Gene repeat expansion and contraction by spontaneous intrachromosomal homologous recombination in mammalian cells

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ABSTRACT

Homologous recombination (HR) is important in repairing errors of replication and other forms of DNA damage. In mammalian cells, potential templates include the homologous chromosome, and after DNA replication, the sister chromatid. Previous work has shown that the mammalian recombination machinery is organized to suppress interchromosomal recombination while preserving intrachromosomal HR. In the present study, we investigated spontaneous intrachromosomal HR in mouse hybridoma cell lines in which variously numbered somatic HR. In the present study, we investigated spontaneous intrachromosomal HR in mouse hybridoma cell lines in which variously numbered tandem repeats of the \(\mu\) heavy chain constant (\(C_\mu\)) region reside at the haploid, chromosomal immunglobulin \(\mu\) heavy chain locus. This organization provides the opportunity to investigate recombination between homologous gene repeats in a well-defined chromosomal locus under conditions in which recombinants are conveniently recovered. This system revealed several features about the mammalian intrachromosomal HR process: (i) the frequency of HR was high (recombinants represented as much as several percent of the total of recombinants and non-recombinants); (ii) the recombination process appeared to be predominantly non-reciprocal, consistent with the possibility of gene conversion; (iii) putative gene conversion tracts were long (up to 13.4 kb); (iv) the recombination process occurred with precision, initiating and terminating within regions of shared homology. The results are discussed with respect to mammalian intrachromosomal HR involving interactions both within and between sister chromatids.

INTRODUCTION

The breaking and rejoining of DNA is a fundamental process in living organisms. It occurs during meiosis, where recombination between homologous chromosomes generates genetic diversity, and during V(D)J recombination, the process of assembling antigen-specific receptors in cells of the mammalian B and T lineage. In addition to these specific reactions, DNA breakage and rejoining is a general feature of cellular DNA metabolic processes, requiring topoisomerases or DNA repair proteins. DNA breakage can also occur during DNA replication, when migrating replication forks are stalled or encounter a single-strand nick, and can be induced when cells are irradiated or exposed to genotoxic chemicals.

A double-strand break (DSB) in duplex DNA consists of two DNA ends at a single location facing each other. DNA DSBs are serious types of lesions, and if not properly repaired, can lead to potentially deleterious chromosomal rearrangements. Cellular repair mechanisms are available for sealing DSBs and nicks (which can be converted to DSBs), and are of two general types: non-homologous-end joining (NHEJ) and homologous recombination (HR). The process of NHEJ simply rejoins the two broken DNA ends often with some loss, or, incidental gain, of genetic material. Thus, rejoining is usually imprecise. Conversely, HR utilizes an identical or nearly identical DNA template for the repair of a DSB, and therefore, is expected to correctly restore the nucleotide sequence. In yeast, HR is considered to be more efficient than NHEJ (1), while in vertebrate cells both mechanisms appear to be important (2–7).

Three HR mechanisms have been proposed for the repair of DSBs in eukaryotes. One mechanism is single-strand annealing (SSA) (Fig. 1A) (8–10). In SSA, a DSB residing between two flanking homologous regions is resected in the 5′-to-3′ direction producing long, 3′-ending complementary single strands that anneal. After removal of non-homologous 3′ ends, and new DNA synthesis, ligation restores two continuous DNA strands. SSA is a non-conservative mechanism of HR because the excision events involved in DSB processing result in the deletion of one of the homologous repeats. A second mechanism proposes that one side of the DSB participates in recombination, and paradigms displaying this feature can be grouped under the category of one-sided invasion (OSI) models (11) (Fig. 1B). The asymmetry associated with OSI models might result from resection on only one side of the DSB. Alternatively, resection might occur on both sides of the DSB, but only one 3′ single strand participates in recombination. Eventually, the newly synthesized DNA from the
The invading strand is displaced from the template, and ligation to the non-invading homologous 3' end, which then directs a second round of repair synthesis. OSI has been proposed to generate primarily non-crossover products in both yeast and mammalian cells (1,11–16). A third mechanism, the DSB repair (DSBR) model (Fig. 1C) (17,18), proposes that the two 3’ single strands produced by DSB resection are recombinogenic, and can invade a homologous template to initiate DNA synthesis that results in formation of the joint molecule, an intermediate bearing a double Holliday junction (HJ). In support of this model, physical evidence suggests that the double HJ structure forms during recombination in Saccharomyces cerevisiae in meiosis (19,20). During meiotic recombination in S.cerevisiae, mitotic recombination in mammalian cells, and recombination in Escherichia coli, DSBR has been proposed to result in crossover products (1,13,14,16,21). Nevertheless, cellular mechanisms can exert control over the resolution of HJs (14,21–23), suggesting that in some circumstances, the recombination outcome might be biased toward non-crossover products (22).

