Structural effect of donor DNA on the initiation of recombination for double strand break repair in human nuclear extracts

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ABSTRACT

The effect of the structure of donor DNA molecules on the initiation of recombination for double strand break repair in human nuclear extracts was investigated here. A unique double strand break was introduced into M13 duplex derivatives by digestion with restriction enzymes. After coincubation of the cleaved DNA in human nuclear extracts, with a plasmid containing M13 sequences spanning the break, double strand break repair was estimated by the plating efficiency in JM109 (RecA1) bacteria. We first confirm that a short heterologous insert (8bp) close to the break on the recipient cleaved M13 DNA inhibits recombination with circular as well as with linear donor molecules. The results indicate that, with these substrates, recombination is initiated at the level of the break, requires uninterrupted homology on both sides of the break, and is associated with a decreasing gradient of gene conversion. When the heterologous insertion is located on the plasmid donor DNA, similar results are obtained with a circular donor DNA. In contrast, with a linear donor molecule, bearing the insert, homology requirements, in the region of the break in M13 DNA, are abolished. This last result suggests that recombination could be initiated at the extremities of the linear donor DNA.

INTRODUCTION

Homologous recombination is a mechanism implicated in fundamental processes determining genome organization. DNA double strand breaks (dsb) stimulate recombination in Escherichia coli (1), in yeast (2), in mammalian cells (3, 4, 5, 6) as well as in cell free systems using yeast (7, 8, 9) or human nuclear extracts (10, 11, 12, 13).

Several models have been proposed to explain recombination-mediated double strand break repair. In Escherichia coli, the dsb serves as an entry site for the RecBCD enzyme and recombination is initiated at internal sequences termed chi sites (for review see 14, 15, 16). In the Double Strand Break Repair model, the dsb has to be located in a region of homology, recombination is initiated at the level of the break and is then followed by polar branch migration in both directions resulting in a decreasing gradient of gene conversion (2). This model was proposed to account for gene conversion after X-ray irradiation, during meiosis and transformation with plasmids in yeast. Results in yeast extracts (9) and during meiosis in Saccharomyces cerevisiae (17, 18, 19, 20, 21) provided support for this model. This model was also proposed in gene targeting experiments in mammalian cells (6) and in human nuclear extracts (10, 13, 22). In contrast, in the Single Strand Annealing model, it is not necessary that the dsb be located in the region of homology: in this model, complementary single strands are created by exonucleolytic (23) or helicase activities (24) initiated at the break. This model was supported by studies with plasmid recombination in mammalian cells (5) and in Xenopus laevis oocytes (25, 26). Although structures predicted by this last model were not observed in yeast extracts (9), Single Strand Annealing model and Double Strand Break repair model can occur for HO induced dsb repair in yeast (27). Recently we have reported results on initiation of dsb repair in human nuclear extracts which support the Double Strand Break Repair model: initiation of recombination at the level of the break and a requirement for uninterrupted homology on both sides of the break (13). However the substrates used (a linear recipient and a circular donor DNA) were not suitable to check the Single Strand Annealing model.

In the present work, we studied the effect of linearization of the donor DNA on the homology requirements in the region of the break, to initiate dsb repair of the recipient molecule, in the human nuclear extracts.

With the linear donor DNA, we observed an asymmetric behavior of the donor and acceptor DNA to promote recombination as reported with plasmids transfected into mammalian cells (3). We describe here that by linearizing the donor DNA, heterologous insertions are more easily recombined when located on the donor than when located on the acceptor DNA. However, we did not observe such a phenomenon with a circular donor DNA. Some of the present results provide support to the Single Strand Annealing model; however the
asymmetric behavior of the donor and acceptor DNA for heteroduplex formation obliges us to envision alternative mechanisms.

