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Review

Analysis of intrachromosomal homologous recombination in mammalian cell, using tandem repeat sequences

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Abstract

In all the organisms, homologous recombination (HR) is involved in fundamental processes such as genome diversification and DNA repair. Several strategies can be devised to measure homologous recombination in mammalian cells. We present here the interest of using intrachromosomal tandem repeat sequences to measure HR in mammalian cells and we discuss the differences with the ectopic plasmids recombination. The present review focuses on the molecular mechanisms of HR between tandem repeats in mammalian cells. The possibility to use two different orientations of tandem repeats (direct or inverted repeats) in parallel constitutes also an advantage. While inverted repeats measure only events arising by strand exchange (gene conversion and crossing over), direct repeats monitor strand exchange events and also non-conservative processes such as single strand annealing or replication slippage. In yeast, these processes depend on different pathways, most of them also existing in mammalian cells. These data permit to devise substrates adapted to specific questions about HR in mammalian cells. The effect of substrate structures (heterologies, insertions/deletions, GT repeats, transcription) and consequences of DNA double strand breaks induced by ionizing radiation or endonuclease (especially the rare-cutting endonuclease ISce-I) on HR are discussed. Finally, transgenic mouse models using tandem repeats are briefly presented.
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Keywords: Homologous recombination; Mammalian cells; Tandem repeat sequences

1. Introduction

Homologous recombination (HR) plays a central role in various fundamental processes determining genome organization and rearrangement such as molecular evolution [1,2] mating type switching in yeast (for review, see Ref. [3]), antigenic variation of the trypanosome (for review, see Ref. [4]), or for diversification of immunoglobulin genes in chicken [5] or in rabbit [6], faithful chromosomal segregation during meiosis in yeast (for review, Refs. [7,8]), and DNA repair (for review, see Ref. [9]).

On one hand, HR is involved in the maintenance of genome integrity by repairing damaged DNA; on the other hand, it may also contribute to genome instability since recombination between homologous

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repeated sequences dispersed through the genome may lead to duplications, inversions, deletions, translocations [10]. Moreover, gene conversion with pseudogenes can inactivate a functional allele [11]. Finally, several lines of evidences connect HR with cancer predisposition or prevention. First, carcinogens stimulate intrachromosomal HR [12–15]. Second, intrachromosomal HR is elevated in DNA repair deficient cell lines, in Ataxia telangiectasia cell lines or p53 defective cells lines, all of these phenotypes being associated with cancer predisposition [16–20]. Third, Rad51, a putative recombination protein in mammalian cells, has been shown to interact with the products of the tumor suppressor genes *p53, BRCA1, BRCA2* [21–25].

The use of intrachromosomal tandem repeat represents a good strategy to measure HR in mammalian cells and has been often used because (i) it is the easiest way to introduce the two partners of recombination in a broad variety of cells and in one round of cell transfection, (ii) these systems permit efficient measure of HR and (iii) it allows to select for gene conversion or crossover and deletion events; (iv) depending on the orientation of the two markers, it can measure recombination arising by different mechanisms such as strand exchange (SE), single strand annealing (SSA) or replication slippage [26]. These mechanisms involving different pathways in yeast, it is thus essential to be able to distinguish between them in mammalian cells also. Here we present mechanisms of HR deduced from the knowledge in yeast, and also some molecular characteristics of HR between tandem repeat in mammalian cells. These data aid in the design of substrates adapted to more specific questions about the regulation of HR in mammalian cells.

1.1. Mechanisms and pathways of homologous recombination between tandem repeat: the knowledge from Saccharomyces cerevisiae

Two different orientations of the tandem repeat recombination substrate can be used: direct repeat (DR) or inverted repeat (IR). In bacteria and yeast, RecA/Rad51 protein promotes strand exchange (SE). SE recombination can act on DR as well as on IR and lead either to gene conversion or to crossing over (Fig. 1). Gene conversion between both DR and IR leaves intact the general structure of the locus. Depending on the orientation of the substrates, crossing over results in different structures: crossing over between IR leads to the inversion of the intervening sequence (Fig. 1B, Fig. 2A); crossing over between DR leads to the deletion of the intervening sequence either by an unequal sister chromatid exchange or by an intrachromatid exchange (Fig. 2B,C). In addition to SE recombination, direct repeats (DR) permit also to monitor deletion events arising by *RAD51* independent mechanisms such as single strand annealing (SSA) (Fig. 3), replication slippage and other mechanisms [26]. Because of their orientation, inverted repeats measure neither SSA nor replication slippage.

