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Noncomplementary DNA double-strand-break rejoining in bacterial and human cells

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ABSTRACT

We examined the rejoining of noncomplementary restriction enzyme-produced DNA double-strand breaks in *Escherichia coli* and in cultured human cells. The enzymes used in this study, *Cla*I, *Bam*HI and *Sal*I, produce double-strand breaks with 5 protruding single strands. The joining of a *Cla*I-produced DNA end to a *Bam*HI-produced end or to a *Sal*I-produced end was examined at the DNA sequence level. End rejoining in *E.coli* was studied by transforming cultures with linear plasmid DNA that was gel purified from restriction digests, and end rejoining in cultured human cells was studied by introducing enzymes into the cells by electroporation. The human cells used contain an Epstein – Barr virus (EBV)-based shuttle vector, pHAZE, that was recovered and introduced into *E.coli* for further analysis. The major products of DNA end-joining processes observed in linear plasmid-transformed *E.coli* and in the human cells exposed to restriction enzymes were identical. Furthermore, the deletions observed in both systems and in the spontaneous mutant plasmids in untreated human cells had a common underlying feature: short stretches of directly repeated DNA at the junction sites.

INTRODUCTION

DNA double-strand breaks are produced *in vivo* during recombinational processes, by X-rays or other DNA-damaging agents, and by single-strand breaks during DNA replication. That cell survival is dependent on the repair of DNA breaks is indicated by the fact that, in yeast, a single unrepaired double-strand break can be a dominant lethal event (1). In a variety of organisms, DNA double-strand breaks can be repaired via homologous recombinational pathways, as exemplified by the *RAD50* group of *Saccharomyces cerevisiae* (for review see ref. 2). DNA double-strand breaks can also be rejoined by end-joining processes that directly ligate ends or are associated with various end modifications, such as exonuclease degradation and fill-in DNA synthesis (reviewed for mammalian cells in refs. 3,4). This type of end joining does not ensure that the joined ends originally came from the same molecule and can lead to the formation of gross chromosomal rearrangements (e.g., translocations and exchange-type aberrations) (5,6). Chromosomal rearrangements can lead to cell death (7) and, in higher eukaryotes, to the formation of cancer cells (8,9).

The rejoining of complementary DNA ends in *E.coli* has been studied by transforming cultures with linearized plasmid DNA (10–13). The most common rejoin product in transformed bacteria results from the faithful ligation of the DNA ends to restore the original plasmid. End modifications, typically deletions, have been observed in several studies (10,13). Several systems have been developed to study the rejoining of complementary and noncomplementary restriction enzyme-produced DNA double-strand breaks in vertebrate cells. One such system uses CV1 monkey kidney cells transfected with linearized SV40 genomes (14,15). An analogous system uses linearized plasmid DNA and extracts from *Xenopus laevis* eggs (16). In these systems a variety of end-modification processes have been inferred on the basis of molecular analysis of the rejoined DNA.

To gain insight into the rejoining of noncomplementary ends in *E.coli*, which to our knowledge has not been examined in any detail, we used gel-purified pBR322 DNA that had been cut with *Cla*I and either *Bam*HI or *Sal*I to transform *E.coli*. We then sequenced the DNA flanking the restriction enzyme sites. To examine the rejoining of *Cla*I ends to either *Bam*HI or *Sal*I ends in human cells, we electroporated these restriction enzymes into Raji F1 cells (17). F1 cells stably maintain as an episome an Epstein–Barr virus (EBV)-based shuttle vector designated pHAZE (18,19). Plasmid DNA was later isolated from these cells and used to transform *E.coli*. The advantage of using the pHAZE system is that it uses nuclear DNA as a target rather than requiring the transfection of naked DNA into cells or cell extracts. With these experiments we extended previous studies that examined mutations in pHAZE caused by the rejoining of complementary and blunt DNA ends (18) or by exposure to radon (19) to include mutations caused by the joining of noncomplementary ends.

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MATERIALS AND METHODS

Preparation of fragment DNA

pBR322 plasmid DNA was digested with appropriate restriction enzymes, and the fragment was purified by gel electrophoresis in 1% agarose. The band containing the fragment with the ampicillin-resistant marker Ap^r and the origin of replication was cut out, and 3% NuSieve low-melting-temperature agarose (FMC BioProducts) in TAE buffer (0.04 M Tris-acetate, 0.001 M EDTA), kept at 50°C, was poured around the plug. The fragment was then electrophoresed into the low-melting-temperature agarose. The band containing the DNA was cut out, the agarose was digested with β-agarase I (New England BioLabs), and the DNA was precipitated with isopropanol.

