *neo* direct repeats in Chinese hamster ovary (CHO) cell chromosomal DNA. One *neo* allele was controlled by the dexamethasone-inducible mouse mammary tumor virus promoter and inactivated by an insertion containing an I-*Sce*I site at which DSBs were introduced in vivo. The other *neo* allele lacked a promoter but carried 12 phenotypically silent single-base mutations that create restriction sites (restriction fragment length polymorphisms). This system allowed us to generate detailed conversion tract spectra for recipient alleles transcribed at high or low levels. Transient in vivo expression of I-*Sce*I increased homologous recombination 2,000- to 10,000-fold, yielding recombinants at frequencies as high as 1%. Strikingly, 97% of these products arose by gene conversion. Most products had short, bidirectional conversion tracts, and in all cases, donor *neo* alleles (i.e., those not suffering a DSB) remained unchanged, indicating that conversion was fully nonreciprocal. DSBs in exogenous DNA are usually repaired by end joining requiring little or no homology or by nonconservative homologous recombination (single-strand annealing). In contrast, we show that chromosomal DSBs are efficiently repaired via conservative homologous recombination, principally gene conversion without associated crossing over. For DSB-induced events, similar recombination frequencies and conversion tract spectra were found under conditions of low and high transcription. Thus, transcription does not further stimulate DSB-induced recombination, nor does it appear to affect the mechanism(s) by which DSBs induce gene conversion.

Double-strand breaks (DSBs) are a form of DNA damage that can arise spontaneously in the genome or may be induced by ionizing radiation and chemicals (for reviews, see references 8, 22, 39, 58, and 75). If misrepaired or unrepaired, DSBs may be mutagenic or lethal to cells. DSBs can be repaired by either homologous or nonhomologous (illegitimate) recombination. Homologous recombination is thought to require at least 200 bp of sequence homology (24, 34). Two models, DSB or gap repair (64) and single-strand annealing (SSA) (2, 31, 33, 57), have been postulated to explain the stimulation of homologous recombination by DSBs. The DSB repair model suggests that broken ends are degraded to form a double-stranded gap. The ends then invade a homologous region of DNA and prime DNA synthesis by using the undamaged allele as a template for repair. In this model, recombination intermediates may be resolved as a reciprocal exchange (crossover) or a nonreciprocal transfer of information from one allele to another (gene conversion). Variations of this model suggest that gaps are not formed, with gene conversion mediated by mismatch repair of heteroduplex DNA (hDNA) intermediates (62). The SSA model, which explains certain types of recombination events between directly repeated sequences, suggests that ends are degraded by a single-strand exonuclease, exposing complementary single strands in two homologous regions, which then anneal to form an apparent crossover product (32). However, this mechanism is nonconservative since only one of two possible crossover products is formed, and it would produce acentric and/or dicentric products if it were to occur between unlinked chromosomal repeats. In both models, repair at break sites occurs with high fidelity since long stretches of sequence homology are involved, but the nonconservative SSA mechanism always leads to deletions of sequences between homologous regions. In *Saccharomyces cerevisiae*, most chromosomal DSBs are repaired by a conservative homologous recombination pathway (39).

Nonhomologous recombination (or end joining) is often imprecise and therefore mutagenic. In vitro studies of DSB repair of plasmid DNA incubated with mammalian cell extracts and in vivo studies of DSB repair at mammalian chromosomal loci revealed an efficient end joining mechanism that involves annealing complementary short sequence repeats of 1 to 5 bp in length (termed "micro-SSA") (14, 19, 42–44, 47, 67). Because this mechanism requires little or no homology, it is considered a nonhomologous recombination mechanism, and like SSA, micro-SSA often results in deletions near DSB sites. Contrasting with yeast cells, studies with mammalian cells have shown that nonhomologous recombination exceeded homologous recombination by 2- to 3-fold (51), 10-fold (56), or more than 1,000-fold (21, 69, 74).

Homologous recombination is also stimulated by transcription. In *S. cerevisiae*, the RNA polymerase I-dependent ribosomal DNA promoter, *HOT1* (26, 70), stimulates recombination 25- to 100-fold, and this stimulation is transcription dependent (60). The galactose-inducible, RNA polymerase II-dependent yeast *GAL1,10* promoter (68) stimulates intrachromosomal recombination. In mammalian cells, transcription enhances DSB-induced extrachromosomal recombination sixfold (40), and product structures were consistent with formation by SSA. Similarly, spontaneous intrachromosomal recombination is stimulated two- to sevenfold by transcription, with most products of direct repeats being gene conversions, while prod-

