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Requirements for Ectopic Homologous Recombination in Mammalian Somatic Cells

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Ectopic recombination occurs between DNA sequences that are not in equivalent positions on homologous chromosomes and has beneficial as well as potentially deleterious consequences for the eukaryotic genome. In the present study, we have examined ectopic recombination in mammalian somatic (murine hybridoma) cells in which a deletion in the μ gene constant (Cμ) region of the endogenous chromosomal immunoglobulin μ gene is corrected by using as a donor an ectopic wild-type Cμ region. Ectopic recombination restores normal immunoglobulin M production in hybridomas. We show that (i) chromosomal μ gene deletions of 600 bp and 4 kb are corrected less efficiently than a deletion of only 2 bp, (ii) the minimum amount of homology required to mediate ectopic recombination is between 1.9 and 4.3 kb, (iii) the frequency of ectopic recombination does not depend on donor copy number, and (iv) the frequency of ectopic recombination in hybridoma lines in which the donor and recipient Cμ regions are physically connected to each other on the same chromosome can be as much as 4 orders of magnitude higher than it is for the same sequences located on homologous or nonhomologous chromosomes. The results are discussed in terms of a model for ectopic recombination in mammalian somatic cells in which the scanning mechanism that is used to locate a homologous partner operates preferentially in *cis*.

Homologous recombination is the process by which genetic information is exchanged between DNA duplexes and can occur by either gene conversion or crossing over. Gene conversion has the property of transferring genetic information in a nonreciprocal manner, while single reciprocal crossover leads to changes in the linkage relationship between genes or groups of genes. Homologous recombination generates new gene combinations that provide the basis for species diversification. It is thought to have played a fundamental role in genome organization and in the maintenance of homogeneity among repeated sequences and members of multigene families (8, 15, 16, 25, 56, 59). As well, it can correct errors of replication and other forms of DNA damage (24, 27). However, recombination can also lead to altered gene function, altered expression of genes, or the loss of genes. These genetic alterations are implicated in the development of several human cancers (32, 46) and other abnormalities (49, 78).

Our laboratory has been investigating homologous recombination in mammalian somatic (murine hybridoma) cells (3–7). Our assay detects homologous recombination between a donor wild-type immunoglobulin μ gene constant (Cμ) region and the mutant Cμ region of the haploid, chromosomal immunoglobulin μ gene in hybridomas which serves as the recipient sequence for recombination. Homologous recombination corrects the mutation in the recipient chromosomal μ gene and restores trinitrophenyl (TNP)-specific immunoglobulin M (IgM) production in mutant cells. Hybridomas making normal TNP-specific IgM are detected as plaque-forming cells (PFC) in a sensitive, TNP-specific plaque assay (4).

In a previous communication (5), we reported that ectopic recombination occurred between homologous Cμ regions present in dispersed chromosomal sites in the hybridoma genome. The analysis of several recombinants revealed that they were generated by a mechanism consistent with gene conversion. In order for such ectopic recombination to occur, the mitotic recombination apparatus must be capable of conducting a search for homology that spans the entire mammalian genome. Thus, our ectopic recombination approach differs from other systems used to study mitotic homologous recombination in mammalian cells. In these other systems, either the two interacting sequences were integrated as closely linked repeats in the genome (within a few kilobase pairs) (37, 39, 51, 58, 63) or one or both of the recombining sequences were present on an introduced multicopy plasmid(s) (12, 14, 18, 30, 36, 51, 57, 72, 73). Since the effective concentration of the recombining sequences in these systems is high, the search for a homologous partner is expected to be less stringent than the global homology search required to mediate ectopic recombination. This provides the opportunity to use the ectopic Cμ region recombination system to investigate the way in which homologous sequences search for each other and undergo recombination in the mitotic mammalian nucleus.

In the present study, we have used the Cμ region recombination system to examine several requirements of the ectopic homologous recombination process. The results of these studies suggest that ectopic recombination is more efficient at converting small chromosomal deletions than larger ones, requires only relatively small segments of homology, and is not dependent on the number of donor copies clustered at one or a few chromosomal sites. Of particular importance is the finding that ectopic recombination occurs much more frequently even for Cμ sequences that are well separated on the same chromosome than it does for matching sequences located on homologous or nonhomologous chromosomes. This suggests that the mammalian homology scanning mechanism is able to preferentially distinguish homologous sequences on the same chromosome from those located elsewhere in the genome.

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MATERIALS AND METHODS

Hybridoma lines. The origin of the Sp6-derived hybridoma lines Sp6/HL, igm482, igm427, igm692, and igm10 along with the methods for cell culture have been described previously (28, 29).

Isolation of hybridoma transformants. Vector DNA (50 μg) bearing the appropriate Sp6 wild-type donor Cμ region fragment was linearized into vector pSV2neo sequences by digestion with *Hpa*I. The vector DNA was then extracted with phenol and chloroform, precipitated with ethanol, resuspended in phosphate-buffered saline, and transferred to 2×10^7 hybridomas by electroporation as described previously (4). Following electroporation, the cells were resuspended in complete DMEM (Dulbecco's modified Eagle medium containing 13% bovine calf serum and 5.3×10^{-4} M 2-mercaptoethanol) in a tissue culture flask at a density of 10^5 cells per ml. The flask was kept at 37°C for 12 h, following which cell survival was determined by trypan blue exclusion. After growth for an additional 2 days, one portion of cells was distributed in microtiter plates at a limiting dilution in complete DMEM containing 600 μg of G418 per ml to measure the frequency of G418-resistant ($G418^r$) transformants. The remaining culture was resuspended in complete DMEM containing G418 for 10 to 14 days to select for stable $G418^r$ igm482 transformants. Independent $G418^r$ igm482 transformants were isolated by cloning in microtiter plates. Genomic DNA prepared from $G418^r$ transformants was digested with various restriction enzymes and analyzed by Southern blotting as described in Results.

Assay for PFC. PFC were detected in cultures of the various hybridomas by a TNP-specific plaque assay (4). The TNP-specific PFC were isolated and purified as described previously (5).

DNA and IgM analysis. Large-molecular-size DNA was prepared from the various cell lines by the method of Gross-Bellard et al. (21). Restriction enzymes were purchased from New England Biolabs, Inc., Boehringer Mannheim Biochemicals, and Bethesda Research Laboratories, Inc., and were used in accordance with the specifications of the manufacturers. DNA electrophoresis was conducted at 2 V/cm through agarose gels of the consistencies described in the figure legends. DNA blotting onto nitrocellulose was performed according to the method of Southern (60). ^{32}P-labelled DNA probe fragments were prepared with the Multiprime DNA-labelling system (Amersham). Hybridizations were conducted as described previously (70).