Cell culture

Raji F1 cells, which stably maintain the shuttle vector pHAZE as an episome, were cultured in RPMI 1640 medium supplemented with 2.0 mg/ml glucose, 0.3 mg/ml L-glutamine, 2.0 mg/ml $NaHCO_3$, 10% fetal bovine serum, 50 units/ml penicillin, 50 μg/ml streptomycin, and 250 μg/ml hygromycin B. pHAZE contains the bacterial origin of replication and Ap^r markers of pBR322, the entire β-galactosidase gene, the EBV origin of replication, and the *EBNA-1* gene (see ref. 17 for a more detailed description). A gene conferring resistance to hygromycin allows for positive selection for pHAZE in cells cultured in medium containing hygromycin B.

Preparation of plasmid DNA from F1 cells

Restriction enzymes were electroporated into 10^7 F1 cells according to the method described by Rufer and Morgan (20). Five hundred units of *Cla*I and 500 units of *Bam*HI or 500 units each of *Cla*I and *Sal*I were used in each experiment. There are three *Cla*I sites in pHAZE and unique *Bam*HI and *Sal*I sites. Twenty-four hours after enzyme treatment, plasmid DNA was isolated from F1 cells for transforming *E.coli* by ion-exchange chromatography with a Qiagen plasmid minikit (Qiagen Inc.). To reduce the number of transformants from undamaged pHAZE molecules, and to make it easier subsequently to isolate mutant molecules, we digested the DNA extracted from the F1 cells with the enzymes *Sac*I and *Bcl*I, which cut between the third *Cla*I site and the *Bam*HI site of pHAZE. *Sac*I and *Bcl*I cut within the *lacZ* gene of pHAZE and will linearize pHAZE but will not linearize derivatives that have undergone deletion between the sites of the enzymes electroporated into the F1 cells. The DNA was heated to 70°C for 10 min to inactivate the enzymes *Sac*I and *Bcl*I, precipitated with ethanol and resuspended in 5 μl of water. One microliter of this DNA solution was used for each *E.coli* transformation. Bluo-gal (Gibco BRL) was used as a substrate for the β-galactosidase enzyme encoded by *lacZ*. A simple color assay permitted identification of mutant derivatives of pHAZE, which give rise to white colonies. Restriction mapping analysis was carried out on DNA purified from 1.5-ml cultures of transformed *E.coli* by using an alkaline lysis miniprep protocol. All restriction enzymes were purchased from Boehringer Mannheim Biochemicals.

E.coli transformations

All *E.coli* transformations were carried out with the Bio-Rad Gene Pulser electroporation device and MC1061, electrocompetent *E.coli* (Bio-Rad Laboratories). The genotype of MC1061 is *ara*D139, Δ*(ara, leu)*7697, Δ*lac*X74, *gal*U, *gal*K, *hsd*R2, *str*A, *mcr*A, *mcr*B1 (21,22). The only modification made to the transformation protocol provided by Bio-Rad was that the post-electroporation incubation time was reduced from 1 h to 40 min to reduce the number of daughter cells produced from a single transformant. In the experiments with the gel-purified linear plasmid DNA, the DNA must be recircularized *in vivo* for the formation of ampicillin-resistant colonies. In the experiments with DNA obtained from enzyme-treated F1 cells, plasmid DNA from individual transformants was mapped with restriction enzymes to identify plasmids that had lost DNA sequences internal to the *Cla*I and *Bam*HI or *Sal*I sites.

DNA sequencing

Sequencing of double-stranded DNA was carried out on DNA prepared from 3-ml cultures of MC1061 by means of Qiagen plasmid minikits (Qiagen Inc.). Sequencing reactions were carried out with the Sequenase version 2.0 DNA sequencing kit from United States Biochemical.