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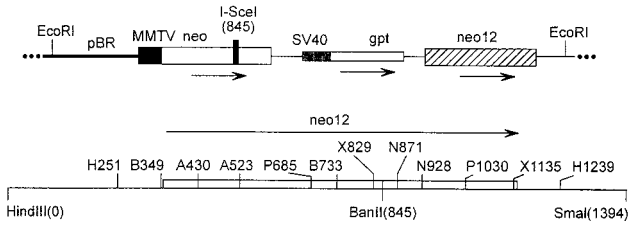

FIG. 1. Recombination substrate structure. Duplicated 1.4-kb *neo* fragments (open and hatched boxes) flank a *gpt* gene driven by the simian virus 40 (SV40) promoter, used to select mycophenolic acid-resistant cells during strain construction. One *neo* allele is driven by the DEX-inducible MMTV promoter (black box) and contains a frameshift mutation at *Ban*II containing the I-*Sce*I site. The second allele, *neo12*, lacks a promoter and contains 12 silent, single-base mutations. *Eco*RI sites are used for plasmid rescue. A map of *neo12* drawn to scale is shown below; numbers in marker names correspond to positions in *neo.* See Table 1 for details.

ucts of inverted repeats showed a variety of rearrangements (38). Transcription is usually thought to enhance recombination by increasing the frequency of initiating events, i.e., by increasing the accessibility of chromatin to recombinational machinery or nucleases.

In yeast, homologous recombination has been studied extensively by using site-specific DSBs as initiating events (39). These recombination assays were modeled on yeast mating type interconversion in which recombination is initiated by an HO nuclease-induced DSB at *MAT*. The discovery of a group of intron-encoded endonucleases (45) has facilitated analogous studies in mammalian cells (23). Since these endonucleases, like HO, have long recognition sites (~18 bp), these sites are expected to occur rarely or not at all in mammalian genomes and are therefore useful tools for the study of recombination induced by specific chromosomal DSBs.

The purpose of the present study was to examine the types of recombination events induced by DSBs at a defined chromosomal locus in Chinese hamster ovary (CHO) cells by the nuclease I-*Sce*I, which is known to stimulate intrachromosomal, extrachromosomal, and plasmid-chromosome recombination in mammalian cells (7, 11, 53, 56). By using substrates having one copy of *neo* driven by the inducible mouse mammary tumor virus (MMTV) promoter and a second transcriptionally silent, multiply marked *neo*, we were able to examine recombination product structures in detail and assess effects of transcription on DSB-induced recombination. I-*Sce*I stimulated homologous recombination 2,000- to 10,000-fold over spontaneous levels, with induced frequencies as high as 1%. In sharp contrast to results with extrachromosomal substrates, nearly all recombinants arose by gene conversion. Transcription had no discernible effect on the frequency of DSB-induced recombination or on conversion tract spectra.

MATERIALS AND METHODS

Plasmid DNA. Plasmids were constructed by standard procedures (55, 65). Derivatives of plasmid pMSG*neo*, carrying *neo* driven by the MMTV promoter (MMTV*neo*) (40), were produced in several steps. All linkers were 10 bp (New England Biolabs) unless otherwise specified. An *Xho*I linker was inserted into the pMSG*neo Eco*RI site, and then an *Eco*RI linker was inserted into the *Nde*I site (both in pBR322 sequences). A second *neo* allele, *neo12* (Fig. 1; Table 1), with 12 silent, single-base mutations that create new restriction sites (restriction fragment length polymorphisms [RFLPs]), was inserted as a 1.4-kbp *Sal*I fragment into the *Xho*I site, destroying both sites. An *Xho*I linker was inserted into the *Ban*II site (made blunt by removing the 4-base, 3′ extension with T4 DNA polymerase) in MMTV*neo*. A 29-bp oligonucleotide with an I-*Sce*I site was inserted into this *Xho*I site; the resulting frameshift insertion inactivates MMTV-*neo*. The I-*Sce*I site was formed by annealing two oligonucleotides (5′ TCGAT GATATCATTACCCTGTATACCCTA and 5′ TCGATAGGGATAACAGGG TAATGATATCA). *Eco*RI linkers were added to a 1.8-kbp *Bam*HI fragment containing the *S. cerevisiae HIS3* gene, which was then inserted into the *Eco*RI linker at *Nde*I, creating pMSG*neo*2S12His. The *HIS3* fragment served as a buffer to prevent loss of the *Eco*RI sites during substrate integration. Plasmids pCMV(I-*Sce*I$^+$) and pCMV(I-*Sce*I$^-$) (11) were a gift from J.-F. Nicolas, Institut Pasteur.