FISH analysis of hybridoma chromosomes. Two DNA probes were used for fluorescence in situ hybridization (FISH) analysis. Probe 1 is a linearized, 10-kb pTCμ transfer vector (4) and was used to identify wild-type donor Cμ region integration. Probe 2 is a unique 10.2-kb *Eco*RI fragment that identifies the haploid chromosomal μ gene in hybridomas. Two-color FISH was performed on metaphase chromosomes counterstained with 4′,6-diamidino-2-phenylindole-dihydrochloride (DAPI) from mouse igm482 hybridoma transformant lines according to published methods (11, 35). Briefly, both probes were labelled by nick translation, probe 1 with digoxigenin (DIG) and probe 2 with biotin. Probe 1 was detected with rhodamine-labelled anti-DIG antibody, followed by DIG-labelled anti-mouse antibody and rhodamine-labelled anti-DIG. Probe 2 was detected with fluorescein isothiocyanate (FITC)-avidin followed by biotinylated antiavidin antibody and avidin-FITC. Separate grey scale images of FITC signals, rhodamine signals, and DAPI-counterstained chromosomes were captured by a thermoelectrically cooled charge-coupled camera (Photometrics, Tucson, Ariz.). These images were overlaid electronically with image analysis software (courtesy of Tim Rand and David Ward, Yale University, New Haven, Conn.) and pseudocolored red (rhodamine), yellow (FITC), and blue (DAPI) as described previously (11).

RESULTS

Ectopic recombination system. We have previously described the system for detecting ectopic homologous recombination in murine hybridomas (5). In brief, it is based upon the ability to detect normal cytolytic, TNP-specific IgM production following homologous recombination between an ectopic wild-type Cμ region donor sequence and the recipient mutant Cμ region of the haploid chromosomal immunoglobulin μ gene. Recombinant cells making normal TNP-specific IgM are detected as PFC in a complement-dependent, TNP-specific plaque assay (4).

Efficiency of correcting chromosomal μ gene deletions by ectopic homologous recombination. For this study, we made use of several mutant hybridoma lines derived from the wild-type Sp6/HL hybridoma (28, 29), which bears a single copy of the TNP-specific chromosomal immunoglobulin μ gene and makes cytolytic, polymeric TNP-specific IgM(κ). Figure 1 presents the structure of the Sp6/HL chromosomal μ gene, the position of various restriction enzyme sites, the extent of the Cμ region deletions affecting the various mutant hybridomas, and the locations of various DNA probe fragments used in Southern (60) blot hybridization and FISH analysis (described below).

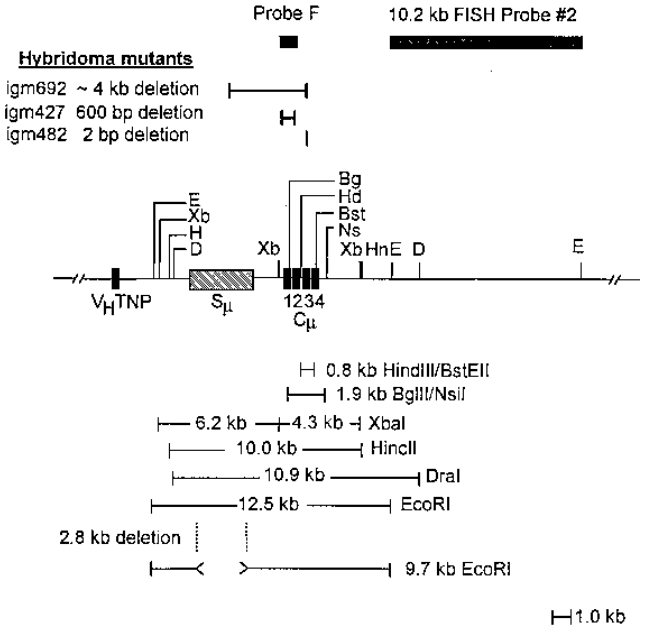


FIG. 1. Structure of the chromosomal μ gene in wild-type Sp6/HL and mutant hybridomas. The wild-type Sp6/HL chromosomal immunoglobulin μ gene along with the sizes of fragments generated by the indicated restriction enzymes and the positions of the various probe fragments used in Southern blot (60) hybridization analysis are shown. The Cμ region deletion in each Sp6-derived mutant hybridoma has been characterized previously (28), and its position in the μ locus is shown at the top. The mutant igm692 hybridoma bears an ~4-kb deletion extending 5′ from its beginning in the Cμ2/Cμ3 intron, while the mutant igm427 hybridoma bears a 600-bp deletion extending 5′ from the beginning of the Cμ2 exon. The mutant igm482 hybridoma bears a 2-bp deletion in the Cμ3 exon which destroys an *Xmn*I site normally present in the Sp6/HL hybridoma. Also shown is the origin of the cloned 9.7-kb *Eco*RI fragment that was used as the recombination donor in ectopic recombination. DNA probe F is an 870-bp *Xba*I-*Bam*HI fragment, FISH probe 2 is a 10.2-kb *Eco*RI fragment, and probe G (not shown) is a 762-bp *Pvu*II fragment from the *neo* gene of pSV2neo (61). Abbreviations: Bg, *Bgl*II; Bst, *Bst*EII; D, *Dra*I; E, *Eco*RI; Hd, *Hin*dIII; Hn, *Hin*cII; Ns, *Nsi*I; Xb, *Xba*I; Sμ, μ gene switch region; V_HTNP, TNP-specific heavy-chain variable region.

The mutant hybridoma line igm482 bears a 2-bp deletion in the exon encoding the third constant region domain of the μ heavy chain (Cμ3). The deletion in the mutant igm427 hybridoma commences at the beginning of the Cμ2 exon and extends for approximately 600 bp in the 5′ direction. In the igm692 mutant hybridoma, the deletion begins in the intron separating Cμ2 and Cμ3 and removes approximately 4 kb of DNA to the 5′ side. The deleted Cμ region in the various mutant hybridomas results in the production of a nonfunctional μ chain as detailed previously (28).

The recombination donor segment for the ectopic recombination studies is derived from the 12.5-kb *Eco*RI Sp6/HL wild-type Cμ region fragment (Fig. 1). Upon cloning into *Escherichia coli*, an internal 2.8-kb segment was deleted from the μ gene switch region (45), generating a cloned 9.7-kb *Eco*RI μ gene fragment. However, the cloned *Eco*RI fragment is (otherwise) fully wild type because when linked to the TNP-specific heavy-chain variable region, it is able to rescue normal, TNP-specific IgM production following transfection into hybridomas expressing the TNP-specific κ chain (45). Mapping of the deletions in the igm692 chromosomal μ gene and the cloned 9.7-kb *Eco*RI μ gene fragment suggests that they overlap by

TABLE 1. Correction of chromosomal deletions by ectopic recombination

Cell line	Size of chromosomal Cμ deletion (bp)	Transformant no.	Wild-type donor Cμ region		No. of cells assayed (10^7)	No. of PFC	Frequency (PFC/cell, 10^{-7})[c]
			Copy no.[a]	No. of integration sites[b]			
igm482	2	1	2.0	1	8.0	25	3.1 ± 1.0
		2	0.9	1	8.0	81	10.1 ± 1.7
		3	0.9	1	6.0	53	8.8 ± 1.1
		4	1.0	1	6.0	28	4.7 ± 0.8
		5	1.3	1	6.0	0	<0.2
igm427	600	1	1.1	1	6.0	4	0.7 ± 0.3
		2	1.8	1	12.0	17	1.4 ± 0.5
		3	1.6	1	6.0	0	<0.2
		4	1.1	1	4.0	1	0.3 ± 0.3
		5	2.3	1	16.0	10	0.6 ± 0.2
igm692	4,000	1	1.9	1	4.0	0	<0.3
		2	2.7	1	6.0	0	<0.2
		3	1.1	1	12.0	2	0.2 ± 0.2
		4	4.0	1	11.0	4	0.4 ± 0.4
		5	4.1	1–2	6.0	0	<0.2
igm482					8.0	0	<0.1
igm427					6.0	0	<0.2
igm692					6.0	0	<0.2
Sp6/HL					600.0	379	6,300,000

