Recombination Analysis of the Human Minisatellite MsH42 Suggests the Existence of Two Distinct Pathways for Initiation and Resolution of Recombination at MsH42 in Rat Testes Nuclear Extracts†

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Received September 19, 2001; Revised Manuscript Received December 6, 2001

ABSTRACT: We have previously described a GC-rich human minisatellite, termed MsH42, which exists in two allelic forms, long and short. Here, we have identified a third allele of medium length and localized the MsH42 locus in the chromosome 15q25.1 inside an intron belonging to a gene of unknown function. The recombinogenic potential of the three alleles was assayed in vitro incubating pBR322-based constructs containing two copies of the minisatellite MsH42 with its flanking sequences, in the presence of rat testes nuclear extracts. This assay system was configured to monitor only reciprocal exchange type events and not gene conversion. All MsH42 allelic sequences enhanced intramolecular homologous recombination promoting high rates (≈76%) of equal crossover, the long allele showing the highest recombinogenic activity. Removal of the MsH42 long allele flanking sequences, which are identical in the three alleles, provoked a decrease in the enhancement of recombination and in the frequency of equal crossovers, suggesting that these sequences are important for the recombinogenic activity and for the correct pairing between homologous sequences. The occurrence of some complex recombination events within the minisatellite MsH42 suggests the existence of processes related to polymerase slippage and unwinding with reinvasion during the repair synthesis. Our findings point toward the existence of two distinct biochemical pathways for initiation and resolution of recombination at the minisatellite MsH42. Finally, the in vitro recombination system employed in this study could provide an approach to dissect processes of repetitive DNA instability and recombination.

Genomes are flexible structures that combine the stability required for inheritance and the plasticity needed for change (*1*). Genetic recombination is a fundamental biological process, common to all forms of life, involving the exchange of DNA sequences between two chromosomes or DNA molecules. This process can occur anywhere along the homologous chromosomes, although there are certain regions of the genome, known as recombination hotspots, which have increased frequencies of genetic exchange (*2*). Recombination is required for the maintenance of chromosomal integrity, through recombinational repair, as well as for the generation of genetic diversity by random assortment of alleles (*3*). It also serves to help ensure the appropriate segregation of chromosomes at the first meiotic division in most organisms (*4*).

The minisatellites, also known as VNTR (variable number of tandem repeats), are arrays of repeat sequences that display length variations. The different minisatellite alleles contain different numbers of repeat units. During the 1990s, evidence has emerged that minisatellites play significant roles in important features of human genome biology, including regulation of transcription, recombination, and imprinting (*5*−*7*). There is evidence indicating that minisatellites are recombination hotspots themselves (*7*, *8*). In this sense, it has been established that gene conversion events (*9*) and crossovers (*10*, *11*) are involved in the interallelic recombinatory events occurring at hypervariable minisatellite sequences in the human germline. Moreover, these kinds of sequences have been identified among DNA undergoing repair in rat meiosis pachytene (*12*). The meiotic instability of the human minisatellite locus CEB1 in yeast (*13*) requires double-strand breaks (DSB),[1] that are essential in the context of the DSB repair models (*14*). Concerning human nonhypervariable minisatellite loci, very few data exist. There is one study on the insulin minisatellite that demonstrates that this sequence appears to evolve by mitotic replication slippage-like events and less frequently by complex recombinational processes probably meiotic in origin (*15*).

† This work was supported by the Spanish Ministerio of Educación y Cultura (PB97-0515) and by the local government Xunta de Galicia (XUGA-20003B98).

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[1] Abbreviations: DSB, double-strand break; MsH42, human minisatellite MsH42; SDSA, synthesis-dependent strand annealing.

10.1021/bi015780i CCC: $22.00
Published on Web 01/24/2002

In a previous work, we have reported the isolation of a human DNA locus (H42) containing a low polymorphic GC-rich minisatellite (MsH42) with two alleles (*16*). The MsH42 long allele includes 36 repeats of closely related decamers and nonamers, whereas the short allele has a deletion of 11 repeats within the tandem array. In this minisatellite region (MsH42 and its flanking sequences), there are several recombination motifs, including two χ octamers, two oligomers described as recombination signals in immunoglobulin VDJ joining, and similar sequences to those involved in immunoglobulin class-switching. The long allele undergoes slipped-strand mispairing during PCR, whereas this process is almost imperceptible with the short allele (*16*). MsH42 is specifically recognized by nuclear proteins in band-shifting experiments (*16*). The MsH42 region strongly promotes in vitro intramolecular homologous recombination in the presence of testes nuclear extracts (*17*), suggesting that this minisatellite region might be involved in recombination in humans.

In the present work, we have characterized more in-depth the MsH42 locus (alleles structure, chromosome localization, population analysis). We found that all MsH42 alleles can enhance intramolecular homologous recombination in vitro, promoting high rates of equal crossover, the long allele being the most recombinogenic. However, with this experimental recombination system, we can only detect reciprocal exchange type events and not gene conversion. The generation of complex recombinants, with the probable involvement of polymerase slippage or misalignments during gap repair, led us to propose a hypothetical process to explain the generation of such recombinants. The in vitro system used in this work could provide an approach to dissect processes related to repetitive DNA instability and recombination.

EXPERIMENTAL PROCEDURES

Materials. Blood or saliva was used as a source of human DNA. The DNAs were obtained from 200 healthy individuals of the Galician population (NW Spain). Rat testes nuclear extracts were prepared from 3-month old Sprague−Dawley rats, as described elsewhere (*17*). The bacteria employed in this study were *Escherichia coli* DH5α cells (*lac*Z$^-$/*rec*A$^-$). QIAamp DNA kit and QIAEX II gel extraction kit were obtained from QIAGEN. T7 Sequenase quick-denature plasmid sequencing kit, PCR product sequencing kit, nucleotides, and DNaseI were purchased from Amersham Pharmacia Biotech. The pGemT vectors, Taq DNA polymerase, and restriction enzymes were obtained from Promega. The X-Gal and IPTG reagents were purchased from Sigma. Radioactive nucleotides were from New England Nuclear. Metaphor agarose was obtained from FMC. The DNA molecular weight marker XIII (a 50 bp ladder) was purchased from Roche Biochemicals, and the 1 kb ladder was from Promega.

Methods: Population Analysis, Allele Characterization, and Chromosome Localization. To estimate the frequency of the MsH42 alleles, human DNAs were prepared using the QIAamp DNA kit. The MsH42 locus was amplified, as described elsewhere (*16*). The middle allele was purified from an agarose gel with the QIAEX II gel extraction kit, cloned in pGemT, and sequenced using the T7 Sequenase plasmid sequencing kit. Chromosome localization of the MsH42 locus was obtained by searching in the ENSEMBL genome server of the EBI/Sanger center (http://www.ensembl.org).

Construction of Homologous Recombination Substrates. The map of the H42 DNA fragment, which includes the MsH42 region and the localization of the PCR primers employed in this study, is shown in Figure 1A. All of the plasmids used as recombination substrates derive from pBR322 and present two identical copies of H42 sequences (see the following discussion), cloned in the same orientation and flanking the *lac*Z gene (Figure 1B). The three different MsH42 allele regions (minisatellite plus flanking sequences) were obtained by PCR of genomic DNAs using primers PS1, 5′-CTG CAG CAA TGG ACT CAA AA-3′, and PS2, 5′-CTG CAG ACT CCA AAT CCT AA-3′, under the conditions described elsewhere (*16*). The construction of pMsH42*Lac*, which has two copies of the MsH42 long allele region (880 bp), and of the control plasmid p5′MsH42*Lac*, which contains two copies of a nonrepetitive control sequence (850 bp) located upstream the minisatellite, was described elsewhere (*17*). Likewise, we constructed pMsH42m*Lac* and pMsH42s*Lac*, which carry two copies of the middle (806 bp) and the short (756 bp) allele region, respectively, and pMsH42sat*Lac* with two copies of a fragment (344 bp) containing the MsH42 long allele without flanking sequences. This fragment was amplified from 1 ng of pRep42 (*16*) using the primers MS1, 5′-TGG GAG AGG CTG GGA TTG CTG GGA G-3′, and MS2, 5′-TCT TTC CCA GTC TTT CCC AGT CTC T-3′ (Figure 1A), during 30 cycles (denaturing step at 95 °C, 1 min; followed by an annealing-extension step at 72 °C, 1 min) and a final cycle with an extension of 5 min, with the aforesaid reaction conditions employed. The corresponding control plasmid, p5′MsH42.1*Lac*, was constructed by cloning two copies of a nonrepetitive sequence (5′MsH42.1 in Figure 1A) with a similar size (356 bp) to that of the MsH42 long allele.

