Strand Invasion and DNA Synthesis From the Two 3′ Ends of a Double-Strand Break in Mammalian Cells

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ABSTRACT

Analysis of the crossover products recovered following transformation of mammalian cells with a sequence insertion ("ends-in") gene-targeting vector revealed a novel class of recombinant. In this class of recombinants, a single vector copy has integrated into an ectopic genomic position, leaving the structure of the cognate chromosomal locus unaltered. Thus, in this respect, the recombinants resemble simple cases of random vector integration. However, the important difference is that the two paired 3′ vector ends have acquired endogenous, chromosomal sequences flanking both sides of the vector-borne double-strand break (DSB). In some cases, copying was extensive, extending >16 kb into nonhomologous flanking DNA. The results suggest that mammalian homologous recombination events can involve strand invasion and DNA synthesis by both 3′ ends of the DSB. These DNA interactions are a central, predicted feature of the DSBR model of recombination.

THE involvement of DNA ends in recombination has been classified according to two types of models. One-sided invasion (OSI) models require the participation of only one 3′ end in recombination and generate primarily noncrossover products (Belmaaza and Chartrand 1994; Pâques and Haber 1999; Allers and Lichten 2001; Cromie *et al.* 2001; Hunter and Kleckner 2001). Consequently, OSI is considered important in the repair of free DNA ends, such as those generated by the reversal of a replication fork, or when a replication fork encounters a single-strand interruption or blockage (Pâques and Haber 1999; Cromie *et al.* 2001). In contrast, the double-strand-break-repair (DSBR) model (Orr-Weaver *et al.* 1981; Szostak *et al.* 1983) and its later revision (Sun *et al.* 1991) require 5′-to-3′ resection on both sides of the double-strand break (DSB) for the strand invasion and DNA synthesis steps to generate the stable, double-Holliday-junction intermediate. In meiotic recombination in *Saccharomyces cerevisiae* and in mitotic recombination in *Escherichia coli* and mammalian cells, DSBR is considered to primarily generate crossover products (Pâques and Haber 1999; Allers and Lichten 2001; Baker and Birmingham 2001; Cromie *et al.* 2001; Hunter and Kleckner 2001), although, through reversal of strand invasion, noncrossover products might also be generated (Gilbertson and Stahl 1996; Foss *et al.* 1999).

Experimental support for OSI has come from gene-targeting experiments (for example, Song *et al.* 1987; Adair *et al.* 1989; Ellis and Bernstein 1989; Belmaaza *et al.* 1990; Jasin *et al.* 1990; Pennington and Wilson 1991; Aratani *et al.* 1992). Similar events are also associated with inter- and intrachromosomal gene conversion (Gloor *et al.* 1991; Malkova *et al.* 1996; Johnson and Jasin 2000; Richardson and Jasin 2000; Kraus *et al.* 2001; Zhou *et al.* 2001). In these studies, recombinants bear genetic information on only one side of the region of homology, suggesting strand invasion and the priming of DNA synthesis by a single, free 3′ end. In a related study, two-strand invasion and DNA synthesis events by the outwardly pointing 3′ ends in transfected DNA ("ends-out"configuration) has been proposed in break-induced replication in *S. cerevisiae* (Morrow *et al.* 1997).

In contrast to OSI, evidence supporting the involvement of the two paired 3′ ends of a DSB ("ends-in" configuration), as proposed in the DSBR model, is lacking. A complication of distinguishing recombination events involving a single, free 3′ end from those involving the two paired 3′ ends of a DSB is that the predicted outcome of both events can be the same when the gene conversion tract is confined within the region of shared homology. For example, DSB-induced bidirectional gene conversion tracts were observed during intrachromosomal recombination in yeast (Pâques *et al.* 1998, 2001; Holmes and Haber 1999) and in mammalian cells (Donoho *et al.* 1998; Elliott *et al.* 1998; Elliott and Jasin 2001). As noted by the authors, conversion tracts did not extend outside the region of homology and therefore the results are consistent with recombination models involving either bistranded invasion or OSI, in which leading-strand synthesis from one invading strand is coupled to lagging-strand synthesis using the D loop as a comple-

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ment (Pâques and Haber 1999, 2001; Cromie *et al.* 2001).

In the present study, we analyzed the crossover products of recombination generated by the interaction of an ends-in vector with its cognate chromosomal locus in mammalian cells. These studies revealed a novel class of recombinant in which the two 3′ ends of the DSB in a single vector molecule copied flanking chromosomal sequences extending well beyond the region of shared homology. Thus, our results support the involvement of the two paired 3′ ends of a DSB in the strand invasion and DNA synthesis steps of homologous recombination, as proposed in the DSBR model.

MATERIALS AND METHODS

Hybridoma cell lines and plasmids: The igm482 hybridoma cell line was used as the recipient for transfection. It bears a single copy of the trinitrophenyl (TNP)-specific, chromosomal immunoglobulin μ heavy chain gene (Figure 1) that serves as the target for homologous recombination and has been described previously (Köhler *et al.* 1982; Baumann *et al.* 1985). The 13.4-kb enhancer-trap sequence insertion vector pCμEn$^{-}{}_{M1-6}$ has been described elsewhere (Ng and Baker 1999). It bears a 5.8-kb segment of homology to the μ-gene constant (Cμ) region inserted into a derivative of pSV2neo from which the SV40 early region enhancer has been deleted. Site-directed mutagenesis was used to create six diagnostic restriction enzyme sites in the vector-borne Cμ region that distinguish it from the corresponding endogenous sites in the igm482 chromosomal Cμ region. Apart from these modifications, the vector-borne and chromosomal Cμ regions are isogenic.

