A model for repair of radiation-induced DNA double-strand breaks in the extreme radiophile *Deinococcus radiodurans*

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Summary

The bacterium *Deinococcus* (formerly *Micrococcus*) *radiodurans* and other members of the eubacterial family Deinococcaceae are extremely resistant to ionizing radiation and many other agents that damage DNA. Stationary phase *D. radiodurans* exposed to 1.0-1.5 Mrad γ-irradiation sustains >120 DNA double-strand breaks (dsbs) per chromosome; these dsbs are mended over a period of hours with 100% survival and virtually no mutagenesis. This contrasts with nearly all other organisms in which just a few ionizing radiation induced-dsbs per chromosome are lethal. In this article we present an hypothesis that resistance of *D. radiodurans* to ionizing radiation and its ability to mend radiation-induced dsbs are due to a special form of redundancy wherein chromosomes exist in pairs, linked to each other by thousands of four-stranded (Holliday) junctions. Thus, a dsb is not a lethal event because the identical undamaged duplex is nearby, providing an accurate repair template. As addressed in this article, much of what is known about *D. radiodurans* suggests that it is particularly suited for this proposed novel form of DNA repair.

Accepted
13 January 1995

What is *Deinococcus radiodurans?*

This remarkable bacterium was originally isolated in Oregon by Anderson and coworkers in 1956 from radiation-sterilized canned meat that had undergone spoilage[1]. Culture yielded a red-pigmented, nonsporulating, non-pathogenic, Gram$^+$ bacterium that was extremely resistant to the lethal and mutagenic effects of ionizing radiation and to many other agents that damage DNA[2-4]. Subsequently, four additional deinobacterial species were isolated from diverse sources ranging from irradiated Bombay duck[5] to weathered granite in Antarctica[6]. These closely related species, all of which are extremely DNA-damage-resistant, have been grouped with *D. radiodurans* to form one of the ten known eubacterial families, Deinococcaceae[4]. Because there has been no systematic search for the deinococci, their natural habitat, or niche, has not been identified. With respect to evolution, as determined by 16S rRNA sequences, the deinobacteria are extremely distant from any well-characterized species, being just as far from *Escherichia coli* as from *Bacillus subtilis*[7]. To date, the deinobacterial species are the most ionizing- and UV-radiation resistant organisms known[3] (Fig. 1).

Radiation-induced dsbs are hazardous to your health, unless you are *D. radiodurans*

Ionizing radiation-induced dsbs pose a formidable challenge for cellular DNA repair processes because both strands of the double helix are broken[8]. Repair of these lesions is more difficult than damage that affects only one strand [e.g. ionizing radiation-induced DNA single-strand breaks (ssbs) or damage to DNA bases][8], which can be repaired by local excision of the damaged single strand while the complementary undamaged strand provides a template to guide accurate resynthesis at the repair site[9]. In contrast, DNA damage-induced dsbs provide little in the

way of guidance for non-mutagenic mending because neither of the two strands are fully informative. In organisms that contain two or more homologous or identical chromosomes (as in all eukaryotes and many prokaryotes), a DNA fragment liberated by damage of one chromosome might provide the necessary information at the site of a given dsb on another, to allow repair by way of recombination. Most organisms can repair and survive no more than two or three ionizing radiation-induced dsbs per chromosome[10]. *E. coli* has been shown to repair a few dsbs per chromosome without lethality only if multiple chromosome copies are present (4 to 5 chromosomes per cell during exponential growth) and there is a functional *recA*$^+$ gene; no dsb repair was detected in *E. coli* during stationary phase, when there is an average of 1.3 chromosomes per cell[11].