Extensive studies in yeast have determined genes involved in the control of HR. SE and SSA represent the main pathways using homologous sequences to achieve double strand break (DSB) repair. Besides DSB repair, replication slippage involved pathways different from those required for SE and SSA [26]. In *Saccharomyces cerevisiae*, the SE mechanism is dependent on the *RAD52* epistasis group including *RAD51, RAD52, RAD54, RAD55, RAD57* genes, whereas SSA does not involve these genes. SSA is dependent upon *SRS2*, on the nucleotide excision repair proteins Rad1 and Rad10 and on the mismatch repair proteins Msh2 and Msh3 [27,28]. Homologues to most of these genes have been described in mammalian cells (Table 1). Clearly, the study of HR in mammalian cells would benefit from the complementary use of the two kinds of substrates (DR and IR).

1.2. Molecular events of intrachromosomal recombination between duplicated sequences in mammalian cells

Two kinds of substrates have been used. One used two *LAC Z* sequences, recombinant cells being detected by the blue coloration. The second type of substrates used selectable genes (herpes simplex virus TK gene in tk^- cells, neomycin resistance gene, hygromycin resistance gene...), recombinant cells

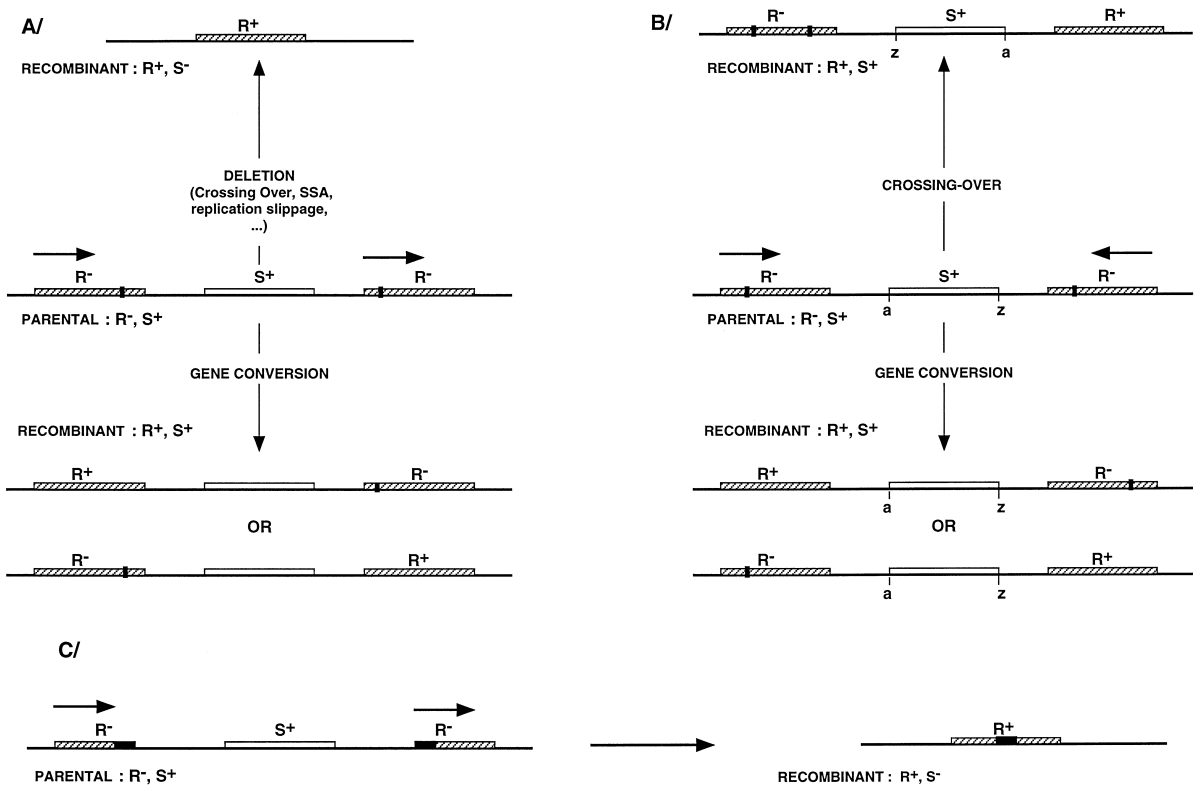

Fig. 1. Principle of the strategy to measure intrachromosomal homologous recombination between tandem repeat sequences. (A) Direct repeats, (B) inverted repeats (the letters a and z show the orientation of the intergenic sequence), (C) truncated repeats. The parental construction contains two inactive copies of a reporter gene (hatched boxes). This reporter gene can confer resistance to selective media. An alternative strategy uses the β-galactosidase gene whose expression can be monitored by histochemistry. In the parental form the two copies are inactivated (R^-) by a mutation, a deletion or an insertion (black boxes) located at different positions on each copy. An alternative strategy uses two truncated but overlapping regions (C) black boxes. In most of the systems, another selection gene (white boxes) is inserted between the two copies. The expression of the selection gene (S^+ or S^-) is used (i) to select the transfected cells with the recombination substrate, (ii) to analyze the distribution of recombination events (deletion or gene conversion). Recombination between the duplicated sequences creates a functional gene (R^+) either by gene conversion (a conservative event) or by deletion of the intergenic sequence (non-conservative event). Gene conversion can occur with direct as well as with inverted repeat sequences. Deletions can only occur with direct repeat sequences by crossing over after strand exchange (SE), by single strand annealing (SSA) or other events such as replication slippage. Crossovers between the inverted repeat sequences do not lead to deletion but to inversion of the intervening sequence. With two truncated copies, a deletion creates a functional gene (R^+).

forming clones when the selection was applied. Additionally, DSB repair has been studied following in vivo endonucleases treatment.

1.2.1. Spontaneous intrachromosomal recombination is a conservative mechanism

Analysis of the structure of recombinants has revealed that spontaneous intrachromosomal recombination is mainly a conservative mechanism in mouse L-cells with gene conversion representing approximately 80% of the recombination events [29]. This was observed using direct repeat or inverted repeat sequences [30]. The majority of the events giving rise to crossover products involved unequally paired of sister chromatids after DNA replication (see Fig. 2) [31]. These results contrast with those of extrachromosomal recombination which follow a non-conservative mechanism in mouse L-cells [32–