Vector transfer and isolation of transformants: Cut vector DNA (8.7 pmol) was introduced into 2×10^7 recipient hybridoma cells by electroporation (Baker *et al.* 1988). Independent G418^R transformants were segregated immediately after electroporation and recovered in 96-well tissue culture plates according to previously described conditions (Ng and Baker 1999).

Southern, PCR, and genetic marker analysis: For Southern analysis, hybridoma genomic DNA was prepared as described (Gross-Bellard *et al.* 1973). Restriction enzymes were purchased from New England Biolabs (Mississauga, ON, Canada) and MBI Fermentas (Burlington, ON, Canada) and used in accordance with the manufacturer's specifications. Gel electrophoresis, transfer of DNA onto nitrocellulose membrane, ^{32}P-labeled probe preparation, and hybridization were performed according to standard procedures (Sambrook *et al.* 1989). Vector copy number was determined by densitometry. In addition to Southern analysis, PCR amplification was used to detect the specific upstream and downstream vector:chromosome junction fragments in recombinants where both 3′ ends of the vector-borne DSB copied sequences from the target chromosomal μ-locus. The upstream vector:chromosome junction fragment was amplified with primers AB9703 (5′-CTACTTGA GAAGCCAGGATCTAGG-3′) and AB8534 (5′-CTTACCGCT GTTGAGATCCAGT-3′) to generate a 4.8-kb product. The downstream vector:chromosome junction fragment was amplified with primers AB22339 (5′-CCAACGGCGACCTGTATAA CGTGT-3′) and AB9438 (5′-GTACCATCAGACTGCACTGTT CCA-3′) to generate a 7.1-kb product or with primers AB22339 and AB5670 (5′-AGGCAGGTGACTGTGGCTGACT-3′) to generate a 3.9-kb product. In all recombinants, the endogenous Cμ region was amplified using primers 5′CμF (5′-TGGACTGTTCT GAGCTGAGATGAG-3′) and AB9438 to generate a specific 9.1-kb product. The PCR primers were synthesized at MOBIX (McMaster University, Hamilton, ON, Canada) and Sigma (Oakville, ON, Canada). Details regarding primer binding sites and PCR amplification conditions have been given previously (Ng and Baker 1999; Li and Baker 2000). For amplification products >5.0 kb, the Expand long template PCR system was used as specified by Roche Molecular Biochemicals (Laval, QC, Canada). The gel analysis methods used in the assignment of Cμ region genetic markers have been detailed previously (Ng and Baker 1999).

RESULTS

Experimental system: The experimental system detects G418^R transformants generated following transfection of an enhancer-trap gene-targeting vector into the igm482 mouse hybridoma cell line containing a single copy of the chromosomal immunoglobulin μ-gene (Figure 1) and has been described previously (Baker *et al.* 1988; Ng and Baker 1998, 1999). In the present study, we present the detailed characterization of a novel class of recombinant in which a single copy of the gene-targeting vector is integrated into an ectopic position in the hybridoma genome, yet bears the signature of a recombinational interaction with the target μ-locus, specifically, the copying of nonhomologous chromosomal sequences by both 3′ ends of the vector-borne DSB.

Bidirectional copying by both 3′ ends of a single vector molecule: A total of 1263 independent G418^R transformants, each arising from the expansion of a single G418^R cell, was generated in hybridoma cell transfections involving the *Xba*I-linearized, enhancer-trap sequence insertion (O-type or ends-in) vector pCmEn$^{-}{}_{M1-6}$ (Ng and Baker 1999; Figure 1). To screen for recombinants in which copying of chromosomal DNA flanking the vector-borne DSB at *Xba*I had occurred, *Eco*RI-digested genomic DNA was analyzed with probe fragments B and XR, which reside 4.7 kb upstream and 5.1 kb downstream of the DSB, respectively. Both probes are specific for chromosomal DNA flanking the region of shared homology (Figure 1). The Southern analysis identified 41 independent G418^R transformants in which the transfected vector had copied endogenous, chromosomal μ-locus sequences. The balance of 1222 G418^R transformants fell into two classes distinct from the above. In 42 cell lines, the transfected vector was correctly integrated into the chromosomal μ-locus by gene targeting as detailed previously (Ng and Baker 1999). The vast majority of the remaining transformants consisted of simple random vector integration events in which the endogenous μ-locus was intact and in which one or more vector bands lacked endogenous μ-gene sequences.

