Promotion of double-strand break repair by human nuclear extracts preferentially involves recombination with intact homologous DNA

Bernard Lopez and Jacques Coppey

Institut Curie, Section de Biologie, 26 rue d'Ulm, 75231 Paris cedex 05, France

Received June 30, 1987; Revised and Accepted August 13, 1987

ABSTRACT
Parameters of DNA double strand break (dsb) repair catalysed by human nuclear extract were analysed using, as substrate, the replicative form (RF) of M13 mp8 in which a single double strand break (dsb) was introduced by restriction. After incubation with the extract, the dsb repair was estimated by the ability of the incubated RF to produce plaques following transfection into JM 109 (Rec A-) bacteria. The possibility of recombination with a purified fragment from M13 mp8 RF enhances up to 20 times the plaquing ability of the RF.

The repair by recombination occurs under several conditions: i) the break in the RF must be located in the region of homology with the fragment, ii) the fragment has to be intact in the region corresponding to the break in the RF, iii) a minimal length of homology between the region surrounding the dsb in the RF, and the fragment is required. The in vitro reaction is ATP dependent and dNTPs partially dependent. Dephosphorylation of the free ends in the RF decreases the repair by ligation but is without effect on the recombination.

INTRODUCTION
It is well demonstrated that the presence of double-strand breaks (dsb) or gaps in DNA gives rise to an increase in genetic recombination (1-6). Two models have been proposed to account for recombination between a double-stranded broken DNA and its intact partner: i) the Double-Strand Break Repair model, which was established from data in yeast (7): recombination is initiated by a double-strand break or a gap in one DNA molecule; the interruption is repaired using the unbroken copy of homologous sequence as template. Therefore the break has to be located in a region of homology between the two DNA's to allow such a process. ii) the Single-Strand Annealing model (8, 9): the ends produced by a dsb are substrates for single strand exonuclease, producing complementary single-stranded regions. These regions are then paired and generate intermediate recombining structures. According to this model, the dsb has not to be necessarily located in a region of homology.

DNA dsb is a critical damage induced by ionizing radiation. Both cell killing and the number of unrepaired dsb are increased as a function of the dose delivered (10, 11). Different rejoining processes can operate for the repair of dsb in irradiated cells (12, 13). In yeast, haploid cells are more radio-sensitive than diploid cells (14 and ref.
therein). An explanation would be that some dsb are responsible for cell death and that the dsb are repaired mainly via a recombinational pathway between homologous sequences. The situation is not clear in mammalian cells since no cell lines with an haploid content are available. X-ray sensitive hamster cells, defective in dsb rejoining, do not exhibit apparent defect in the ability to perform homologous recombination (15, 16).

It therefore appeared of interest to ask if homologous recombination promoted by mammalian nuclear extracts enhances the rate of dsb repair. The strategy depicted in fig. 1 was used in order to answer this question. The test chosen was based upon the restoration of viability of repaired molecules and not on the selection of a recombined phenotype.

MATERIAL AND METHODS

Strategy

We used, as substrate, the replicative form (RF) of the bacteriophage M13 mp8 digested by the restriction endonuclease Smal. M13 mp8 RF possesses an unique Smal endorestriction site (Fig. 1A). The Smal digestion results in a homogenous population of linearized RF's. Following digestion, the RF exhibits a drastic reduction in its ability to produce plaques after transfection into host bacteria. The digested RF was incubated with human nuclear extract, then purified, ethanol precipitated and transfected into JM 109 (rec A⁻) bacteria. Two assays were simultaneously carried out: a) the digested RF was incubated alone in the extract, then transfected into bacteria. The number of plaques formed is referred as P1. b) the digested RF was coincubated in the extract with the 1 kb AvaII-BglII fragment (Fig. 1B) purified from M13 mp8 RF. The whole DNA's were then transfected into bacteria. The number of plaques formed is referred as P2.

The contribution of the fragment to the double-stranded break repair is given by the reactivation rate (RR), i.e., the ratio P2/P1. A RR < 1 indicates that the presence of the fragment in the incubation mixture has no effect on repair of the dsb created in the RF. A RR > 1 indicates that the presence of the fragment leads to enhanced repair of the dsb.