In Vitro Recombination Assays. To study the recombinogenic capacity of the MsH42 allelic sequences, we performed three different recombination experiments. First, each plasmid construct containing two copies of the minisatellite region (pMsH42*Lac*, pMsH42m*Lac*, pMsH42s*Lac*) was incubated with the same amount of the control plasmid (p5'MsH42*lac*) in the presence of a rat testes nuclear extract. Second, likewise, we performed experiments with mixes of two minisatellite-bearing plasmids (pMsH42*Lac*/pMsH42s*Lac*, pMsH42*Lac*/pMsH42m*Lac*, pMsH42s*Lac*/pMsH42m*Lac*, and pMsH42*Lac*/pMsH42sat*Lac*). Third, the recombinogenic capacity of the minisatellite without flanking sequences was assayed incubating together pMsH42sat*Lac* with p5′MsH42.1*Lac* (control) under the same experimental conditions. In all experiments, after the incubation with the testis nuclear extract, samples were phenol extracted, ethanol precipitated, and used to transform *E. coli* DH5α cells. The bacteria were plated onto LB agar plates containing X-Gal and IPTG as *lac*Z gene indicators. Because the intramolecular recombination between the two homologous copies produces the excision of the *lac*Z gene (Figure 1C), transformation with the recombinant products generated ampicillin-resistant white colonies. In contrast, the original recombination substrates that did not undergo intramolecular recombination generated ampicillin-resistant blue colonies. To differentiate the clones containing the MsH42 alleles from those generated by the control sequences, colonies were blotted and hybrid-

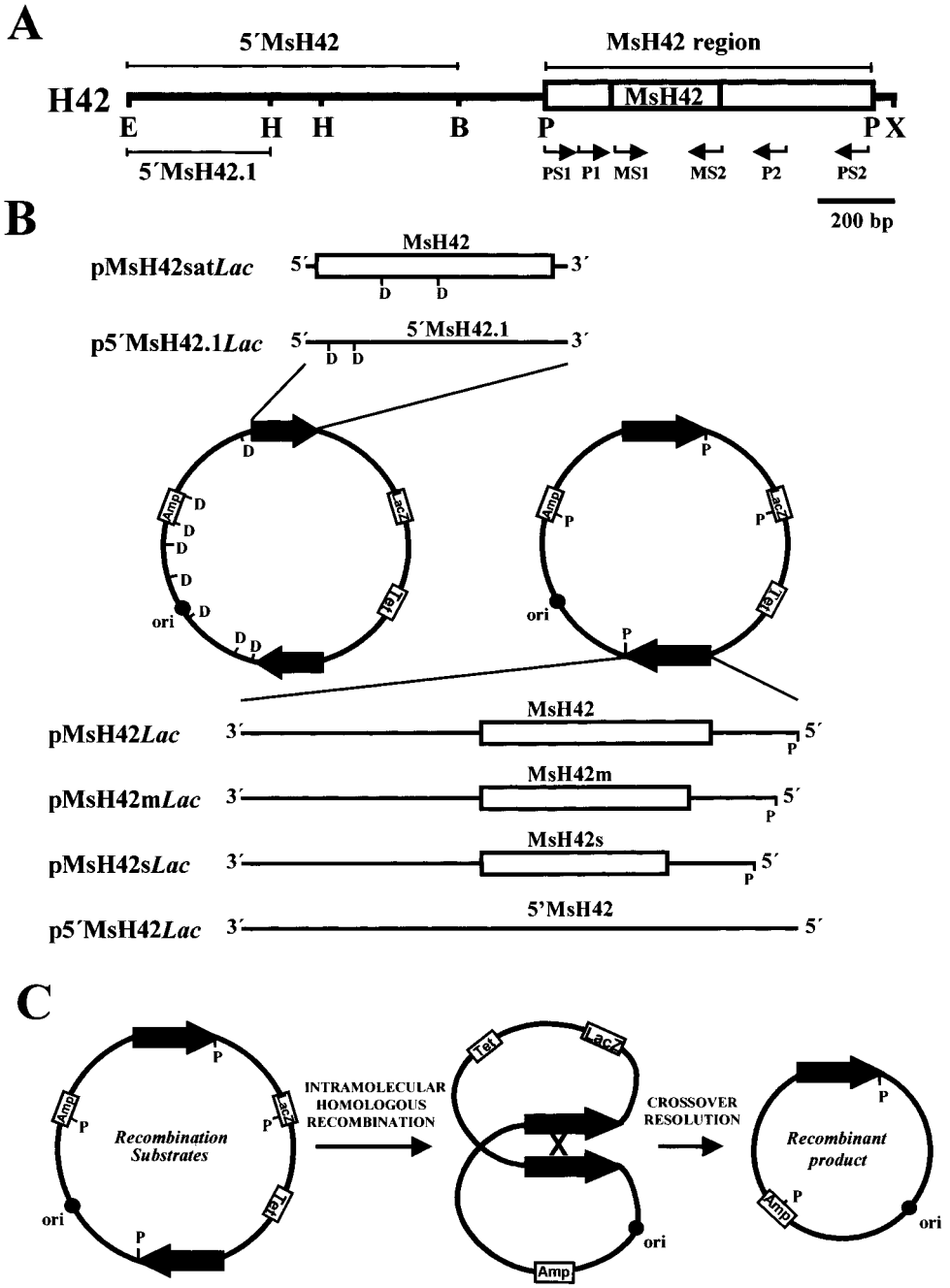

FIGURE 1: Recombination substrates. (A) Map of the H42 DNA fragment showing the minisatellite MsH42 region, which comprises the minisatellite MsH42 and its proximal flanking sequences, delimited by two *Pst*I sites. The segment 5′MsH42 represents a nonrepetitive sequence delimited by *Eco*RI and *Bgl*II sites, and the segment 5′MsH42.1 represents a nonrepetitive sequence delimited by *Eco*RI and *Hae*III which is situated inside the 5′MsH42 sequence. Positions of the different primers (P1, P2, PS1, PS2, MS1, MS2) employed in the PCR experiments are marked by arrows below the MsH42 region. (B) Overall design of the homologous recombination substrates. All plasmids have identical backbones and derive from pBR322. They contain the replication origin (ori), the ampicillin (Amp) and tetracycline (Tet) resistance genes, the *Lac*Z gene (LacZ), and two identical copies (black arrows) of different H42 inserts cloned in the same orientation. A higher-resolution linear scheme of each of the various MsH42 and control inserts is shown. The plasmids are pMsH42*Lac* (long allele region), pMsH42m*Lac* (middle allele region), pMsH42s*Lac* (short allele region), pMsH42sat*Lac* (long allele without its flanking sequences), p5′MsH42*Lac* (control sequence), and p5′MsH42.1*Lac* (control sequence situated inside 5′MsH42). The open box represents the minisatellite region of each allele. Plasmids are not drawn to scale. (C) Representation of an intramolecular homologous recombination event showing one of the two plasmids generated in the recombination (the other plasmid cannot propagate in *E. coli* because it lacks a replication origin). The restriction enzymes shown in the three panels are as follows: B, *Bgl*II; E, *Eco*RI; H, *Hae*III; P, *Pst*I; X, *Xba*I, D, *Dde*I.