RESULTS

Transformation of *E.coli* with linear DNA

The transformation frequency obtained from 0.1 μg of uncut pBR322 DNA per transformation was 7.7×10^8 transformants per μg. Using 0.1 μg of the *Cla*I-*Bam*HI fragment, we estimated the transformation frequency to be 4.0×10^2 transformants per μg, and with the *Cla*I-*Sal*I fragment we estimated 1.6×10^3 transformants per μg. Transformation frequencies were estimated from three independent experiments. These transformation frequencies are extremely low, but electroporation is such an efficient transformation technique that large numbers of transformants were easily obtained. Ten of the *Cla*I-*Bam*HI-transforming plasmids and 10 of the *Cla*I-*Sal*I-transforming plasmids were analyzed by conventional gel electrophoresis. All 20 plasmids were smaller in size than monomeric pBR322, indicating that they were not the result of intermolecular recombination. Sequence analysis of these plasmids and 50 other transforming plasmids supported this view.

The junctions of 35 *Cla*I-*Bam*HI and 35 *Cla*I-*Sal*I *E.coli* transformants were sequenced. Fifteen of the *Cla*I-*Bam*HI transformants rejoined with the terminal G of the *Bam*HI end, aligning with the terminal C of the *Cla*I end (Figure 1). The most common *Cla*I-*Sal*I rejoin (Figure 2) aligned with the nucleotides TCG pairing to the nucleotides AGC. This pairing resulted in the loss of a T nucleotide from either the protruding single strand of the *Sal*I end or the recessed strand of the *Cla*I end. This product was observed in 18 of the *Cla*I-*Sal*I transformants sequenced.

The remaining transformants sequenced had deletions of 14 to >2,000 base pairs (bp) (approximately the maximum deletion that can occur if Ap^r and the origin of replication are retained). The sequences of the *Cla*I-*Bam*HI deletions (plasmids with nucleotides deleted from either or both of the enzyme-produced DNA ends) are shown in Figure 3. Eleven rejoined at a single complementary base, either a G or a C nucleotide; two of the 11 aligned with a terminal G, and these had not lost nucleotides from the *Cla*I end. Two of the rejoins occurred with 3 complementary bases, one with 8, and one with none; the remaining seven rejoins had 2 bp of complementarity.

The *Cla*I-*Sal*I deletions are shown in Figure 4. Eight of these transformants had a single complementary base, six had 2, and the remaining three had 3 bp of directly repeated DNA sequence. Of the six *Cla*I-*Sal*I rejoins that had lost DNA from both ends

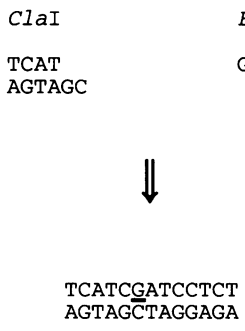

Figure 1. Most common product resulting from the joining of a *Cla*I to a *Bam*HI DNA end. The C nucleotide of the 5′ protruding single strand of the *Cla*I-produced end joins with the G nucleotide of the 5′ protruding single strand of the *Bam*HI-produced end. The product is shown below with the complementary base underlined.

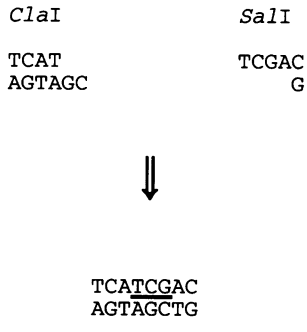

Figure 2. Most common product resulting from the joining of a *Cla*I to a *Sal*I DNA end. The *Cla*I-produced end rejoins with the *Sal*I-produced end with 3 complementary bases. The product is shown below with the complementary bases underlined.

plasmid	sequence of junction	bases deleted from: *Cla*I end	*Bam*HI end
CB1	GGGGTTCTGCCTC	133	1,691
CB2	ATCATCGCATGCA	0	186
CB3	GTTTGACTACGCC	12	6
CB4	ATCATCGCAGCCC	0	1,267
CB5	TATCATCAGCTCC	1	308
CB6	GGGGTTCTGCCTC	133	1,691
CB8	TGACAGCGGCTGC	9	2,018
CB11	ATCATCGGTCCTG	0	1,063
CB12	TATCATCGGGCAG	0	1,050
CB14	GGGGTTCTGCCTC	133	1,691
CB16	CATGAGCCGGCGC	179	35
CB17	GACAGCTACGCCG	8	6
CB18	TATCATCAGCTCC	1	308
CB20	TATCATCGCTTGT	0	119
CB21	ATAGGCGGTTATC	54	2,060
CB23	TATCATCGCTTTC	0	427
CB24	GGGGTTCTGCCTC	133	1,691
CB25	TATCATCGTGGCC	0	22
CB30	GGGGTTCTGCCTC	133	1,691
CB32	TTTGACAGCTTCA	7	710
CB33	TCATGACTGCGCT	75	2,003
CB35	AAAACCATGAGCG	89	114