With the exception of the non-conservative SSA mechanism, the repair of DSBs by HR is expected to be conservative, faithfully restoring the continuity of the broken DNA. However, in mammalian cells, conservative repair of the DSB is found in association with another class of recombinant, in which a homology-driven repair event extends beyond the flanking homologous sequences into non-homology. Thus, an initial HR event is completed by NHEJ, and recombinants of this type have been interpreted according to a non-crossover, OSI mechanism. Examples of this class include those in which double-stranded gaps in transfected plasmids sustain only partial homology-directed repair (24), as well as cases where the DNA synthesis associated with DSB repair during plasmid transformation, or during intra- and inter-chromosomal HR, has extended beyond the region of homology into flanking non-homologous sequences (7,12,24–28).

In contrast to DSB-induced repair events, recombination between homologous sequences also occurs spontaneously. While spontaneous HR may also be initiated by a DSB, the circumstances involved in the generation of the DSB and/or the outcome of its repair might well differ from the examples of DSB-induced HR reviewed above. In this study, we investigated spontaneous HR in mouse hybridoma cell lines in which variously numbered tandem repeats of the m heavy chain constant (Cm) region reside at the haploid, chromosomal immunoglobulin m heavy chain locus. Recombinant products were generated at high frequency, and for the most part, were largely non-reciprocal, consistent with the possibility of gene conversion. In contrast to the association between HR and NHEJ that is observed in the instances of DSB-induced repair.
recombination described above, our analysis suggests that the initiation and termination of HR is confined within the region of homology. These results are discussed with respect to mechanisms involving recombinational interactions within and between sister chromatids.

MATERIALS AND METHODS

Cell lines

The origin of the hybridoma cell lines Sp6/HL, igm482 and igm10, and the methods used for cell culture have been described previously (29,30). The E14 hybridoma cell line is based on the Sp6/HL-derived hybridoma cell line igm482, which bears a 2 bp deletion in the third exon of the \( \mu \) gene constant (C\( _\mu \)) region. The igm482 hybridoma cells were transfected with the 10 kb vector pRC\( _\mu \), which bears a 4.3 kb C\( _\mu \) segment from the wild type Sp6/HL \( \mu \) gene inserted into the plasmid pSV2neo (31). Screening of the G418-resistant (G418R) transformants by Southern analysis (data not shown) revealed the E14 hybridoma, in which three tandemly oriented copies of the pRC\( _\mu \) vector reside 3' of the igm482 \( \mu \) gene (Fig. 2). Hybridoma cell lines 43 and 122 (Fig. 2) were generated by targeted integration of a single copy of the C\( _\mu \)-bearing vector, pC\( _\mu \)palEn\(^{-}\) into the haploid chromosomal \( \mu \) locus of the recipient Sp6/HL hybridoma cell line as described (32) [the vector pC\( _\mu \)palEn\(^{-}\) is a pSV2neo derivative and, for other purposes, bears an inserted HSV-1 thymidine kinase (tk) gene (32)]. The two cell lines are distinguished by the replacement of endogenous SacI, Al\( _\mu \)III and Ap\( _\mu \)I sites in one member of the C\( _\mu \) region pair with a 30-bp palindromic genetic marker bearing a diagnostic NotI site. The endogenous SacI, Al\( _\mu \)III and Ap\( _\mu \)I sites are located at genomic C\( _\mu \) positions 503, 1053 and 1765 bp, respectively, according to the numbering system presented in Goldberg et al. (33).

DNA and IgM analysis

High-molecular weight DNA was prepared from the various hybridoma cell lines according to Gross-Bellard et al. (34). Restriction enzymes were purchased from New England Biolabs Inc. (Beverley, MA), Amersham Pharmacia Biotech Inc. (Baie d’Urfé, Quebec), and Canadian Life Technologies Inc. (Burlington, Ontario), and used in accordance with the specifications of the manufacturer. Gel electrophoresis, DNA blotting onto nitrocellulose, acid treatment of blots,
32P-labeled DNA probe fragment preparation and hybridization were performed by standard procedures (35). Quantification of band intensity in Southern analysis was performed by phosphorimager and densitometric analysis.

**PCR amplification**

PCR was used to specifically amplify the recombinant Cμ regions according to conditions described previously (36). The sequence of oligonucleotide primers AB9703, AB9745, AB22339 and AB9438, and their binding site locations have been presented earlier (21,32,36). A new primer synthesized for this study, AB15321 (5’ CAGACAGAGAAAGCCAGACTCATA 3’), binds 5’ of the chromosomal μ gene switch (Sμ) region.