TABLE 1. Silent mutations in *neo12*

Mutation[a]	Change[b]	Site[c]
H251	G→C	*Hin*dIII
B349	G→C	*Bam*HI
A430	A→C	*Apa*I
A523	C→A	*Apa*LI
P685	C→A	*Pst*I
B733	T→G	*Bam*HI
X829	G→A	*Xba*I
N871	C→G	*Nru*I
N928	C→A	*Nsi*I
P1030	C→A	*Pml*I
X1135	T→A	*Xba*I
H1239	G→C	*Hin*dIII

[a] Numbers in mutation designations indicate position in *neo*, numbering from the *Hin*dIII site in pSV2*neo* (59).

[b] The wild-type base in the coding strand is listed first; the mutant base is second.

[c] Enzyme recognizing RFLP mutation. The X829 *Xba*I site overlaps a *dam* methylation site and is mapped in PCR-amplified DNA.

Construction and characterization of CHO cell lines. Cell culture and electroporation conditions were described previously (38). From 0.1 to 1 μg of pMSG*neo*2S12His was linearized in *HIS3* with *Bst*XI and electroporated into CHO cell line K1c, producing mycophenolic acid-resistant transfectants, and structures of integrated recombination substrates were characterized by Southern hybridization as described previously (38). Total RNA was prepared from cultures inoculated with 1×10^6 to 1.5×10^6 cells in 100-mm (diameter) dishes and incubated for 2 days. RNA preparation and Northern hybridization analyses were performed as previously described (38).

Recombination assays. To perform recombination assays, 2×10^6 parent cells were seeded into eight 100-mm dishes. Twenty-four hours later, dexamethasone (DEX; 1 μM) was added to half of the dishes and cells were incubated for 4 h to induce transcription. Cells were transfected with 10 μg of either pCMV(I-*Sce*I$^+$) or pCMV(I-*Sce*I$^-$) by using lipofection, as recommended by Gibco-BRL. Thirty-six hours posttransfection, cells were trypsinized, counted, and transferred to 24-well plates. G418 (500 μg/ml, 50% active; Gibco-BRL) was added 24 h later, and G418-resistant (G418^r) colonies arose after 14 days. Viability was determined by seeding appropriate dilutions of cell suspensions into nonselective medium, incubating them for 10 to 12 days, and staining colonies with 1% crystal violet in 70% ethanol. Recombination frequencies were calculated as the ratio of G418^r colonies to the total number of live cells plated in selective medium. G418^r colonies were dispersed within the wells and expanded to confluence, and genomic DNA was prepared. Ten micrograms of genomic DNA was digested with *Eco*RI (which excises recombinant *neo* alleles linked to bacterial vector sequences) and treated with T4 DNA ligase at a low DNA concentration (50 μg/ml) to favor recircularization of the excised plasmid over ligation to other DNA fragments. Ligation reaction mixtures were ethanol precipitated and electroporated into recombination-defective *Escherichia coli* HB101 cells, and plasmid DNA was isolated from kanamycin-resistant (Kan^r) *E. coli* transformants. Two types of products, gene conversions and pop-outs, were recovered. All markers were scored in both *neo* alleles in rescued gene conversion products by using RFLP mapping strategies analogous to those described previously (63), except that the X829 *Xba*I site is blocked by an overlapping *dam* methylation site and was mapped in PCR-amplified DNA.

RESULTS

Experimental design. To gain detailed information about DSB-induced recombination in CHO cells, we constructed lines with recombination substrates consisting of two inactive, multiply marked *neo* alleles in direct orientation flanking a selectable *E. coli gpt* gene driven by the simian virus 40 early promoter (SV*gpt*) (Fig. 1). The regulated MMTV*neo* allele was inactivated by an insertion mutation that included the recognition sequence for the highly specific endonuclease, I-*Sce*I (12). The 18-bp I-*Sce*I recognition sequence was inserted into an *Xho*I linker present in a natural *Ban*II site. *Ban*II yields

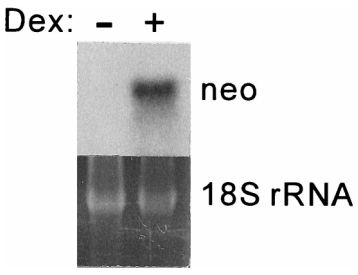

FIG. 2. DEX-induced transcription of MMTV*neo*. Total RNA was isolated from cultures incubated without (−) or with (+) 1 μM DEX for 4 h. Northern hybridization was performed with a ^{32}P-labeled *neo* probe; the autoradiograph is shown above. RNA loading is shown below in a photograph of ethidium bromide-stained 18S rRNA. Longer exposures show faint signals in the absence of DEX.