ized with the ^{32}P-labeled pRep42 insert, which carries the MsH42 region (*16*). In the recombination experiments with mixes of two minisatellite-bearing plasmids, the blue colonies corresponding to each plasmid were distinguished by *Eco*RI digestion. Digestion with *Pst*I was used to distinguish between the original recombination substrates (plasmids with the MsH42 region and control plasmid) and their recombinant products. Thus, *Pst*I digestion of the pMsH42*Lac*, pMsH42m*Lac*, and pMsH42s*Lac* yields six restriction fragments (two of them identical), whereas digestion of their respective recombinant products generates only three fragments (Figures 1B and 2A). In the case of p5′MsH42*Lac*, digestion with *Pst*I generates four fragments, whereas digestion of its recombinant products originates only

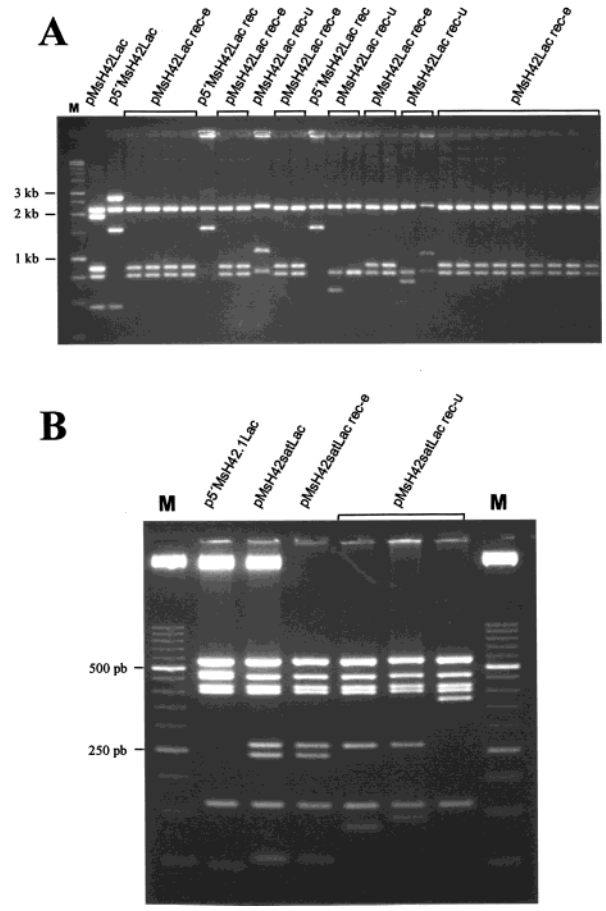

FIGURE 2: Restriction analysis of recombinant products. (A) *Pst*I restriction enzyme analysis of plasmids obtained from *lac*Z$^-$ colonies in recombination experiments carried out with pMsH42*lac* and p5′MsH42*Lac* as original recombination substrates. Plasmid DNAs were prepared from 5 mL cultures following standard procedures; aliquots of 0.3−0.5 μg were digested with *PstI*, electrophoresed through a 1.5% agarose gel, and stained with ethidium bromide. The *Pst*I restriction patterns shown correspond to the original plasmids pMsH42*Lac* and p5′MsH42*Lac*, to recombinants products derived from equal (pMsH42*Lac* rec-e) and unequal (pMsH42*Lac* rec-u) crossovers in pMsH42*Lac*, and to recombinant products (p5′MsH42*Lac* rec) obtained from the control plasmid p5′MsH42*Lac*. Lane M has the 1 kb molecular weight marker. Similar patterns were obtained with the middle and the short allele plasmids (not shown). (B) *Dde*I restriction enzyme analysis of plasmids obtained from *lac*Z$^-$ colonies in recombination experiments carried out with pMsH42sat*Lac* and p5′MsH42.1*Lac*. Aliquots of 0.3−0.5 μg, prepared as indicated for A, were digested with *DdeI*, separated on 2.5% Metaphor agarose gels, and stained with ethidium bromide. The *Dde*I restriction patterns shown correspond to the original plasmids p5′MsH42.1*Lac* and pMsH42sat*Lac* and to recombinant products derived from equal (pMsH42sat*Lac* rec-e) and unequal (pMsH42sat*Lac* rec-u) crossovers in pMsH42sat*Lac*. Lane M has a 50 bp molecular weight marker.

two fragments. *Dde*I digestion was employed to differentiate between pMsH42sat*Lac* and p5′MsH42.1*Lac* and to identify their recombinant products because they lack the restriction fragment that harbors the *lac*Z gene (Figures 1B and 2B). When we incubated pMsH42*Lac* and pMsH42s*Lac* together, the recombinants derived from each plasmid were identified by *Nco*I digestion because pMsH42s*Lac* has an extra *Nco*I site generated during the cloning procedure. Similarly, the recombinant products from the pMsH42*Lac*/pMsH42m*Lac* and pMsH42s*Lac*/pMsH42m*Lac* reactions were differentiated by restriction with *Eco*RI. The recombinant products obtained from pMsH42*Lac*/pMsH42sat*Lac* reactions were identified with *Pst*I. In all experiments, several representative recombinant products were amplified by PCR with the primers PS1/PS2, for the products generated by the plasmids bearing the MsH42 allele regions, and the MS1/MS2 primers, in the case of the products generated by the long allele without flanking sequences. The amplified DNAs were sequenced using the PCR product sequencing kit.

The *lac*Z$^-$ colonies, originated by mutations in the *lac*Z gene, were identified by restriction analysis and tetracycline resistance, and they made up less than 1% of the total *lac*Z$^-$ colonies. The frequency of the *lac*Z$^-$ colonies in transformations with the substrate plasmids not exposed to the nuclear extract or with heat-inactivated extract (15 min, 100 °C) was about 2×10^{-5}. Because the testis extract can induce lesions (single- or double-stranded breaks) in the plasmid molecules which may be repaired by the RecA-independent recombination machinery of the bacteria generating recombinant products, we carried out incubations of pMsH42*lac* (1 μg) with several amounts (10, 50, and 100 pg) of DNaseI in the recombination buffer at 37 °C during 30 min. Aliquots of these reactions were analyzed by electrophoresis in agarose gels and used to transform *E. coli* DH5α cells. The estimation of recombination frequency was performed as that for recombination experiments. Finally, it should be emphasized that the original plasmid constructs and their recombinant products showed a very similar transformation efficiency of *E. coli.*

RESULTS

Population Study and Chromosome Localization of the MsH42 Locus. We have previously reported the characterization of two alleles belonging to the MsH42 locus (*16*). During the screening of the Galician population, we found a new allele (middle allele) with a deletion of 57 bp within the repeat array respect to the long allele (Figure 5B, line 1). The results obtained in the population analysis showed that the long allele is the most abundant (long allele = 0.834 ± 0.019, middle allele = 0.005 ± 0.0035, short allele = 0.161 ± 0.018) and that the genotype distribution is in Hardy−Weinberg equilibrium ($\chi^2 = 0.76$, $P > 0.05$). The minisatellite MsH42 (short allele) was localized in the contig AC027808.5.1.207303 (positions 163 518 to 163 756) at the chromosome 15q25.1 (*18*). The MsH42 region is situated within an intron, between the exons 5 and 6, that belongs to the gene ENSG00000103888, whose function is still unknown.