These observations suggest that homologous interchromosomal recombination may play an important role in dsb repair. *D. radiodurans* is eligible for this form of repair since it contains multiple identical chromosomes: about 4 in stationary phase and 8-10 during exponential growth[12]. This mode of repair is distinct from the better-studied mechanism of 'post-replication repair', which uses recombinational processes between the two new daughter duplexes immediately behind the semiconservative replication fork, for the purpose of filling any single-stranded gaps left behind by the replication fork[13,14]. The mechanistic difference between 'interchromosomal recombination' and 'post-replication repair' is profound. The former repairs dsbs using two separate homologous chromosomes, presumably at any site and without a requirement for semiconservative chromosomal DNA replication, while the latter is limited to repair of single-stranded gaps in daughter duplexes just behind the replication fork of a single replicating chromosome[14].

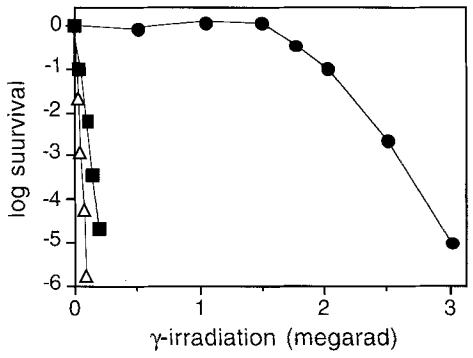

Fig. 1. Survival curves of various strains. Stationary phase *D. radiodurans* and *E. coli* (both *recA*$^+$ and *recA*) were exposed to ^{60}Co-irradiation at 1.33 Megarad per hour at 0°C. Appropriate dilutions were then spread on nutrient agar and grown for 3 days at 32°C (*D. radiodurans*) or for 1 day 37°C (*E. coli*), prior to counting of colonies. Filled circles, stationary phase *D. radiodurans*; filled squares, *recA D. radiodurans*; open triangles, *E. coli*. The survival of *recA E. coli* is not shown, since the very large ordinate scale of the figure renders the graphic illustration of the survival of the *recA E. coli* indistinguishable from the *y* axis.

Of the variety of lesions in cellular DNA induced by ionizing radiation, dsbs are repaired the least, and their frequency is related directly to cell death[10,11,13,15]. An exception to this generality is *D. radiodurans*: although DNA damage can lead to death of this organism, its DNA repair capabilities are so extremely efficient that at high fluences direct protein damage is also a significant contributor to cell death[16].

Extent of DNA damage and repair following irradiation

We routinely find that in stationary phase cultures exposed to 1.0-1.5 Mrad γ-irradiation (1 Mrad is equal to 10 kGray in SI units) *D. radiodurans* sustains >120 dsbs per chromosome, which it repairs over a period of hours with 100% survival and virtually no mutagenesis[2,17,18]. It is well established that the extraordinary resistance of *D. radiodurans* to DNA damaging agents is due to extremely efficient DNA repair[2,3] and not to some intrinsic physical property of the DNA itself or to radioprotectant compounds. Conceptionally, it would appear to be an insurmountable task to reconstruct the 3 Mbp deinococcal chromosome[12] from many hundreds of small (approx. 25 kbp) overlapping DNA fragments. Not only are the number of fragments generated per chromosome enormous, but from the logistical point of view of a particular searching fragment, there is no obvious way of eliminating from the search other fragments that have already been scrutinized, resulting in repetitive and futile reinspection. This is in stark contrast to the classical 'search for homology' involving unfragmented DNA that proceeds rapidly and processively along an uninterrupted duplex DNA substrate[13,19,20]. Furthermore, in *D. radiodurans* it appears that dsbs are not subject to blunting of the broken ends followed by blunt-end ligation, a process that would produce mutations and DNA rearrangements[21]; these events are extraordinarily rare following ionizing radiation in *D. radiodurans*, even among survivors of lethal exposures[3,22]. Following the extreme radiation-induced fragmentation of deinococcal DNA within surviving cells it is likely that many hundreds of recombinational events occur before regenerating a single intact chromosome.

Two necessary proteins: are they sufficient?