Figure 3. DNA sequences of junctions formed by deletions in *Cla*I-*Bam*HI fragments used to transform *E.coli*. The number of bases deleted from each end is shown at the right. Counting starts at the most terminal 5′ residue of each end. Complementary bases are underlined.

and had a single complementary base at the junction site, two had a complementary C and two had a complementary G. There may be some preference for aligning at C or G residues. Additional homology was not seen to any significant extent in the DNA sequences adjacent to the junctions.

All the deletions were unique except for those in transformants CB5 and CB18, which were identical, in CS18 and CS24, which were identical, and in CS12, CS20, CB1, CB6, CB14, CB24, and CB30, which were also identical (Figures 3 and 4). The identity of CB5 and CB18, and of CS18 and CS24, may be the result of single transformants' giving rise to pairs of daughter cells. The *Cla*I-*Bam*HI transformants came from a single experiment in which approximately one third of the total number of transformants were sequenced. Approximately one fifth of the *Cla*I-*Sal*I transformants were sequenced. In the case of the six plasmids with identical deletions, represented by CB1, it is unlikely that they arose by duplication because they were observed in two different experiments. These deletions may represent the product of a site-specific recombination process or a favored rejoin product, or the plasmid may be present in very low quantities in the pBR322 DNA used in this experiment.

The rejoining of pHAZE ends in human cells

Twenty-four hours after restriction enzyme treatment, pHAZE was recovered from the human cells and digested with enzymes that cut between the *Bam*HI site and the nearest *Cla*I site. This DNA was then used to transform *E.coli*, and plasmid DNA was later isolated from white colonies and analyzed by restriction mapping. Plasmids that were missing enzyme sites between the *Cla*I sites and either the *Bam*HI or the *Sal*I site, yet had not undergone a large deletion (>200 bp), were sequenced. Eighteen plasmids were isolated from two experiments in which a *Cla*I end had rejoined to a *Bam*HI end. Sixteen of these plasmids had rejoin junctions that were identical to the most common rejoin junctions from the *E.coli* transformants produced by linearized pBR322 DNA (Figure 1); all but one rejoined at the most proximal *Cla*I site, and the other rejoined at the most distal *Cla*I site. The two plasmids that were different from the major product of the *E.coli*-catalyzed rejoins had identical 50-bp deletions that had a junction with a CG direct repeat. These two plasmids were isolated from the same experiment and could have arisen by duplication either in the F1 cells or in *E.coli*.

Twelve plasmids that had undergone rejoining between *Cla*I- and *Sal*I-produced DNA ends were recovered and sequenced. All junctions were identical to the most common rejoin junction observed in the *E.coli*-catalyzed reactions (Figure 2). These plasmids originated from five separate experiments. Seven of the plasmids rejoined at the most proximal *Cla*I site, and five rejoined at the most distal *Cla*I site. No rejoins involving the middle *Cla*I site were isolated. Similarly, no mutations were observed at this site by Winegar *et al.* (18). We do not know if the lack of rejoining at this site is due to methylation or to modification of

plasmid	sequence of junction	bases deleted from: *ClaI* end	*SalI* end
CS2	ATCATCGGTGGGC	0	42
CS3	ATCATCGCTTCTG	0	1,324
CS7	TATTTAGGAAACG	155	977
CS12	GGGTTCTGCCTCG	133	1,415
CS13	CCGCGCAGTCACG	127	1,569
CS17	TTTCCCGACCGAT	118	1
CS18	ATCATCGTTTCGG	0	250
CS20	GGGGTTCTGCCTC	133	1,415
CS21	ATCATCGCAGCGC	0	121
CS22	ATCATCGTGATTT	0	1,095
CS23	CCCTTTCGACCGA	33	0
CS24	ATCATCGTTTCGG	0	250
CS26	ATATTTGCGCTGG	166	1,076
CS28	TATCATCGCTCTG	0	125
CS29	TATCATCGCTCAA	0	219
CS30	TATCATCGCGACG	0	320
CS34	TCATGACGCAGGA	75	1,025

Figure 4. DNA sequences of junctions formed by deletions in *ClaI-SalI* fragments used to transform *E.coli*. The number of bases deleted from each end is shown at the right. Counting starts at the most terminal 5′ residue of each end. Complementary bases are underlined.