In Vitro Recombinogenic Activity of the Minisatellite MsH42 Alleles. The MsH42 region (Figure 1A) comprises the minisatellite MsH42 plus its flanking sequences which are identical in the three alleles. The flanking sequences are arbitrarily limited by the most proximal *Pst*I sites situated at both sides of the minisatellite. To study the recombinogenic activity of each MsH42 allelic region with respect to a nonrepetitive sequence located upstream of the MsH42 region (control sequence), we constructed several pBR322-based vectors bearing two copies, cloned in the same orientation, of the minisatellite allelic region (pMsH42*Lac* contains the long allele, pMsH42m*Lac* contains the middle allele, and pMsH42s*Lac* contains the short allele) or two copies of the control sequence (p5′MsH42*Lac*). The map of

Table 1: Effect of the Minisatelite MsH42 Region on Recombination

recombination substrates[a]	*Lac*Z$^-$ colonies[b]	*Lac*Z$^+$ colonies[b]	recombinant frequencies (%)[c]	recombination enhancement[d]	equal recombinants	equal crossover frequency (%)[e]
pMsH42*Lac*/p5′MsH42*Lac*	60/5	8895/8328	0.670/0.060	11.166	55	91.667
(long allele)/(control)	89/6	7266/7494	1.210/0.080	15.126	54	60.674
	95/10	11633/11755	0.810/0.085	9.530	77	81.053
			0.897 ± 0.280/0.075 ± 0.013	11.941 ± 2.877		77.798 ± 15.751
pMsH42m*Lac*/p5′MsH42*Lac*	38/8	9010/6146	0.420/0.130	3.231	33	86.842
(middle allele)/(control)	56/12	8179/6864	0.680/0.175	3.897	34	60.714
	25/7	7328/7929	0.340/0.088	3.855	21	84.000
			0.480 ± 0.178/0.131 ± 0.043	3.661 ± 0.373		77.185 ± 14.335
pMsH42s*Lac*/p5′MsH42*Lac*	31/9	8127/8173	0.380/0.110	3.455	27	87.097
(short allele)/(control)	28/7	8457/8743	0.330/0.080	4.125	21	75.000
	49/16	9374/10651	0.520/0.150	3.467	28	57.143
			0.410 ± 0.098/0.113 ± 0.035	3.682 ± 0.384		73.080 ± 15.069
pMsH42sat*Lac*/p5′MsH42.1*Lac*	27/9	11712/9565	0.230/0.094	2.447	8	29.630
(MsH42)/(control)	15/5	11523/9254	0.130/0.054	2.407	5	33.333
	16/6	8873/10163	0.180/0.059	3.051	5	31.250
			0.180 ± 0.050/0.069 ± 0.022	2.635 ± 0.361		31.404 ± 1.875

[a] The recombination substrates employed in these experiments are shown in Figure 1. Long, middle, and short alleles represent their respective allelic regions (minisatellite plus flanking sequences), and MsH42 is the minisatellite alone. The data were collected from three independent experiments. In the recombination assays, the plasmid containing the MsH42 allele region and the control plasmid were incubated simultaneously with testes nuclear extracts. [b] The plasmids undergoing intramolecular homologous recombination yield *lac*Z$^-$ colonies, whereas the nonrecombinant ones generate *Lac*Z$^+$ colonies. [c] The recombinant frequencies are given as the ratio between the number of *Lac*Z$^-$ colonies and the total colonies obtained from each recombination substrate. For columns 4, 5, and 7, mean ± SD of the data is provided. [d] The recombination enhancement is the ratio between the recombinant frequencies obtained from each recombination substrate. [e] The equal crossover frequency represents the percentage of equal crossovers from the total MsH42 sequence crossovers in each experiment.

these plasmids is shown in Figure 1B. Because we cloned the *lac*Z gene between the two copies of the H42 inserts, the intramolecular homologous recombination produces the excision of the *lac*Z gene from the original constructs (Figure 1C). Consequently, the recombinant products will yield *lac*Z$^-$ bacteria (white colonies) after transformation.

In the recombination experiments, each plasmid substrate was incubated with an equal amount of the control plasmid in the presence of testes nuclear extracts. After incubation, DNAs were extracted and used to transform *E. coli* DH5α cells. The identification of the recombinant (from equal and unequal crossovers) and nonrecombinant plasmids was carried out by restriction analysis. Figure 2A illustrates a representative restriction analysis of a recombination experiment carried out with the MsH42 long allele region (pMsH42*Lac*) and the control sequence (p5′MsH42*Lac*). As shown in Table 1, the three alleles were able to stimulate the intramolecular homologous recombination. The enhancement of recombination promoted by the middle and the short alleles was 3.6 times higher than that produced by the control sequence (lines 2 and 3 in column 5) and approximately 3.2 times lower than that exhibited by the long allele (line 1 in column 5). According to these results, the long allele is the most efficient in the stimulation of recombination, and the middle and the short alleles have a similar capacity to enhance recombination in vitro. Because both the original plasmid constructs (pMsH42*Lac*, pMsH42m*Lac*, pMsH42s*Lac*, and p5′MsH42*Lac*) and their recombinant products have a very similar transformation efficiency of *E. coli* (data not shown), the differences among the three alleles should be attributed to their capacity of enhancing in vitro recombination. Nevertheless, the analysis of the data shown in Table 1 does not allow for the conclusion that the recombinant frequencies of the long and middle alleles are significantly different (Table 1, column 5). Some of the control crosses exhibited recombinant frequencies that are significantly different from other control crosses (column 4, right numbers). This heterogeneity in the recombinant frequencies of the control sequence might be the consequence of the highest recombinogenic capacity of the long allele region, which would compete more efficiently for the recombination machinery than the control sequence.

An alternative approach to compare more directly the recombinogenic potential of the MsH42 alleles consisted in incubating together combinations of two plasmids, each of them representing an allelic region of MsH42 (long/middle, long/short, middle/short, and long/long without flanking DNA). In these experiments, mixes of two plasmids (pMsH42*Lac* and pMsH42m*Lac*; pMsH42*Lac* and pMsH42s*Lac*; pMsH42m*Lac* and pMsH42s*Lac*; pMsH42*Lac* and pMsH42sat*Lac*) were incubated in the presence of the rat testes nuclear extract as described previously. The recombinant frequencies were obtained from three different experiments (data not shown). The results of these assays (Figure 3) provided strong evidence that the long allele region is the most recombinogenic and that the middle and the short alleles have a similar recombinogenic capacity. Because all alleles share the same flanking sequences, the structure of the minisatellite array seems to be very important for the enhancement of recombination observed in our in vitro experiments.

To determine the percentage of recombination events that occurs during plasmid propagation in bacteria, hence contributing to the observed recombinant frequencies (Table 1), we carried out the transformation of *E. coli* DH5α with the different recombinogenic substrates without exposing them to the testis extract. In these experiments, the frequency of the *lac*Z$^-$ colonies was about 2×10^{-5}. Likewise, incubation of the plasmid constructs with heat-inactivated testis extracts produced a frequency of *lac*Z$^-$ colonies again in the range of 2×10^{-5}. These control experiments provide bona fide evidence that the plasmid intramolecular recombination was catalyzed by the recombination machinery present in the testis extract and not during the cloning in *E. coli*. However, these controls did not completely rule out the implication of the transformed bacteria in the outcome of the recombination results. In fact, our observations could be also attributed to a specific nuclease activity present in the testis extract that

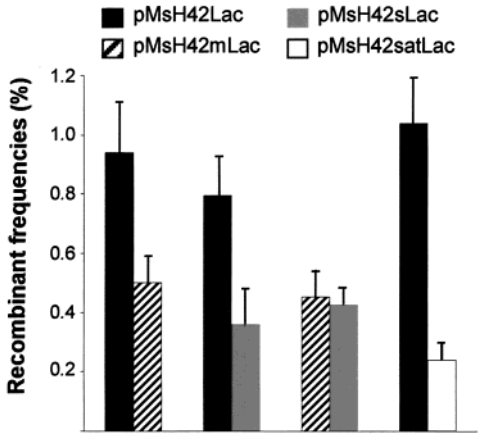

FIGURE 3: Recombination assays with combinations of MsH42 allelic regions. In these experiments, mixes of two plasmids (pMsH42*Lac* and pMsH42m*Lac*; pMsH42*Lac* and pMsH42s*Lac*; pMsH42m*Lac* and pMsH42s*Lac*; pMsH42*Lac* and pMsH42sat*Lac*) were incubated together in the presence of the testes nuclear extract. Each pair of bars represent the recombinant frequencies obtained from three independent recombination assays carried out with combinations of two plasmids which are indicated at the top of the figure. Data are shown as mean ± SD of three independent experiments.