Since *D. radiodurans* is resistant, generally, to mutagenesis, we are fortunate that Moseley identified a mutagen to which this organism is sensitive, namely, N-methyl-N′ nitro-N-nitrosoguanidine (MNNG)[22]. Consequently, there

is a variety of MNNG-mutagenized strains that are ionizing radiation-sensitive[3,23]. These strains are proving useful in isolating genes by complementation assays that restore wild-type ionizing radiation-resistance[23-25]. To date, two genes have been identified and characterized in terms of both their DNA sequence and corresponding proteins; the remaining mutant strains are subject to ongoing research. These two genes are the deinococcal *polA*$^+$ (DNA Pol I) and *recA*$^+$ (RecA protein) genes[24,25]. Deinococcal strains that are *polA*$^-$ are very sensitive to a variety of DNA damaging agents including ionizing radiation[24]. However, when the *E. coli polA*$^+$ gene was expressed in *polA*$^-$ *D. radiodurans* there was complete restoration of wild-type deinococcal resistance to all tested forms of damage, including ionizing radiation[26]. This observation indicates that the deinococcal DNA Pol I is not uniquely qualified, since *E. coli* DNA Pol I can do the same job, i.e. DNA Pol I is necessary, but not sufficient[26].

The evidence with respect to the deinococcal RecA homologue is quite different from the DNA Pol I homologue. Strains defective in the *recA* gene are the most ionizing radiation-sensitive deinococcal strains discovered to date, approaching the radiation sensitivity of *E. coli recA*$^+$ cells (Fig. 1). Expression of the *Shigella flexneri recA* protein in *D. radiodurans recA*$^-$ cells results in no increase of DNA damage resistence*, even when expressed at high levels as determined by western blotting (K. W. Minton and M. J. Daly, unpublished results). The reciprocal experiment, i.e. expression of the *D. radiodurans recA* gene in *recA*$^-$ *E. coli*, results in severe toxicity or death of the *E. coli* recipient, even at low levels of expression[25]. This is unusual, since the majority of *recA* genes of various bacterial species typically complement *E. coli recA* strains, and *vice versa*[13].

The central hypothesis

It is proposed here that in *D. radiodurans* pairs of double-helical chromosomes are closely associated with each other. The presence of 4-10 chromosomal copies is not in itself nearly sufficient to impart to *D. radiodurans* its DNA damage-resistance[28]. For example, all eukaryotic cells in G_2 are tetraploid, but very damage-sensitive. Bacteria with many chromosomes, such as *M. luteus* and *M. sodonensis*, are also very sensitive[3]. *Azotobacter vinelandii*, that contains 40 to 80 chromosomes per cell[29,30], is UV-sensitive[31]. These observations address the question of why diploid or polyploid organisms other than the deinobacteria are *not* resistant to radiation. We suggest that *D. radiodurans* makes use of redundant information in a manner that these other organisms do not.

While investigating recombinational repair in *D. radiodurans-E. coli* shuttle plasmids damaged *in vivo* by ionizing radiation[17,18], observations were made in both wild-type and *recA D. radiodurans*, compatible with the presence of four-stranded junctions (Holliday junctions) between plasmids (K. W. Minton and M. J. Daly, unpublished results). We have so far no evidence for such junctions when these same plasmids were purified from *E. coli* of either genotype. This possibility has led us to speculate that, like plasmids, the chromosomes of *D. radiodurans* might also be linked by Holliday junctions, giving rise to the following hypothesis:

Specifically, that pairs of the deinococcal chromosomes are joined to each other at thousands of sites by four-stranded junctions (Holliday junctions).

A refresher course in four-stranded junctions

Four-stranded junctions, also known as Holliday junctions[32,33], are generally thought of as intermediate structures in genetic recombination occurring between homologous or identical regions of two DNA duplexes (Fig. 2). Their presence in both prokaryotes and eukaryotes[34,35] and their properties *in vitro*[36] have been extensively studied, and they are an integral part of virtually all models of genetic recombination. *In vitro* studies on Holliday junctions indicate that they possess no single-stranded character and all bases are paired[36] (as in Fig. 5, top). They are able to move freely in regions of identity since there is no net gain or loss of base pairs, and their movement is typical of a 'random walk'[34,37,38]. Migration of Holliday junctions, either spontaneously or accelerated enzymatically, is referred to as 'branch migration' (Fig. 2). Movement in a given direction is greatly impeded if branch migration encounters heterologies including base mismatches, unless the junction is enzymatically driven across such heterologous regions at the expense of either ATP or dATP[37,38].