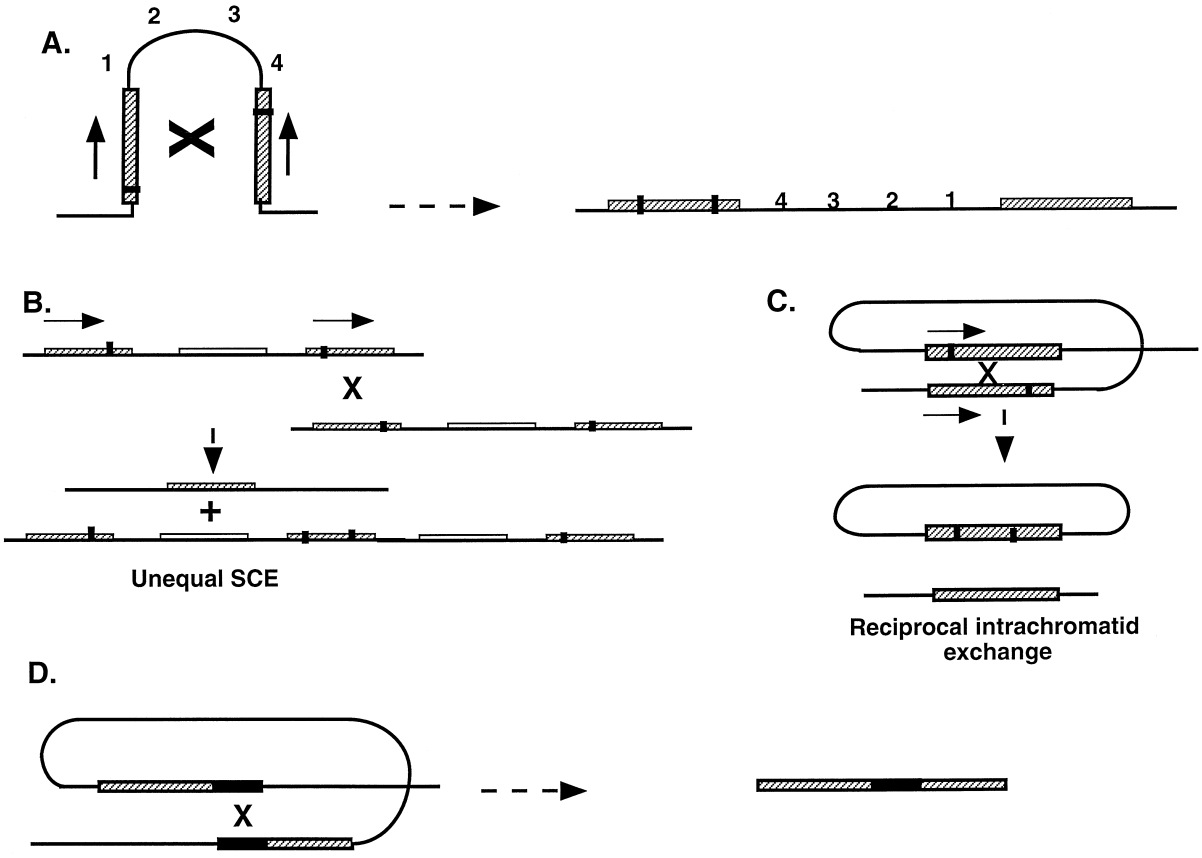

Fig. 2. Models for crossing-over events after SE. (A) Crossing over between two inverted repeats leads to the inversion of the intervening sequence. The arrows indicate the orientation of the two copies, (×) symbolizes the exchange and the numbers are here to orient the intergenic sequence. (B) and (C) recombination between two direct repeats (hatched boxes). The arrows indicate the orientation of the two copies. The lines represent the duplex DNA. (B) Recombination between two mispaired chromatids. (C) Intrachromatid recombination. The two copies (hatched boxes) are paired. After the exchange (×), the resolution of the Holliday junction can lead to gene conversion (see Fig. 1) or to a crossing over event. In (B), the crossing over leads to the formation of a unique active gene and to the deletion of the intergenic sequence on one chromatid and to a triplication on the other chromatid. In (C), the crossing over leads to the maintenance of only one copy on the chromosome and one loop that can be eliminated. In some cases, the loop can be integrated elsewhere in the genome. (D) Crossover between two truncated copies. The black box represents the overlapping sequence. (A, C, D) correspond to reciprocal intrachromatid exchanges.

34]. It cannot be excluded that these ratios may differ in other cell types.

1.2.2. Heteroduplex formation

The different models for HR involved intermediate structures in which DNA-strand exchanges create hybrids and heteroduplex DNA between the two recombining molecules [35–37]. Biochemical studies of mitotically dividing mammalian cells indicate that exchange between sister chromatids involved interstrand transfer of DNA [38,39]. In addition, heteroduplex formation during HR promoted by human nuclear extracts was observed in a cell free system [40]. The analysis of the intrachromosomal recombination products between tandem repeats has shown that recombinant colonies result from unrepaired heteroduplex DNA [31,41]. Since particular structures may affect the formation, the stability or the resolu-