**RESULTS**

**Experimental set up**

The mouse hybridoma cell lines E14, 43 and 122 (Fig. 2), are based on the wild type hybridoma Sp6/HL, which bears a single copy of the chromosomal immunoglobulin μ heavy chain gene and synthesizes IgM (6) specific for the hapten trinitrophenyl (TNP) (29,30). The haploid chromosomal μ heavy chain gene in these cell lines hosts variously numbered tandem repeats of the μ heavy chain constant (Cμ) region, which function as substrates for spontaneous HR. In the E14 hybridoma cell line, the chromosomal μ locus contains four tandem Cμ region copies integrated 3’ of the TNP-specific, heavy chain variable (VH/TNP) region, while in hybridoma cell lines 43 and 122, the chromosomal μ locus contains two tandem Cμ region copies. A distinguishing feature of the chromosomal μ gene in hybridoma cell lines 43 and 122 is the replacement of endogenous SacI, AflII and Apal sites in one member of the Cμ region pair with a 30-bp palindromic marker containing a diagnostic NotI restriction enzyme site. Small palindromic sequences can form hairpin loops when encompassed in heteroduplexes with the wild type sequence (37). These structures are semi-refractory to mismatch repair (MMR) (32,37–39), which can result in two distinct daughter genotypes after replication. Thus, a colony derived from a single cell in which hDNA has formed during recombination may have a mixed genotype, consisting of two cell populations; one bearing the NotI palindromic marker, and the other, the corresponding endogenous restriction enzyme site. Such mixed or ‘sectored’ genotypes have been observed in yeast after postmeiotic segregation (40) or mitotic recombination (41).

**Spontaneous intrachromosomal HR between Cμ repeats in E14**

The structure of the E14 chromosomal μ gene is based on detailed Southern analysis (data not shown) performed with restriction enzymes recognizing internal sites within the transfected pRCμ vector used in E14 construction (XmnI, HincII, BglII and ScaI), as well as restriction enzymes whose recognition sites are external to the Cμ region (Xbal, EcoRI) (Fig. 2). This analysis suggests that the tandem Cμ array at the chromosomal μ locus in E14 resides on EcoRI and Xbal fragments of 42.5 and 34.3 kb, respectively. However, digestion with the non-cutters EcoRI and Xbal also revealed several secondary μ bands that were not visible with any of the internal cutters. In the case of the EcoRI digest, secondary μ bands of 22.5, 32.5, ~52 and ~62 kb were visible, while for the Xbal digest, they were present on fragments of 14.3, 24.3, ~44 and ~54 kb (Fig. 3A). The same results were obtained using neo probe G and probe N, of which the latter probe has a single binding site within the major μ gene intron (Fig. 2). The visible μ bands form a ladder that varies by multiples of 10 kb according to the unit length of the transfected pRCμ vector. Thus, the μ bands differ in size, and not with respect to internal detail, consistent with the structures presented in Figure 3B. The results suggest that the secondary μ bands arise spontaneously in the E14 culture as a result of HR between members of the tandem Cμ array, and that they represent deletion and amplification recombinants in which the size of the chromosomal μ gene is reduced or increased, respectively, by an incremental unit of the pRCμ vector.

In the blots, control lanes include genomic DNA from the igm482 hybridoma cell line in which the single copy chromosomal immunoglobulin μ gene resides on the 12.5 kb EcoRI fragment, as well as the igm10 hybridoma cell line (29,30), an Sp6-derived mutant that has lost the chromosomal μ gene and serves as a negative control for probe specificity. Absent in the digests are deletion products indicative of a μ gene bearing a single Cμ region without associated pSV2neo vector sequences, namely, the 12.5 kb EcoRI fragment and the 4.3 kb Xbal fragment. However, this was expected since hybridoma cells lacking the neo gene would not survive in the G418-supplemented medium used in culturing E14.