[a] The various transformant cell lines were isolated from G418^r populations of the indicated hybridoma lines generated following transfer of the pSV2neo vector bearing the 9.7-kb *Eco*RI wild-type Cμ region donor (Fig. 1). The frequency of transfection among the various cell lines averaged 0.8×10^{-3} G418^r transformants per cell, and individual transformants were isolated from pools of about 5×10^3 G418^r cells. Transformant donor Cμ region copy number was estimated following Southern blot analysis by direct counting of radioactivity in the ectopic wild-type donor (D) Cμ region and the haploid, recipient (R) chromosomal μ gene in the various hybridoma mutants and determination of the D/R ratio. The deletions in the mutant hybridomas igm482 and igm427 result in *Dra*I chromosomal μ gene fragments of 10.9 and 10.3 kb, respectively, compared with the 10.9-kb *Dra*I fragment found in the Sp6/HL wild-type μ gene (Fig. 1). These *Dra*I chromosomal μ gene fragments are conveniently distinguished from the integrated pSV2neo transfer vector, which harbors the wild-type donor Cμ region on an 8.1-kb *Dra*I fragment. The deletion in the chromosomal μ gene of the igm692 hybridoma results in conversion of the adjacent 6.2- and 4.3-kb Sp6/HL *Xba*I fragments (Fig. 1) to a single 6.5-kb *Xba*I Cμ region fragment. The igm692 recipient chromosomal μ gene can be distinguished from the ectopic wild-type donor Cμ region because the latter is present on an internal 4.3-kb *Xba*I fragment derived from the 9.7-kb *Eco*RI fragment contained on the integrated pSV2neo transfer vector.

[b] The number of ectopic donor Cμ region integration sites was determined by Southern blot analysis of transformant DNA digested with *Sca*I or *Eco*RI, enzymes which cut once in the transfected DNA. In the case of transformants with one donor Cμ region copy, a single novel band was visible, suggesting integration at one site. For transformants with >1 donor Cμ region, all except igm692 no. 5 showed a single novel band and a unit length vector band, implying vector integration at a single ectopic chromosomal site. In igm692 no. 5, a unit length vector band and 2 novel bands were visible, suggesting the possibility of donor Cμ region integration at one or two chromosomal sites.

[c] Aliquots of the indicated cell lines ($\sim 5 \times 10^4$ cells) were inoculated into media in separate flasks and grown to a density of $\sim 10^8$ cells per culture and assayed for PFC. For most cell lines, PFC from at least two independent cultures were enumerated. The frequency (10^{-7}) is reported as means ± the standard errors of the means.

approximately 800 bp (9, 10). This makes the functional difference between the cloned *Eco*RI μ gene fragment and the igm692 chromosomal μ gene approximately 6.0 kb. However, as is evident from Fig. 1, the cloned *Eco*RI recombination donor fragment still bears at least 1.6 and 4.4 kb of homology on the 5′ and 3′ sides of the deleted Cμ region in each of the mutant hybridomas, respectively. This amount of homologous flanking DNA is greater than that present on the 4.3-kb *Xba*I Sp6 wild-type donor Cμ region fragment, which is able to undergo ectopic recombination with the recipient mutant igm482 chromosomal Cμ region (1.6 and 2.7 kb of homologous donor DNA flanking the 5′ and 3′ sides of the deletion in the recipient mutant igm482 Cμ region, respectively) (see Tables 2 and 3). This argues that sufficient homologous flanking DNA is present on the cloned *Eco*RI donor Cμ fragment to permit recombination with the deleted chromosomal Cμ region in the various mutant hybridomas.

The 9.7-kb *Eco*RI fragment was inserted into the *Eco*RI site of the vector pSV2neo (61) and transferred to the various mutant hybridoma lines by electroporation as described previously (4). For each mutant hybridoma line, several independent G418^r transformants containing the integrated ectopic wild-type Cμ region were isolated. The transformants were screened by Southern blot analysis to identify those bearing the ectopic wild-type donor Cμ region in low copy numbers at (usually) a single independent chromosomal position as described in the Table 1 footnotes. In the transformants, homologous recombination between the ectopic donor wild-type *Eco*RI Cμ region fragment and the recipient mutant chromosomal μ gene was detected by the generation of TNP-specific PFC as detailed previously (4, 5). The results suggested a noticeable trend toward poorer correction of the larger chromosomal deletions. However, precise quantification of the effect was complicated somewhat by the finding that a slight but significant variation was observed among the various transformants (about threefold) ($P = 0.05$ according to analysis of variance and least significant difference tests). As controls, Table 1 also presents the frequencies of detection of TNP-specific PFC in the various recipient mutant hybridoma lines and the wild-type Sp6/HL hybridoma.

Homology requirement for ectopic recombination. To examine the homology requirement for ectopic recombination, Sp6/HL wild-type donor Cμ region fragments bearing increasing lengths of overall homology to the igm482 chromosomal Cμ region were isolated: the 0.8-kb *Hin*dIII-*Bst*EII fragment, 1.9-kb *Bgl*II-*Nsi*I fragment, 4.3-kb *Xba*I fragment, and 9.7-kb *Eco*RI fragment (Fig. 1). The various Cμ fragments were inserted into vector pSV2neo (4) (described in the Table 2 foot-

TABLE 2. Influence of wild-type donor Cμ region homology on ectopic recombination

Sp6/HL wild-type donor Cμ region fragment	Length of homology (kb)	Transformant no.[a]	Wild-type donor Cμ region		No. of cells assayed (10^7)	No. of PFC	Frequency (PFC/cell, 10^{-7})[d]
			Copy no.[b]	No. of integration sites[c]			
*Eco*RI	9.7	1	2.0	1	8.0	25	3.1 ± 1.0
		2	0.9	1	8.0	81	10.1 ± 1.7
		3	0.9	1	6.0	53	8.8 ± 1.1
		4	1.0	1	6.0	28	4.7 ± 0.8
		5	1.3	1	6.0	0	<0.2
*Xba*I	4.3	1	0.9	1	8.0	7	0.9 ± 0.5
		2	1.1	1	12.0	12	1.0 ± 0.5
		3	1.8	1	8.0	19	2.4 ± 0.5
		4	1.8	1	12.0	17	1.4 ± 0.3
*Bgl*II-*Nsi*I	1.9	1	2.3	1	4.0	2	0.5 ± 0.5
		2	3.8	1–2	6.0	2	0.3 ± 0.2
		3	1.9	1	6.0	0	<0.2
*Hin*dIII-*Bst*EII	0.8	1	3.3	1	6.0	0	<0.2
		2	3.6	1	6.0	1	0.2 ± 0.1
		3	6.8	1	6.0	0	<0.2
		4	7.6	1–2	6.0	3	0.5 ± 0.5
		5	8.4	1	6.0	1	0.2 ± 0.1
Control igm482 cells					8.0	1	0.1 ± 0.1

[a] The various igm482 hybridoma transformants were generated following transfer of pSV2neo-derived vectors bearing the indicated Sp6/HL donor wild-type Cμ region fragments by electroporation as detailed in Materials and Methods. The frequency of transfection for the various vectors averaged 0.7×10^{-3} G418^r transformants per cell. Individual transformants were isolated from pools of approximately 5×10^3 G418^r cells. Transformants derived from transfer of the *Eco*RI donor fragment are those reported for igm482 in Table 1. Transformants 1, 2, and 3 derived from transfer of the *Xba*I fragment are the transformants E29, E15, and E26 described previously (53).

