Molecular recombination and the repair of DNA double-strand breaks in CHO cells

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ABSTRACT

Molecular recombination and the repair of DNA double-strand breaks (DSB) have been examined in the G-0 and S phase of the cell cycle using a temperature-sensitive CHO cell line to test i) if there are cell cycle restrictions on the repair of DSB's; ii) the extent to which molecular recombination can be induced between either sister chromatids or homologous chromosomes and iii) whether repair of DSB's involves recombination (3). Mitomycin C (1-2 µg/ml) or ionizing radiation (50 krad) followed by incubation resulted in molecular recombination (hybrid DNA) in S phase cells. Approximately 0.03 to 0.10% of the molecules (number average molecular weight: 5.6×10^6 Daltons after shearing) had hybrid regions for more than 75% of their length. However, no recombination was detected in G-0 cells. Since the repair of DSB was observed in both stages with more than 50% of the breaks repaired in 5 hours, it appears that DSB repair in G-0 cells does not involve recombination between homologous chromosomes. The possibility is not excluded that repair in G-0 cells involves only small regions (less than 4×10^6 Daltons).

INTRODUCTION

The induction of molecular and cytological exchange by mutagens and radiation has been examined in a variety of mammalian cells (4, 5, 6, 7). In Chinese hamster cells sister chromatid exchanges (SCE) can be enhanced as much as 10 times by various mutagens. Ultimately it should be possible to characterize the cytological changes by the primary molecular events. Using mitomycin C, Moore and Holliday (7) have shown a correlative increase of presumed molecular exchanges with SCE's. The molecular recombinational events were identified by the appearance of a small amount of heavy-heavy DNA⁸ (HH-DNA) following mutagenic treatment of cells in which one strand of the DNA was heavy (containing bromouracil) and one strand was light in each chromatid. In a similar manner, Rommelaere and Miller-Faures (9) were able to show that UV irradiation of stationary phase cells led to the formation of HH-DNA during the subsequent round of replication in the

presence of bromouracil. Recent results of Waters, Regan, and German (10) have further substantiated this with human fibroblasts.

Contrary to the above results, Loveday and Latt (11) were unable to detect HH DNA resulting from molecular exchanges due to the presence in CHO cells of a small amount of satellite DNA of a similar density to that expected for HH DNA in their experiments. Using different techniques, the possibility that there might be some exchange of UV-induced pyrimidine dimers between sister chromatids has been indicated by the work of Meneghini and Hannawalt (12) and Waters and Regan (13); the nature and extent of this exchange is still under investigation (see reference 14).

Although there is evidence in mammalian cells for molecular and cytological recombination between sister chromatids, there have been no reports of recombination prior to DNA synthesis between homologous chromosomes. In lower eukaryotes such as the fungi Aspergillus nidulans (15), Saccharomyces cerevisiae (16, 17) or Ustilago maydis (18) the induction by mutagens of genetic recombination between homologous chromosomes has been well established. Contrary to these results, it has not been possible in mammalian cells to induce genetic recombination in mitotic cells (19, 20).

The failure to detect genetic recombination between homologous chromosomes does not exclude the occurrence of such recombination since at present only a few genetic systems have been examined. A more general approach would be to examine total molecular recombination (see reference 3) between homologues. We have extended the method of Moore and Holliday (7) to cells in which the induction of molecular recombination could be determined in the early G-1 or the G-0 phase of the cell cycle. This technique of detecting HH-DNA was developed to measure recombination between sister chromatids; however, if one mitosis is allowed to occur after the labeling of the DNA, HH-DNA can then be detected due to recombination between homologous chromosomes.

In this study the recombinogenic effects of mitomycin C and ionizing radiation have been examined. Mitomycin C was used to determine if the recombination induced between sister chromatids (7) could also be induced between homologous chromosomes. Ionizing radiation has been employed for two reasons: i) to determine if it, too, can induce molecular recombination in mammalian cells, and ii) to examine whether the proposed recombination mechanism (3) for the repair of DNA double-strand breaks (DSB) in lower eukaryotes also applies to mammalian cells. In terms of lethality, ionizing

radiation has similar effects in both vertebrates and fungi (21). For the case of fungi, ionizing radiation is an efficient recombinogen with a correlation of approximately one DSB per genetic recombinational event (3) and the repair of DSB's in fungi and bacteria (22) is considered to involve recombination (3, 3a), or at least an association of homologous chromosomes or sister chromatids (Brunborg and Williamson, preparation [23]). As proposed by Resnick (3, 3a) DSB's could be rejoined through processes involving recombination intermediates including Holliday type structures (24); in this model there is no loss of genetic material.