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). The cells were grown in 150 mm diameter Petri dishes (Nunc). 2 to 5 x 10⁸ cells were collected by scraping with a rubber policeman after three washings with cold phosphate buffered saline (PBS). The cells were centrifuged and suspended in 10 ml of buffer A (20 mM Tris-HCl pH 7.5, 0.5 mM MgCl₂, 0.5 mM dithiotreitol, 5 mM KCl,
SUBSTRATE:
RF of M13 mp8
digested by one restriction enzyme

SUBSTRATES:
RF of M13 mp8
digested by one restriction enzyme +
1 kb AvaII - BglII fragment

Incubation
with nuclear extract

Transfection of JM 109 (recA-)
bacteria and plating

Plaque counting

P₁ = number of plaques

Incubation
with nuclear extract

Transfection of JM 109 (recA-)
bacteria and plating

Plaque counting

P₂ = number of plaques

REACTIVATION RATE = P₂/P₁

Fig. 1: A) Restriction map of M13 mp8. Dark box : lacZ⁺; dotted box : lacT⁻. B) Strategy used.
Fig. 2: Reactivation rate (see Methods) as a function of the extract protein concentration (incubation time: 30 min).

2 mM CaCl$_2$ and 250 mM sucrose). After 15 minutes, the cells were collected by centrifugation and resuspended in buffer A without sucrose but with 1 mM phenylmethylsulfonyl fluoride (PMSF). Nuclei were released by 40 strokes in a Dounce homogenizer and collected by centrifugation for 1 min at 2000 g. The nuclei were washed 4 times in buffer A with 0.5% NP40, then 3 more times in buffer A without NP40. Nuclei were resuspended in 2 ml of 500 mM NaCl, 10 mM EDTA, 0.1 mM PMSF, sonicated at 0-4°C for 30 sec using a Branson sonifier (output 6, duty cycle 50%) and ultracentrifuged at 40,000 rpm for 1 h in a SW 56 rotor. The supernatant was passed through a DEAE sepharose column equilibrated with 500 mM NaCl. Proteins were precipitated with (NH$_4$)$_2$SO$_4$ (50%) and centrifuged at 10,000 rpm for 10 min. The pellets were dissolved in 1 ml of 50 mM Tris (pH 7.5), 0.1 mM EDTA, 10 mM β-mercaptoethanol, 0.1 ml PMSF, 10% glycerol, and dialyzed overnight against the same buffer. Protein concentration was determined by the Biorad protein assay.

**DNA manipulations**

Isolation, purification and modification of DNA's from phage M13 mp8 were carried out using standard procedures (17). All enzymes were used in conditions specified by the manufacturers.

**In vitro assay**

500 ng of digested RF and various amount of Avall-BglII fragment were incubated with nuclear extracts, for different time, in a 100 μl reaction mixture containing 20 mM Tris-HCl (pH 7.5), 10 mM MgSO$_4$, 1 mM ATP, 100 μM each dNTP. The DNA's were then purified by phenol extraction and ethanol precipitation.
Transfection of bacteria

The bacteria JM 109 (\textit{recA}, \textit{lacz}) (18) were grown to a DO of 0.8-0.9 (\textit{A} = 550) and were transfected according to the method of Hanahan (19). The transfected bacteria were plated on Petri dishes with IPTG and X-gal in 3 ml of Top Agar.

RESULTS AND DISCUSSION

Following incubation with the nuclear extract, the RR is increased up to a value of 15 according to the protein concentration (Fig. 2), the time of incubation (Fig. 3) and to the relative amount of fragment (Fig. 4). When the DNA's are transfected without prior incubation with the nuclear extract, the RR presents a maximal value of 1.5 (table 1 and background values of Fig. 2 and 3). This indicates that the plaque forming efficiency of the digested RF transfected in the bacteria (which are \textit{recA}) is not increased by the presence of the co-transfected AvaII-BglII fragment. Boiling the extract abolishes its effect. Moreover there was no increase of the RR when the two DNA's were incubated separately with the extract, mixed after purification and then incubated in the absence of extract (table 1).

These data show that the addition of the fragment leads to an enhanced efficiency in the dsb rejoining in the RF only when the two DNA's are coincubated in presence of the extract. We can conclude that the effects are brought about by activities present in the nuclear extract.

The break created in the RF has to be located in the region of homology with
the added fragment to obtain an enhancement of RR. This is shown by the lack of effect when the RF is digested by Ball, i.e. outside of the region of homology (table 1). In addition, when the fragment is digested at the same site (SmaI) as the RF, no enhancement can be detected (table 1). These data are suggestive of a recombinational pathway catalysed by the nuclear extract which could be responsible for the enhancement in RR. It is important to point out that we have previously demonstrated using the same nuclear extract preparation the promotion of strand exchange and homologous pairing between the fragment and the intact circular RF (20). Interestingly the homologous pairing is independent of exogenous ATP (20). In contrast, the enhancement of RR requires exogenous ATP (table 2). This may indicate the existence of distinct recombination pathways, one involved in strand exchange which is ATP independent and the other in the repair of breaks by recombination which is ATP dependent. An alternative explanation would be that several activities are required in dsb rejoining and that at least one of them, ATP dependent, is limiting. The requirement in exogenous ATP for the promotion of strand exchange (rec A-like activities) by mammalian cells extracts has not be found in all cases (21-23).