[b] The copy number of the *Eco*RI donor fragment was determined as described in the footnotes to Table 1. To measure the donor Cμ region copy number in the other transformants, we made use of the fact that the various homologous donor Cμ regions are inserted as *Sal*I fragments into the *Sal*I site of a derivative of vector pSV2neo (61). The recognition sequence for *Sal*I also contains the site for the enzyme *Hin*cII. Therefore, following *Hin*cII digestion, the D/R ratio was determined by counting the ^{32}P radioactivity present in the various ectopic wild-type donor (D) *Hin*cII Cμ region fragments and the single-copy 10.0-kb *Hin*cII fragment from the recipient (R) igm482 chromosomal μ gene (Fig. 1).

[c] The conclusions are based on experiments similar to those described in the footnotes to Table 1.

[d] Determined as described in the footnotes to Table 1.

notes). The plasmid vector DNA was linearized by digestion with *Hpa*I and transferred to igm482 cells by electroporation as described previously (4). For each vector, several representative G418^r transformants were isolated. The procedures for analyzing donor Cμ region copy number and the number of vector integration sites in the various transformants are presented in the Table 2 footnotes. The results suggested a lower efficiency of ectopic recombination as the overall length of homology decreased. A low level of PFC was detected with fragments bearing the shortest length of homology to the igm482 chromosomal μ gene, the 1.9-kb *Bgl*II-*Nsi*I and 0.8 kb *Hin*dIII-*Bst*EII fragments. However, these frequencies were similar to the frequency of PFC detected in the control igm482 hybridoma. Therefore, the minimum length of overall homology required for ectopic recombination is between 1.9 and 4.3 kb.

Influence of donor copy number on ectopic recombination. The transformants in Tables 1 and 2 typically contain <4 wild-type donor Cμ regions integrated at (usually) one ectopic site. It was therefore of interest to determine whether the frequency of ectopic recombination was influenced by greater numbers of wild-type donor Cμ regions integrated at one or several chromosomal sites. To investigate this, G418^r igm482 transformants bearing the integrated 4.3-kb *Xba*I Sp6/HL wild-type donor Cμ region fragment (Fig. 1) (contained on the pSV2neo-derived vector pTCμ [4]) were generated. From the pool of G418^r hybridoma transformants, 60 G418^r cell lines were isolated by cloning.

Table 3 presents representative transformants and their donor copy number, number of donor integration sites, and ectopic recombination frequencies. The structure of the wild-type donor Cμ region in several transformants is presented in Fig. 2. As is evident from Table 3, transformant E69 is exceptional in that it displays an ectopic recombination frequency which is approximately 156-fold higher than the mean frequency of PFC in the other transformants (approximately 2.0×10^{-7} PFC per cell). The same high frequency of recombination was also observed in E69 subclones (data not shown), indicating that it was a stable property of the cell line. With the exception of E69, the PFC frequencies in the cell lines were similar. In fact, analysis of recombination frequencies by analysis of variance and least-significant difference tests revealed that only E32 and E46 differed slightly (about threefold) from the remaining transformants. Thus, for the most part, the frequency of ectopic recombination appeared not to be strongly affected by chromosomal position. Also, the frequencies of ectopic recombination among the various transformants were similar in spite of their widely differing copy numbers and the fact that in some, the donors were distributed in more than one chromosomal site. The data clearly suggest the absence of a simple relationship between the frequency of ectopic recombination and wild-type donor Cμ region copy number.

Ectopic recombination with an enhancer-trap vector. In the ectopic recombination studies described above, the expression of the *neo* gene in the various pSV2neo-derived vectors is driven by the simian virus 40 (SV40) early region promoter and enhancer sequences (61). In contrast to the high transfection

TABLE 3. Influence of wild-type donor Cμ region copy number on ectopic recombination

Cell line[a]	Wild-type donor Cμ region		No. of cells assayed (10^7)	No. of PFC	Frequency (PFC/cell, 10^{-7})
	Copy no.[b]	No. of integration sites[c]			
E29	0.9	1	8.0	7	0.9 ± 0.5
E69	1.1	1	8.0	2,492	311.5 ± 21.2
E26	1.8	1	8.0	19	2.4 ± 0.5
E75	1.8	1	12.0	17	1.4 ± 0.3
E3	3.0	1	8.0	19	2.4 ± 0.4
E21	3.2	1	8.0	10	1.3 ± 0.4
E2	5.1	1	6.0	9	1.5 ± 0.6
E32	7.1	1	8.0	34	4.3 ± 0.6
E46	8.6	1	8.0	30	3.8 ± 1.0
E23	9.5	2	6.0	4	0.7 ± 0.3
E4	11.7	2	10.0	21	2.1 ± 0.6
E25	15.6	1	8.0	10	1.3 ± 0.4
E27	20.7	2 or 3	8.0	10	1.3 ± 0.3

[a] The various hybridoma lines were isolated following transfer of the wild-type donor Cμ region contained on the pTCμ transfer vector (4). From the frequency of G418[r] transformants (1.1×10^{-3} transformants per cell) and the number of cells surviving electroporation (1.0×10^7), we calculate that the cell lines were isolated from a pool of approximately 10^4 G418[r] transformants. The hybridoma lines E26, E29, and E69 have been described previously (53) and are included here for comparison.

[b] Equal to the D/R ratio as described in the legend to Fig. 2.

[c] The genomic DNA of some transformants was digested with *Sca*I or *Bam*HI, enzymes which cut once within the 10-kb transfer vector, whereas for other transformants, *Eco*RI and *Xba*I, enzymes which do not cut the transfer vector, were used followed by hybridization analysis with probe fragments F or G (described in the Fig. 1 legend). To summarize the results (data not shown), transformant E75 contained two novel bands with the noncutters *Eco*RI or *Xba*I, suggesting that each wild-type donor Cμ region is integrated at a single genomic site. Analysis of the remaining transformants with single cutters revealed the following. Six transformants (E3, E21, E2, E32, E46, and E25) contained the repeated 10-kb vector band and a novel band, implying that in these independent transformants, the wild-type donor Cμ regions are integrated at a single ectopic site in the igm482 genome. Two transformants (E23 and E4) contained the repeated 10-kb vector band and, depending on the enzyme and probe fragment used, two or three novel bands, suggesting the likelihood of two sites of ectopic donor Cμ region integration. Transformant E27 contained the repeated 10-kb vector band and five novel bands, suggesting that the ectopic donor Cμ regions are probably integrated in at least two or three different chromosomal sites.