Both repair of DSB's and the appearance of molecular recombination between homologous chromosomes and sister chromatids after ionizing radiation were examined in Chinese hamster ovary (CHO) cells cs4-D3. This line of cells (25) is temperature-sensitive for growth and at the restrictive temperature is arrested in a post-mitotic stage considered to be early G-1 or G-0 (26). While DNA synthesis is prevented at the restrictive temperature, RNA synthesis is normal and protein synthesis is approximately 40% that of normal cells.

MATERIALS AND METHODS

Cells

In order to obtain a population of cells synchronized in the G-0/G-1 phase of the cell cycle, we have used cs4-D3, a cold sensitive mutant derived from a CHO cell line (25). When shifted to the restrictive temperature (33°), the cells complete the cell cycle upon which they have already embarked, undergo mitosis, and arrest in G-0, thereafter remaining viable for many days (26). Upon return to the permissive temperature (39°), the cells resume a normal cell cycle, after a lag period which is dependent on the time spent at 33°. To obtain G-0 cells with HL-DNA, BU/³H was added to an exponentially growing population of cells at 39°. After four hours the cells were shifted to 33° and incubated two more hours. They were then washed, returned to unlabeled medium containing thymidine (5 μ C/ml) and subsequently kept at 33° for 48-60 hours before treatment with ionizing radiation or mitomycin C.

During the labeling period each chromatid can only become labeled with BU/³H in one strand of the DNA. All those cells that were in S phase when label was present undergo mitosis during the period at 33° and by random assortment of chromosomes, each resulting G-0 cell

will have one-half of its pair of homologous chromosomes with BU/ ^3H in strands of opposite polarity. Thus HH-DNA which is a measure of recombination can be detected in half the pairs of labeled chromosomes (those with strands of opposite polarity; see Fig. 1 in reference 7). Recombination in chromosomes labeled in strands of the same polarity would only lead to HL-DNA.

In some experiments cells were kept at 33° throughout, while in others they were shifted back to 39° two hours prior to irradiation or mitomycin C, treatment and incubated at 39° for the subsequent "repair period." Control cells were treated identically to corresponding irradiated cells in all respects other than the exposure to ionizing radiation.

In experiments carried out on exponential cells growing at 39°, cells were treated with BU/ ^3H for six hours and chased one hour in unlabeled medium containing 5 $\mu\text{g/ml}$ thymidine. This protocol eliminates the possibility of HH-DNA being formed by branch migration at sites of replication (4). Previously (7), these cells were regarded as being G-2. Although all the label is in regions of the chromosomes that have already replicated, it is probable that many of the cells that have incorporated label have not completed S phase when treated with X-rays/ionizing radiation or mitomycin C; therefore the cells are considered as being in S or early G-2 (S/G-2 cells).

Cells to be used for sucrose gradient analysis were incubated in the same manner as above except that BU was omitted.

Media and Growth Conditions

These were as previously reported (7).

Labeling with ^3H and BU

In experiments to detect molecular exchange (HH-DNA) cells were incubated with BUdR, 3 $\mu\text{g/ml}$, FUDR, 0.25 $\mu\text{g/ml}$, rU, 1.5 $\mu\text{g/ml}$, CdR, 25 $\mu\text{g/ml}$ and ^3H -thymidine 20 $\mu\text{Ci/ml}$ (40-60 Ci/mole). At the level of detection in these experiments, the presence of BU in one strand of the DNA did not significantly alter the production of DSB's or interfere with their repair (unpublished observations).

Irradiation

For irradiation the medium was removed and replaced with phosphate buffered saline. While still attached to the bottle, the cells were irradiated (^{60}Co) at 15 krad per minute.

CsCl Gradients and Size of DNA

DNA extraction and analysis on CsCl gradients was carried out as

described previously (7), except that (1) cells were harvested by scraping with a silicone rubber scraper instead of trypsin treatment and (2) the DNA was treated with S1 deoxyribonuclease (kindly provided by Dr. Ian Molineux) after shearing to remove any single stranded regions that would otherwise interfere with the detection of HH-DNA (see reference 7). After shearing and S1 treatment the number average molecular weight, M_n , of the DNA was determined by slab gel electrophoresis (see legend to Fig. 3) using the techniques of McDonnell, et.al., (27). Bacteriophage lambda DNA digested with endonuclease Eco RI was used as a standard (28).