**Analysis of μ gene structure in individual E14 subclones**

It was important to determine whether all of the recombinant μ genes resided within every cell in the E14 culture, or, whether E14 became heterogeneous during growth in culture as a consequence of the segregation of the various recombinant μ gene products to different daughter cells. To distinguish between these alternatives, E14 was cloned at 0.1 cell/well in 96-well tissue culture plates in medium lacking G418, and a total of 37 independent subclones were isolated. Approximately 28 hybridoma culture doublings elapsed from the time of plating until the recovery of individual subclones. This suggested that there were no obvious differences in the growth characteristics and/or the relative stability of the subclones. Genomic DNA prepared from each subclone was digested with EcoRI and Southern analysis was performed using Cμ probe F. Representative examples are presented in Figure 4. Each subclone is characterized by a primary μ gene corresponding in size to one shown in Figure 3A and B. The frequency of subclones in which a particular size μ gene is the primary fragment is in general agreement with the intensity of that particular fragment in E14 genomic DNA (Table 1). Thus, the heterogeneity in the E14 culture results from intrachromosomal HR within the tandem Cμ array generating secondary μ genes that segregate to different daughter cells. In addition, given that the E14 hybridoma culture was grown for no more than ~35 doublings prior to the preparation of genomic DNA for Southern analysis (~18 h per doubling), the data also indicate a high frequency of intrachromosomal HR within the tandem Cμ array (~65% total rearrangement products after 35 hybridoma culture doublings).
Figure 3. Analysis of secondary chromosomal \( \mu \) genes in E14. (A) The blot presents the results of Southern analysis of E14 genomic DNA digested with either EcoRI or XbaI and hybridized with probe fragment F. The size (in kb) of each \( \mu \) gene of interest is shown on the left of the blots while high-molecular weight marker bands (denoted M) are presented on the right. (B) Structure of the secondary chromosomal \( \mu \) genes in E14. The diagrams illustrate the proposed overall structure of the secondary recombinant \( \mu \) based on the results of Southern analysis. Abbreviations and symbols are as indicated in the legend to Figure 2.
Figure 4 reveals that the primary μ gene characterizing each E14 subclone is itself prone to ongoing intrachromosomal HR. In some subclones, the primary tandem Cm array was rearranged to generate both deletion and amplification products. For example, in subclones 6 and 14, the primary μ gene is the 32.5 kb EcoRI fragment consistent with three tandem Cm regions (Fig. 3B), and in these subclones, amplification and deletion products present on 42.5 and 22.5 kb EcoRI fragments, respectively, are visible (Fig. 4). Similarly, in E14 subclones 9 and 10, in which the primary μ gene resides on the 22.5 kb EcoRI fragment (from Fig. 4, subclones 4, 5, 9, 10, 16 and 17), secondary EcoRI fragments of 12.5 or 32.5 kb are visible, consistent with Cm deletion and triplication products, respectively (Fig. 3B). Unlike the parental E14 cells grown under G418-selective conditions (Fig. 3A), the spontaneous rearrangement products observed in the various E14 subclones arise in the absence of G418 selection, and this provides an explanation for the presence of the unit size chromosomal μ gene on the 12.5 kb EcoRI fragment in several of the subclones (for example, subclones 1, 8, 9 and 10) (Fig. 4).

Analysis of intrachromosomal HR in hybridoma cell lines bearing a chromosomal Cm region duplication

To examine the intrachromosomal HR process in more detail, further studies were performed with hybridoma cell lines 43 and 122, in which the primary chromosomal μ gene is simpler consisting of only a pair of recombining Cm regions. Thus, in this respect, it is similar to the chromosomal Cm region duplication in E14 subclones 4, 5, 9, 10, 16 and 17 (Fig. 4). However, a defining characteristic of the Cm region duplication in hybridomas 43 and 122, is that it resides on 16.2 and 9.6 kb EcoRI fragments (Fig. 2). Another distinguishing feature is the replacement of three endogenous restriction enzyme sites in one of the Cm regions (SacI, AluI and ApaI) by a poorly repairable, small palindrome genetic marker containing a diagnostic NcoI site. The primary Cm duplication in hybridomas 43 and 122 is expected to undergo rearrangement to generate deletion and/or triplication products of recombination; deletion products bear a single Cm region and reside on a 12.5 kb EcoRI fragment, while triplication recombinants are identified by the middle Cm region segment residing on the diagnostic 13.4 kb EcoRI fragment (Fig. 5A).
To recover recombinants, hybridoma 43 and 122 cell lines (grown for approximately 22 culture doublings in the absence of G418 selection) were cloned at 0.1 cell/well in medium lacking G418. Of the 2254 wells plated, 213 individual colonies were recovered, following which, Southern analysis of EcoRI-digested genomic DNA was performed with Cμ-specific probe F. As summarized in Table 2, in 191 of the 213 subclones, the parental 16.2 and 9.6 kb EcoRI Cμ fragments were the main bands. The remaining 22 subclones bear EcoRI fragments that distinguish them as recombinants. Therefore, a high frequency of HR was also a characteristic of these cell lines, resulting in ~10% rearrangement after 22 culture doublings. The diagnostic 13.4 kb EcoRI μ gene fragment defined 10 subclones as triplication recombinants, while the 12.5 kb EcoRI μ band in nine recombinants identified these as deletion recombinants. The triplication and deletion recombinants were verified by the expected 41.6 and 14.8 kb bands, respectively, in Southern analysis with the non-cutters PacI/PaeR71 (Fig. 5A) (data not shown). The EcoRI digests also revealed three clones that were heterogeneous, consisting of both a parental cell and a deletion recombinant in equal frequency, according to Southern analysis performed on individual subclones (data not shown). The cell lines are included under the column designated ‘other’ in Table 2, and are identified in Figure 5B(i) as hybridoma 43 recombinants, 68 and 85, and in Figure 5B(ii) as hybridoma 122 recombinant, 1. The heterogeneity in these three cultures is interesting. On the one hand, it might suggest the occurrence of a new recombination event in each of the culture wells. On the other hand, from the plating data and the Poisson distribution, the heterogeneity is also consistent with the chance deposition, in the same culture well, of a pre-existing deletion recombinant and a parental duplication.