[d] Determined as described in the footnotes to Table 1.

frequencies obtained with this vector system (as indicated in the footnotes of Tables 1 to 3, approximately 10^{-3} G418[r] stable transformants per cell), a much lower frequency of stable transfection is observed if the SV40 early region enhancer is deleted (26; see also below). This likely occurs because most chromosomal sites in the mammalian genome do not supply the enhancer (or equivalent) activity required for expression of the enhancerless *neo* gene in the integrated vector. The activity associated with chromosomal integration sites sampled with the enhancer-negative vector might have the capacity to promote ectopic Cμ region recombination. Therefore, the frequency of ectopic recombination was examined in transformants containing the wild-type donor Cμ region on a pSV2neo vector in which the SV40 enhancer had been deleted.

For this study, the 501-bp *Sfi*I-*Nde*I fragment bearing the SV40 enhancer region was excised from the pTCμ vector used in the ectopic recombination experiments whose results are presented in Table 3. The resulting vector was transferred to igm482 cells by electroporation (4). In two experiments, the frequency of G418[r] transformants averaged 8.0×10^{-7}, approximately 10^3-fold lower than that obtained with the SV40-enhancer containing pTCμ vector. The frequencies of ectopic recombination in eight G418[r] transformants isolated in these experiments were examined. The results revealed that ectopic recombination frequencies for seven of the eight transformants ranged from 0.2×10^{-7} to 1.2×10^{-7} PFC per cell (data not shown). Thus, with the caveat that only a few cell lines were examined, the results suggested that ectopic recombination frequencies for transformants bearing the enhancerless pTCμ vector were similar to those presented in Table 3 for transformants in which the pTCμ vector contained the SV40 enhancer. However, one transformant (E9) bearing a single functional wild-type donor Cμ region (Fig. 2) demonstrated an exceptionally high frequency of ectopic recombination ($1.8 \times 10^{-3} \pm 0.05 \times 10^{-3}$ PFC per cell). The high frequency of ectopic recombination was also a characteristic of E9 subclones originating from a single cell (data not shown).

Characterization of the wild-type donor Cμ region integration site in the E9 and E69 hybridomas. The high frequency of homologous recombination in the E9 and E69 hybridomas was intriguing and might have resulted from the serendipitous integration of the wild-type donor Cμ segment near the recipient igm482 chromosomal Cμ region. The haploid igm482 chromosomal Cμ region was determined to be present on a 12.5-kb *Eco*RI fragment when analyzed with probe fragment F (Fig. 1 legend). Since the transferred 10-kb pTCμ vector containing the wild-type donor Cμ region lacks the recognition site for *Eco*RI, the size of the igm482 chromosomal *Eco*RI fragment is expected to increase in proportion to the number of copies of the integrated vector. Therefore, as a first step in analyzing the position of the integrated wild-type donor Cμ region in the E9 and E69 hybridomas, an *Eco*RI blot of the transformant genomic DNA was analyzed with probe fragment F (Fig. 3).

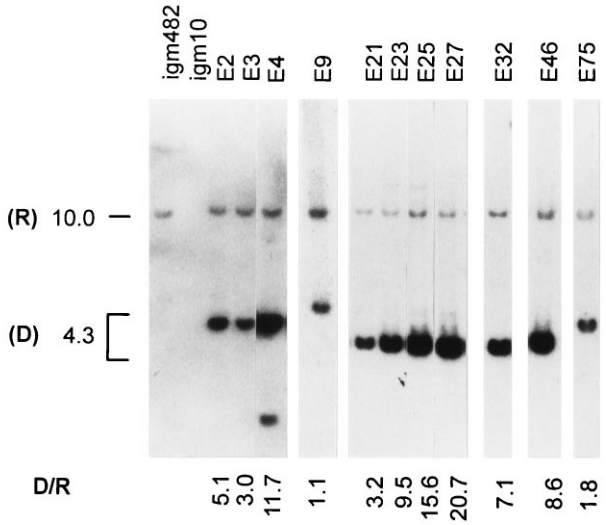


FIG. 2. Analysis of ectopic donor Cμ region copy number in igm482 transformants. As described previously (4), vector pTCμ contains the 4.3-kb *Xba*I wild-type donor Cμ region fragment (Fig. 1) inserted via *Sal*I ends into the *Sal*I site of a derivative of vector pSV2neo. The *Sal*I site bears the recognition sequence for *Hin*cII, a feature which enables determination of the donor Cμ region copy number. In these experiments, igm482 transformant DNA was digested with *Hin*cII, electrophoresed through 0.7% agarose, blotted onto nitrocellulose, and hybridized with probe F (Fig. 1). The ectopic wild-type donor Cμ region (D) is detected as a 4.3-kb fragment, whereas the recipient igm482 chromosomal μ gene fragment (R) is 10.0 kb. Measurement of the radioactivity in each band and determination of the D/R ratio gives the ectopic wild-type donor Cμ region copy number (numbers below lanes). The single, functional donor Cμ region in the E9 hybridoma is present on a 6.0-kb *Hin*cII fragment as a result of the loss of the *Hin*cII site 5′ to Cμ1 following pTCμ transfer or integration (data not shown).

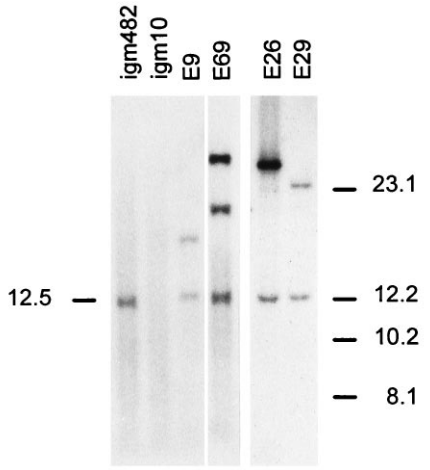


FIG. 3. Analysis of chromosomal μ gene structure in transformants. Shown are the results of Southern blot analysis of transformant genomic DNA digested with *Eco*RI and probed with fragment F (Fig. 1). The 12.5-kb *Eco*RI fragment bearing the recipient igm482 chromosomal μ locus is indicated on the left, while the positions of the relevant DNA molecular size markers are shown on the right.

For comparison, DNA from the low-frequency transformants E26 and E29 was included (Table 3). The results revealed that transformants E9, E26, E29, and E69 retained the 12.5-kb *Eco*RI igm482 chromosomal Cμ region fragment. Additional mapping of E9 and E69 genomic DNA digested with the rare cutter *Cla*I suggested that the μ locus in these cell lines was identical to that found in the igm482 hybridoma over a distance spanning approximately 55 kb (data not shown). Thus, the transferred wild-type donor Cμ region is not closely linked to the mutant igm482 chromosomal μ locus in the hybridomas.

A larger *Eco*RI fragment(s) is visible in the various transformants (Fig. 3), and all but the 12.5-kb chromosomal μ band were detected following reprobing with *neo* probe G (data not shown). The extra *Eco*RI band(s) represents the site(s) of pTCμ vector integration in the igm482 genome. Thus, in transformants E9, E26, and E29, the transferred vector integrated in a single genomic site, whereas in transformant E69, there were two sites of vector integration. Since E69 appears to contain a single copy of the full-length wild-type Cμ region (53), only one of the vector integration sites contains the functional Cμ donor. The same conclusion regarding the number of vector integration sites was reached following digestion of transformant genomic DNA with the enzyme *Xba*I, which also does not cut within the integrated pTCμ vector (data not shown).