Sucrose Gradient Centrifugation

Cells were collected, washed and resuspended at about 2×10^5 per ml; 0.1 ml was then gently layered on 5-20% sucrose gradients (at room temperature) containing 1.0 M NaCl and 0.02 M EDTA pH7 that had an 0.1 ml lysing layer which contained 1.0 M NaCl, 0.02 M EDTA, 0.01 M Tris Buffer (pH8), 2% Sarkosyl, 3% sodium deoxycholate, 5% sodium dodecyl sulfate (29). After approximately one hour they were centrifuged in a Beckman SW50.1 rotor at less than 9 krpm to avoid speed dependence artifacts (30). The gradients were fractionated (equal volumes) on to Whatman No. 3 paper strips. Subsequent treatment has been described (29).

RESULTS

Molecular Exchanges

The isolation of DNA with HH regions from cells which have undergone one round of replication in BU is an indication of recombination at the molecular level (7). Normally some DNA is AT rich in base composition (Kommelaere and Miller-Faures, 1975) so that its density after BU incorporation is as much as 0.025 g/cc higher than that of HL-DNA (density = 1.748 g/cc). In order to avoid inclusion of this, DNA of density greater than 1.785 g/cc was chosen for further examination by rebanding (see Fig. 1 & 2). This is slightly higher than that used previously (7).

Since DNA of density greater than 1.785 g/cc was being used, one strand would be expected to be heavy and the other at least 75% heavy. (The density of DNA which is totally heavy is 1.798 g/cc). Therefore, the size of recombination regions could be determined from the density of the DNA analysed on the CsCl gradient and the length of the DNA. The DNA size was determined using gel electrophoresis and Eco RI digested bacteriophage lambda DNA as markers. The M_n calculated from the distribution of counts in the gel presented in Fig. 3 was 5.6×10^6 Daltons.

Figure 1. Distribution in a CsCl gradient of DNA from S/G-2 cells.

Cells were labelled as described in Methods and then treated with mitomycin C, $1 \mu\text{g/ml}$, for 5 hr. All initial gradients irrespective of cell stage or treatment gave essentially the same profile with the exception of the region of the gradient corresponding to densities greater than 1.785 g/cc, which for this gradient is shown in Figure 2b with an expanded ordinate scale.

Therefore, in the density range analysed the molecular exchanges (i.e., the HH-DNA) would involve at least 6500 base pairs.

As previously reported, treatment of S/G-2 cells with mitomycin C results in an increase in the amount of HH-DNA (Fig. 2, Table 1) over the control level. When HH-DNA was rebanded it was clear that there was at least a 2-3 fold increase in the amount of HH-DNA with density of 1.785 - 1.805 g/cc over the very low control values of about 0.03% (lines

Figure 2. Detection of molecular exchanges in S/G-2 cells.

The figures 2a, 2b, and 2c are regions of the initial CsCl gradients corresponding to densities greater than 1.785 g/cc (see Figure 1). The Figures 2d, 2e, and 2f correspond to recentering of DNA from fractions indicated by horizontal bars (1.785-1.805 g/cc) on gradients shown in a, b, and c. The Figures 2a and 2d correspond to untreated cells, 2b and 2e to cells treated with mitomycin C, $1 \mu\text{g/ml}$ for 5 hrs, 2c and 2f to cells irradiated with 50 krad and incubated for 2.5 hrs. The total counts on the gradients (d to f) and the counts in the density range 1.785-1.805 g/cc are as follows:

	CPM	
	Total	Between 1.785-1.805
Control	276	142
Mitomycin C	1033	414
50 krad + 2.5 hrs	1645	328

Figure 3. Gel electrophoresis of DNA recovered from CsCl gradients.