On the other hand, we observed that homologous pairing between uncut RF and the fragment was independent of exogenous dNTP's (20), whereas the enhancement of RR was partially dependent of exogenous dNTP's (table 2). This appears to indicate that a limited polymerization has to occur for an efficient dsb rejoining although the RF contains a punctual dsb and not a gap. In accordance with the model of Double-Strand Break Repair (7), we can postulate that the dsb has to be enlarged prior to its repair.
Table 1: Reactivation rate as a function of different conditions

<table>
<thead>
<tr>
<th>Substrates</th>
<th>Cleavage by</th>
<th>Incubation condition</th>
<th>Value</th>
<th>Rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>M13 mp8 RF</td>
<td>Ball</td>
<td>30 min with extract</td>
<td>P1=185</td>
<td>0.8</td>
</tr>
<tr>
<td>plus fragment</td>
<td>none</td>
<td></td>
<td>P2=148</td>
<td></td>
</tr>
<tr>
<td>M13 mp8 RF</td>
<td>Smal</td>
<td>30 min with extract</td>
<td>P1=165</td>
<td>0.8</td>
</tr>
<tr>
<td>plus fragment</td>
<td>Smal</td>
<td></td>
<td>P2=132</td>
<td></td>
</tr>
<tr>
<td>M13 mp8 RF</td>
<td>Smal</td>
<td>30 min without extract</td>
<td>P1=215</td>
<td>1.5</td>
</tr>
<tr>
<td>plus fragment</td>
<td>none</td>
<td></td>
<td>P2=322</td>
<td></td>
</tr>
<tr>
<td>M13 mp8 RF</td>
<td>Smal</td>
<td>separate incubation of each</td>
<td>P1=153</td>
<td>1.1</td>
</tr>
<tr>
<td>plus fragment</td>
<td>none</td>
<td>substrate with extract for 30 min*</td>
<td>P2=170</td>
<td></td>
</tr>
<tr>
<td>M13 mp8 RF</td>
<td>Smal</td>
<td>boiled extract</td>
<td>P1=114</td>
<td>1</td>
</tr>
<tr>
<td>plus fragment</td>
<td>none</td>
<td></td>
<td>P2=125</td>
<td></td>
</tr>
<tr>
<td>M13 mp8 RF</td>
<td>Smal</td>
<td>30 min with extract</td>
<td>P1=253</td>
<td>15</td>
</tr>
<tr>
<td>plus fragment</td>
<td>none</td>
<td></td>
<td>P2=3790</td>
<td></td>
</tr>
</tbody>
</table>

By "fragment" we intend: the Avall/BglI restricted sequence purified from M13 mp8 RF.

The transfection frequency of uncut DNA without incubation with the extract is \(10^7\) pfu/µg DNA. The transfection efficiency of uncut DNA after incubation with the extract is about \(5 \times 10^6\) pfu/µg DNA.

* refers to an experiment in which the two DNA's are separately incubated with extract, then purified and coincubated 30' at 37°C without extract.

** The P1 and P2 values represent the sum of values obtained from, at least, 3 independent experiments.

One possible mechanism could be that a cut represents the initiation point of exonuclease activity leaving complementary single-stranded regions which can anneal by pairing. This possibility is excluded by the lack of enhancement of the RR when the two substrates are separately incubated (table 1). Therefore, our system does not monitor a process corresponding to the Single-Strand Annealing model (8, 9). This could be due either to loss of the required exonuclease activity during the preparation of the extract and/or to the fact that our system which involves a fragment of short length is not suitable for the detection of such a process.
Table 2: Requirement in exogenous cofactors

<table>
<thead>
<tr>
<th>Substrates</th>
<th>Cleavage by</th>
<th>Conditions</th>
<th>Value</th>
<th>Rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>M13 mp8 RF plus fragment</td>
<td>Smal</td>
<td>complete</td>
<td>P1* 253</td>
<td>P2* 3790</td>
</tr>
<tr>
<td></td>
<td>none</td>
<td>no ATP</td>
<td>P1* 125</td>
<td>P2* 120</td>
</tr>
<tr>
<td></td>
<td>none</td>
<td>no dNTP's</td>
<td>P1* 90</td>
<td>P2* 453</td>
</tr>
</tbody>
</table>

P1* and P2* values represent the sum of the values obtained from 3 independent experiments.