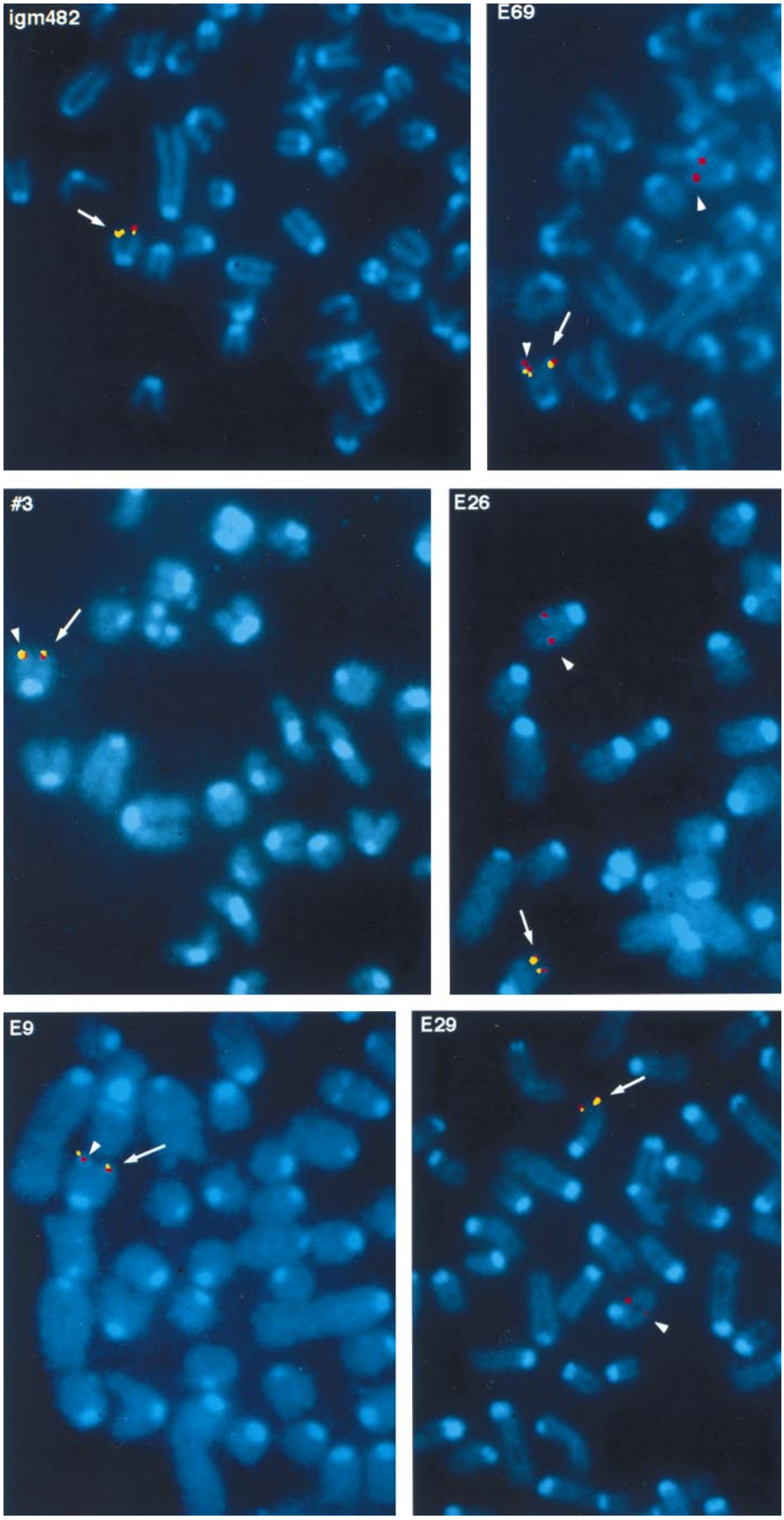
To determine the site of vector integration, we performed FISH on hybridoma metaphase chromosomes (Fig. 4). Two DNA probe fragments were used for FISH. Probe 1 is the linearized, 10-kb pTCμ vector (4) labelled with DIG. It reveals the ectopic, wild-type donor Cμ region (vector) integration site as a red signal in Fig. 4 through the use of rhodamine-labelled anti-DIG antibody. Probe 2, the biotinylated unique 10.2-kb *Eco*RI fragment located 3′ of the igm482 chromosomal Cμ region (Fig. 1), identifies the haploid, igm482 chromosomal μ locus through the use of avidin-FITC. It is revealed by a yellow signal in Fig. 4. Unavoidably, as shown in Fig. 4, the Cμ portion of probe 1, which is required to identify the site(s) of ectopic wild-type donor Cμ region integration, cross-reacts with the Cμ region of the endogenous haploid chromosomal μ gene in the igm482 hybridoma. Thus, for each hybridoma, assignment of the chromosomal location of the wild-type donor Cμ region must be done by considering the results of FISH in conjunction with the number of wild-type donor Cμ region integration sites identified in Fig. 3. As a means of assessing the physical intrachromosomal distance between the wild-type and donor Cμ regions as denoted by the FISH signals, we have included hybridoma line 3, in which the single-copy wild-type donor is known to be 5.7 kb from the recipient igm482 chromosomal Cμ region (6). The close proximity of the two Cμ regions is visualized by the fused probe signals in cell line 3 (Fig. 4).

In the E9 hybridoma, the FISH analysis revealed a single site of wild-type donor Cμ region integration, confirming the data from Southern blot analysis (Fig. 3). The integration site is on the same chromosome and fairly closely linked to the recipient igm482 μ locus (Fig. 4). For E69, the FISH analysis confirmed the two vector integration sites identified by Southern blot analysis (Fig. 3). As shown in Fig. 4, one vector integration site is on the same chromosome as the igm482 μ locus whereas the second vector is integrated on a different murine chromosome. Molecular analysis of several independent PFC derived from both the E9 and E69 hybridomas (to be published in detail elsewhere) reveals that they are generated by intrachromosomal recombination between the wild-type donor and recipient igm482 Cμ regions. Thus, in both E9 and E69, high-frequency ectopic recombination results from the serendipitous integration of the functional, wild-type donor Cμ region on the same chromosome as the recipient igm482 μ locus. In contrast to the closely linked donor and recipient Cμ regions visible as a fused signal in the control hybridoma 3, the wild-type and donor Cμ regions in both E9 and E69 are visible in Fig. 4 as more discrete FISH signals. This suggests that the functional, wild-type donor Cμ region in the E9 and E69 hybridomas resides at least 1 to 2 Mb from the recipient igm482 chromosomal Cμ region (33, 69, 77). For purposes of comparison, we have also included hybridoma lines E26 and E29, which Southern blot experiments show to contain the single-copy wild-type donor Cμ region at one site (Fig. 3). In both cell lines, FISH reveals the ectopic wild-type donor Cμ region on a chromosome different from that of the igm482 μ locus. Thus, the low frequency of ectopic recombination in E26 and E29 (Table 3) results from interchromosomal recombination.