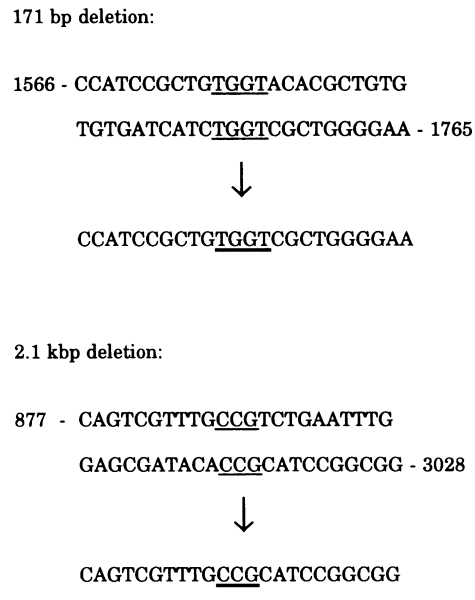

Figure 5. pHAZE spontaneous mutants. The DNA sequences on both sides of the joining sites are shown above the deletion products. Numbers adjacent to sequences correspond to positions in pHAZE. Complementary bases are underlined.

the site, but we have verified the presence of the site by sequence analysis (data not shown).

Spontaneous mutations in F1 cells

To gain further insight into the products of a breakage-and-rejoin reaction in the human cells, we sequenced the junctions of two plasmids that had deletions in the *lacZ* gene and that appeared several times in different experiments with untreated F1 cells (Figure 5). The deletions, one of 171 bp and the other of 2.1 kbp, formed between direct repeats of 3 and 4 bp. White (mutant) colonies were observed at a rate of approximately 5 per 10,000 blue colonies; light blue colonies were not scored as mutant. In contrast, the enzyme-treated DNA gave rise to about one white colony for every 10 blue colonies, and about 10% of the white colonies appeared to have undergone deletion between the sites of the enzymes electroporated into the F1 cells. Because the two spontaneous mutant plasmids appeared in separate experiments of untreated cells, it is unlikely that they were formed in *E.coli*.

DISCUSSION

A question of vital interest to us was whether the rejoined plasmids isolated from the F1 cells were catalyzed in *E.coli*. There are three reasons why this is unlikely. The first two reasons are based on cytological studies of restriction enzyme-induced damage. Restriction enzymes have a finite lifetime in a cell (1 to 3 h), and the DNA breaks they produce are rejoined after a few hours (23,24). It is also unlikely that a significant number of linear fragments would survive for over 20 h in the cells because they would be exposed to degradation by endogenous cellular processes. The third, and most compelling, reason is the extremely low transformation frequency of linear DNA with noncomplementary ends. Linear molecules would need to outnumber recircularized molecules by approximately one million to one to give rise to a significant fraction of transformants. Therefore, based on approximately 80 copies of pHAZE per cell (17), to produce the number of transformants that rejoined the noncomplementary ends in pHAZE, nearly every copy of pHAZE present in the F1 cells would have to be cut by both enzymes and remain intact during the subsequent incubation period. We know this is not the case because, even after incubation of plasmid DNA from F1 cells with additional restriction enzymes, 9 of 10 transformants had a functioning *lacZ* gene. However, for a single unique mutant plasmid it is impossible to discern if the rejoining occurred in *E.coli* or in the F1 cells.

pBR322 DNA, linearized with a single enzyme, has been used successfully to transform *E.coli* in several investigations (10,12,13,25). These studies predominantly revealed faithful ligations that restored the enzyme sites, but they also showed deletions that typically had 4 to 10 bp of directly repeated DNA at the junction site. The recircularization process was found to be independent of *recA*, which is required for homologous recombination in *E.coli*. Consequently, we did not carry out our experiments in a *recA*-defective host, and in light of the results obtained, we feel that having done so would not have made a difference; clearly, homologous recombination is not necessary for end rejoining.