MATERIALS AND METHODS

Cells and nuclear extracts

Hela cells were maintained in RPMI medium supplemented with 10% fetal calf serum and checked for the absence of mycoplasma contamination (BRL-mycotest). 2 to 5 x 10⁸ cells were collected by scraping with a rubber policeman after three washings with cold phosphate buffered saline (PBS). Nuclei were isolated and nuclear extracts prepared as already described (12, 28). After DEAE chromatography, proteins were precipitated by adding 0.33g of ammonium sulfate per ml and stirring for 1 hr at 0°C. Pellets were collected by centrifugation, 10 min at 10,000 rpm (rotor Sorvall, HB-4), the pellets were then dissolved in 1 ml of buffer C (50mM Tris-HCl pH 7.5, 0.1 mM EDTA, 10 mM β-mercaptoethanol, 0.1 mM PMSF, 10% glycerol) and dialyzed overnight in the same buffer. Protein concentration was determined by the Biorad protein assay. The extracts were checked for the absence of excessive nuclease activities.

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**Figure 1.** Strategy and substrates used. A) Strategy: linear duplex M13 derivatives were incubated alone (I) or with pCZ plasmid derivatives (II) linear or circular. Thin lines: M13 sequences; heavy lines: pBR sequences. c/b: ClaI/BamHI junction. B) Substrates used: The recipient DNA's were cleaved M13 derivatives. The donor molecules were pCZ derivatives (see Material and Methods) circular or cleaved at the ClaI site (at 352bp and 4009bp from the BamHI/ClaI junctions). Thin lines represent non homologous sequences (M13 in the recipient molecule and pBR322 sequences in pCZ DNA). The box represent the region of homology (in grey: lac Z' sequence) and the white triangle indicates the location of the insert. C) Name of the substrates and corresponding sizes of the inserts (white triangles in panel B).
DNA manipulations
Isolation, purification and modifications of DNA were carried out using standard procedures (29). All enzymes were used in conditions specified by the manufacturers. Cleaved DNA's were treated with T4 DNA polymerase and dNTP's as described (29), in order to produce blunt ends. The linear molecules were then purified by agarose gel electrophoresis and electroelution.

Construction of the substrates
M13 refers here to M13 mp8. M13-8 was obtained by insertion of a 8bp XbaI linker (CTCTAGAG), into the Smal site of M13. The pCZ derivatives were constructed by ligation of the Clal fragment from the corresponding M13 derivatives into the BamHI site of pBR322, after end-filling by DNA poll large fragment.

In vitro assay
Each substrate (0.5μg) was incubated with nuclear extract (final protein concentration at 20mg/ml), for 30 minutes at 37°C, in a 100 μl reaction mixture containing 20 mM Tris-HCl (pH 7.5), 10 mM MgSO4, 1 mM ATP, 100 μM each dNTP. The DNA was then extracted with phenol, ethanol precipitated and suspended in 100 μl of 10 mM Tris-HCl (pH 7.5), 1 mM EDTA.

Transfection of bacteria
JM 109 (rec A1) (30) were grown to an OD of 0.8-0.9 (λ = 550 nm) and transfected according to the method of Hanahan (31). For transfection of the DNA from in vitro assay, 10 μl of the DNA solution (see above) were incubated with 100 μl of competent bacteria.