DISCUSSION

In the present study, we examined some of the requirements for ectopic homologous recombination in murine hybridomas in which the recipient for genetic recombination, the mutant igm482 chromosomal Cμ region, was corrected by a wild-type donor Cμ region integrated elsewhere in the genome. Recombinant cells were detected as PFC in a sensitive TNP-specific plaque assay (4, 5). Molecular characterization of several recombinants in previous studies suggested that gene conversion was an important mechanism of recombination between dispersed Cμ sequences in the hybridomas (5).

We first examined the capacity of the ectopic recombination process to correct deletions in mammalian chromosomes. For these studies, we made use of Sp6-derived mutant hybridomas bearing well-characterized deletions in the Cμ region of the haploid chromosomal μ gene as recipients for gene conversion with an integrated ectopic Sp6 wild-type donor Cμ region. The results revealed a trend toward poorer correction of larger deletions, although some ectopic recombination was still apparent even with large deletions of 600 bp and 4 kb. In these studies, the ectopic wild-type donor Cμ region shares at least 1.6 and 4.4 kb of homologous DNA flanking the 5′ and 3′ sides of each Cμ region deletion in the mutant hybridomas, respectively. This length of homology is well above that present in the


igm482
E69
#3
E26
E9
E29

recipient mutant igm482 chromosomal Cμ region, which is corrected by ectopic gene conversion with the 4.3-kb *Xba*I Sp6 wild-type donor Cμ segment (1.6 and 2.7 kb flanking the 5′ and 3′ sides of the 2-bp igm482 mutation, respectively) (Fig. 1; Tables 2 and 3) or that required to mediate intrachromosomal recombination (38, 74) or gene targeting (23, 54, 67) in mammalian cells. Also, since all mutants are derived from the Sp6 wild-type hybridoma (28, 29), the chromosomal DNA flanking the various Cμ gene deletions is expected to be isogenic with the ectopic Sp6 wild-type donor Cμ sequence. Thus, the ectopic *Eco*RI donor segment appears to contain adequate homologous flanking DNA to mediate genetic recombination with the various μ gene deletion mutants.

Thus, what might explain the reduced efficiency for correcting large chromosomal deletions? Current recombination models suggest two ways by which chromosomal deletions might be corrected. In one model, gene conversion results from repair of heteroduplex DNA (40). In the second model, gene conversion is the result of the processing of double-stranded DNA breaks (the double-strand break repair model [65]). In this model, a double-stranded break is made in the recipient duplex and the break is enlarged to a gap by exonuclease activity. The 3′ tails of the gapped duplex invade the unbroken donor duplex, which serves as the template for gene conversion. The large chromosomal deletions in the igm427 and igm692 hybridomas might destabilize or impede the correction of heteroduplex DNA. Alternatively, in the case of the double-strand break repair model, difficulties associated with synthesizing large tracts of donor DNA sequence might reduce the efficiency of correcting large chromosomal deletions.

The homology requirement for mitotic recombination has been investigated for a number of organisms, with the general finding that recombination is sensitive to the length of homology of two sequences. The minimum length of homology has been estimated at 20 to 50 bp in *E. coli* (52, 55, 75) and between 63 and 89 bp in *Saccharomyces cerevisiae* (1, 41, 64). In *Drosophila melanogaster*, the minimum length of homology required for recombination is no more than 999 bp and probably less than 115 bp (43). The homology requirement for recombination in mammalian cells has been studied in a variety of ways. The homology requirement for efficient intra- and extrachromosomal recombination between closely linked homologous sequences ranges from 163 to 300 bp (38, 50, 74). In the case of extrachromosomal recombination, this can still occur, albeit at a much-reduced frequency, with as little as 20 bp (2, 50). Recombination between transferred DNA and its cognate chromosomal sequence (gene targeting) in mammalian cells requires larger regions of homology, ranging from about 0.5 to 2.0 kb (23, 54, 67).

In the present study, we investigated a different type of homology requirement for mammalian genetic recombination, namely, that required to mediate ectopic recombination. The results revealed a trend toward poorer efficiencies of ectopic recombination with decreasing lengths of wild-type donor Cμ region homology to the recipient igm482 chromosomal Cμ region. The data suggest that the minimum length of homology required for ectopic recombination is between approximately 1.9 and 4.3 kb and that recombination frequency and the length of donor homology might be related in a linear fashion. The length of homology required for ectopic recombination is greater than that needed to mediate intra- and extrachromosomal recombination in the mammalian cell studies described above. However, in these systems, the two recombining regions are closely linked (within a few kilobase pairs) either on an integrated plasmid vector or on an extrachromosomal multicopy plasmid (2, 38, 50, 74). Since the local concentration of the recombining sequences in these systems is expected to be high, the search for homology might be less demanding. Our Cμ region ectopic recombination assay requires a search for homology that spans the entire mammalian genome. A global homology search might be expected to be more stringent, perhaps explaining the requirement for a somewhat larger region of homology. This idea is consistent with the observation that the homology requirement for mammalian gene targeting (0.5 to 2 kb) (23, 54, 67), which is also expected to result from a global homology search, is not substantially different from the minimum required to mediate ectopic recombination.

It is evident from the analysis of cell lines with different numbers of wild-type donor Cμ regions (with the exception of the E9 and E69 hybridomas) that the frequency of ectopic recombination for a single donor does not differ significantly from that for multiple donors clustered at one or a few (two or three) chromosomal sites. Similar results have been reported for ectopic recombination in the murine germ line (42), mammalian gene targeting studies (68, 79), and *S. cerevisiae* for ectopic meiotic recombination between *leu2* alleles (22) and Ty elements (31). In the present study, the independence of ectopic recombination and donor Cμ region copy number might be expected if, following chromosome interaction, the recipient igm482 Cμ sequence conducted a processive search along the DNA for a homologous Cμ donor region. Several lines of evidence suggest that recombination occurs following a processive search for a homologous partner. The data in the present study and those from mitotic ectopic recombination between *P* elements in *D. melanogaster* (17, 43) suggest that the recipient sequence can locate a single homologous donor anywhere in the genome with only a relatively small segment of homologous DNA. Furthermore, several studies described above for bacterial yeast, *D. melanogaster* and mammalian cells suggest that the search for homology is highly discriminatory in that it is hypersensitive to even a small proportion of DNA mismatches. Such a powerful mechanism for locating a homologous sequence tends to suggest that it functions by a processive search of the genome rather than simply a series of