As an example of the Southern analysis, Figure 6 presents an EcoRI digest of genomic DNA prepared from 12 representative subclones of hybridoma cell line 43. In each subclone, the characteristic 16.2 and 9.6 kb EcoRI fragments that define the Cμ duplication in hybridoma cell line 43 are visible (Fig. 2). In the blot, DNA samples from the Sp6/HL and 19/9 hybridoma cell lines are included as controls. In Sp6/HL, the single Cμ region of the haploid chromosomal μ gene resides on the 12.5 kb EcoRI fragment, and this band serves as the size marker for deletion recombinants. The chromosomal μ gene in hybridoma cell line 19/9 bears a tandem, Cμ region triplication (42), identifiable by the three Cμ region EcoRI fragments of 16.2, 13.4 and 9.6 kb (see also Fig. 5A), of which
Figure 5. Analysis of recombination in hybridoma cell lines 43 and 122. (A) Structure of chromosomal μ gene triplication, duplication and deletion products. Abbreviations: Pc, PacI; Pr, PaeR71; tk, thymidine kinase-1 gene. The various PCR primer pairs are designated by the prefix, AB and the sizes (in kb) of the amplification products are indicated. For further details regarding the primers, refer to the Materials and Methods section. All abbreviations and symbols are as indicated in the legend to Figure 2. (B) Genetic marker analysis in hybridoma 43 and 122 recombinants. The figures indicate the positions of the vector-borne NotI palindrome genetic marker as well as the endogenous SacI, AflIII and Apal restriction enzyme sites in the deletion, duplication and triplication products of HR recovered from the subcloning analysis of the parental hybridoma cell lines, (i) 43 and (ii) 122.
the single Cμ region on the middle 13.4 kb EcoRI fragment is diagnostic (Fig. 5A). The single Cμ region in the 12.5 and 13.4 kb EcoRI bands is readily detected with Cμ-specific probe F. Therefore, the intensity of the 12.5 and 13.4 kb EcoRI bands is expected to reflect the copy number of deletion and triplication recombinants, respectively, accumulating in the hybridoma 43 and 122 cell lines. The blot suggests two things about the HR process in this system. First, it shows that like E14 and its subclones, the primary chromosomal μ gene in hybridoma cell lines 43 (and 122) is prone to HR, generating secondary deletion and/or triplication products of recombination. Second, it suggests that the deletion and/or triplication products of recombination are not always generated in equal frequency within the subclones, as was also suggested from the analysis of the E14 subclones in Figure 4. For example, in subclones 2, 7, 9, 10 and 12, the products are largely triplication or deletion products. In other subclones where both products are visible, they are sometimes (subclones 4, 5 and 6), but not always (subclones 1, 3 and 11) of a similar intensity. Overall, such clonal variation could well account for the accumulation of deletion and triplication products of recombination in similar frequency in the hybridoma 43 and 122 cell lines as indicated in Table 2.

**Table 2.** Southern analysis of EcoRI-digested genomic DNA from subclones of hybridoma 43 and 122

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Frequency and size of EcoRI Cμ bands</th>
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<tr>
<td>Parental (16.2 kb; 9.6 kb)</td>
<td>Triplication (16.2 kb; 13.4 kb; 9.6 kb)</td>
</tr>
<tr>
<td>43</td>
<td>79/93</td>
</tr>
<tr>
<td>122</td>
<td>112/120</td>
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**Figure 6.** Southern analysis of hybridoma 43 subclones. The blot presents an EcoRI digest of genomic DNA from representative hybridoma 43 subclones hybridized with Cμ-specific probe F. In the blot, DNA samples from the hybridoma cell lines Sp6/HL (29,30) and 19/9 (36) were included. The 12.5 kb EcoRI chromosomal μ gene fragment in Sp6/HL serves as the size marker for deletion recombinants, while the 13.4 kb EcoRI μ gene fragment in 19/9 is the band diagnostic of a μ gene triplication (see also Fig. 5A), and each are denoted by an asterisk. The imm10 hybridoma is an Sp6-derived mutant that has lost its chromosomal μ gene (30), and serves as a control for Cμ probe specificity. The size (in kb) of each μ gene of interest is shown on the left of the blot while relevant molecular weight marker bands (denoted, M) are presented on the right.