4-base, 3′ extensions; when this site is made blunt with T4 DNA polymerase prior to insertion of the 10-bp *Xho*I linker, a 4-base deletion of the *neo* coding sequence results. Micro-SSA occurring between flanking 4-bp repeats (created from linker DNA upon insertion of the I-*Sce*I site) would yield a *neo* allele with a 6-bp insertion that does not reform the *Ban*II site (changing the amino acid sequence from the wild-type. . .Gly-Leu. . .to. . .Ala-Leu-Glu-Gly. . .). The second *neo* allele (*neo12*) has 12 single-base RFLP mutations that serve as markers for recombination events. All 12 markers are phenotypically silent, so *neo12* has wild-type coding capacity, although it is inactive because it lacks a promoter. MMTV*neo* is transcribed at low and high levels in the absence and presence of DEX, respectively. Thus, this system also allows us to determine whether varying transcription levels in MMTV*neo* influence DSB-induced recombination.

Four strains harboring single integrated copies of the recombination substrate were isolated, and recombination levels were measured after transfection with the I-*Sce*I expression plasmid or with a nonexpressing control plasmid (with the I-*Sce*I gene in reverse orientation) in the presence or absence of DEX. In theory, DSBs could be repaired by nonhomologous recombination via micro-SSA (30) or homologous recombination, including gene conversion, pop-outs via crossing over or SSA, or unequal sister chromatid exchange (41). However, G418 selection pressure prevents detection of certain of these events (see below). In any case, G418^r recombinants were expected to carry an MMTV*neo* lacking the I-*Sce*I site. Gene conversion could result in loss of the I-*Sce*I site by transfer of information from *neo12*, either intrachromosomally or from the sister chromatid, and such transfer may or may not include RFLPs from *neo12*. Recombinant products were isolated by plasmid rescue, and restriction mapping was used to characterize both gross product structures and RFLP configurations.

Transcription levels in MMTV*neo*. Constitutive, low-level MMTV*neo* transcription (in the absence of DEX) was shown previously to confer full resistance to G418 in cells with similar single-copy integrated recombination substrates (38), and data shown below indicates that this is also true for strains constructed for the present study. Steady-state transcription levels in strain 33 were induced ~50-fold in DEX-treated cells (Fig. 2). This level of induction is highly reproducible; similar induction levels were seen in a previous study (38) and in more than 40 CHO cell lines carrying related substrates (unpublished results). This increase in transcription was shown to stimulate spontaneous homologous recombination between direct and inverted chromosomal repeats by approximately sixfold (38).

TABLE 2. Spontaneous and DSB-induced recombination frequencies

Expt	Strain[a]	pCMV (I-*Sce*I)[b]	DEX[c]	G418^r frequency (10^5)[d]	Increase[e]
1	33	No DNA	−	<0.01	
2	33	No DNA	+	<0.01	
3	33	−	−	<0.01	
4	33	−	+	<0.01	
5	33	+	−	610 ± 100	6,000
6	33	+	+	610 ± 50	6,000
7	17	−	−	<0.01	
8	17	+	−	210 ± 14	2,000
9	18	−	−	<0.01	
10	18	+	+	440 ± 38	4,000
11	30	−	−	<0.01	
12	30	+	+	1,000 ± 620	10,000

[a] Each strain has a single copy of the *neo* direct repeat substrate (Fig. 1) integrated into the CHO genome.
[b] Cells were transfected with no DNA or with plasmids carrying the I-*Sce*I nuclease gene, including pCMV(I-*Sce*I$^+$) (indicated by "+"), which expresses I-*Sce*I, and the negative control plasmid pCMV(I-*Sce*I$^-$) (indicated by "−"), which has the I-*Sce*I gene in the opposite direction.
[c] Transfected cells were preincubated with (+) or without (−) DEX.
[d] Frequencies were calculated as the number of C418^r colonies divided by the total number of viable cells plated in G418 medium. Values are means ± ranges for two determinations.
[e] Frequency in the presence of I-*Sce*I divided by frequency in its absence.

DSBs strongly enhance homologous recombination. In previous studies with strains harboring identical recombination substrates but lacking the closely spaced RFLP markers, spontaneous recombination levels were $\sim 5 \times 10^{-6}$ (38), but in all four strains with the multiply marked *neo12* allele, levels were at least 50-fold lower and thus below the detection limit ($<10^{-7}$), whether MMTV*neo* was transcribed at low or high levels (Table 2). Reductions in spontaneous direct repeat recombination with additional markers have been reported previously (71).

When cells were transfected with the I-*Sce*I expression plasmid to induce DSBs in MMTV*neo* alleles, G418^r colonies were recovered at levels more than three orders of magnitude higher than spontaneous levels, approaching 1% for some strains. This increase was dependent on I-*Sce*I expression, since no recombinants were recovered when cells were transfected with the negative control plasmid, pCMV(I-*Sce*I$^-$), or when transfecting DNA was omitted (Table 2). Data presented below clearly indicates that these G418^r colonies resulted from homologous recombination. Similar recombination levels were seen for DSB-induced events in the presence and absence of DEX. We draw several conclusions from these results. First, high-level transcription alone does not provide a sufficient stimulus to overcome the strong block to spontaneous recombination imposed by the closely spaced RFLPs. Second, DSBs provide a strong stimulus that is sufficient to overcome the inhibitory effects of the RFLPs. Third, increasing transcription in alleles suffering a DSB does not further enhance DSB-induced recombination.