RESULTS
a) Experimental strategy
The strategy and the substrates are depicted in Figure 1. The recipient DNA was the replicative form (RF) of the bacteriophage M13 mp8 or the derivative M13-8, digested by a restriction enzyme that cleaves at a unique site. The linearized products were then treated with T4 DNA polymerase (+ dNTP's) to produce blunt ends in order to have similar ends in each condition. Linear M13 exhibited a drastic reduction in its ability to produce plaques after transfection into host bacteria. The linear M13 derivative was incubated with a human nuclear extract, then purified and transfected into JM 109 bacteria (recA1). Double strand break repair, i.e. recircularization of the duplex M13 replicative form, was estimated by the plating efficiency. Two assays were performed in parallel: i) the cleaved DNA was incubated alone in the extract, then isolated and transfected into bacteria (Figure 1. I). P1 defined the number of plaques; ii) the cleaved recipient DNA was coincubated in the extract with the pCZ plasmid (Figure 1. II). DNA was then isolated and transfected into bacteria; P2 defined the resulting number of plaques. The contribution of homologous recombination to double strand break repair was estimated by the Reactivation Rate due to recombinational repair (RR), i.e. the ratio P2/P1. A RR >2 was considered to indicate significantly the involvement of homologous recombination in double strand break repair in human nuclear extracts. We have previously shown that the enhancement of RR depends on the concentration of extract protein, the incubation time and the relative amount of each substrate (12). Moreover, RR was fairly well correlated with the frequency of lacZ' phenotype modification, named here 'recombined phenotype', due to the implication of the insert in recombination (13). To study the homology requirements in the region of the break, for dsb repair, we used the strategy depicted in Figure 2. Length of homology refers here to the distance (in bp) from the break to the insert which disrupts the homology. The RR and the 'recombined phenotype' were measured using different substrates bearing an interruption of homology on the recipient (M13) or on the donor (pCZ) derivatives, at various distances from the break on the M13 DNA. We have previously shown that an heterologous insert close to the break (at 7 or 15 bp) in the recipient DNA, inhibited recombination. Increasing the length of uninterrupted homology enhanced RR (13). We compared here the effect on recombination i) of a circular or linear donor DNA, ii) of the location of the heterologous insert on the donor or on the recipient DNA.

Figure 2. Strategy used to determine the length of homology required in the region of the break, for double strand break repair. The upper molecule represents the circular or linear pCZ donor plasmid. The other molecules represent the M13 duplex acceptor molecules cleaved in the region of homology with the donor DNA, at different distances from the insert. The black triangles indicate the position of the insert. M13 was cleaved with AvaII (a), BalI (l), BamHI (h), BglI (bg) or PstI (p). The lengths (in bp) from the cut to the insertion (black triangle), i.e. the length of contiguous homology in the region of the break, are indicated for each molecule. Black box: lac Z' sequence; solid lines: M13 sequence; dotted lines: pBR sequence.
b) Inserts on the recipient DNA

When the 8 bp insert was located on the recipient M13 DNA and circular (black histograms) or linear (grey histogram) pCZ donor DNA. P1 and P2 values are the means of four independent experiments (150 to 200 plaques for P1 value). Frequency of 'recombined phenotype' indicates the ratio of the number of blue plaques to the total number of plaques. Controls (no extract or separate incubation of the substrates) gave RR < 1 and a frequency of blue plaques < 10^-5.

Previously described with circular donor DNA (13). These histograms are characteristic of a recombination initiation in the region of the break on the recipient M13-8 DNA, as discussed elsewhere (13). In the present case, the same behavior was observed with circular as well as with linear donor DNA and the results indicated the requirement of uninterrupted homology in the region of the break.

c) Insert on the donor DNA

We used cleaved M13 as recipient DNA and pCZ-8 as donor. In this case, the recipient M13 DNA produced blue plaques. Recombination between the cleaved M13 and the donor pCZ-8 would i) enhance RR, ii) enhance the frequency of white plaques...
by inserting the 8 bp insert into the LacZ' sequence of the M13 DNA. In this case, 'recombined phenotype' referred to white plaques.

The results show that the homology requirements, in the region of the break on the recipient molecule, were affected by linearization (in a non-homologous region) of the donor pCZ-8 plasmid (Figure 4). When pCZ-8 donor DNA was circular, an insert located close to the region corresponding to the break in M13 DNA, inhibited recombination, and we obtained the same pattern of histograms as obtained when the insert was borne on the recipient DNA (compare Figure 3A, 3B and black histograms in Figure 4A, 4B). In contrast, when the pCZ-8 donor DNA was linearized, we did not observe any correlation between the length of homology and the efficiency of recombination (grey histograms in Figure 4). A high level of RR and 'recombination phenotype' frequency was observed even with only 7 and 15bp of homology (Figure 4). With different sizes of inserts (8, 28 and 163 bp), we obtained similar results (data not shown). Particularly, with M13 cleaved at 7bp (BamHI site) from a 163bp long insert on the donor DNA, we obtained a RR of 5 and 20% of 'recombined phenotype' among the repaired population. The sequence analysis of 30 repaired clones harboring a 'recombined phenotype' (white plaques) indicated that the entire 163bp long insert was transferred from the donor DNA into the recipient M13.