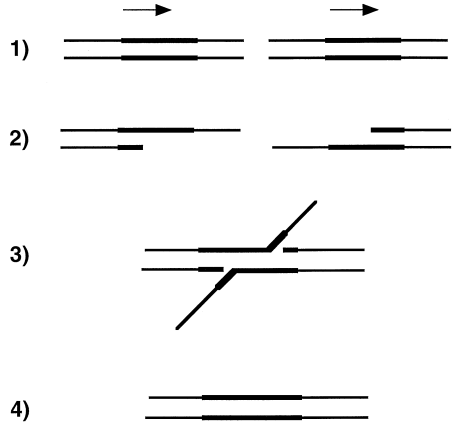

Fig. 3. The single strand annealing model [32]. The two complementary strands of the duplex DNA are drawn here. The heavy lines symbolized the homologous repeat sequences and the arrows the orientation of the two repeats. (1) A double strand break can occur even in the non-homologous intervening sequence. (2) After degradation by a single strand exonuclease, single stranded tails (ss-tails) are created. If the repeats are in a direct orientation, complementary ss-tails are created. (3) Annealing of the two complementary single stranded regions results in a structure leading to the deletion of the intervening sequence after the resolution of this structure. (4) SSA is exclusively a non conservative mechanism. If the repeats are in an inverted orientation, the ss-tails (step 2) are identical but not complementary and SSA cannot act.

tion of the heteroduplex recombination intermediates, the effect on recombination of the DNA structure (insertions or deletions leading to loops formation into the heteroduplex molecule the homology requirements . . .) have been studied.

1.2.3. Effect of the sequence structure: insertions, deletions, sequence homology requirements, microsatellites, transcription

1.2.3.1. Insertions and deletions. The molecular nature of insertion or deletion mutations (corresponding to the black boxes in Figs. 1 and 2) in the copies of the duplication can influence the efficiency of HR. DNA strand exchange is able to propagate through heterologous sequences forming heteroduplex DNA bearing loops. The resolution of such intermediates leads more frequently to the excision of the loop with an efficiency correlated to the size of the loop [42].

1.2.3.2. Homology requirements. Two alternative strategies can be drawn to address the question of how much homology is required for HR in mammalian cells. The first uses two truncated molecules with different lengths of overlapping of uninterrupted homology (Fig. 2D). When the two interacting molecules share length of homologies between 295 bp and 1.8 kb, the rate of gene conversion is

Table 1
Comparison between strand exchange (SE) and single strand annealing (SSA)

Process	Products	Orientation of the substrates	Pathway in *S. cerevisiae*	Homologues in human cells
Strand exchange	Gene conversion	Direct repeat	*RAD51*[a]	*HsRAD51*[a]
		Inverted repeat	*RAD52*[b]	*HsRAD52*
			RAD54	*HsRAD54*
	Crossing over		*RAD55*[a]	see:[a]
	Deletions	Direct repeat	*RAD57*[a]	see:[a]
	Inversions	Inverted repeat	*RAD59*	
Single strand annealing	Deletions	Direct repeat	*RAD1*	*XP-F*[c]
			RAD10	*ERCC1*
			MSH2	*HsMSH2*
			MSH3	*HsMSH3*
			SRS2	

[a] In yeast *RAD55* and *RAD57* share homologies with *RAD51*. In mammalian cells, beside *HsRAD51*, at least 6 other *RAD51* homologues have been described: *XRCC2, XRCC3, RAD51B / HREC2, RAD51H3, RAD51C, RAD51D.* Some of these homologues presumably correspond to *RAD55* and to *RAD57*.
[b] Defines the epistasis group for homologous recombination in yeast.
[c] *Xeroderma pigmentosum* group F.

directly proportional to the length of uninterrupted homology. This rate is reduced 7 fold with 200 bp homology and 100 fold with 95 bp length of homology [43].

The second strategy uses two molecules of approximately the same size but containing sequence polymorphism. Waldman and Liskay used a HSV-*tk* duplication in which one *TK* copy came from HSV type 1 and the other copy from HSV type2 (homeologous recombination). These *TK* genes share 81% of homology. The authors observed that with 19% divergence, the rate of intrachromosomal HR was reduced 1000 fold relative to the rate of HR between two identical HSV1-*tk* sequences. In contrast, the rate of intramolecular or intermolecular extrachromosomal recombination was only reduced by a factor 3 to 15 [44]. These results also argue in favor of distinct mechanisms between extrachromosomal and intrachromosomal recombination. Moreover, if efficient intrachromosomal recombination required a minimum of 134 to 232 bp of uninterrupted homology, a single-nucleotide heterology in this minimal region of homology was sufficient to inhibit efficient recombination [45]. In addition, when recombination initiates in a perfectly homologous sequence, it is able in a second step to propagate through an adjacent sequences exhibiting 19% heterologies [45]. Finally, Yang and Waldman [46] show that gene conversion involved transfer of uninterrupted blocks of information from 35 to more than 330 bp. Taken together, these results are consistent with the notion that more than 200 bp of homology are required to initiate efficient gene conversion in mammalian cells. This has also been found in in vitro reactions in cell free systems [47].