The HL fractions of a control gradient were pooled and dialysed against 1.5 SSC buffer. A sample of DNA was loaded onto a 0.5% agarose slab gel and electrophoresis was at 4.6 volts/cm for 3.5 hrs in 40 mM Tris acetate, pH 8.0, 5 mM sodium acetate, 1 mM EDTA. The portion of the gel containing DNA was visualized by ethidium bromide staining and was sliced into 2.5 mm slices which were boiled in 0.5 ml water for 5 min. before counting the radioactivity. The position and molecular weight of the three EcoRI restriction fragments of lambda DNA run on the same gel are indicated by arrows. Mobility was measured relative to the 1.33×10^7 Dalton fragment. The number average molecular weight (M_n) over the fractions containing significant counts was determined by the formula

$$M_n = \frac{\sum C_i}{\sum C_i / M_i}$$

3, 4, and 5 in Table 1 correspond to the results in Fig. 2; see the legend of Fig. 2 for examples of the counts in the rebanded material). In absolute terms the amount detected in the present experiments is somewhat lower than that found previously (lines 1 & 2 of Table 1) although in both sets of data at least a 2-3 fold increase in HH-DNA is found after mitomycin C treatment (control values are a maximum estimate). The more restricted range of densities examined here probably contributes to the

Table 1. HH-DNA in Untreated and Treated CHO Cells

Cells	Treatment	Incubation Time (hr.)	Total ^b cpm ($\times 10^{-5}$)	HH-DNA	
				I ^c (% cpm)	II ^c (% cpm)
S/G-2 ^a	0	5	1.00	0.18	0.11
	Mitomycin C (2 μ g/ml)	5	1.19	0.46	0.31
S/G-2 (39°)	0	5	2.20	0.05	<0.03
	Mitomycin C (1 μ g/ml)	5	3.00	0.15	0.07
	50 krad	2.5	2.79	0.21	0.06
	50 krad	5	1.98	>0.20 ^d	>0.06
G-0 (33°)	0	4	0.29	0.30	
	50 krad	4	0.57	0.39	
	0	5	3.32	0.24	0.11
	50 krad	5	2.90	0.35	0.14
	0	5	3.29	— ^e	0.12
	50 krad	5	3.49	— ^e	0.10
G-0 (39°)	0	5	1.06	0.11	
	Mitomycin C (1 μ g/ml)	5	1.18	0.12	<0.025
	50 krad	2.5	1.21	0.15	<0.035
	50 krad	5	1.50	0.10	

- Results of Moore and Holliday (7); the DNA examined by them was greater than 1.775 g/cc.
- For the initial gradients 1/3 or 1/2 of each fraction was counted and the remainder was used for rebanding. The figures are for the cpm actually counted.
- I = per cent of total cpm on initial gradients at densities 1.785 to 1.805 g/cc (see Fig.2).
II = per cent of total cpm still found at this density after rebanding DNA from fraction I. The total counts in the density range 1.785 to 1.805 g/cc after rebanding is divided by the corresponding total cpm on the initial gradient (note b above).
- Since a high background was observed on the initial gradient, the figure given is a minimum estimate.
- Single-stranded heavy DNA on the initial gradient gave rise to high background and, therefore, DNA greater than 1.785 g/cc was rebanded after further S_1 treatment.

smaller amounts of HH-DNA observed. In some cases the use of a different cell line, a higher temperature and S1 nuclease, which eliminates spurious background counts due to single-stranded heavy DNA, may also contribute to this difference. Ionizing radiation also induced the formation of HH-DNA in S/G-2 cells; within 2.5 hours after a 50 krad dose there was an increase to 0.06% and by 5 hours even more HH-DNA was observed (see Table 1). Irradiation without subsequent incubation did not increase the HH-DNA over control values (results not shown).

Contrary to these results, when cells in G-0 were treated with mitomycin C or ionizing radiation, no increases in HH-DNA over control values were observed (Table 1). In experiments where the cells were kept at the restrictive temperature (33°) after treatment, the amount of HH-DNA recovered was greater than that for S/G-2 cells. However, if cells were shifted to 39° two hours prior to treatment, the amount of HH-DNA found was comparable to S/G-2 controls (Table 1). The reason for a difference in the amounts obtained from cells held at the restrictive versus permissive temperature could be related to the physiology of the cells (25,26).