As an example of the Southern analysis, Figure 2 presents *Eco*RI-digested genomic DNA from representa-

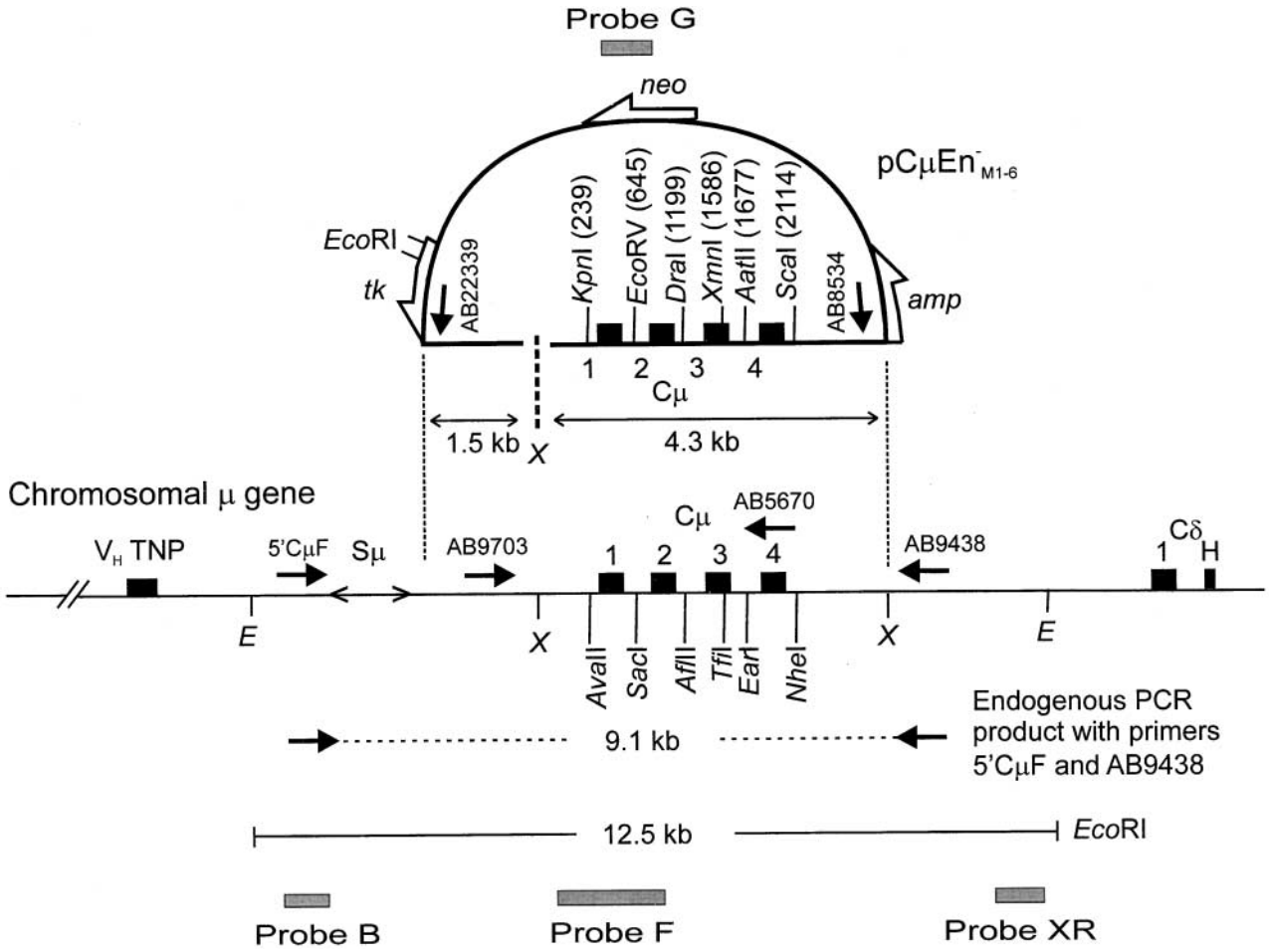

FIGURE 1.—Recombination between transfected and chromosomal DNA. The 13.4-kb enhancer-trap sequence insertion vector pCμEn$^{-}_{M1-6}$ (NG and BAKER 1999) shares a 5.8-kb region of homology with the chromosomal μ-gene beginning downstream of the Sμ region and extending to the *Xba*I site downstream of the μ-gene Cμ region, which was converted to a *Sal*I site for cloning (BAKER *et al.* 1988). Homology lengths of 1.5 and 4.3 kb reside to the left and right, respectively, of the unique DSB site at *Xba*I. The indicated restriction enzyme site polymorphisms distinguish the vector-borne and chromosomal Cμ regions as described previously (NG and BAKER 1999). The distance (in base pairs) of each marker from the DSB at *Xba*I is presented. The location of the vector-specific PCR primers AB22339 and AB8534 is shown. The structure of the recipient haploid TNP-specific chromosomal immunoglobulin μ heavy chain gene is presented below pCμEn$^{-}_{M1-6}$. Relevant regions of the chromosomal μ-gene include the TNP-specific heavy-chain variable region (V_HTNP), switch μ (Sμ) region, Cμ region exons 1–4, and the δ gene constant (Cδ) region exons 1 and H. The positions of primers AB9438, AB9703, AB5670, and 5′ CμF are indicated. For further details regarding the primers, refer to MATERIALS AND METHODS. Probe B is a 915-bp *Sst*I fragment; probe XR is a 913-bp *Xho*I/*Eco*RI fragment; probe F is an 870-bp *Xba*I/*Bam*HI fragment; probe G is a 762-bp *Pvu*II fragment from the *neo* gene of pSV2neo. *E*, *Eco*RI; *X*, *Xba*I; *tk*, thymidine kinase; *neo*, neomycin phosphotransferase; *amp*, ampicillin. The figure is not drawn to scale.