**Initiation and resolution of intrachromosomal recombination within homologous sequences**

The diagnostic Cμ region genetic markers in hybridoma cell lines 43 and 122 were exploited for the purposes of understanding the recombination process in greater detail. The Cμ regions in all deletion, duplication and triplication recombinants were amplified with specific primers to yield the PCR products indicated in Figure 5A. The individual PCR products were digested separately with SacI, AluI and ApaI, diagnostic for the endogenous Cμ region sites, and NotI, diagnostic of the small palindrome genetic marker. The restriction enzymes cleave the various Cμ region PCR products into diagnostic fragments (ranging in size from ~0.4 to ~4 kb) that can be resolved by standard electrophoresis using 0.7% agarose gels. The analysis of fragment sizes permits individual genetic markers to be assigned to each of the Cμ region sites as detailed previously (32,36). The results of the various digests (data not shown) are summarized in Figure 5B(i) for hybridoma cell line 43, and in Figure 5B(ii) for hybridoma cell line 122. As the figures show, individual Cμ region sites contain either the NotI palindrome marker or the corresponding endogenous marker; there were no recombinants exhibiting ‘patchy’ Cμ regions composed of both chromosomal and vector-borne markers, or, recombinants in which one or more Cμ region sites were mixed or ‘sectored’, both of which would have suggested the presence of hDNA formation during the recombination process. This notwithstanding, both the correct size of the PCR amplified bands and the various restriction enzyme digestion products, together with the lack of any genetic marker alterations, supports a molecular process that has faithfully reconstructed the various Cμ regions in each of the recombinants. Thus, the results suggest that intrachromosomal recombination initiates and terminates within homologous sequences.

**DISCUSSION**

In this study, we describe mouse hybridoma cell lines in which variably numbered tandem Cμ regions reside at the haploid, chromosomal immunoglobulin μ heavy chain locus. This organization provides the opportunity to investigate spontaneous recombination between homologous Cμ repeat members in a well-defined chromosomal location. Through Southern analysis with restriction enzymes recognizing internal sites and those cleaving outside the tandem Cμ regions, HR was shown, most frequently, to involve the loss or gain of one or more Cμ region copies. Ordinarily, for repeated
sequences, the possibility of recombinational exchanges resulting from intra- and/or interchromosomal interactions would require consideration. However, in the hybridoma cell lines reported here, the chromosomal μ gene is present in only one copy per genome, and therefore rearrangements resulting from HR between the Cμ regions can only result from intrachromosomal HR.

A high frequency of spontaneous intrachromosomal HR was observed between the Cμ regions in the various hybridoma cell lines in this study. As noted earlier (43,44), this is unusual, since typically, spontaneous intrachromosomal HR between defective drug-resistance heteroalleles in other mammalian cell lines is orders of magnitude lower (45–48).

The high frequency of intrachromosomal HR in this system can be explained on the basis of our recent work showing that the chromosomal μ locus promotes HR between repeated sequences (49). The rearrangement frequency in E14, whose μ locus contains four tandem Cμ regions, was ~65% after 35 culture doublings (~2%/culture doubling). This frequency is ~4-fold higher than the corresponding value in hybridoma cell lines 43 and 122 (~0.5%/culture doubling), in which the μ locus contains only a pair of Cμ regions. The higher rearrangement frequency in E14 might be explained on the basis of the greater number of potential Cμ region pairing possibilities and/or potential sites for recombinogenic lesions afforded by the increase in Cμ region copy number.

Potentially, intrachromosomal HR can involve intramolecular interactions occurring within the chromosome (or chromatid), or, intermolecular events involving sister chromatids, and proceed according to the general models shown in Figure 1. With respect to intramolecular interactions, deletions might result from mispairing between homologous segments and crossing-over, perhaps, according to events depicted in the DSBR model. In the event of such intramolecular crossing-over, the reciprocal excision product must have been lost, since no unaccounted for μ bands are observed in the recombinants. Alternatively, deletion formation is also consistent with the possibility of SSA. Although well capable of explaining size reductions, neither of the above mechanisms explains the several instances of array amplification during replication, strand slippage between short direct repeats can generate deletion and amplification products (50,51) and this mechanism has been proposed to explain contractions and expansions in the copy number of microsatellite sequences (52). However, in this study, strand slippage would have to involve distances spanning adjacent Cμ repeats (10 or 13.4 kb, or multiples thereof, depending on the cell line), which seems unlikely.