Why are pre-existing Holliday junctions useful in repair of dsbs?

With respect to the ability of *D. radiodurans* to repair myriad dsbs, the essential property endowed by the presence of numerous persistent Holliday junctions is that their occurrence between homologous regions serves to organize the genetic material in space such that pairs of chromosomes of *D. radiodurans* are aligned. If so, then the 'search for homology'[13] for repair of dsbs becomes simpler due to a pre-existing alignment. The alignment of homologous chromosomal regions is often tacitly

*The *S. flexneri* RecA protein is identical to the *E. coli* RecA protein[13,27]. The *S. flexneri recA* gene was used for gene expression in *D. radiodurans* because of convenient restriction sites (and was a gift of K. McEntee).

assumed in models of recombination (both single- and double-strand break models) as the starting point for a given model, yet little is known about how this fortuitous alignment occurs in most forms of recombination.

If the central hypothesis stated above is correct, then many mechanisms of repairing large numbers of dsbs can be postulated, given the alignment of chromosomes and RecA protein-mediated strand invasion. Of the numerous mechanisms that can be advanced, two examples are shown (Fig. 3). The first example (Fig. 3A) is an adaptation of a model of dsb-initiated generalized recombination in meiotic cells[39]. The other example (Fig. 3B), is an adaptation of the Meselson-Radding single strand invasion model of recombination[40]. This latter model achieves repair of dsbs by a mechanism that not only exploits the pre-existing alignment of the chromosomes, but also exploits the flanking Holliday junctions themselves in the repair process by way of branch migration. Indeed, the advantage of alignment is evident in the fact that post-replication repair is only possible because the two daughter duplexes are aligned as a natural consequence of semiconservative replication.

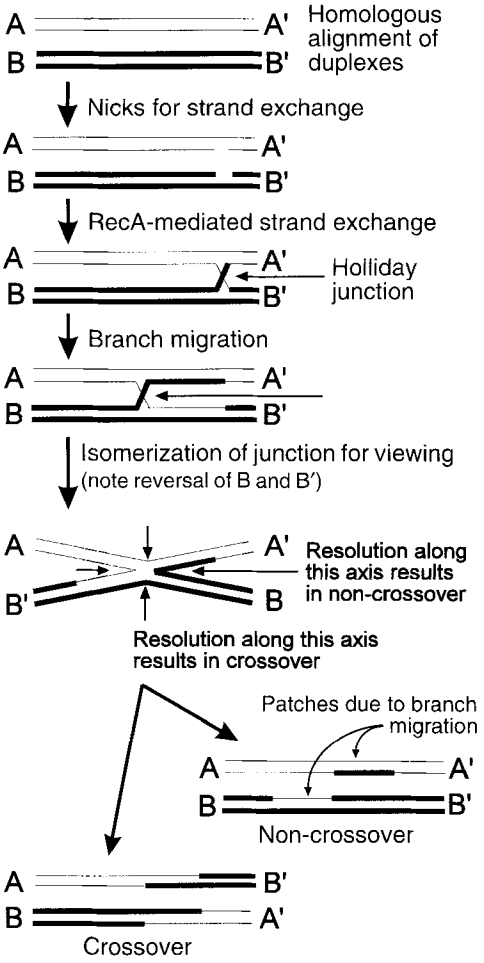

Fig. 2. A simple model for genetic recombination, based on the original proposal of J. Holliday[32,33]. The first step is single-stranded nicking of both chromosomes, respectively, at the same site, followed by RecA-mediated strand exchange, thereby forming the four-stranded Holliday junction. The Holliday junction is able to migrate spontaneously (branch migration) because the identity of the two strands results in no net loss in base pairing. If migration is impeded by heterologies, this can be overcome if migration is driven enzymatically. Resolution of the Holliday junction is by specific nucleases, and can be categorized as productive (crossover) or non-productive (non-crossover). In the case of non-crossover events, it is still possible to transmit information from one duplex to the other by means of branch migration (gene conversion).