**DISCUSSION**

The strategy used here permits the study of the initiation of recombination in human nuclear extracts. Although elongation and resolution of the cross-junction could occur in bacteria, human nuclear extracts were also shown to be proficient to perform all the steps for double strand break repair (10). Little is known about the early events initiating recombination in human nuclear extracts. The assay used here, focused on the initiation steps by using a strategy that allowed comparison of the recombinational repair (RR) and a 'recombined phenotype' (modification of the lacZ' phenotype) independent of selection pressure. The results indicate that, with a circular donor DNA, recombination is initiated in the region of the dsb, requires a length of uninterrupted homology on both sides of the break, and is then followed by a polar elongation of the exchange from the break to the distal regions of the DNA's (13). These data provide support for the Double Strand Break Repair model (2) based on studies in yeast. With circular donor pCZ derivatives, we confirm here our previous results with the insert located on the recipient as well as on the donor DNA. In contrast, with a linear donor pCZ plasmid, the homology requirement was affected, depending on the location of the heterologous insert on the donor or on the recipient DNA. Indeed, with a linear donor DNA, a length of homology in the region of the break was required only when the insert was located on the recipient molecule but not when it was located on the donor DNA. Such asymmetric behavior has been reported in cultured mouse L cells, in which formation of heteroduplex, was more easily accommodated when the insertion was located on the donor molecule (3). In vitro, with the bacterial RecA protein, heteroduplex formation is affected by the position of heterologous inserts: long inserts on the single-stranded initiating molecule were easier handled by RecA protein than inserts located on the non-initiating double-stranded DNA (32). In these two cases, large inserts block the process when located on the acceptor molecule but not when located on the donor DNA. In our system, we studied inhibition of recombination by interruption of homology close to the dsb. Nevertheless, a similar result was obtained: there was no inhibition of recombination when the insert was located on the linear donor DNA. However, this effect depended on the structure of the donor DNA as it occurred only with a linear donor. We postulate that helicase or exonuclease activities could act on the ends of the cleaved donor, producing single-stranded tails that include the M13 sequences; recombination could be then initiated in the region of the Clal site and would not be inhibited by the heterologous insert distant of more than 300 bp. Such a mechanism could be consistent with the Single Strand Annealing model (23).

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recombination would be initiated in this region, with the single stranded donor DNA as the invading molecule. According to this hypothesis, insertions on the invading DNA would readily be accommodated, as it is the case with RecA protein (32). However, with RecA, heteroduplex formation was inhibited by large inserts. In our experiments, recombination was aborted by a short insert (8 bp) located close to the break on the recipient molecule. Therefore, in our hypothesis, if recombination is initiated in the ClaI site region and is then elongated in the direction of the break (see Figure 5), we must postulate that a length of homology is required beyond the heterologous insert when it is borne on the recipient molecule. Requirements of homology beyond the insert have also been observed for heteroduplex formation promoted in vitro by RecA protein (Radding, CM., personal communication).

In conclusion, recombination by human nuclear extracts is very efficient when the donor molecule is linear, and enables efficient insertion of heterologous sequences into the recipient DNA. In agreement with this, we note that in yeast, One Step Disruption efficiently promotes integration of large inserts into the chromosome (33), provided that the exogenous DNA presents ends homologous to the chromosomal sequence. In contrast, for recombination in mammalian cells using insertion vectors, homology is not required at the ends of the donor DNA (34) as it is the case with our in vitro system.

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