In contrast, intermolecular interactions involving sister chromatids can account for both deletion and amplification products of HR. Indeed, a role for the sister chromatid in recombination has been suggested previously in yeast and mammalian cells (12,38,53,54). One possible mechanism is unequal sister chromatid exchange (USCE) (55–57), a crossing-over event between unequally paired sister chromatids that generates reciprocal deletion and amplification products, which segregate into different daughter cells. USCE has been invoked as a mechanism to explain changes in gene dosage, as observed for example, in the ribosomal RNA gene family of Drosophila (58) and S.cerevisiae (59,60), and in the CUP1 locus of S.cerevisiae (61). Several recombinants in this study are consistent with the possibility of USCE. That is, in E14 subclones 6 and 14, the primary μ gene is present on the 32.5 kb EcoRI fragment and secondary μ genes of the sizes expected for USCE, namely, the amplification and deletion products present on 42.5 and 22.5 kb EcoRI fragments, respectively, are visible (Fig. 4). Similarly, in E14 subclones 9 and 10, where the primary μ gene resides on the 22.5 kb EcoRI fragment, the expected reciprocal products of USCE namely, EcoRI fragments of 32.5 and 12.5 kb, are visible. Nevertheless, the observed recombinant products in several other subclones do not appear to be consistent with USCE. For example, in E14 subclones 2, 8, 11, 12, 13 and 15, secondary rearrangement products are primarily deletions (Fig. 4), while in others (E14 subclones 14, 16 and 17), the products appear to be primarily amplifications.

While not entirely consistent with USCE, the apparent lack of reciprocity in HR can be accounted for by gene conversion, a mechanism of recombination that involves the non-reciprocal exchange of genetic information (62). Gene conversion events between repeated sequences have been important over evolutionary time in shaping eukaryotic genomes (63–65). Gene conversion also generates sequence diversity in a variety of genes in response to developmental or environmental stimuli (65,66), and is an important DNA repair mechanism (1). Gene conversion events are considered a frequent form of recombination between repeated sequences in eukaryotic cells (12,43,44,45,67–71). DSBs are known to be potent inducers of both meiotic and mitotic HR (4,72,73), and might initiate gene conversion in this system. Gene conversion in the context of an initiating DSB might be an asymmetric process. That is, strand invasion and DNA synthesis might involve only one side of the DSB as proposed in OSI (Fig. 1B). Gene amplification may result from unequal pairing between sister chromatids, and the acquisition of an extra repeat(s) by DNA synthesis from the invading 3' end, while deletions can result from strand invasion of a repeat on the same chromosome (or chromatid) (11). Alternatively, gene conversion might involve both 3' ends of the DSB, eventually forming the double HJ (joint molecule) intermediate, which is the centrepiece of the DSBR model of recombination (Fig. 1C). Array amplification could result from the two 3' ends of a single recipient interacting with different donors, while a deletion event could arise if the two 3' ends of different recipients interacted with a single donor (61,67,68,74,75). Cellular mechanisms can exert control over how the HJs are resolved (14,21–23), and during mitotic intrachromosomal recombination, might bias the outcome toward non-crossover (conversion) products. In either mechanism, repair might also involve the use of equivalent regions on sister chromatids, or, be confined to a single Cμ region, in which case, the size of the array would not be altered (43,69–71).

Conceivably, gene conversion could also be initiated by a single-strand nick. In the event a single strand is displaced, it may invade a homologous sequence on the same chromosome (or chromatid), or on the sister chromatid, and initiate
leading-strand synthesis. If pairing involves a homologous sequence on the same chromosome (or chromatid), leading-strand synthesis from the invading end coupled to lagging strand synthesis on the unbroken, displaced loop (1), could amplify the array according to the number of repeat segments enclosed in the loop. Alternatively, the D-loop generated by the initial single-strand invasion event might pair with the exposed complementary region in the unbroken strand. A second nick in the unbroken strand could generate the double HJ intermediate, and lead to deletion formation via crossing over.

Assuming a gene conversion mechanism, the amplification products recovered from hybridomas 43 and 122 suggest that such conversion tracts may well have exceeded 13.4 kb, the unit length of the integrated vector in these cell lines. Depending on the donor choice and the number of conversion events occurring in a particular clone of cells, the deletion and amplification products of recombination might differ in frequency or be approximately equivalent. To summarize, although we cannot rule out the possibility that the deletion and amplification products of HR are generated by multiple mechanisms, they could also be generated by a single mechanism of gene conversion occurring within the chromosome (or chromatid) and/or between sister chromatids.