Bergsma *et al.* (11) treated a *Bam*HI-linearized, pBR322-based plasmid (pMK2004) and a *Pvu*II-*Bgl*II DNA fragment containing the SV40 origin of replication with T4 DNA ligase. They isolated 12 plasmids that had undergone ligation between the complementary *Bam*HI and *Bgl*II sites and rejoined the *Pvu*II-produced end to the remaining *Bam*HI end. Among these clones were three that had undergone rejoining between a blunt (*Pvu*II) end and a 5′ (*Bam*HI) end. The remaining clones had deletions in which 7, 9 or 13 bp of homology were present at the junction site. In the plasmids that we sequenced that had undergone

deletion, there were typically only 1 to 3 bp of complementary DNA at the junction sites.

Transformation with linear plasmid DNA with blunt or complementary ends is typically two to three orders of magnitude less efficient than transformation with circular DNA (10,12,13). In our experiments, transformation with linear plasmid DNA with noncomplementary ends was approximately six orders of magnitude less efficient than transformation with circular DNA. Conley *et al.* (13) found that the efficiency of transformation depended on the type of ends produced by the enzyme used for linearization. A 4-base 5′ protruding single strand produced by *Sal*I was circularized more efficiently than a 2-base 3′ protruding single strand produced by *Pvu*I, which in turn was circularized more efficiently than blunt-end DNA produced by *Nru*I or *Pvu*II. The inefficiency of transformation with linear DNA may be largely due to its degradation by endogenous exonucleases. *recB*$^-$ *recC*$^-$ strains that are proficient in recombination because of a second mutation, e.g., *sbcB*, transform at a 10-fold higher rate than strains that have a functioning *recBC* nuclease (26).

Vertebrate cells have been shown to rejoin a variety of restriction enzyme-produced DNA double-strand breaks with blunt, complementary, or noncomplementary ends (reviewed in refs. 3,27). The joining of complementary and blunt-end DNA double-strand breaks has been studied with SV40 in cultured monkey cells (14) and with pHAZE in cultured human cells (18). In the SV40 system, most of the transfecting molecules were recircularized without end modification, as determined by restoration of the restriction enzyme site. Approximately one sixth of the genomes had undergone small deletions, and a few had undergone larger deletions (>25 bp) or had an insertion. Winegar *et al.* (18) used the pHAZE system to examine rejoining of restriction enzyme-induced complementary and blunt-end DNA breaks. They electroporated *Pvu*II, *Cla*I or *Pvu*I into F1 cells and later recovered plasmid DNA from the cultured cells and screened for mutations in *E.coli*. They observed end modifications indicative of terminal addition of nucleotides, exonucleolytic degradation and fill-in DNA synthesis of protruding single strands. Our study extended this work to include the examination of noncomplementary end rejoining.

Noncomplementary ends cannot be joined to form an intact double-stranded molecule with Watson–Crick base pairing without some processing. Roth *et al.* (15) used the SV40 system to examine the rejoining of a *Fnu*DII-produced blunt end to a *Taq*I-produced 5′ protruding single strand. This system was later modified to permit the use of several combinations of enzymes (28). An analogous system uses *Xenopus* egg extracts (16,29); linearized plasmid DNA is exposed to the extracts, and the reaction products are analyzed by gel electrophoresis and cloning in *E.coli*. DNA sequence analysis of circular molecules produced in these systems revealed several interesting features (28,29). In both systems 3′ and 5′ protruding single strands could be joined to blunt-end DNA. Furthermore, 5′ protruding single strands could be joined to 3′ protruding single strands. In the joining of 5′ to 5′ or 3′ to 3′ protruding single stands, or if there was exonucleolytic degradation, only 1 or 2 bp of terminal homology was necessary for end joining to occur. If nucleotides were deleted from one or both ends, 1 to 4 complementary bases were typically present at the junction site. In our experiments *Cla*I ends were joined to *Bam*HI ends with a single terminal complementary base, and *Cla*I ends were joined to *Sal*I ends with 3′ complementary bases, deleting a T nucleotide in the process. The mutant plasmids from untreated F1 cells had 3 or 4 complementary bases at the junction sites.

In all these systems—SV40, *Xenopus*, pHAZE and *E.coli*—noncomplementary ends typically join with short stretches of directly repeated DNA at the junction sites. These results illustrate that the products of rejoining broken DNA in bacterial and vertebrate systems are very similar. These similarities, coupled with the extensive genetic and enzymatic knowledge of *E.coli*, make *E.coli* an ideal model system for studying DNA break rejoining in higher eukaryotes.

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