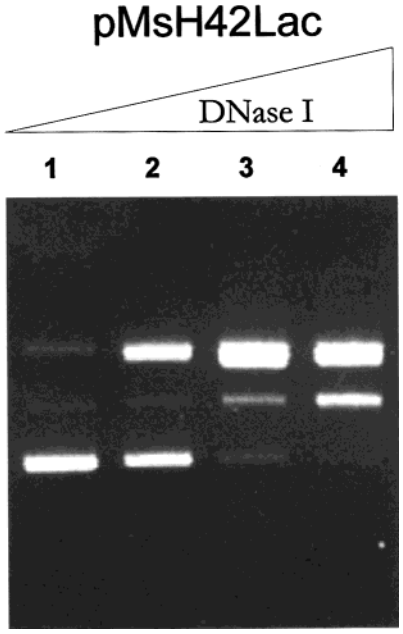

FIGURE 4: DNaseI nicking of pMsH42*Lac*. Aliquots of 1 μg of supercoiled plasmid pMsH42*Lac* (lane 1) were partially digested (nicked) with increased amounts of DNaseI (10 pg, lane 2; 50 pg, lane 3; 100 pg, lane 4), as described in Experimental Procedures. Aliquots of the digestions were analyzed by electrophoresis in 1% agarose gels. The nicking activity of the DNaseI was monitored by the conversion of supercoiled DNA into open circular molecules.

introduces lesions (nicks or double-stranded breaks) into plasmid molecules, which are then repaired by recombination in *E. coli* after transformation. When this reasoning is followed, the very low recombination activity found in heat-treated extracts may simply show that such nuclease activity is inactivated. To test this possibility, we performed experiments consisting in the introduction of lesions in the original recombination substrate pMsH42*Lac* by limited treatment with DNaseI (Figure 4). The activity of the DNaseI on the plasmid molecules was observed by the variation in the amount of supercoiled pMsH42*Lac*.

After incubation with the DNaseI, the nicked and the original plasmids were used to transform bacteria. We found that the recombinant frequencies were quite similar ($\sim 2 \times 10^{-5}$) for both untreated and DNaseI-treated plasmids. Therefore, a recombinogenic lesion model does not seem to be adequate to explain our results with the testis extract. In addition, we have previously reported that liver, which has a high endogenous nuclease activity, and NC-37 cell extracts did not provoke an increase in the recombinant frequencies of the minisatellite over the control, whereas the testis extract produced a strong enhancement of recombination (*17*). Moreover, other authors have previously demonstrated with a similar in vitro system, which also used the same *E. coli* strain, a different recombinogenic capacity of extracts derived from mouse testes and Ehrlich ascites cells (*19*).

The existence of a low frequency of recombination in bacteria indicates that, in some cases, the recombination machinery of *E. coli* might be acting on those plasmids. Whereas it is reasonable to assume that the recipient bacteria cannot support the initiation of recombination because RecA deficient cells should be defective for strand invasion, those plasmids that have initiated but not completed recombination before transformation into *E. coli* could be influenced by a number of factors other than RecA. For example, RecG and RuvABC can restart repair synthesis that has paused at a polymerase-blocking lesion by generating Holliday junctions (*20*). On the other hand, the mutant *lac*Z$^-$ colonies because of spontaneous mutagenesis in the *lac*Z gene (the background of *lacZ*$^-$ colonies) were identified by restriction analysis and tetracycline resistance, and they made up less than 1% of the total *lac*Z$^-$ colonies.

Recombination Analysis of the MsH42 Long Allele without Flanking Sequences. Because the flanking sequences of MsH42 contain some signals that could contribute to the stimulation of recombination (*16*), we evaluated the recombinogenic activity of the minisatellite (long allele version) without its flanking sequences. The recombination experiments were carried out by incubation of equal amounts of pMsH42sat*Lac* (the MsH42 long allele without its flanking sequences) and p5'MsH42.1*Lac* (its corresponding control sequence). The map of these plasmids is shown in Figure 1B. We found that the capacity of MsH42 long allele to enhance intramolecular homologous recombination is lower than that observed when the flanking sequences are present (compare lines 1 and 4 in Table 1). Moreover, incubation of equimolecular amounts of pMsH42*Lac* and pMsH42sat*Lac* showed that pMsH42*Lac* provokes an enhancement that is 4.3-fold higher than that produced by the other plasmid (Figure 3), confirming that the flanking sequences are required for the fully recombinogenic activity of the MsH42 region.

A linear relationship between the recombination frequency and the length of homology has been reported (*21*−*23*). Because the plasmids pMsH42*Lac* and pMsH42sat*Lac* contain inserts differing by 534 bp, it was possible that these differences in the enhancement of recombination were influenced by the different insert sizes. To check this possibility, we performed recombination assays incubating together the control plasmids p5′MsH42*Lac* (850 bp) and p5′MsH42.1*Lac* (356 bp). Our results showed that the recombinant frequencies with p5′MsH42*Lac* were approximately 1.8 times higher than those found with p5′MsH42.1*Lac*.

According to this, the differences in size cannot explain the higher enhancement provoked by the MsH42 long allele region (4.3-fold) as compared to the MsH42 long allele.

Crossover Analysis. The intramolecular homologous recombination generates recombinant plasmids that can be recognized by their different restriction patterns and by PCR analysis. Thus, in the equal recombinants, the fragment containing the minisatellite remains unaltered in length, whereas the existence of unequal pairings or alterations during the recombination process produces size variations. Obviously, the equal crossover events can occur within the minisatellite as well as in the flanking DNA. We have sequenced five equal recombinants and found that there are no alterations either in the minisatellite array or within its flanking sequences, corroborating previous observations from our laboratory (*17*). Nonetheless, it is possible that some recombinants showing no variations in length, classified as equal recombinants, present alterations in the minisatellite array as a consequence of polymerase slippage or another kind of recombination process.

To estimate the frequency of equal and unequal crossovers, we inspected for the *Pst*I restriction fragment that contains the minisatellite: when this fragment showed no size variation, we scored it as an equal crossover, and when its size changed, it was scored as an unequal crossover. We analyzed the same number of *lac*Z$^-$ colonies that were used to quantify the enhancement of recombination (Table 1). Figure 2 shows two representative agarose gels of routine restriction analyses of the white colonies obtained in the recombination experiments. The equal and unequal crossovers were always corroborated by PCR analysis of the recombinant products. Among the crossover products that suffered size variations, the number of recombinants showing minisatellite shortening or growing was about the same. Thus, the number of unequal recombinants detected for each allelic region and for the long allele without flanking sequences was 58 for the long allele region (27 short and 31 long), 31 for the middle allele region (16 short and 15 long), 32 for the short allele region (15 short and 17 long), and 40 for the long allele without flanking sequences (21 short and 19 long). The frequency of equal crossovers was very high for the three allelic regions (Table 1, last column). In contrast, when the recombination experiments involved the long allele without its flanking sequences, the frequency of equal crossovers strongly decreased (Table 1, last line in last column), suggesting that the presence of the sequences flanking the minisatellite MsH42 is important for the accuracy of the recombination process.

The existence of recombinants with alterations in the minisatellite structure allows for the search of the repeat where the intramolecular exchange took place. In addition, we have previously reported that the third repeat area of the minisatellite array is involved in the resolution of unequal crossovers leading to minisatellite shortening (*17*). Therefore, we decided to investigate the behavior of the three alleles in the generation of short recombinants. For this purpose, we amplified and sequenced several representative minisatellite short recombinants: 7 from the long allele (total of 27), 5 from the middle allele (total of 16), 6 from the short allele (total of 15), and 8 from the long allele without flanking sequences (total of 21). The sequences of these short recombinants are shown in Figure 5. To make sure that the different sequence arrays found in the recombinant products are stable, we subcloned the amplification products in the pGemT-Easy system, and after propagation in bacteria, the inserts were sequenced with the M13 universal primers. The results of these experiments showed that the recombination products were not altered during cloning and, hence, that they should be considered as stable.