1.2.3.3. Effect of GT repeats. A variety of DNA sequences may play direct or indirect roles in recombination by their effects on the DNA structures. It was proposed that GT and GC repeats, which can form Z-DNA, may influence recombination [48]. It has been shown that GT, GC repeats and minisatellite repeats, stimulated extrachromosomal recombination of transfected DNA [49,50]. However, using an intrachromosomal assay, a $(GT)_{29}$ repeat has been shown to be unable to stimulate recombination and to influence the distribution of the recombination events [51].

1.2.3.4. Transcription. Transcription stimulates homologous recombination in *S. cerevisiae* [52,53]. Stimulation by transcription of extrachromosomal recombination [54] and intrachromosomal recombination [55] has been reported in CHO cells. Alleles transcribed at high levels recombined about 2 to 7-fold more frequently than identical alleles transcribed at low level. In line with this, preferential repair of UV damage has been shown to abolish the transcription–stimulation of HR [56]. Finally, transcription has no effect on recombination induced by a DNA double strand break [57].

1.3. Stimulation of homologous recombination by DNA double strand breaks

In different species, DNA-damaging agents were shown to stimulate homologous recombination. DNA double strand breaks (DSB) are the main lesion involved in the stimulation of homologous recombination in different organisms. It is used to initiate recombination during meiosis in yeast *S. cerevisiae* (for review, see Refs. [7,8]). The DSB is also one of the most genotoxic lesion induced by ionizing radiation and can be repaired by two general mechanisms: (i) non homologous end joining (NHEJ), which does not necessarily involve sequence homologies and promote the end-joining of the broken extremities; (ii) homology-directed repair which involves homologous sequences and corresponds to SE and SSA mechanisms. Ionizing radiation stimulates interallelic recombination in the endogenous *TK* locus in a human lymphoblast cell line [58]. However, the effect of ionizing radiation on intrachromosomal HR between tandem repeat sequences varies according to the cells and/or to the substrates used. Although, HR between two LacZ sequences in CV-1 cells is stimulated by ionizing radiation [59], it does not stimulate HR between HSV-TK sequences in mouse L-cells [12]. The fact that the cell lines used are different could explain this result. Another explanation could involved the differences in the substrates, LacZ vs. TK sequences. In the former case, recombinant are scored by coloration (β-galactosidase activity) some hours following the treatment. In contrast TK^+ recombination events can be scored on surviving colonies several weeks after the treatment. Thus,

in the former system, recombination events can be scored before the death of the cells generally arising several days after exposure to radiation. In contrast, the second system scores only surviving colonies.

Another way to produce DSB in target DNA uses nucleases. Restriction enzymes corresponding to single sites present in the target were electroporated into CHO cells or human cells [42,60]. In these cases, recombination was increased by more than 10-fold. Electroporation of the rare cutting yeast endonuclease PI-*Sce*I also stimulated recombination provided there was a corresponding cleavage site in the duplication [60]. The yeast endonuclease I-SceI has provided a useful tool to study targeted DSB in mammalian cells, as already reviewed [61]. I-SceI recognizes a cleavage site of 18 bp long. Due to this large restriction sequence, there is probably no I-SceI site in the mammalian genome and expression of the I-SceI enzyme in mammalian cells is not toxic. However, when the I-SceI restriction site is present in the duplication, at least 80% of the molecules are cleaved after transfection of a plasmid expressing I-SceI enzyme [62]. Induction of a site-directed DSB into the recombination substrates strongly stimulates both homologous and non-homologous recombination [63,64,57]. HR is stimulated 100 times but non-homologous recombination is stimulated 1000 times [64]. However, using a physical analysis, homology-directed repair of I-SceI-induced DSB's is found to account for 30–50% of the observed events [65]. ISce-I induced recombination produced mainly deletion events (80%), that are interpreted as a result from SSA. Finally, in contrast with spontaneous HR, transcription does not stimulate DSB-induced recombination [57], although we cannot exclude that the efficiency of DSB-stimulation is so great that it would mask the effect of transcription.