Nearly all DSB-induced recombinants arose by gene conversion. Because only one *neo* allele carried a promoter, the various types of events that could produce a G418^r product yield distinctive product structures. Among homologous recombination products, gene conversions retain the gross structure of the parental substrate, whereas pop-outs, resulting from either SSA or crossing over, have deleted one copy of *neo* and SV*gpt*. An unequal sister chromatid exchange yields two products, one with a *neo* triplication and the other indistin-

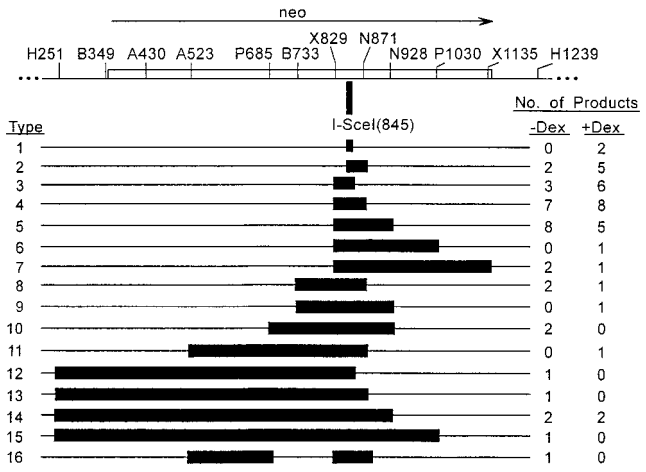

FIG. 3. DSB-induced gene conversion tract structures. *neo12*, shown above, is the information donor for conversions of MMTV*neo*. Conversion tracts shown below are indicated by black bars. The relative position of the I-*Sce*I site is shown above; this site is always converted among *neo*$^+$ products. The number of products of each tract type isolated under conditions of low or high MMTV*neo* transcription (−Dex or +Dex, respectively) is given. Tract type 16 is discontinuous; all others are continuous.

guishable from a pop-out. Nonhomologous recombination could conceivably yield products with structures similar to gene conversions or pop-outs. However, fine structure analysis of rescued plasmids can distinguish all of these possibilities. Plasmids were rescued from 67 DSB-induced G418^r products from strain 33 cultures grown in the presence or absence of DEX. Plasmid rescue was highly efficient: only 10% of initial rescue products did not have structure expected for either gene conversion, pop-out, or triplication events. In these cases, a secondary rescue always gave one of the expected structures. The success of secondary rescue, plus the fact that rescued plasmids with unexpected structures usually had an additional *Eco*RI fragment, indicates that unexpected structures reflected ligation of random genomic fragments to the plasmid backbone during ligation and were not representative of the structure of the recombinant in the genome. Thus, the rescue procedure does not impose a bias against particular classes of products. Of the 67 rescued products, 65 had parental structures (two *neo* genes) consistent with gene conversion and 2 had pop-out structures (one *neo* gene); none had *neo* triplications.

Rescued products were characterized by RFLP mapping. Pop-outs may result from crossing over, SSA, or nonhomologous recombination and are therefore less informative than other types of recombination products. Since we found only two pop-out products, these were not characterized further. The remaining 65 products with two *neo* genes were fully analyzed to determine whether these had arisen by gene conversion or by nonhomologous recombination. All products lost the I-*Sce*I site in MMTV*neo*, and most had one or more RFLPs transferred from *neo12* to MMTV*neo* (Fig. 3). Such RFLP transfer cannot occur via nonhomologous recombination. There were two products that only lost the I-*Sce*I site and may have arisen by conversion or nonhomologous recombination. In these products (and in those with RFLP conversions), the wild-type *Ban*II site had transferred from *neo12* to MMTV*neo*. Thus, all 65 products arose by gene conversion. A limited analysis of products from a different strain showed similar results (data not shown).