FIG. 4. FISH of hybridoma chromosomes. FISH was performed as described in Materials and Methods on metaphase chromosomes from six different hybridoma lines. The single-copy endogenous, recipient igm482 μ locus, identified by FITC-labelled probe 2 (yellow signal), is indicated by the arrow for each cell line. The integration site of the wild-type donor Cμ region, identified by rhodamine-labelled probe 1 (red signal), is shown by the arrowhead for each cell line. Probes 1 and 2 mapped to the same chromosome in hybridoma lines igm482, 3, E26, and E29. Positive hybridization signals for both probes on the same chromosome were noted in >80% of 30 well-spread metaphases. Both signals were present on both chromatids in >80% of the positive spreads. The signals for both probes mapping to the same chromosome were very close, appearing most frequently as a fusion signal. For cell lines E26 and E29, probe 1 also localized to a second single chromosome different from that to which probe 2 hybridized. For both cell lines, the frequency of appearance of the second chromosomal signal was >90% for 30 well-spread metaphases. For hybridoma lines E9 and E69, probes 1 and 2 mapped to the same chromosome in approximately 80% of 30 and 20 well-spread metaphases, respectively. Both signals were present on both chromatids in all of the positive spreads and appeared as discrete signals, suggesting that they are located at least 1 to 2 Mb apart (33, 69, 77). In the case of E69, positive signals for probe 1 on a second hybridoma chromosome were visualized in 65% (13 of 20) of the spreads. Note that in hybridoma lines igm482, E26, and E29, the red signal that colocalizes with the endogenous igm482 μ locus (yellow signal) is the result of unavoidable cross hybridization of the Cμ portion of probe 1 with the endogenous igm482 Cμ region. For this reason, the FISH results for wild-type donor Cμ region integration must be analyzed in conjunction with the number of vector integration sites identified by Southern blot analysis of transformant genomic DNA (Fig. 3) (described further in Results).

time-consuming, random collisions. Studies of the way in which the RecA protein of *E. coli* mediates the pairing of homologous DNA are consistent with a processive search mechanism (20), as are experiments with *S. cerevisiae* which suggest that a molecule of DNA is scanned preferentially when the target sequence is embedded in a longer region of relatively weak homology (41).

Our results reveal a very high frequency of ectopic recombination in cell lines in which the recombining Cμ sequences are separated on the chromosome by about 1 to 2 Mb. The *cis* preference for recombination is striking, being as much as 10^4-fold higher than recombination between the same homologous Cμ sequences present on nonhomologous chromosomes or in their normal position on homologous chromosomes (53). A similar preference for intra- over interchromosomal recombination was also observed by us (3, 7, 53) and by others (19, 39) when homologous repeats were closely linked in the mammalian genome. The results of the present study are significant and have important implications for our understanding of the mechanism of recombination in mammalian somatic cells. The search for homology is the only component of the recombination process that is expected to involve the entire genome. Thus, our results suggest that the search for homology is more effective for sequences that are physically connected on the same chromosome than it is for matching sequences located elsewhere in the genome.

A *cis* preference for mitotic homologous recombination has also been observed, but to a lesser degree, in *D. melanogaster* (17, 43) and *S. cerevisiae* (34). In *D. melanogaster*, *P* element-induced mitotic recombination is 6- and 2.5-fold more efficient when the donor and recipient sequences are present in *cis* on the X chromosome than when the homologous template is located in *trans* on either an autosome or the homologous X chromosome, respectively (17, 43). A homolog preference or X-specific factors might explain the slight preference for a homologous template located on the X chromosome versus an autosome (17). Thus, in *D. melanogaster*, the *cis* advantage in recombination is a very long range, operating over more than 15 Mb on the X chromosome. In *S. cerevisiae*, a 6- to 13-fold stimulation in mitotic recombination was observed for *leu2* sequences separated by an intrachromosomal distance of only 20 kb compared with allelic copies of *leu2*. However, the *cis* effect on mitotic recombination diminished with intrachromosomal distances of more than 70 kb to a level which was similar to the frequency of mitotic recombination between allelic *leu2* sequences or *leu2* sequences located on nonhomologous chromosomes (34). Similar results have been reported for mitotic recombination between Ty elements in *S. cerevisiae* (31, 48). Interestingly, as in our homologous recombination studies, a *cis* preference has also been reported for site-specific recombination in mammalian cells. With the Cre recombinase, site-specific recombination between *loxP* sites well separated on the same chromosome is on average about 90-fold more frequent than between *loxP* sites situated on homologous chromosomes (47).

It is evident that the *cis* over *trans* preference we have observed for homologous recombination between well-separated Cμ regions in hybridomas can be as much as 10^3-fold higher than the corresponding ratio for recombination in *D. melanogaster* and *S. cerevisiae* and about 10^2-fold higher than for the Cre/*loxP* site-specific recombination system. There is strong evidence from a number of studies to suggest that transcriptional activity and/or the binding of proteins to genetic regulatory sequences can stimulate genetic recombination, perhaps by increasing the accessibility of the locus to recombination (44, 62, 66, 71, 76). Although the state of the ectopic wild-type donor Cμ segments is not known, the recipient igm482 chromosomal μ gene in the hybridomas is highly transcribed (13). Thus, it might be argued that increased accessibility of the chromosomal μ locus favors intra- over interchromosomal recombination. However, as discussed previously (53), the chromosomal μ gene segments involved in interchromosomal allelic recombination are expected to be at least as accessible as those involved in ectopic and intrachromosomal recombination. This suggests that the preference for intra- over interchromosomal recombination is not the result of increased accessibility of the chromosomal μ locus to recombination.

While homologous chromosomes must pair in meiosis, there appears to be no such requirement for somatic pairing of mammalian chromosomes in mitosis. Thus, why do mitotically dividing mammalian cells conduct a search for homology and what advantage would be derived by conducting it with such efficiency, preferentially in the *cis* configuration? Errors in DNA replication and other forms of DNA damage occur in mitotically dividing cells and are potentially deleterious if not repaired prior to cell division. In *S. cerevisiae*, mitotic recombination is primarily a result of DNA repair processes (24). Homologous DNA which is potentially capable of repairing DNA damage in vegetatively growing cells is available in G_1 of the cell cycle on the homologous chromosome or in G_2 on the sister chromatid. Kadyk and Hartwell (27) found that sister chromatids are preferred over homologous chromatids as substrates for recombinational repair in *S. cerevisiae*. This recombination pathway is expected to be of significant benefit in all replicating cells. Thus, the finding that mammalian mitotic cells undergo high-frequency recombination preferentially in the *cis* configuration might reflect a homology search mechanism operating within a chromosome (or chromatid) or between sister chromatids (if it occurs after the S phase of the cell cycle) that is part of the DNA repair process in mitotically dividing mammalian cells. In contrast, a homology search that occurs with a similar high efficiency in mammalian somatic cells in *trans* confers no corresponding advantage and, in fact, might lead to the generation of chromosomal rearrangements which predispose the cells to cancer and other genetic abnormalities (32, 46, 49, 78).

In summary, we have used our Cμ region recombination assay to probe some of the molecular requirements for ectopic recombination in mammalian somatic cells. The conclusions of this work are that (i) ectopic recombination corrects small chromosomal deletions more efficiently than larger ones, (ii) homologous segments of between 1.9 and 4.3 kb are required for ectopic recombination, (iii) the frequency of ectopic recombination does not depend on donor copy number, and (iv) homologous recombination is much more frequent for sequences physically connected on the same chromosome than it is for matching sequences located elsewhere in the genome. The latter finding has important implications for the way in which mammalian somatic cells conduct the search for homology. It suggests that the scanning mechanism is capable of searching the genome for a homologous partner sequence but is involved much more frequently in searches among nearby sequences on the same chromosome. This suggests that a sequence which has suffered a recombinogenic lesion is more likely to encounter and undergo recombination with a homologous partner that is physically connected on the same chromosome than it is with one that is unlinked.

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