tive transformants probed with fragment XR, diagnostic of copying events downstream of the DSB. The endogenous igm482 μ-gene resides on the 12.5-kb *Eco*RI fragment. The blot shows random transformants (denoted R) bearing the endogenous μ-gene and correctly targeted recombinants (denoted T), in which the gene-targeting event has replaced the endogenous μ-gene fragment with a specific 9.6-kb *Eco*RI fragment diagnostic of the linkage between the vector-borne *Eco*RI site and the chromosomal *Eco*RI site downstream of Cμ (Figure 1; as indicated in the targeted control cell line, 118-2; NG and BAKER 1999). In addition, the blot reveals transformants 7-1, 26-1, 43-1, 62-1, and 77-2 in which the endogenous 12.5-kb *Eco*RI μ-gene fragment is accompanied by another μ-hybridizing fragment of similar intensity. In recombinants 26-1 and 43-1, the second μ-band is 9.6 kb, consistent with a copying event extending to at least the endogenous *Eco*RI site residing downstream of Cμ (Figure 1). In cell lines 7-1, 62-1, and 77-2 the second band is of a variable size, suggesting that copying has not reached this *Eco*RI site. Hybridoma cell line igm10 is an igm482-derived mutant (KÖHLER and SHULMAN 1980; KÖHLER *et al.* 1982) that has lost the endogenous μ-gene and was included as a control for probe specificity.

Of the 41 transformants that had copied nonhomolo-

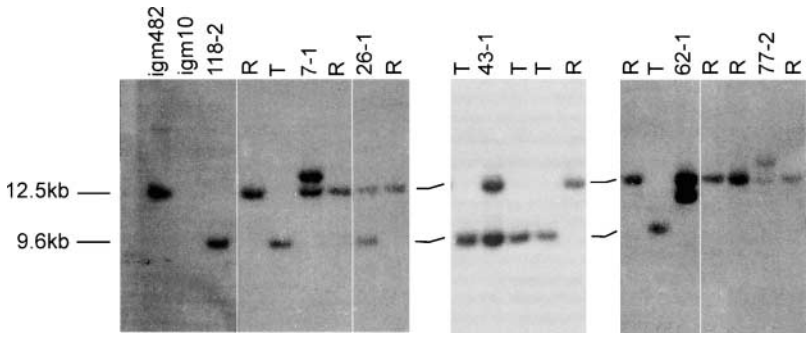

FIGURE 2.—Representative Southern blot of transformant screening. Transformant genomic DNA was digested with *Eco*RI and analyzed by Southern blotting with probe XR, which is diagnostic for copying events downstream of the DSB. The endogenous μ-gene in igm482 resides on the 12.5-kb *Eco*RI fragment. Random transformants bear only the 12.5-kb endogenous fragment, while, in targeted recombinants, this fragment is replaced by a 9.6-kb *Eco*RI band as shown in the control cell line 118-2. Transformants 7-1, 26-1, 43-1, 62-1, and 77-2 bear an endogenous 12.5-kb *Eco*RI μ-gene fragment as well as another μ-hybridizing fragment of similar intensity. Hybridoma cell line igm10 is an igm482-derived mutant that has lost the endogenous μ-gene and was included as a control for probe specificity. T, targeted recombinant; R, random integrant.

gous chromosomal sequences, seven cell lines contained three distinct *Eco*RI fragments: the endogenous μ-gene present on the 12.5-kb fragment (Figure 1) and two additional bands hybridizing to probes B and XR. These features are expected of a novel class of recombinant in which both 3′ ends of the DSB in the transfected vector have participated in the copying of nonhomologous chromosomal sequences. In the remaining 34 transformants, the endogenous 12.5-kb *Eco*RI μ-gene fragment was accompanied by a second probe B- or XR-hybridizing fragment. The frequency of cell lines bearing either of the latter fragments was equivalent. These features are consistent with the copying of nonhomologous chromosomal sequences on only one side of the vector-borne DSB. To investigate the possibility that some of the cell lines displaying unidirectional copying might have performed bidirectional copying that simply failed to extend far enough to incorporate one of the probe binding sites, PCR analysis was used to test for the presence of upstream or downstream vector:chromosome junction fragments. Primers AB22339 and AB9438 or AB22339 and AB5670 were used to amplify the downstream junction fragment, while primers AB8534 and AB9703 were used to amplify the upstream junction fragment. Details pertaining to each primer are presented in MATERIALS AND METHODS and elsewhere (NG and BAKER 1999; LI and BAKER 2000; LI *et al.* 2001). This analysis revealed a further 6 recombinants (bringing the total to 13) displaying evidence of bidirectional copying.

Out of concern that primary screening by Southern analysis might have underestimated the frequency of recombinants displaying bidirectional copying, a representative sample composed of 258 of the original 1263 $G418^R$ transformants was screened by PCR for the specific upstream and downstream vector:chromosome junction fragments. However, the PCR screening did not reveal any additional recombinants that had not been initially identified by Southern analysis. Thus, we are relatively certain that the Southern screening identified the vast majority of recombinants in which copying extended into chromosomal sequences flanking the vector-borne region of homology.