As discussed below, it is mechanistically unnecessary to invoke a role for RecA protein in maintaining the Holliday junctions during semiconservative replication. However, given the extreme radiosensitiviy of *D. radiodurans recA* strains (Fig. 1), it is likely that RecA plays a crucial role in pre-aligned repair reactions by mediating strand invasion. This is consistant with the observation that RecA is not detectable in *D. radiodurans* during normal growth, and can only be observed (by western and northern blots) after a substantial DNA damaging exposure (J. D. Carroll, M. J. Daly and K. W. Minton, unpublished results). Perhaps deinococcal RecA is as toxic to *D. radiodurans* as it is to *E. coli*, and is only tolerated by the host during DNA repair itself.

Is the model compatible with the conformation of Holliday junctions?

How can two double helices with negatively charged backbone phosphates be aligned? The shape of the Holliday junction is strongly influenced its ionic environment. In *in vitro* studies in the absence of salts, junctions form a planar cross in which the arms are fully extended and unstacked[36], rendering any alignment between double helices difficult to imagine structurally. However, with the addition of metallic salts (Mg^{2+} being the most studied) the junction assumes the more compact 'stacked X' conformation (Fig. 5, top) due in part to shielding of backbone phosphates and stabilization of base-stacking interactions[36]. The cross of the stacked X can only be appreciated from a frontal or rear view (i.e., just as we see the frontal view of 'X' on this page). From a side view, the junction appears flat, composed of two double-helices that are side-by-side without visible divergence. The angles, apparent only when the stacked X is viewed from the front or back, are 120° (top and bottom obtuse angles) and 60° (the lateral acute angles)[36] (Fig. 5, top).

This arrangement, in which the two helices are side by side, is favored thermodynamically in the antiparallel configuration *versus* the parallel configuration by about –1.5 kcal/mol per junction; this means that in an unrestrained

configuration, virtually all Holliday junctions would be antiparallel[36]. However, the relatively small difference in free energy could be overcome readily by a variety of factors, including restraining DNA binding proteins or prohibitive transition energies. In the current context, 'antiparallel' means that the two *duplexes* are oriented in opposite directions with respect to each other, and 'parallel' indicates alignment of two duplexes in the same direction[36,38,41]. Both branch migration and the proposed model of alignment of chromosomes by Holliday junctions are much easier to reconcile as occurring between helices that are both parallel. However, the antiparallel configuration might also be acceptable, since this would call only for small reversals localized to the sites of the Holliday junctions themselves.

While the effect of Mg^{2+} on four-stranded junctions has been studied extensively, the consequence of Mn^{2+} has not. This overlooked cation is of special significance to *D. radiodurans* because this organism appears to be unique in that it has an exceptionally high intracellular Mn^{2+} content, 100-fold greater than in *E. coli*[42]. Furthermore, Mn^{2+} binds DNA with an affinity about 5-fold greater than Mg^{2+}[43]. Localization of this Mn^{2+} has determined that DNA is the prime repository, and it is possible to estimate that there is approximately one Mn^{2+} ion bound by DNA per every 7 base pairs. The effect of this large amount of associated Mn^{2+} on the structure of chromosomes and Holliday junctions, while unknown, may be important in several ways, including the exact configuration of the stacked X, the free energy and transition energy of parallel and antiparallel configuration, and the distance of approach of the paired double helices, due to the enhanced backbone shielding.