None of the products had alterations in the unbroken allele (*neo12*). Thus, *neo12* acted solely as a donor of information during conversion of MMTV*neo*. This result and the fact that 64 of the 65 gene conversion products had continuous conversion tracts are reminiscent of results of DSB-induced recombination in yeast (39) (see Discussion). There are 83 possible types of continuous conversion tracts for events initiated at the I-*Sce*I site, but only 15 types were found among the 64 products (Fig. 3), the majority of which (75%) were relatively short (types 1 to 5 and 8, averaging <200 bp in length). These data can be used to classify products according to several tract parameters, including directionality, length, and structure (Table 3). Most products had tracts extending bidirectionally from the I-*Sce*I site (72%). Distributions of individual tract types were quite similar in the presence or absence of DEX, which translates into similar average tract lengths and similar distributions of uni- and bidirectional tracts. Plots of conversion frequencies of individual markers versus distance from the I-*Sce*I site reveal no differences for 5′ versus 3′ markers and, again, no effects of transcription (Fig. 4). Thus, varying transcription levels in the recipient MMTV*neo* allele did not alter gene conversion tract lengths, directionality or structure, or conversion rates of individual markers.

TABLE 3. Tract parameters for DSB-induced products

DEX	n^a	Tract length^b (bp)	Continuous tract distribution (%)^c			
			I-*Sce*I only	3′	5′	Bidirectional
−	31	277	0	6	9	85
+	33	183	6	15	18	61

[a] Number of products analyzed. One product isolated without DEX had a discontinuous tract and is not included.

[b] Average tract lengths estimated for each type of tract shown in Fig. 3 as the means of the longest and shortest possible tract lengths. These values were used to calculate an average tract length for each set of products.

[c] Percentage of products with particular tract structures. Bidirectional tracts are those in which at least one marker on either side of the I-*Sce*I site had converted. Unidirectional tracts include three subgroups: tracts extending either 5′ or 3′ from the I-*Sce*I site and short tracts (<42 bp) in which only the I-*Sce*I site converted.

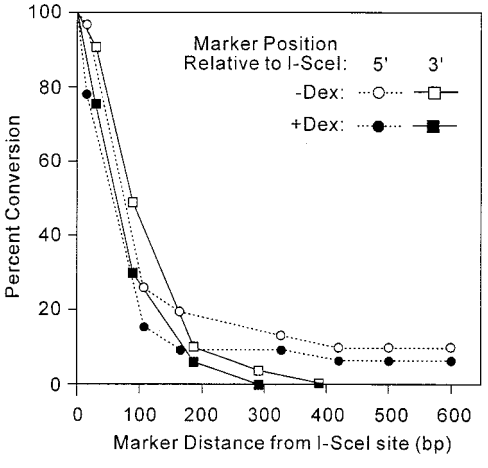

FIG. 4. Conversion frequencies for each marker as a function of distance from the I-*Sce*I site. Each line represents conversion frequencies for markers 3′ or 5′ of the I-*Sce*I site in product sets isolated in the presence or absence of DEX. Data is from Fig. 3.

DISCUSSION

We constructed CHO cell lines carrying direct repeats of multiply marked *neo* alleles to perform a detailed examination of DSB-induced homologous recombination in mammalian cells. In mouse LTK$^-$ cells, a minimum of approximately 200 bp of uninterrupted homology is required for efficient spontaneous homologous recombination (72). The *neo12* allele in our substrate contains 12 silent single-base changes (RFLPs) spaced at ~100-bp intervals, and so it is not surprising that we did not recover spontaneous recombinants. However, DSBs introduced into one *neo* repeat stimulated homologous recombination by at least 3 to 4 orders of magnitude, with frequencies approaching 1% (Table 2), suggesting that once an event is initiated (i.e., by a DSB), the closely spaced markers may have little or no effect on later steps. It is possible that higher levels of DSB-induced recombination would be seen in the absence of these markers. Liang et al. (30), using a similar *neo* direct repeat assay in CHO cells but one lacking closely spaced markers, also found I-*Sce*I-induced recombination levels near 1%. However, that study is not directly comparable to the present study since they used a different I-*Sce*I expression vector and at threefold-higher levels, and transfection was performed by calcium phosphate coprecipitation instead of lipofection. If the closely spaced markers do not impose a block to DSB-induced recombination, it would suggest that the block seen for spontaneous events acts at the level of initiation. In any case, chromosomal DSBs are highly effective at stimulating conservative, homologous recombination in mammalian cells, even between alleles lacking long stretches of uninterrupted homology.

Transcription stimulates spontaneous intrachromosomal recombination, but only by 5- to 10-fold in both yeast and mammalian cells (38, 68), and thus has a much smaller effect than DSBs. The present study suggests that this low-level stimulation is not sufficient to overcome the block to spontaneous recombination imposed by closely spaced markers. In addition, DSB-induced recombination frequencies were similar when recipient alleles were transcribed at high or low levels, indicating that once recombination is initiated by a DSB, transcription has no further stimulatory effect. Also, the similar gene conversion/pop-out ratios, and similar conversion tract spectra obtained under conditions of low and high transcription, suggest that transcription does not influence later steps during conservative, chromosomal gene conversion induced by DSBs. If DSB-induced gene conversion proceeds by similar mechanisms in mammalian cells and in yeast (see below), later steps would include strand invasion, hDNA formation, and mismatch repair (46, 50, 73). In contrast, transcription does stimulate DSB-induced extrachromosomal recombination in CHO cells (40). However, the current study indicates that there are fundamental mechanistic differences between extrachromosomal and chromosomal recombination, since extrachromosomal events proceed primarily by SSA (9, 13, 32, 36, 57). It is unclear how transcription might stimulate DSB-induced SSA-mediated, extrachromosomal recombination.