We next addressed the issue of whether chromosomal sequences flanking the DSB were attached to a single vector molecule. As shown in Figure 1, probe F detects both the vector-borne and chromosomal Cμ regions. Therefore, in Southern analysis of *Eco*RI-digested genomic DNA, within-lane comparison of the intensity of the vector-borne band(s) with the single copy, endogenous μ-locus reveals the plasmid copy number. Further, cell lines bearing a single vector copy can be verified independently by rehybridization with *neo*-specific probe G since it is expected to cohybridize to the same *Eco*RI fragment detected with probe F (Figure 1). These determinations revealed that of the 41 recombinants identified originally, 17 contained a single vector copy. Within this group were seven examples of bidirectional copying (cell lines 8-2, 25-2, 26-1, 30-2, 68-4, 88-2, and 116-6), with the remaining 10 cell lines displaying unidirectional copying.

To summarize, of the 41 recombinants in which copying of nonhomologous chromosomal sequences was originally detected, in 13 copying proceeded bidirectionally from the vector-borne DSB. Vector copy number determinations revealed the unique property of copying from the two 3′ ends of a single vector-borne DSB in the 7 recombinants indicated above. This may have been also true for the other 6, but in the presence of more than one vector copy, this was not easily determined. The remaining 28 recombinants displayed copying on only one side of the DSB. Such unidirectional copying of nonhomologous chromosomal sequences is consistent with OSI by a single 3′ end (BELMAAZA and CHARTRAND 1994; PÂQUES and HABER 1999; CROMIE *et al.* 2001).

Genetic marker analysis: As indicated above, the identification of additional bidirectional recombinants was aided by the use of PCR analysis with primers AB9703 and AB5670, which are not chromosome specific. Thus, it might be argued that the observed PCR amplification was not the result of bidirectional copying, but rather made possible by the acquisition of a primer binding site from another plasmid through nonhomologous recombination as suggested by the studies of SAKAGAMI *et al.* (1994). However, the following results rule out this interpretation and strongly support bidirectional copying in the recombinants.

As illustrated in Figure 1 and described previously

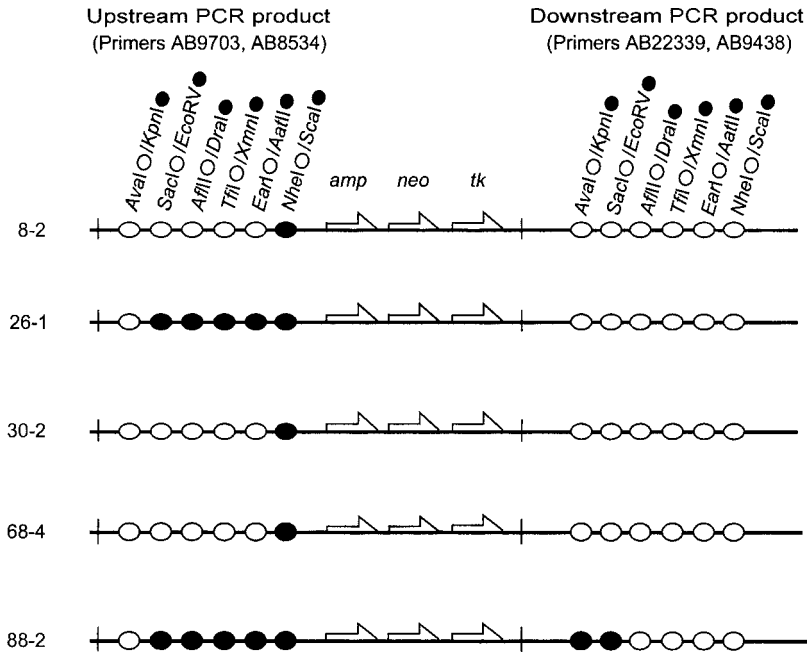

FIGURE 3.—Genetic marker analysis. Analysis of Cμ region genetic markers in the upstream and downstream PCR products produced by primer pairs AB9703 and AB8534 and AB22339 and AB9438, respectively. PCR products were digested with each of 12 restriction enzymes to determine the contribution of chromosomal (open ovals) and vector (solid ovals) sequences. The position corresponding to the *Xba*I site of vector linearization is indicated by the vertical line. The figure is not drawn to scale.

(NG and BAKER 1999), six pairs of restriction enzyme site polymorphisms distinguish the vector-borne and endogenous Cμ regions. Thus, chromosomal markers are expected to be linked to 3′ vector ends that have invaded and copied chromosomal Cμ region sequences. To examine this, PCR was used to amplify the upstream and downstream junction fragments in the representative recombinants 8-2, 26-1, 30-2, 68-4, and 88-2 as described above. As part of this analysis, a 9.1-kb endogenous Cμ region product was also amplified from each recombinant using primers 5′CμF and AB9438 (Figure 1). All PCR products were digested separately with the 12 diagnostic restriction enzymes. The gel analysis methods used to assign chromosome- and vector-specific markers to their respective Cμ region positions have been detailed previously (NG and BAKER 1999).