Semiconservative DNA replication and preservation of Holliday junctions

How are thousands of Holliday junctions preserved during semiconservative DNA replication? Are they first resolved, followed by chromosomal replication, and then finally reintroduced by RecA and associated proteins? This is an issue to our knowledge that has never been addressed[44], and the mechanism suggested above seems ponderous indeed. Instead, we suggest that *D. radiodurans* is capable of copying Holliday junctions along with the rest of the chromosome during semiconservative DNA synthesis. The ability to do so is not inherent in what is already known regarding replicative DNA synthesis pathways[44], but can be deduced, as follows.

With simultaneous initiation of semiconservative DNA synthesis at each of the chromosomal origins of replication in a given linked pair of chromosomes (Fig. 4), the replication forks will encounter a given Holliday junction simultaneously (assuming the junction is not resolved or pushed

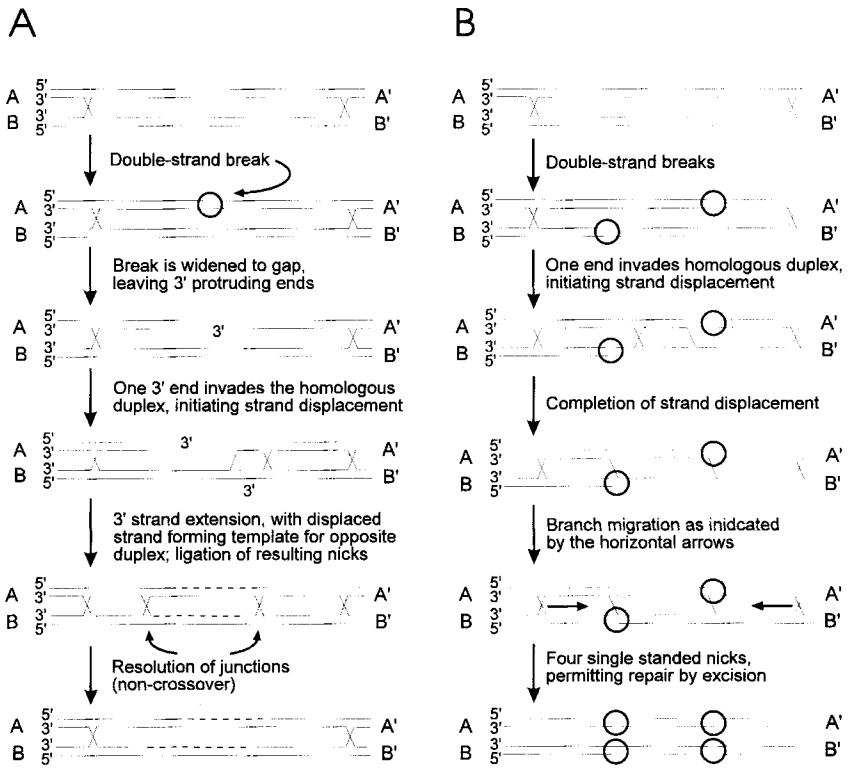

Fig. 3. (A) A model for dsb repair adapted from proposed models of dsb-induced generalized homologous recombination. (B) A model for dsb repair adapted from models of ssb-initiated homologous recombination.

ahead) (Fig. 4). When the replication forks encounter each other at the junction there is a limited set of four routes (referred to as patterns or rules) for negotiating the Holliday junction in such a way that continuation of semiconservative DNA synthesis is uninterrupted (Fig. 4, rules 1–4). As it turns out, all four patterns yield four daughter duplexes originating from the two parental duplexes. However, only one of the options (rule #4) will preserve the Holliday junctions in the newly created daughter duplexes, retaining the four-stranded DNA structures during semiconservative replication without the aid of RecA protein.