Because of the duplication of repeat blocks along the minisatellite (*16*), it was not always possible to determine the exact point where the crossover occurred as well as whether they came from single or complex recombination events. With the long allele, most of the crossovers that produced minisatellite shortening were between the third repeat or its vicinity and another repeat inside MsH42 (Figure 5A). Interestingly, one of the recombinants generated by the long allele (12 in Figure 5A) is identical to the short allele, suggesting that the natural origin of the short allele might have been a recombination event. The long allele also generated recombinant products showing complex rearrangements (deletions and duplications) that could not be explained by a simple unequal crossover (7 in Figure 5A). In the short recombinants generated by the middle and the short alleles, there is no predominance of the third repeat area, and a clear polarity toward the 5′ end of the minisatellite could not be observed (Figure 5B,C). In the recombinants obtained with the MsH42 without its flanking sequences (9−16 in Figure 5A), we found a more random distribution of deletions, some of them with complex rearrangements (14−15 in Figure 5A) probably provoked by the lack of flanking sequences. It is worth noting that all recombinant products analyzed contain the group of repeats A-A3-A4-A5 situated at the 3′ end of the minisatellite, unaltered.

DISCUSSION

In this work, we have employed a biochemical approach to study mechanisms of function of the MsH42 region in recombination. However, with this in vitro recombination system, we can only detect reciprocal exchange type events and not gene conversions. The results presented here demonstrate that the three alleles of the MsH42 locus are able to enhance in vitro intramolecular homologous recombination, the long allele showing the highest recombinogenic activity. Because the three alleles share the same flanking sequences, the distinct degree of recombination enhancement should be attributed to differences in the organization of the minisatellite. If the main factor involved in the recombinogenic potential was the number of repeats, then a linear gradient (long−middle−short) in the capacity of recombination enhancement should be expected. However, our results showed that an increase in repeat length is not enough to explain the differences in the capacity of recombination enhancement. Thus, the middle and the short alleles displayed very similar recombinant frequencies in experiments with the control sequence (Table 1) as well as in combinations of the two MsH42-bearing plasmids (Figure 3), although they have different number of repeats. Furthermore, the recombinogenic capacity of the long allele (36 repeats) with respect to either the short (25 repeats) or the middle (30 repeats) alleles is very similar (Figure 3). It is possible that the repeat

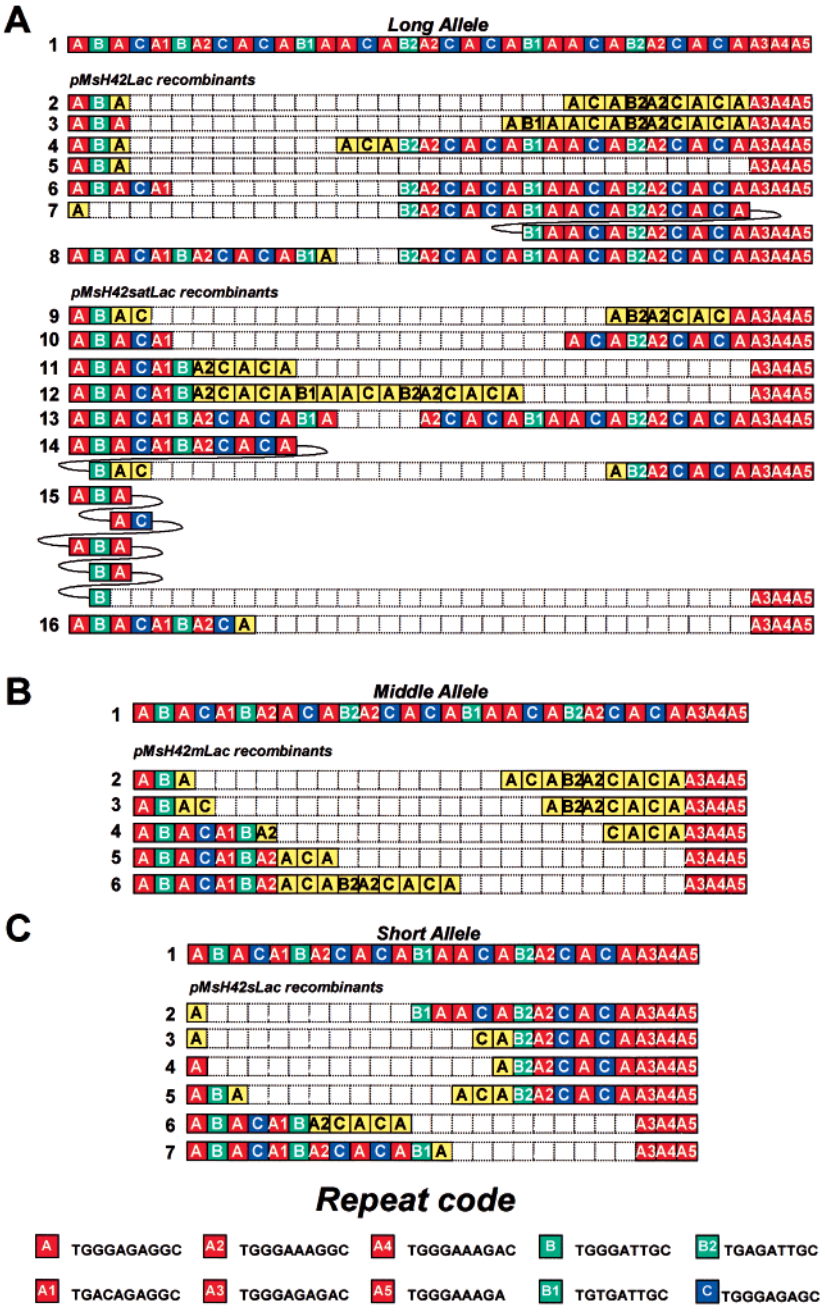

FIGURE 5: Sequence analysis of several MsH42-derived short recombinants. (A) Line 1 shows the repeat array of the MsH42 long allele, lines 2−8 represent the repeat array of some long allele short recombinants obtained in experiments carried out with pMsH42*Lac*, and lines 9−16 represent the repeat array of some short recombinants obtained in experiments carried out with pMsH42sat*Lac* (the minisatellite without the flanking sequences). (B) Line 1 shows the repeat array of the MsH42 middle allele, and lines 2−6 represent the repeat array of some middle allele recombinants obtained in experiments carried out with pMsH42m*Lac*. (C) Line 1 shows the repeat array of the MsH42 short allele, and lines 2−7 represent the repeat array of some short allele recombinants obtained in experiments carried out with pMsH42s*Lac*. In the three panels, the recombinants are shown aligned with their respective progenitor allele with empty boxes indicating repeat blocks lost. Because of the structure of the minisatellite repeats, it was not always possible to know the precise localization of one repeat or a block of repeats, and such repeats are shown in yellow.

array of the long allele permits the formation of secondary structures, such as those described in other minisatellites (*24*), producing a pause in the DNA polymerase and slippage events similar to what has been found in microsatellites (*25*). Single-stranded DNA can act as a substrate in homologous recombination (*26*), serving as a pre-existing intermediate in the recombination process, either to generate a free end that would invade an homologous duplex or to provide a D-loop structure that would facilitate such strand invasion (*14*, *27*−*29*). In this sense, it is tempting to speculate that the lower capacity to stimulate recombination of the short and the middle alleles compared to the long allele might be related to their diminished ability to generate single-stranded conformations (slippage capacity).

We have found a remarkably high rate of equal crossovers (Table 1) despite the many degrees of freedom available for allele mispairing. Interestingly, in humans (*11*, *17*) and yeast (*30*), the generation of equal crossovers from minisatellite recombination events has also been reported. Furthermore, there is a notable parallelism between the present results and

Table 2: Equal and Unequal Recombinant Frequencies Produced by the Different Recombination Substrates

recombination substrates	equal recombinants fraction[a]	unequal recombinants fraction[a]	equal recombinant frequencies (%)[b]	unequal recombinant frequencies (%)[b]
pMsH42Lac	0.78	0.22	0.70	0.20
pMsH42mLac	0.77	0.23	0.37	0.11
pMsH42sLac	0.73	0.27	0.30	0.11
pMsH42satLac	0.31	0.69	0.05	0.12

[a] Recombinant fractions are given as the ratio of equal or unequal crossovers in the total crossovers detected for each experiemental recombination substrate. The data employed to ellaborate this table have been taken from Table 1. [b] The recombinant frequencies were obtained by multiplying the average of the recombinant frequency by the fraction of equal or unequal recombinants.

the findings obtained in previous experiments of intermolecular recombination in mammalian cells using plasmids containing minisatellite DNA sequences (*31*). In these experiments, it was demonstrated that a consensus hypervariable minisatellite sequence stimulates homologous recombination, preferentially by reciprocal exchanges, and that the recombinants did not exhibit significant levels of allele expansion or contraction. It is noteworthy that our findings contrast with some extant models of minisatellite DNA variability that predict high frequencies of unequal crossovers (*6*, *14*).