For events between direct repeats, recombinants may arise by gene conversion, pop-out, or unequal sister chromatid exchange (41); these events are distinguishable by combined analysis of gross product structure (i.e., triplications and pop-out structures result from unequal exchange) and detailed mapping of the recombinant *neo* genes (gain and loss of RFLP markers). Of the 67 products we analyzed, 65 were gene conversions, 2 were pop-outs, and none had a triplication. We cannot determine whether the two pop-outs resulted from crossing over or from SSA. In yeast direct repeat recombination, both mechanisms are operative: crossovers are found when a break occurs within homology (49), and SSA is found when a break occurs between homologous regions (54, 61). Since the DSBs studied here occurred within homology, the two pop-outs may have resulted from crossing over.

The predominance of gene conversions seen here is consistent with the ~10-fold-higher frequency of gene conversions than of pop-outs for DSB-induced recombination at a duplicated *aprt* locus (56) and for spontaneous *neo* recombination products (38). In yeast, crossovers are highly associated with gene conversions, approaching 50% for some loci in meiosis (46). This association has been explained by models that include Holliday junctions in intermediates, which are thought to be symmetric and therefore resolved without bias in either of two senses leading to crossover or noncrossover products at equal frequencies (e.g., see reference 64). Although this idea is consistent with the ~50% association at some yeast loci in meiosis, most studies in yeast and mammalian cells have shown that associated crossovers occur at frequencies much lower than 50% (this study and references 5, 27, 38, and 56), suggesting either that Holliday junctions are resolved with bias or that they are not common in recombination intermediates. Some one-ended invasion models do not invoke Holliday junctions (3). Gene conversion is thought to be important for maintaining genetic homogeneity between members of multigene families (26) and large tandem repeat stability (20). Regardless of the mechanism by which crossovers are suppressed during gene conversions, this suppression may be an evolutionary adaptation that serves to prevent large-scale genetic alterations while allowing the benefits of gene conversion. Interestingly, for allelic events such as during meiosis, crossing over does not lead to deleterious genome rearrangements, and meiotic conversion appears to be less restricted for crossing over.

We found that conversion tract spectra had a high degree of symmetry. Of the unidirectional tracts that were recovered, equal numbers extended in the 5′ and 3′ directions (Table 3), and conversion frequencies as a function of distance from the DSB were similar for 5′ or 3′ markers (Fig. 4). The closest markers flanking the I-*Sce*I site, 16 and 26 bp away, converted at very high frequencies (86 and 82%, respectively), and both converted in most products (72% of tracts were bidirectional). It is likely that conversion tracts usually extend at least 26 bp into homologous DNA on both sides of a DSB. This contrasts with results in yeast for DSB-induced plasmid-chromosome and intrachromosomal recombination, in which ~60 to 80% of tracts were unidirectional, extending less than 23 to 31 bp in one or both directions (10, 63). In two yeast studies, tracts that extended less than 6 to 8 bp into homology in one or both directions were identified (37, 48). A significant proportion of conversion tracts in yeast are short (~20% were <53 bp), but in the present study only 2 of 65 products (3%) had tracts of <42 bp. The average tract length in the present study (230 bp) was similar to that in yeast (264 bp), measured with direct *ura3* repeats with similar repeat length (1.2-kbp *ura3* versus 1.4-kbp *neo*) and marker spacing. Thus, the greater fraction of bidirectional tracts in mammalian cells is not reflected in greater average tract length, indicating that conversions are less extensive on either side of a DSB in mammalian cells than in yeast. Although it is tempting to speculate that uni- and bidirectional tracts reflect one- and two-ended invasions, respectively, one-ended invasion models do not preclude bidirectional conversion tracts, and at least in yeast, it appears that other factors are important (37). Interestingly, crossovers in yeast are less likely to occur if tracts are short (1), providing a potential clue as to why crossovers may be so infrequent in mammalian cells. Each of the curves in Fig. 4 is bimodal, with an initial steep

decline that sharply changes (at ~100 bp from the DSB) to a much shallower slope. Remarkably similar bimodal curves, also having inflection points ~100 bp from a stimulating DSB, were obtained from gene conversion tract spectra of gene targeting experiments in mouse embryonic stem cells, leading Jasin and coworkers to speculate that conversion of near and distant markers may involve distinct mechanisms (17). DSB-induced gene conversion tract spectra in yeast do not display bimodal curves (10, 63).