Another interesting observation concerns the structure of the recombinant products. From the combination of Southern analysis with restriction enzymes recognizing internal sites and those cleaving outside the tandem Cμ array, as well as more detailed, fine structure analysis of the individual recombinant Cμ regions through PCR amplification and restriction enzyme mapping, the deletion and amplification products of HR were shown to represent precise, integral unit length of the integrated vector in these cell lines. The extent of DNA synthesis that would have supported hDNA formation during recombination, which report that conservative repair of the DSB is observed in association with another class of recombinant, in which an initially homology-directed repair event is completed by NHEJ. In the recombinants, DNA synthesis directed by an invading homologous strand could extend beyond the cognate region into flanking non-homologous sequences. Thus, a homologous junction can be present on one side of the DSB, while a non-homologous junction resides on the other side, consistent with the possibility of a non-crossover, OSI mechanism (24–28).

The fidelity of the spontaneous intrachromosomal HR process described above differs from previous studies examining DSB-induced HR in plasmid transformation experiments, which report that conservative repair of the DSB is observed in association with another class of recombinant, in which an initially homology-directed repair event is completed by NHEJ. In the recombinants, DNA synthesis directed by an invading homologous strand could extend beyond the cognate region into flanking non-homologous sequences. Thus, a homologous junction can be present on one side of the DSB, while a non-homologous junction resides on the other side, consistent with the possibility of a non-crossover, OSI mechanism (24–28). This ‘coupling’ between homologous and non-homologous repair is also observed in experiments examining I-SceI-mediated, DSB-induced recombination between defective neo gene heteroalleles in mammalian cells (7,12). In particular, during intrachromosomal recombination, copying of homologous and non-homologous sequences from the sister chromatid by the cut, recipient locus was observed in a substantial fraction (~41%) of clones (12). The extent of DNA synthesis that included non-homologous sequences ranged from ~0.3 (the amount required to reach flanking non-homology) to ~3.3 kb. The short (~0.3 kb) interval between the DSB site and the flanking region of non-homology was suggested as an explanation for the rearrangements. In the present study, neither the nature of the recombination-initiating lesion nor its position within the homology region are known. However, copying from the 3’ end of an invading homologous strand need only progress through ~4 kb of Cμ region sequence, or, the ~5 kb of intervening pSV2neo-derived sequence [distances that do not differ enormously from the longer conversion tracts reported above (12)], before encountering flanking non-homology. In principle, copying could have terminated anywhere within the adjacent non-homology, generating recombinants with illegitimate junctions as observed (7,12). However, despite extensive dissection of the homologous μ gene in the recombinants, no variable-sized Cμ bands, that might have included adjacent vector sequences, were observed.

The above results suggest that spontaneous HR and DSB-induced HR might differ. Interestingly, comparison of spontaneous and I-SceI-induced intrachromosomal HR between aprt repeats suggested that rearrangements were rare for spontaneous recombination, but high for I-SceI-induced events (3). In the event spontaneous recombination involves an initiating DSB, then perhaps the location of the DSB, or the configuration of its ends, differs from those generated by
enzymes, including I-SceI. Another possibility is that of a difference in the circumstances associated with spontaneous DNA break repair. For example, as suggested above, spontaneous HR might normally be activated by a single-strand nick, which is processed to a DSB, permitting recombination to proceed with fidelity. In principle, the spontaneous recombination observed in this study, can be explained by OSI or DSBR mechanisms. If OSI is the mechanism, then in order to avoid unrestricted leading-strand DNA synthesis and consequently, the outcome whereby an initially homology-directed repair event terminates in NHEJ, the newly synthesized, extruded DNA must have been efficiently captured by the non-invading 3' end. In contrast, in DSBR, formation of the double HJ intermediate requires that DNA synthesis from the invading 3' end need only progress until the extruded D-loop undergoes complementary base-pairing with the non-invading 3' end, in effect, stabilizing the dHJ intermediate so that its resolution generates HJ on both sides of the DSB. In the event spontaneous HR involves an initiating DSB, our results support a mechanism whereby the two 3' ends of the break are co-ordinated to interact systematically with homologous sequences during HR, to ensure that both junctions are homologous.

In conclusion, this study shows that tandemly repeated Cμ segments integrated at the chromosomal immunoglobulin μ locus can undergo a high frequency of spontaneous intra-chromosomal HR. The recombination process generates distinct rearrangements that consist of integral increases and/or decreases in Cμ region copy number. Thus, the recombination process appears to initiate and be resolved within homologous sequences. Based on what would appear, for the most part, to be a non-reciprocal mode of recombination, gene conversion is a most likely mechanism. Conversion tracts may be long and continuous, and can be explained on the basis of interactions occurring both within and between sister chromatids.

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