The observation that the three MsH42 alleles produced similar high levels of equal recombinants and the fact that they share identical flanking sequences indicates a synapsis function for this flanking DNA. In this sense, it would be possible that the flanking sequences facilitate the pairing in register (i.e., the exact alignment of the minisatellite allele region), favoring the maintenance of the minisatellite array, producing the same results if the crossover occurs either within the minisatellite or in its flanking DNA. In agreement with this role, the elimination of the MsH42 long allele flanking sequences provoked a decrease in the frequency of equal crossovers (Table 1, last column). The comparison of the equal and unequal recombinant frequencies (Table 2) clearly illustrates that the presence of the flanking sequences leads to a massive increase in the frequency of equal recombinants (6-fold for the short and middle alleles to 14-fold for the long allele) and that the recombination substrates produce a similar frequency of unequal recombinants, regardless of whether this flanking DNA is present. Moreover, the data in Table 2 corroborate that the repeat length has a modest (2-fold) effect upon equal recombinant frequencies.

The elimination of the MsH42 long allele flanking sequences provokes a marked reduction in the enhancement of recombination, indicating that the flanking sequences are important not only for the exact pairing between the two homologues but also for the stimulation of recombination. This descent in the recombinogenic capacity could be due to a length of homology smaller than that necessary for an efficient homologous recombination (*23*, *32*). Nevertheless, the results obtained in the experiments carried out with the control plasmids (p5′MsH42*Lac* and p5′MsH42.1*Lac*) indicate that the differences in size are not enough to explain the decrease in the enhancement of recombination shown by pMsH42sat*Lac*. It is possible that the MsH42 flanking sequences could confer stability to the recombination intermediate structure or to serve for leading the DSB inside the minisatellite or in its immediate vicinity. In fact, in another minisatellite, it has been proposed that cis-acting motifs are present in the flanking DNA that would control their recombinogenic activity (*33*−*36*). One of the arguments favoring a function of minisatellite flanking sequences in recombination is the polarity toward an extreme observed in the recombinatory events (*37*). Interestingly, in the recombinants derived from pMsH42*Lac*, there is a polarity toward the 5′ end of the minisatellite (inside or around the third repeat), whereas such bias is not seen in the recombinants obtained from pMsH42sat*Lac* (Figure 5A). Perhaps the 5′ flanking DNA is driving the introduction of a DSB inside or near the minisatellite, as has been proposed for another repetitive sequences (*6*, *38*). If the DSB does not depend on specific sequences (*39*), some of the repeats of MsH42 could display an structural motif that would be used as a breakpoint signal.

The appearance of MsH42 recombinants showing size variations or complex rearrangements could be explained in a number of different ways. The explanation by means of single-strand annealing (*40*) is unlikely for two reasons: the percentage of short recombinants should be higher (in our experiments there is about the same number of shortening and growing), and the proportion of equal crossovers should be much lower. The generation of the majority of MsH42 short recombinants may be also explained by simple unequal crossovers (*41*). However, the generation of some recombinants that present complex rearrangements (lines 7, 14, and 15 in Figure 5A) would be very difficult to explain by several rounds of unequal crossovers. It has been proposed that multiple pathways are present and overlap in the recombination process (*42*). In our in vitro assay, we have found that there is a considerable increase in the equal recombinant frequencies when the MsH42 flanking DNA is present. This result led us to postulate the existence of two biochemical pathways working in our recombination system. The first pathway uses resolution of Holliday junctions at minisatellite DNA, as proposed in a previous model (*7*, *8*). In this pathway, the 5′ flanking DNA stimulates the initiation of recombination, and the Holliday junctions branch migrate from that initiation point along the DNA into the MsH42 array and are resolved to produce equal recombinants. This pathway fits the data for approximately 75% of the recombinants. The second pathway uses synthesis-dependent strand annealing (SDSA) and leads predominantly to unequal recombinants (*43*). This SDSA pathway is unaffected by the presence of flanking sequences and, thus, almost certainly reflects repair of the DNA breaks that occur within the MsH42 repeats themselves. In Figure 6, we depict a model in the context of the DSB repair mechanism (*28*, *44*) to illustrate the second pathway. This model assumes some of the features proposed in the SDSA models to explain tandem