Semiconservative replication at the Holliday junction according to rule #4 is illustrated in greater detail in Fig. 5. Surprisingly, rule #4 works out very well, yielding a replicated structure of simple conformation. There are no topological obstructions, and following the synthesis of the two new daughter duplex junctions, they can be simply separated, producing two duplexes identical to the starting material (Fig. 5). Furthermore, the parallel (or antiparallel) configuration of the Holliday junction is preserved. Hence, if the stacked X structure has a significant transition energy *in vivo*, conversion from the parallel to antiparallel stacked X orientation after DNA replication will be very unlikely.

Fig. 6 shows a simplified illustration of two separate chromosomes connected by three Holliday junctions, two between 'Watson' strands and one between the 'Crick' strands of the two double-helices. Replication from the origins is simultaneous and shown as unidirectional, but bidirectional replication yields an identical outcome. If Holliday junction replication pattern #4 is adhered to at all junctions, then two daughter duplexes are produced (Fig. 6; note that the third structure from the bottom is composed of the two structures below it), each of which is an exact replica of the starting material, including the Holliday junctions. Once again, other than the usual topological manipulations carried out during replication of conventional circular DNAs (elongation, termination and disengagement), there are no special enzymatic activities required. Thus, the problem of retaining Holliday junctions during semiconservative repli-

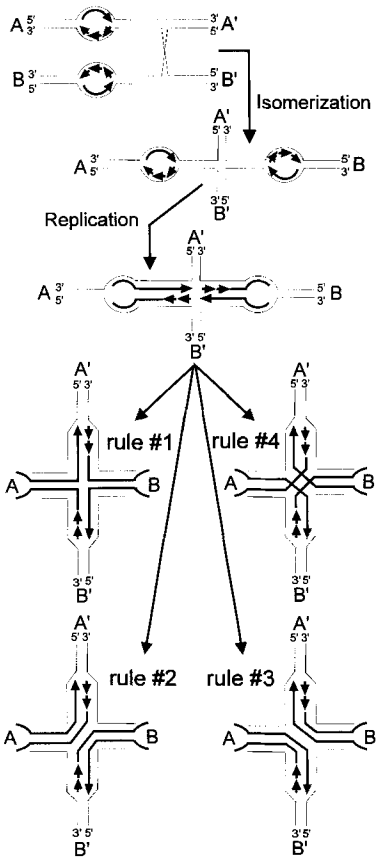

Fig. 4. The consequences of semiconservative replication forks simultaneously meeting at a Holliday junction. The thin lines represent the pre-existing DNA, while the thick lines indicate newly replicated material. Four patterns (or 'rules') will permit uninterrupted semiconservative replication to continue beyond the encounter, as shown. An arrowhead indicates a 3′OH end, while two or more contiguous reversed arrowheads indicate a 5′-P end.

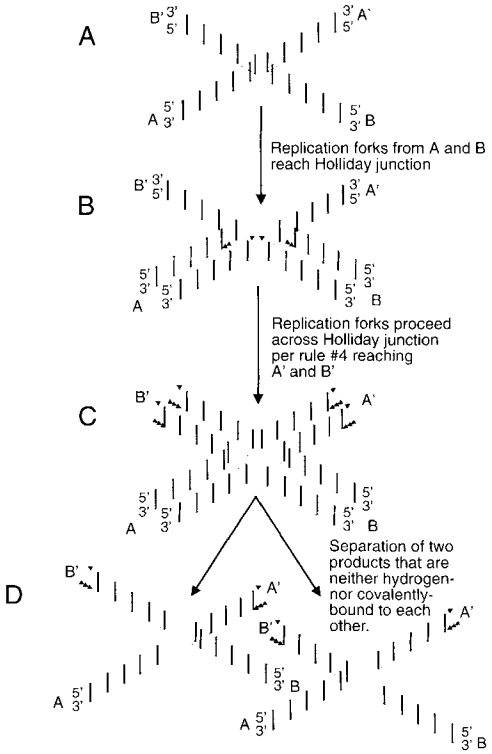

Fig. 5. 'Stacked X' model of a Holliday junction (A). (B) shows semiconservative synthesis up to the junction, while (C) shows the results of completed replication through the junction according to rule #4 of Fig. 4. The separated products are shown in (D). Arrowheads indicating 3′ and 5′ ends are as in Fig. 4.

cation in the absence of RecA does not appear to require a complicated solution.