1.4. Transgenic mice models

The assay using duplicated repeats to measure intrachromosomal recombination can be envisioned in vivo in transgenic mice. In that case, recombination can only be recorded using genes giving a coloration such as the β-galactosidase gene, but not with a gene conferring resistance to a drug. Two models were developed to measure recombination in specific tissues. One model has been developed to analyze the lineage of cells in the myotome; thus, the expression of the recombination substrate was driven by a promoter which confers expression specifically to cells of this compartment: the promoter of the α-subunit of acetylcholine receptor. The descendants of the recombinant cells are histochemically identified, permitting the analysis of the lineage of cells in the intact embryos [66]. It appears that the frequency of recombination in this tissue is similar to the frequency measured in cultured cells: between 1 and 2×10^{-6} [66].

Another transgenic model also uses a duplication of β-galactosidase genes driven by a meiosis specific promoter. Germ line gene conversion was analyzed in transgenic male gametes. Spermatids which undergo intrachromosomal gene conversion produce functional β-galactosidase ($lacZ^+$), visualized by histochemical staining. Approximately 2% of the spermatids, produced by a combination of meiotic and mitotic conversion events, were $LacZ^+$ [67].

2. Conclusions

Numerous differences exist between intrachromosomal and extrachromosomal HR. For example, intrachromosomic recombination is conservative whilst plasmid recombination is non-conservative in mouse L-cells; in addition heterologies and GT repeats differently affected plasmid and intrachromosomal recombination. Moreover, the number of plasmid copies is not controlled and it is well established that plasmids are submitted to extensive nuclease attack after transfection. Chromatin structure and the nuclear organization may also account for the differences observed between plasmid and intrachromosomal recombination. Thus, we believe it is most suitable to measure HR between two intrachromosomal tandem repeat sequences.

The other possibilities to measure HR between two intrachromosomal sequences are interallelic and ectopic recombination, which are however technically complicate to set up. Furthermore, interallelic HR as well as ectopic HR are rather inefficient in mammalian cells [68,42,69]. Finally, the possibility of using two different orientations of tandem repeats

(direct or inverted) and the use of the rare-cutting endonuclease ISce-I provide a set of complementary approaches to study HR in mammalian cells.

Using these of strategies, connections between HR and other fundamental cellular processes have been described. Firstly, transcription stimulates HR but not DSB-induced HR [57]. Connections with cell cycle control and DNA repair have also been studied: alteration of *ATM* or p53 functions lead to an increase of HR [18–20]; carcinogens and DNA damaging agents treatments stimulate both gene conversion and deletion [12]. More generally, the efficiency of repair of DNA damages diminishes the stimulation of recombination [17,56]; finally, inhibition of Poly(ADP-ribose)polymerase (PARP) by 3-methoxybenzamide increases recombination between 3 and 4-fold in mouse L-cells [70].

Very little is known on the genetic control of homologous recombination in mammalian cells. Overexpression of human *RAD52* stimulates recombination between two lacZ direct repeats about 3-fold, in monkey cells and confers resistance to ionizing radiation [59]. The Rad52 stimulation acts via the single stranded DNA binding protein RpA which is also involved in replication and DNA excision repair [71]. One question would be to understand how overexpression of only one component of a multiprotein complex can stimulate the whole process, i.e., homologous recombination. One explanation could be that Rad52 is the limiting factor of the complex. However, it has recently been shown that overexpression of the Hamster Rad51 protein into CHO, also confers resistance to ionizing radiation and stimulates HR between direct repeat sequences [72]; this result is not in accordance with this hypothesis. The combined analysis of HR between direct repeats, between inverted repeats and of the DSB-induced HR should afford essential information to understand these process in mammalian cells.

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