Repair of Double-strand Breaks

The induction and repair of DSB's was examined in S/G-2 and G-0 phase cells; two hours prior to irradiation the G-0 cells were shifted to the permissive temperature (39°). Regardless of stage in the cell cycle we observed that DNA from unirradiated cells sediments as large complexes so that less than 5% of the counts layered were found in the gradient after centrifugation (data not shown). Similar observations have been reported with exponentially growing cells of other mammalian lines (31, 32). Irradiation of cells results in the DNA being released from the complex and with increasing doses the DNA sediments freely at decreasing sedimentation rates (Fig. 4 and 5). There appears to be no difference in sensitivity to DSB break production between G-0 and S/G-2 phase cells. By way of comparison, results are included for yeast cells (Fig. 4) which were also exposed to 50 krad and sedimented under the same conditions. The comparable profiles indicate a similar efficiency of DSB production in the two very different eukaryotes. (A more complete description of the response of yeast cells to ionizing reduction is contained in reference 29).

The DNA from cells which had been irradiated and subsequently incubated was found to increase in size and ultimately develop into a complex. Within 2.5 hours after a dose of 50 krad, the DNA sedimentation pattern approximated that of the 25 krad dose (Fig. 4). By 5 hours a

Figure 4. The repair of DNA from irradiated G-0 cells.

Cells which had been labelled with ^3H thymidine in S at 39° were subsequently incubated at 33° (restrictive temperature). Two hrs prior to irradiation the temperature was raised to 39° . Just before irradiation the cells were washed with phosphate buffered saline; after irradiation the growth medium was added back and the cells were then kept at 39° . (Not included are results from cells which were not irradiated since greater than 95% of the counts were recovered on the tube or in the bottom two fractions.)

..... 25 krad only 50 krad only
 _____ 50 krad + 2.5 hrs post-irradiation incubation
 - - - - - 50 krad + 5 hrs post-irradiation incubation
 _____ The sedimentation profile of DNA from yeast cells which
 were irradiated with 50 krad (see reference 29).

large amount of radioactivity was found in the bottom two fractions (Fig. 4 and 5). Since both the G-0 and the S/G-2 phase cells exhibited this pattern of repair, we conclude that an efficient mechanism of DSB repair exists in both types of cells and that within 2.5 hours at least 50% of the breaks are rejoined.

DISCUSSION

In these experiments with CHO cells we have examined whether recombination as measured by molecular exchanges between sister chromatids could also be observed between homologous chromosomes. Molecular exchanges be-

Figure 5. The repair of DNA from irradiated S/G-2 cells.

Cells which were grown at 39° were labelled with ³H thymidine for 5 hrs and "chased" for one hr. They were then washed with buffered saline and after irradiation, growth medium was added back and the cells were incubated at 39°. (Not included are results from cells which were not irradiated since greater than 95% of the counts were recovered on the tube or in the bottom two fractions.)

25 krad only

50 krad only

25 krad + 5 hrs post-irradiation incubation

50 krad + 5 hrs post-irradiation incubation

between sister chromatids can be detected by the formation of a small amount of HH-DNA after one round of replication in BUdR (7, 9, 10). Recently Loveday and Latt (11) have reported that they were unable to detect HH-DNA in similar experiments due to the presence of a small amount of heavy satellite DNA in CHO cells. They suggested that this satellite would obscure even smaller amounts of HH-DNA on CsCl gradients and questioned whether previous reports (7, 9) had indeed identified HH-DNA. In the experiments of Loveday and Latt the cells were labeled with BUdR without the addition

of FudR and they obtained a density for HH-DNA near 1.765 g/cc which, if correct, would indicate a shift from HL of substantially less than 0.05 g/cc suggesting incomplete substitution of bromouracil for thymine in the DNA. Thus the presence of a satellite which is about 0.025 g/cc heavier than main band DNA (also reported in reference 9) would not allow detection of HH-DNA under these conditions. In previous studies (7, 9) and in the experiments reported here, the use of FudR results in complete substitution with bromouracil and a shift of 0.05 g/cc between HL- and HH-DNA thus allowing HH-DNA to be distinguished from heavy satellite DNA (see results).

In the present experiments we have modified the techniques of Moore and Holliday (7) to detect exchange regions which are at least 6,500 base pairs in length. Recombination as measured by molecular exchanges occurs in S/G-2 phase cells after exposure to mitomycin C and ionizing radiation. As previously reported (7) this recombination is associated with the production of cytologically observable sister chromatid exchanges; however, the possibility of exchanges between homologous chromatids is not excluded (33). In contrast to the S/G-2 results we have been unable to detect the induction of recombination between homologous chromosomes in G-0 cells even at a level of discrimination which would have revealed a frequency corresponding to at least one hybrid molecule per thousand.