Other than the higher frequencies of bidirectional tracts and the bimodal conversion of near and distant markers, the events observed here are quite similar to those seen in yeast (39). Because both alleles were analyzed from recombination products, formation of a *neo*$^+$ allele by a double crossover could be distinguished from a gene conversion. However, double crossovers were not detected, the donor allele was never changed, the allele suffering the DSB was preferentially converted, and almost all conversion tracts were continuous. These features are consistent with conversion via gap repair (64) but can also be explained by models suggesting that conversion is mediated by mismatch repair of hDNA. For hDNA models, preferential conversion and tract continuity require biased, long-tract mismatch repair tracts, as seen in bacteria (29), yeast cells (15, 16), and mammalian cells (36). Current evidence strongly indicates that hDNA repair mediates most or all DSB-induced conversion in yeast, including meiotic conversion (46), *MAT* switching (50), and general mitotic conversion (73). Spontaneous gene conversion in mammalian cells revealed sectored products at low frequency, providing direct evidence for hDNA formation during these events (4). For DSB-induced events, discontinuous conversion tracts provide evidence of hDNA, as these cannot arise by a single gap repair event. Although we found only 1 of 65 products with a discontinuous tract, this is not unexpected in light of evidence for efficient mismatch repair in recombination intermediates with strand breaks in mammalian cells (36, 66). Thus, although nearly all of the products described here can be explained as arising from either gap or long tract mismatch repair, the evolutionary conservation of repair functions among prokaryotes and eukaryotes (18) would argue that mammalian DSB-induced gene conversion is also a consequence of mismatch repair.

In the present system, the I-*Sce*I site was inserted such that DSB repair by micro-SSA would produce a short in-frame insertion. However, this insertion may not be silent, as we found no G418^r products with structures consistent with such an event. Thus, our construction differs from those in which *neo* sequences are duplicated on either side of an I-*Sce*I site, as these readily produce *neo*$^+$ products via micro-SSA (23). Other types of micro-SSA events involving shorter repeats could also restore the reading frame, but no evidence for these was obtained. We constructed several modified *neo* genes for mismatch repair studies, and in all cases, even short in-frame insertions inactivated *neo* (66), despite being located in regions that are not highly conserved among *neo* family members and distant from the coding region for the putative active site (28). These results indicate that *neo* is quite intolerant of coding sequence changes and that functional alleles may rarely be produced by nonhomologous recombination. Thus, the present system does not provide a measure of nonhomologous recombination. This is in contrast to several other systems (21, 35, 52, 56). For example, in a study of DSB-induced intrachromosomal recombination between duplicated *tk* genes in CHO cells, seven of seven products were formed through nonhomologous events (21). However, it was pointed out that this data may underestimate the number of homologous events, since functional *tk* genes could not be recovered from gene conversions mediated by sister chromatids, which are thought to be preferred templates for recombinational repair (6, 25). Unlike *neo*, *tk* tolerates many in-frame changes; one *tk*$^+$ product had a 33-bp insertion and others had short in-frame deletions (21). Thus, at least three factors influence the relative rates at which selected products arise from homologous and nonhomologous recombination in mammalian cells, including substrate location (episomal or chromosomal), type and location of inactivating mutation (insertion versus deletion), and the intrinsic capacity of a gene to tolerate subtle sequence changes.

Three previous studies have examined homologous recombination between chromosomal alleles stimulated by a single DSB (induced by I-*Sce*I) in mammalian cells. The first study was restrictive, as only pop-out recombination between duplicated *HPRT* exons was detectable (7); we show here that these events are in the minority. Liang et al. (30) assayed both homologous and nonhomologous recombination in direct *neo* repeats in CHO cells. That study focused on the key role that Ku80 plays in micro-SSA-mediated nonhomologous recombination, and although homologous recombination products were detected, it was not determined whether these products resulted from gene conversion, SSA, or unequal exchange; our data would suggest that they are primarily gene conversions. Sargent et al. (56) selected for loss of function at a partial, direct duplication of *APRT*, which could occur by nonhomologous or homologous recombination (including gene conversion and pop-outs), and concluded that nonhomologous recombination was more frequent than homologous recombination. However, gene conversion required a minimum tract length of 700 bp, and we found that 88% of products had tracts of <700 bp, suggesting that the *APRT* system imposed strong pressure against the dominant homologous recombination mechanism. Our data further shows that nonconservative SSA, the primary mode of homologous recombination in extrachromosomal substrates (9, 13, 32, 57), may occur rarely or not at all in chromosomes. Instead, DSB-induced homologous recombination in chromosomes is mediated almost exclusively by a conservative mechanism that leads to gene conversion.

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