Nevertheless, Holliday junctions will be lost over time due to resolvase(s), DNA repair or annihilation when two like Holliday junctions meet. It may be the case that RecA amounts that are less than detectable, are sufficient to mediate initiation of new Holliday junctions. A novel possibility is that *D. radiodurans* harbors an enzyme of the λ integrase family, which produces Holliday junctions at specific sites as its primary activity[45]. *D. radiodurans* contains numerous repeated sequences of 100-200 bp of unknown function[46], and these might serve as integrase-specific sites.

Predictions and experimental approach

This model makes two straightforward predictions: First, electron microscopic examination of adequately spread chromosomal DNA from *D. radiodurans* should reveal evidence of Holliday junctions or possibly other structures linking chromosomes or chromosomal fragments; and second, that chromosomal restriction fragments linked by Holliday junctions should migrate aberrantly and, therefore, be detectable by neutral agarose gel electrophoresis and Southern blotting.

Holliday junctions could be lost during purification of chromosomal DNA processes related to branch migration, e.g. by migration off the ends of the two linked double-helices. One remedy for this potential complication could be to treat the cells *in vivo* with the DNA crosslinking agent trimethylpsoralen plus UV_{360}, immediately before isolation of the DNA. This technique is commonly used to generate interstrand crosslinking *in vivo*[47]. Crosslinking should inhibit branch migration of Holliday structures because a junction cannot proceed in a given direction if one of the two double-helices contains an interstrand crosslink at that site.

Results of studies such as those described above will be presented in due course.

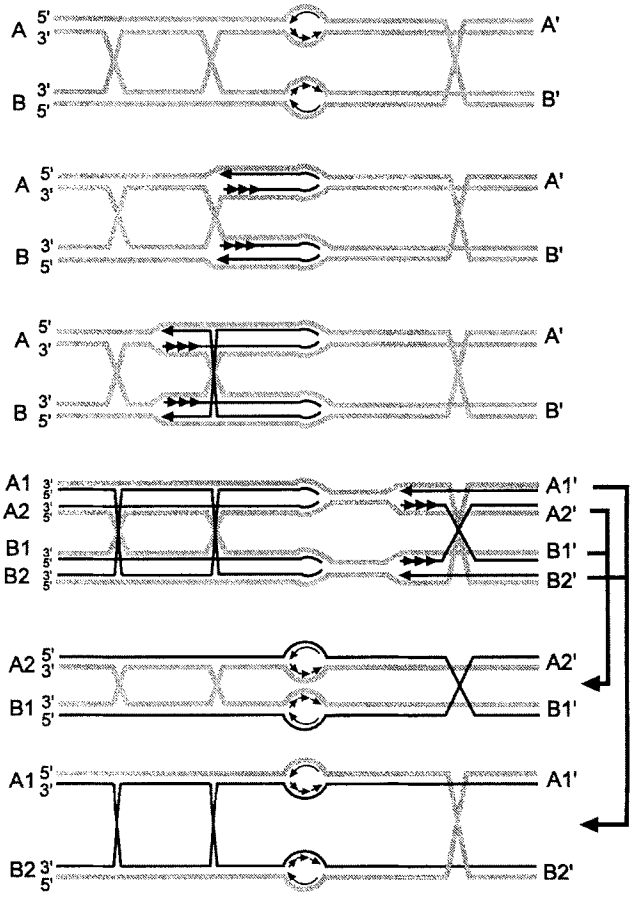

Fig. 6. A cartoon of two complete double-helical chromosomes attached to each other by three Holliday junctions. The parental chromosomes are shown in broad grey lines, while the newly synthesized DNA is represented by thin black lines. Arrowheads as in Fig. 4.

Acknowledgements

This work was supported by USPHS grant no. GM39933.

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