Because of the importance of recombination between homologues, it is necessary to assess the reasons for its absence or for the lack of detection if, in fact, it does occur. In the CHO cell lines from which cs-4 is derived there are chromosome rearrangements (34) which could interfere with homologous chromosome pairing. Nevertheless there is nearly a complete diploid content of chromatin as measured cytologically (34), and genetically (35) and mitomycin C induced chromatid interchanges are frequently seen at metaphase (reference 6 and Moore, unpublished observations). Therefore, the absence of recombination in G-0 cells is not due to lack of DNA homology; however, physical restraints such as DNA membrane attachments (32, 36) could preclude pairing. Another reason for the absence may relate to the physiological block in these cells at the restrictive temperature: more than two hours at the permissive temperature may be required prior to treatment. It is important to emphasize that only exchanges involving regions of 6,500 base pairs or greater can be observed in the present system. Therefore, it is quite possible that recombination between homologous chromosomes involves

regions smaller than this and, therefore, they go undetected.

The second major aspect of these experiments has been to examine DSB repair in CHO cells and to determine whether it is related to molecular recombination as suggested from work with lower eukaryotes. The repair of DSB's is an efficient process in CHO cells that are in either the S/G-2 or G-0 phase of the cell cycle. Following the doses of radiation used in these experiments, no survivors would be expected. The reason for cellular inactivation could be due to a few DSB's per cell remaining unrepaired since, as shown for the case of the diploid yeast *S. cerevisiae*, a single unrepaired DSB appears to be lethal (29). While very little is known about the actual mechanism of DSB repair, the results of this study and those obtained with yeast in the G-2 phase of the cell cycle (Resnick, Brunborg, and Williamson, submitted for publication) show that there is no requirement for an S-phase to carry out repair.

The absence of molecular exchanges in G-0 suggests that recombination is not associated with DSB repair contrary to what appears to be the case in lower eukaryotes and bacteria. From the size of molecules analyzed (Fig. 3) it is possible to estimate the number of DSB's expected per molecule, and if recombination were involved in their repair, the minimum number of detectable recombinational events. Although the efficiency of DSB induction was not determined in this report, the comparison with yeast (Fig. 4; reference 29) and other systems (see references in 37) indicates an efficiency of approximately $0.5 - 1 \text{ break} / 10^{10} \text{ Daltons/krad}$. Since the M_n of DNA from the CsCl gradients was $5.6 \times 10^6 \text{ Daltons}$, approximately 1.5 to 3% of the molecules would have had a DSB induced in them. Based on Figs. 4 and 5 at least 50 to 75% of the molecules which had DSB induced in them had been subsequently repaired. In these experiments we have been able to detect molecules with exchanges at a frequency of less than 0.1%. If repair can occur through recombination between homologous chromosomes, then in G-0 cells there cannot be more than 1 recombinant per 10 DSB's. [Other types of lesions such as single-strand breaks or base damage must be even less recombinogenic since they are induced with a twenty times greater frequency than DSB's (38, 39).] In S/G-2 phase cells there are 1 or 2 recombinants per 10 DSB's repaired. (It is interesting that this frequency is similar to that for sister chromatid exchange production; Perry and Evans (6) found that irradiation of S phase CHO cells gave rise to 35-40 sister chromatid exchanges per 100 rads. Using our estimate of DSB production, this corresponds to one sister chromatid

exchange for every 8-15 DSB's).

Although these results argue against repair of DSB's through a recombinational process, the possibility is not excluded that only small regions of hybrid DNA are involved (see above) or alternatively there is a transient association of DNA of one chromosome with another as has been proposed elsewhere (3). Another possibility is that repair occurs by blunt-end ligation (40, 41) as has been observed in vitro for the joining of "clean ends." If this were the case, then the nucleosome rather than hybrid regions of DNA could provide the structure required for the correct rejoining of broken ends. Occasional "incorrect" rejoining could lead to chromosomal rearrangements and interchanges; these occur at a frequency much lower than DSB production (42). However, blunt-end ligation as a means of repair would be inconsistent with results in lower eukaryotes and *E. coli*, since there is a requirement in these organisms for duplicate chromosomes (22; 23).

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