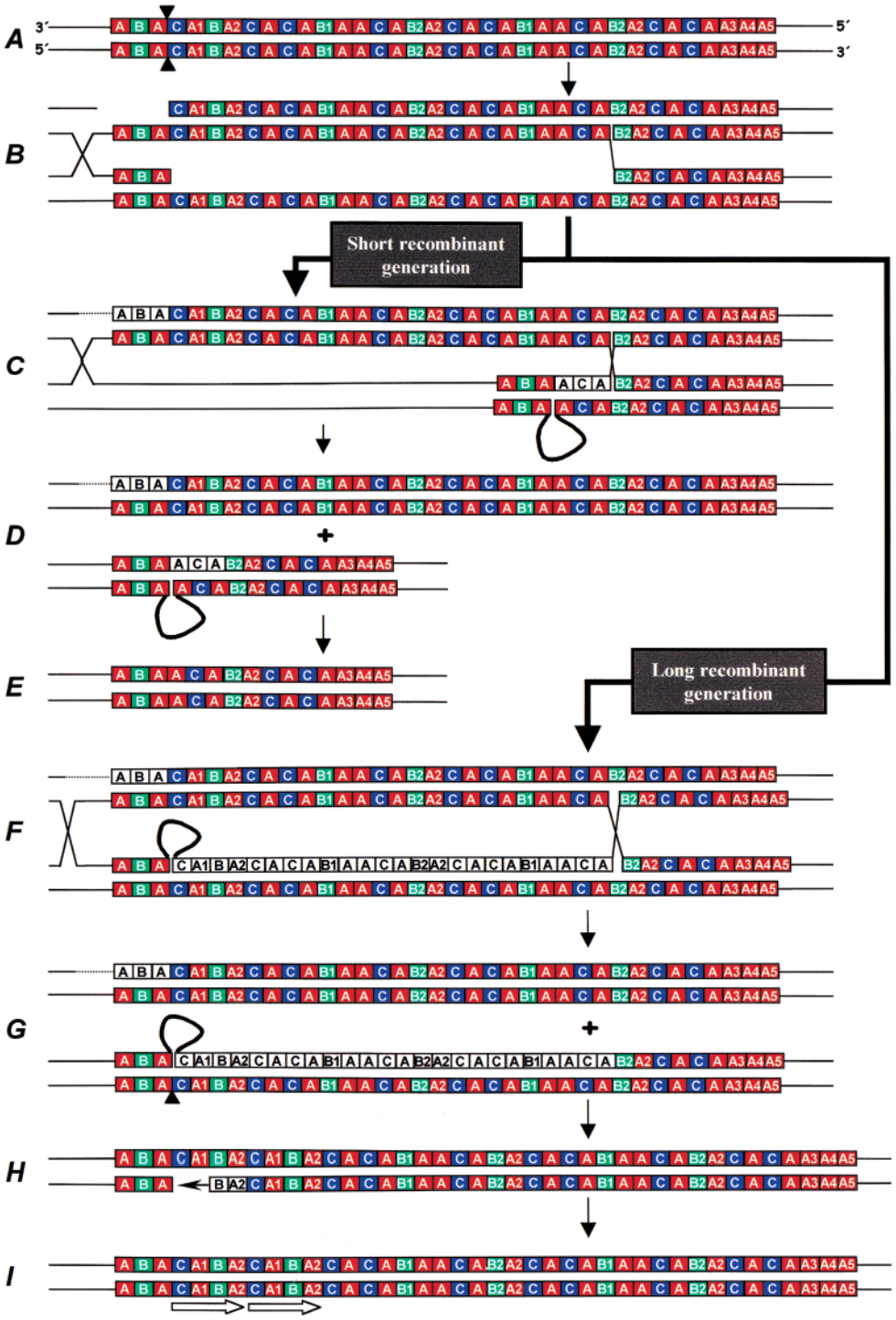

FIGURE 6: Hypothetical recombination model involving polymerase slippage to generate unequal recombinants. An initial equal pairing of the two homologous sequences is followed by a DSB within the long allele repeat array (A). The breakpoint (arrowheads) was situated at the third repeat according to the polarity observed in some recombinant events. After the break, both 5′ ends undergo resection, yielding 3′ single-stranded tails that can invade the homologous duplex. After the invasion (B), during the DNA synthesis, polymerase slippage generating a loop in the template strand could occur, which would produce the shortening of the newly synthesized strand (C); the same effect could be produced by the unwinding of the newly synthesized strand, followed by a posterior reinvasion on any repeat of the template. The recombinant products are generated after the formation and resolution of the Holliday junctions (C and D). The loop is probably eliminated by a mismatch repair system capable of correcting large DNA loops (*2*, *45*), generating a short recombinant (E). In a similar way and equally likely to occur, the slippage can produce a loop in the synthesis repairing strand (F). The loop can be corrected by a single-strand cut on the opposing DNA strand (G), as has been proposed to explain minisatellite variations (reviewed in ref *7*). The posterior repairing by filling in (H) provokes a direct duplication (open arrows) in the minisatellite array generating a long recombinant (I). According to this model, the complex recombinants could be originated by several rounds of slippage in the same or in both strands. Colorless boxes represent the newly synthesized DNA repeats. The repeats code is the same as in Figure 5.

repeat variations compatible with crossovers. In our model, we explain the generation of a short and a long recombinant, each of these events (shortening or growing) being equally probable, starting from an initial equal pairing between the two copies of the minisatellite region and followed by DSB inside the minisatellite.

ACKNOWLEDGMENT

We want to thank to the Instituto do Carballiño (Ourense) and the Xunta de Galicia for facilitating the participation of F.B. in the present research.

REFERENCES

1. Leach, D. R. F. (1996) *Genetic Recombination*, Blackwell Science Ltd., London, U.K.
2. Lichten, M., and Goldman, A. S. H. (1995) *Annu. Rev. Genet. 29*, 423−444.
3. Kowalczykowski, S. C. (1994) *Experientia 50*, 204−215.
4. Moore, D. P., and Orr-Weaver, T. L.(1998) *Curr. Top. Dev. Biol. 37*, 263−299.
5. Nakamura, Y., Koyama, K., and Matsushima, M. (1998) *J. Hum. Genet. 43*, 149−152.
6. Jeffreys, A. J., Barber, R., Bois, P., Buard, J., Dubrova, Y. E., Grant, G., Hollies, C., May, C., Neumann, R., Panayi, M., Ritchie, A., Shone, A. C., Signer, E., Stead, J., and Tamaki, K. (1999) *Electrophoresis 20*, 1665−1675.
7. Vergnaud, G., and Denoeud, F. (2000) *Genome Res. 10*, 899−907.
8. Wahls, W. P. (1998) *Curr. Top. Dev. Biol. 37*, 37−75.
9. Jeffreys, A. J., Tamaki, K., MacLeod, A., Monckton, D. G., Neil, D. L., and Armour, J. A. L. (1994) *Nat. Genet. 6*, 136−145.
10. Jeffreys, A. J., Murray, J., and Neumann, R. (1998) *Mol. Cell 2*, 267−273.
11. Jeffreys, A. J., Neil, D. L., and Neumann, R. (1998) *EMBO J. 17*, 4147−4157.
12. Ramachandra, L., and Rao, M. R. S. (1994) *Chromosoma 103*, 486-501.
13. Debrauwère, H., Buard, J., Tessier, J., Aubert, D., Vergnaud, G., and Nicolas, A. (1999) *Nat. Genet. 23*, 367−371.
14. Pâques, F., and Haber, J. E. (1999) *Microbiol. Mol. Biol. Rev. 63*, 349−404.
15. Stead, J. D. H., and Jeffreys, A. J. (2000) *Hum. Mol. Genet. 63*, 349−404.
16. Boán, F., González, A. I., Rodríguez, J. M., and Gómez-Márquez, J. (1997) *FEBS Lett. 418*, 251−257.
17. Boán, F., Rodríguez, J. M., and Gómez-Márquez, J. (1998) *J. Mol. Biol. 278*, 499−505.
18. International Human Genome Sequencing Consortium (2001) *Nature 409*, 860−921.
19. Edelmann, W., Kröger, B., Goller, M., and Horak, I. (1989) *Cell 57*, 937−946.
20. McGlynn, P., and Lloyd, R. G. (2000) *Cell 101*, 35−45.
21. Bi, X., and Liu, L. F. (1994) *J. Mol. Biol. 235*, 414−423.
22. Liskay, R., Letsou, A., and Stachelek, J. (1987) *Genetics 115*, 161−167.
23. Shen, P., and Huang, H. V. (1986) *Genetics 112*, 441−457.
24. Coggins, L. W., O'Prey, M., and Akhter, S. (1992) *Gene 121*, 279−285.
25. Kang, S., Oshima, M., Shimizu, S., Amirhaeri, S., and Walls, R. D. (1995) *J. Biol. Chem. 270*, 27014−27021.
26. Rauth, S., Young Song, K., Ayares, D., Wallace, L., Moore, P. D., and Kucherlapati, R. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 5587−5591.
27. Meselson, M. S., and Radding, C. M. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 358−361.
28. Szostak, J. W., Orr-Weaver, T., Rothstein, R. J., and Stahl, F. W. (1983) *Cell 33*, 25−35.
29. Stahl, F. (1996) *Cell 87*, 965−968.
30. Bishop, A. J. R., Louis, E. J., and Borts, R. H. (2000) *Genetics 156*, 7−20.
31. Wahls, W. P., Wallace, L. J., and Moore, P. D. (1990) *Cell 60*, 95−103.
32. Reiter, L., Hastings, P., Nelis, E., Jonghe, P., Broeckhoven, C., and Lupski, J. R. (1998) *Am. J. Hum. Genet. 62*, 1023−1033.
33. Andreassen, R., Egeland, T., and Olaisen, B. (1996) *Am. J. Hum. Genet. 59*, 360−367.
34. He, Q., Cederberg, H., Armour, J., May, C., and Rannug, U. (1999) *Gene 232*, 143−153.
35. Monckton, D. G., Neumann, R., Guram, T., Fretwell, N., Tamaki, K., MacLeod, A., and Jeffreys, A. J. (1994) *Nat. Genet. 8*, 162−170.
36. Buard, J., Shone, A. C., and Jeffreys, A. J. (2000) *Am. J. Hum. Genet. 67*, 333−344.
37. Bois, P., and Jeffreys, A. J. (1999) *Cell. Mol. Life Sci. 55*, 1636−1648.
38. Appelgren, H., Cederberg, H., and Rannug, U. (1999) *Gene 239*, 29−38.
39. Smith, K. N., and Nicolas, A. (1998) *Curr. Opin. Genet. Dev. 8*, 200−211.
40. Lin, F.-L., Sperle, K., and Sternberg, N. (1984) *Mol. Cell. Biol. 4*, 1020−1034.
41. Smith, G. P. (1976) *Science 191*, 528−535.
42. Mosig, G. (1998) *Annu. Rev. Genet. 32*, 379−413.
43. Pâques, F., Leung, W., and Haber, J. E. (1998) *Mol. Cell. Biol. 18*, 2045−2054.
44. Sun, H., Treco, D., and Szostak, J. W. (1991) *Cell 64*, 1155−1161.
45. Kirkpatrick, D., and Petes, T. D. (1997) *Nature 387*, 929−931.

BI015780I