In each recombinant, the chromosomal Cμ region retained all of the original markers (Figure 1), indicating that it was unmodified by the 3′ end invasion and copying events. As shown in Figure 3, a chromosome-specific marker(s) resided at the terminus of both the upstream and downstream PCR products as expected for bidirectional copying of chromosomal sequences by the two 3′ ends of the DSB. Varying amounts of degradation from the terminus of the vector arm residing to the right of the DSB followed by repair synthesis provide an explanation for the acquisition of one or more chromosomal markers in the upstream PCR product. Similarly, copying of chromosomal sequences by the vector arm to the left of the DSB accounts for the chromosomal markers in the downstream PCR product of recombinants 8-2, 26-1, 30-2, and 68-4. The downstream PCR product of recombinant 88-2 bears both vector-borne and chromosomal markers, suggesting a contribution of sequence information to the newly synthesized strand by the vector arm residing to the right of the DSB. This may have occurred through mismatch repair of the endogenous μ-locus or during DNA synthesis by template switching (PÂQUES and HABER 1999). The unaltered endogenous μ-locus in the recombinants favors the latter explanation.

Extensive copying of chromosomal sequences: To characterize the copying of flanking chromosomal sequences more fully, more extensive Southern analysis with additional restriction enzymes was performed (data not shown). Figure 4 presents the structure of the entire chromosomal μ-region and summarizes the extent of bidirectional copying in the recombinants. For clarity, the vector-borne region of homology shared with the chromosome is represented by the thick solid lines. Shaded lines denote the length of copied chromosomal sequences, while their extension as dashed lines indicates the position at which copying terminates or the known restriction enzyme map ends. The results indicate extensive copying of nonhomologous chromosomal sequences by both 3′ ends of the transferred vector. In recombinant 26-1, chromosomal sequences extending beyond the downstream *Psh*AI site were attached to the vector, indicating that copying extended >16.2 kb from the vector-borne DSB. In the remaining recombinants, the extent of chromosomal sequences acquired by the two 3′ ends of the vector was less extensive, but still considerable—on average, ~6 kb.

DISCUSSION

In the present study, the analysis of the crossover products of recombination generated by the interaction of a sequence insertion (O-type or ends-in) vector with its cognate chromosomal locus in mammalian cells has revealed a novel class of recombinant, one in which the two 3′ ends of the DSB have both participated in the