Another possibility could be the repair of the Smal restriction cut via illegitimate recombination between the RF and the fragment. It is worth to note in this context that X-ray sensitive hamster mutant cells, defective in dsb rejoining,

---

Fig. 5: The two possible processes of non homologous recombination. 
A) Terminal pairing-like process. B) Insertion of the fragment into the RF. 
Heavy line: AvaII-BglII fragment; thin lines: RF.
present a reduced efficiency to perform integration of exogenous plasmids into the cellular genome (16), a process involving illegitimate recombination. Such a recombination between the fragment and the restricted RF could result in either structure depicted in Fig. 5: i) a process (Fig. 5A) similar to terminal pairing described by Anderson and Eliason (24) which would give linear molecules, the viability of which (Fig. 5A) would not be increased compared to that of the original restricted RF. Therefore this could not account for the enhancement of the RR. ii) integration of the fragment at the cleavage site. In accord with this last possibility, the enhancement of RR would occur with the RF restricted at the Bann site as well as at the SmaI site. This is not the case (see table 1). Moreover, this integration would lead to a modification of the restriction map of the repaired RF. Restriction analysis of 10 repaired clones show no modifications as compared to the normal M13 mp8 RF (Fig. 6). These different results allow to exclude a mechanism of illegitimate recombination for the enhancement of the RR.

In order to definitively exclude a mechanism of non homologous recombination, we decided to test whether the enhancement of RR is submitted to sequence homology constraints between the fragment and the region surrounding the break in the RF. For this purpose, we used a M13 mp8 RF in which we inserted an octonucleotide linker (Xbal) at 7 nucleotides downstream the EcoRI restriction site (Fig. 7). The result (table 3) shows that an interruption of the sequence homology (8

![Fig. 6: Clal restriction map of ten clones arising from repaired RF molecules.](image)
Table 3: Effect of a short sequence heterology on the reactivation rate (+)

<table>
<thead>
<tr>
<th>Substrates</th>
<th>Cleavage</th>
<th>Length of homology from the cut up to the linker Xba</th>
<th>Value</th>
<th>Rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>M13 mp8 RF</td>
<td>EcoRI</td>
<td>(no heterology)</td>
<td>205</td>
<td>1025</td>
</tr>
<tr>
<td>plus fragment</td>
<td>none</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M13 mp8-Xba RF</td>
<td>EcoRI</td>
<td>7 nucleotides</td>
<td>193</td>
<td>96</td>
</tr>
<tr>
<td>plus fragment</td>
<td>none</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M13 mp8 RF</td>
<td>BglI (+++)</td>
<td>(no heterology)</td>
<td>187</td>
<td>935</td>
</tr>
<tr>
<td>plus fragment</td>
<td>none</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M13 mp8-Xba RF</td>
<td>BglI (+)</td>
<td>148 nucleotides</td>
<td>192</td>
<td>960</td>
</tr>
<tr>
<td>plus fragment</td>
<td>none</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(+) Incubation was carried out for 1 hr with ATP, dNTP's and extract (20 µg/ml)
(++) The ends of BglI restricted RF were treated with S1 nuclease to produce blunt ends.
(+++) $P_1$ and $P_2$ values represent the sum of the values of 3 independent experiments.

nucleotides long) located at 7 nucleotides from one of the end of the dsb abolishes the RR enhancement effect of the added fragment. In contrast, an enhancement of RR is obtained when the M13 mp8-Xba1 RF is restricted by BglI (table 3), i.e. at 108 base pairs from the heterology region. Three mechanisms can account for these data: i) recombination is initiated at the level of the restriction cut with a progressive extension of the pairing reaction. Such a process would be stopped if the homology (hence the strength of pairing) is disrupted within a few nucleotides from the initiation site. It is worth to note that non homologous recombination can be initiated with only 1 to 6 complementary bases to position the junction (25). In the present case, the homology is only 7 nucleotides. The negative response recorded here is therefore not compatible with a mechanism of non homologous recombination. It is interesting to point out in this context that the minimal length of homology for recombination in *E. coli* is comprised between 30 and 150 base pairs (26). ii) prior to the pairing, an enlargement of a few nucleotides occurs at the restriction site up to the region of heterology. This would inhibit the initiation of the pairing process. If this is the case, it indicates that the enlargement extends over a short length, less than $7 + 8 = 15$ nucleotides. iii) resolution of intermediates structures would be achieved in bacteria and the *E. coli* cells could not resolved the intermediates structures formed...
Table 4: Effect of dephosphorylation of the RF on the reactivation rate

<table>
<thead>
<tr>
<th>Substrates</th>
<th>P1 value</th>
<th>P2 value</th>
<th>Reactivation rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>M13 mp8 RF digested by Smal plus fragment</td>
<td>307*</td>
<td>3360*</td>
<td>11</td>
</tr>
<tr>
<td>M13 mp8 RF mp8 digested by Smal and dephosphorylated plus fragment</td>
<td>24*</td>
<td>2412*</td>
<td>100</td>
</tr>
</tbody>
</table>

Incubation for 30 min with 20 μg/ml of extract, ATP and dNTP's.
* The P1 and P2 values represent the sum of the values obtained from 3 independent experiments.

with M13 mp8 Xba RF. This is be surprising since it would be not the case with M13 mp8 Xba RF cut with BgIII. On the other hand, we have previously observed a resolution activity of intermediate structure, with an intact M13 mp8 Xba RF, in the extract.

The structure of the restricted RF harboring a Xbal linker presents an heterology only on one side of the cut, whereas the other side is perfectly homologous to the corresponding sequence of the fragment. We can deduce from the results that homology is required on both sides of the cut for an efficient rejoining. Both ends are therefore involved in the initiation of the process.

The viability of the restricted RF incubated alone (the P1 value, Fig. 1) can result from ligation activities present either in the extract or in the host bacteria. Ligation can be prevented by dephosphorylation of the 5' ends at the cut. It can be seen that dephosphorylation of the RF causes a decrease of its viability when incubated alone but has no effect on its viability when coincubated with the fragment (P1 and P2 values, table 4). This results in a strong increase of the RR. These data indicate that ligation plays a role in the rejoining of a dsb in our system and that the enhancement of dsb rejoining by recombination is not significantly affected by dephosphorylation of the dsb ends. This lack of inhibitory effect in condition 2 (Fig. 1) can result from the removal of the dephosphorylated ends caused by the enlargement of the break, as discussed above. This would indicate that the enlargement is strongly associated to the recombination process. Indeed if enlargement was only caused by the presence of a dsb, it should take place in either experimental condition, 1 or 2 (see material and methods, and Fig. 1). The dephosphorylated ends would be therefore removed in either of the two conditions and ligation could occur in condition 1. This possibility is ruled out by the important decrease of the P1 value (table 4). We can propose that enlargement exonuclease activity is coupled to the recombination process.
CONCLUSION

The present data indicate that the efficiency of DSB repair is improved when homologous recombination can take place. A multienzymatic complex seems to be involved in the steps of this process (summarized in Fig. 8). Such a process is in agreement with the model of Szostak et al. (7) which was derived from genetical data in yeast. In accord with the finding that homologous recombination increases DSB repair, a highly increased frequency of a recombined phenotype among the repaired molecules is expected and is indeed observed (2-5).

DSB rejoining can occur either by direct ligation of the breaks and/or by recombinational/ligation. Both processes seem to occur in bacteria and in mammalian cells following their exposure to ionizing radiation (13). The in vitro system used here allows to detect the occurrence of the two repair processes and to demonstrate that the recombination pathway is predominantly involved. This can be connected to data
showing that rec− bacteria are fourfold more radiosensitive than wild type strains (28), although their ligation activity is not impaired (13).

Moreover we show here that the efficiency of dsb repair is increased as a function of the relative number of intact DNA copies (see Fig. 4). This can account for observations done in living cells: i) yeast cells are more radioresistant in a diploid state than in a haploid state (14 and ref. therein). ii) dsb's are successfully resealed in irradiated mammalian cells during their G2 phase, i.e. when they possess a duplicated DNA content (29 and ref. therein).

All these data indicate that, for dsb repair and survival, the availability of a system of homologous recombination, namely the concomitant presence of enzymatic activities and of the suitable substrates, is more important than ligation alone.

ACKNOWLEDGEMENTS

This work was supported by grants from the Commission des Communautés Européennes (B16-151F), the Institut National de la Santé et de la Recherche Médicale (n° 87460/BCR/NT/MG), the Commissariat à l'Energie Atomique (France) and the Association de la Recherche sur le Cancer (n° 6381). We thank Dr. E. Moustacchi for critical reading of the manuscript and D. Chardonnieras for helpfull assistance.

REFERENCES