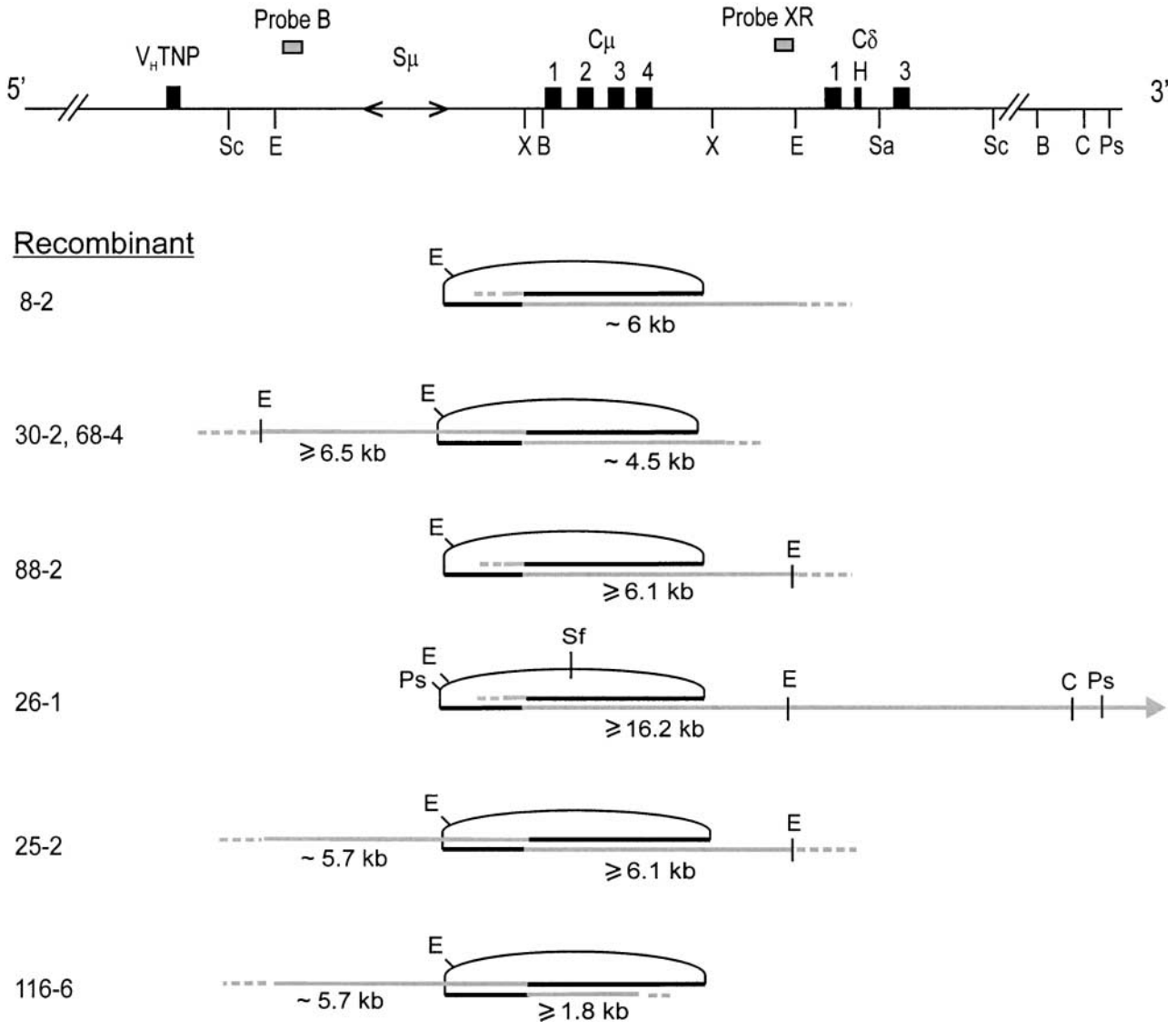

FIGURE 4.—Detailed Southern analysis of bidirectional copying events. (Top) The structure of the haploid chromosomal μ-δ constant region is a reference for (bottom) the extent of copying of nonhomologous chromosomal sequences in each recombinant. The vector-borne region of homology shared with the chromosome is represented by the thick solid lines. Shaded lines denote the length of copied chromosomal sequences, and their termination in dashed lines indicates the position at which copying terminates or at which the known restriction enzyme map ends. The approximate or minimum distance of copying from the DSB is indicated below each recombinant. B, *Bst*Z171; C, *Cla*I; E, *Eco*RI; Ps, *Psh*AI; Sa, *Sac*I; Sc, *Sca*I; Sf, *Sfi*I; X, *Xba*I. The figure is not drawn to scale.

strand invasion and DNA synthesis steps of homologous recombination. Potentially, the involvement of the two 3′ ends of the DSB in recombination can generate an intermediate bearing two Holliday junctions, which is the centerpiece of the DSBR model (SZOSTAK *et al.* 1983). It is relevant to consider the bidirectional recombinants in the context of the DSBR model because our previous studies of mammalian gene targeting are consistent with the requirement for an intermediate bearing two Holliday junctions in the step preceding crossover (LI and BAKER 2000; BAKER and BIRMINGHAM 2001).

In this study, the number of recombinants demonstrating uni- and bidirectional copying are similar, and both types are recovered alongside recombinants that have undergone proper crossing over with the chromosome. Although the recombinants might result from completely unrelated genetic events, it seems more likely that they represent different potential outcomes of one process. The crossing-over reaction associated with DSBR would appear to require proper regulation between the two 3′ ends to generate the double Holliday junction intermediate and to avoid extensive leading-strand DNA synthesis (SZOSTAK *et al.* 1983; GILBERTSON and STAHL 1996; CROMIE *et al.* 2001). Therefore, in this assay for

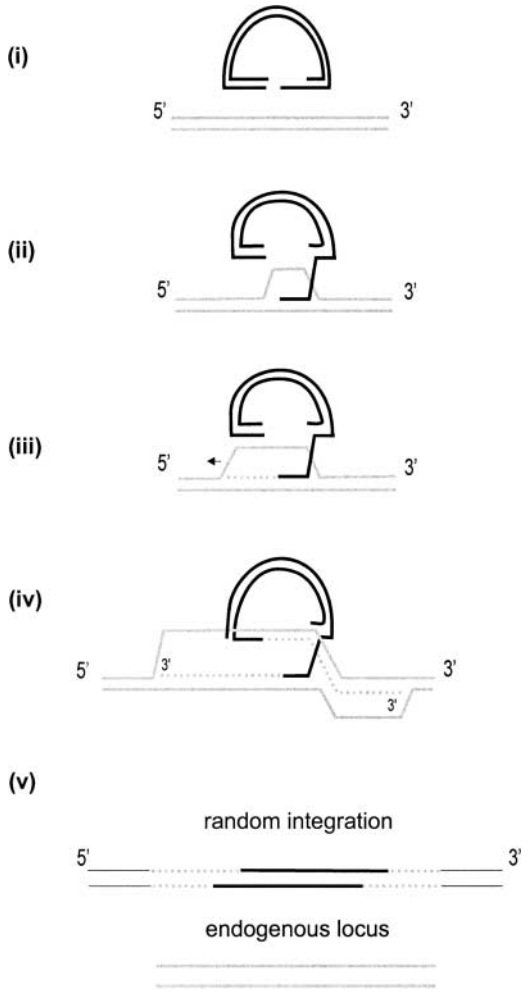

Figure 5.—Proposed model for bidirectional copying of nonhomologous chromosomal sequences. In the intermediate presented in i, only one side of the vector-borne DSB is available for strand invasion. Strand invasion of the homologous chromosomal duplex (shaded line) by a single-strand, 3′-ending vector tail (solid line) initiates recombination, and ii is accompanied by D-loop displacement. In iii, leading-strand DNA synthesis (dashed shaded lines) primed from the invading 3′ end proceeds into nonhomologous chromosomal sequences prior to the involvement of the second 3′ end. Eventually, the second 3′ end may anneal with the extended D loop and initiate the second round of leading-strand DNA synthesis, but at this stage, the stable double Holliday junction (crossover) intermediate cannot form. In iv, the D loop is further enlarged as copying on both sides of the vector-borne DSB proceeds into regions of nonhomology, and finally, in v, the vector unwinds from the target locus and integrates elsewhere in an ectopic genomic position.

crossover recombinants, bidirectional copying would be consistent with the failure to properly regulate the two sequentially recombining 3′ ends of the DSB. A proposed mechanism is presented in Figure 5. Leading-strand DNA synthesis might initiate from one invading 3′ end before the second 3′ end is properly formed or available for complementary base pairing. The failure of the second 3′ end to properly engage would be expected to generate recombinants bearing unidirectional conversion tracts as observed. Eventually, the second 3′ end might become available for recombination, following which it pairs with the D loop and initiates DNA synthesis. However, at this late stage, copying from the first invading strand might have progressed beyond the 3′ end of the second strand, precluding formation of a stable Holliday junction intermediate. In this case, recombination is aborted, and the plasmid unwinds from the locus and integrates elsewhere in the genome. While all recombinants displaying bidirectional copying are consistent with the events depicted in Figure 5, it should be noted for completeness that the rather limited copying that is confined to one vector arm in recombinants 8-2, 26-1, and 116-1 is also consistent with another explanation. In these latter recombinants, one 3′ DNA end may have invaded and copied nonhomologous chromosomal sequences and then ejected from the target locus. Following this, limited invasion and synthesis of the end-extended arm by the remaining 3′ vector end might have occurred.

A contrasting view to the events depicted in Figure 5 is that of two independent strand invasion and copying events. According to this scheme, one 3′ end would initiate strand invasion and DNA synthesis, after which it would be ejected from the endogenous locus. Following this, the second 3′ end would initiate strand invasion and DNA synthesis, followed by its ejection from the target locus. In this study, the frequency of unidirectional, single-copy recombinants was 0.0079 (10/1263) and therefore two independent events are expected at the frequency of $0.0079^2 = 0.000062$. However, bidirectional, single-copy recombinants were observed at the frequency of $7/1263 = 0.0055$, a value ~89-fold higher than expected. This suggests that the two 3′ ends of the DSB do not behave independently and supports the concept that they are regulated by the cellular recombination machinery.

It is interesting to note that bidirectional copying events have not been reported previously in mammalian cells. In studies where ends-in vectors were used, the failure to detect bidirectional events may be attributed to a requirement for the recombination event to reconstitute a selectable genetic marker, to inadequate homology on both sides of the DSB, or simply, to recombinant screening procedures that were performed on only one side of the DSB (Song *et al.* 1987; Ellis and Bernstein 1989; Belmaaza *et al.* 1990; Pennington and Wilson 1991; Richard *et al.* 1994; Villemure *et al.* 1997; Adair *et al.* 1998). Bidirectional copying has also not been observed in the case of ends-out vectors, even though some studies screened specifically for these events (Adair *et al.* 1989, 1998; Jasin *et al.* 1990; Aratani *et al.* 1992; Scheerer and Adair 1994; Waldman *et al.* 1996). In an ends-out configuration, the two DNA ends point away from each other and are expected to interact independently of each other with homologous se-

quences at different genomic locations (*i.e.*, they are free ends). In contrast, in an ends-in configuration, the two DNA ends are at a single location and face each other (*i.e.*, they are paired ends; Cromie *et al.* 2001). The information presented above suggests that the DNA ends in the ends-out configuration may behave differently from those in the ends-in configuration as a consequence of differences in the way the two types of ends are regulated during recombination.

In essence, the events illustrated in Figure 5 depict the perturbation of a normal ends-in reaction required for proper crossing over with the chromosome. In the perturbed reaction, the DNA ends are now free to initiate unrestricted DNA synthesis. In gene targeting, the failure to properly regulate the two ends of the DSB appears to result in the recovery of recombinants as simple random vector integration events. In a cellular context, the impact of unrestricted DNA synthesis is expected to be more severe, leading, potentially, to the formation of an entire chromosome with associated changes in gene dosage and chromosome segregation difficulties. Thus, in both prokaryotes and eukaryotes, cellular mechanisms are in place to ensure proper regulation between the two recombining ends of a chromosomal DSB (Cromie *et al.* 2001). Complexes consisting of Mre11/Rad50/Xrs2 in *S. cerevisiae* or Mre11/Rad50/Nbs1 (MRN) in mammalian cells are leading candidates for this role and might regulate the reactivity of the DNA ends by controlling formation of 3′ single-strand tails and/or by sequestering the two 3′ ends from the potentially lethal consequences of acting like free ends (Cromie and Leach 2000; Cromie *et al.* 2001). Thus, one possible explanation for the bidirectional copying observed in this study is the failure of the MRN complex to properly regulate ends-in gene targeting either as a result of topological constraints imposed by the vector or as a consequence of reduced reactive capacity of the DNA ends, perhaps resulting from their alteration during DNA transfer. In any case, the results are consistent with the scenario whereby the failure to properly coordinate DSB ends during gene targeting abrogates formation of the stable Holliday junction intermediate proposed in the crossover reaction.

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LITERATURE CITED

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