DISCLAIMER

This report was prepared as an account of work sponsored by an agency of the United States Government. Neither the United States Government nor any agency Thereof, nor any of their employees, makes any warranty, express or implied, or assumes any legal liability or responsibility for the accuracy, completeness, or usefulness of any information, apparatus, product, or process disclosed, or represents that its use would not infringe privately owned rights. Reference herein to any specific commercial product, process, or service by trade name, trademark, manufacturer, or otherwise does not necessarily constitute or imply its endorsement, recommendation, or favoring by the United States Government or any agency thereof. The views and opinions of authors expressed herein do not necessarily state or reflect those of the United States Government or any agency thereof.

DISCLAIMER

Portions of this document may be illegible in electronic image products. Images are produced from the best available original document.

Award Information
Grant No. DE-FG02-91ER61235
ID:ER61235-1004037-0000103
Principal Investigator: James E. Haber 781-736-2462
PIs: Institution: Brandeis University
Title: Nonhomologous Mechanisms of Repair of Chromosomal Breaks
SC Division:SC-72
Program Manager: Arthur M. Katz 301-903-4932
Research Areas: StrBio-Bio

DOE Patent Clearance Granted
MPDvorscak 12-19-01
Mark P Dvorscak Date
(630) 252-2393
E-mail: mark.dvorscak@ch.doe.gov
Office of Intellectual Property Law
DOE Chicago Operations Office

Project Progress

Most recent report of results to date:

1. In a published paper (Lee et al., 1999), we showed that NHEJ was strongly regulated by yeast cell mating-type and that the effects of sir mutations was entirely explained by an indirect change in mating-type. We further showed that the balance between NHEJ and homologous recombination could be shifted by changing the cell from either a or alpha mating-type to a-alpha, as is normally seen in diploids. We argued that this is a logical process, as a diploid always has a homologue with which to repair a DSB whereas a G1 haploid would be entirely dependent on NHEJ.

2.. Screening for genes involved in NHEJ. We have carried out a microarray screening in collaboration with Pat Brown,s lab, to identify genes that are a-alpha regulated, as our previous work (Lee et al., 19999 (above), showed that mating-type plays a key role in the regulation of NHEJ. We identified about 15 unknown genes turned off by mating-type and are systematically testing them for their effect on NHEJ. To date none of 8 that we have tested has had a very profound effect on this process, but the screening continues. We also have examined the role of MSH2 mismatch repair protein in NHEJ, on the assumption that the absence of MMR proteins might enhance the use of microhomologies during end-joining. There was however no effect of deleting MSH2.

We also showed that deleting RAD9 checkpoint gene had less than a two-fold effect on NHEJ. This is important for two reasons. First, it means that NHEJ must usually be over before cells enter mitosis, as rad9 cells do not show cell cycle arrest that would give NHEJ more time to occur (see also 3 below). Second, several labs reported that the Ku protein needed for NHEJ "left" the telomeres and could be found at sites of NHEJ, after a single DSB, but that this relocalization did not occur in rad9 cells.

3. We have now examined the kinetics of NHEJ by creating a DSB on two different chromosomes and using PCR to score the kinetics of appearance of the translocation that arises from this process. Reciprocal translocations occur 5-10% of the time these ends are all rejoined. Translocations are visible almost as soon as the HO endonuclease expression is turned of and the entire process is complete within 30 min, despite the fact that not all ends are joined. We believe that extensive 5, to 3, resection of the ends discourages NHEJ and have confirmed this by creating such substrates in vitro and examining their successful re-joining in vivo after transformation.

4. We have developed two assays for scoring the competition between NHEJ and homologous recombination and are using these to test the effects of various mutations on these two processes. In one, we measure how often a DSB at MAT is simply re-ligated versus how often it gene converts. This has proven to be a very useful assay for examining putative mutants identified by microarray screening of mating-type regulated genes, for example.
Using a selectable marker that lacks any homology in the genome, and with relatively short homologous segments on either end homologous to the THR4 gene, we have shown that the proportion of accurately targeted events increases as the lengths of homology increase from 30 to about 400 bp. A yku70 mutant eliminates nearly all non-targeted events. This is an important finding that may help improve gene targeting in mammalian cells. Conversely, a rad51 deletion reduces but does not eliminate targeting, supporting our previous work that shows Rad51p is important but not essential for several types of recombination events. The most surprising result comes so far from analysis of a DNA ligase 4 deletion, which was expected to eliminate nonhomologous targeting (as it eliminate NHEJ of cut DNA ends), but in fact there was a profound reduction in targeted transformants. We are now checking to make sure that a lig4 deletion does not re-route repair into a different pathway that might for example cause the replicative duplication of the entire targeted chromosome. We will pursue this intriguing and unexpected result, testing strains deleted for lig4-associated lif1 (xrcc4).

5. Using chromosome separating gels we are identifying the locations of "hit-and-run" transformation events that we have been studying. As soon as this analysis is completed and we sequence a few junctions to know the nature of the sites of insertion, we will complete a manuscript on these transformants, where only one end of the fragment shares homology with a genomic target.

6. Tid1p has been shown to play a very important role in meiosis, where it interacts with the meiosis-specific Dmc1p. In mitotic cells it has a rather minor role in recombination, a result we have confirmed in our strains with HO induction. Our finding that TID1 mRNA was 4 times as abundant in haploid cells than diploids suggested to us that it might have a more important role in mitotic cells than had been suspected. At the same time we found that a tid1 mutant was more resistant to hydroxyurea (HU) than wild type, a phenotype shared by yku70. We found that a similar phenotype was displayed by a srs2 mutant in a helicase implicated in recombination. Another helicase mutation, sgs1, does not display this phenotype. We therefore tested both tid1 and srs2 mutants for similarities with yku70. We were surprised to discover that both mutants were defective in adaptation to a single DSB.

Adaptation is defined as the ability of checkpoint-protein arrested cells with unrepaired DNA damage to resume cell cycle progression. Our previous work (Lee et al. 1999b) showed that cells were very sensitive to the extent of single-stranded DNA produced by resection of DSBs. Hence a cell with one DSB could adapt, but a cell with 2 DSBs as well as a cell with one DSB, resected twice as fast as normal (in yku70), could not. We further showed that the permanence of cell cycle arrest was suppressed by a point mutation in the large subunit of the single-strand DNA binding complex RPA.

We realized that other proteins must compete with RPA for ssDNA, including Rad51p. We therefore tested if a rad51 deletion would cause a more permanent arrest of cells with one DSB, on the assumption that more RPA might be bound. This proved to be the case. So now we have three more DNA-interacting proteins (Tid1p, Srs2p and Rad51p) that all affect adaptation, but not the initial arrest of cells with one DSB. Unlike yku70, none of these mutations affect the 5, to 3, resection rate of the DSB ends. We postulate that Tid1p and Srs2p play roles in loading and unloading RPA and/or Rad51p and perhaps other proteins on ssDNA.

7. Damage signal is nuclear limited. In a collaboration with Tim Stearns (Stanford), we have made the surprising and important observation that DNA damage arrest is mediated by a signalling process that is nuclear-limited. We created a zygote in which the two nuclei could not fuse because of kar1 mutation. One nucleus expresses HO endonuclease but has no HO cleavage site. The second nucleus (marked by LacI::GFP bound to a LacO array) has a MATalpha locus that is cut. In a haploid this would be sufficient to cause cell cycle arrest. Indeed in the heterokaryon, the damaged nucleus fails to enter anaphase whereas the undamaged nucleus proceeds through mitosis. This asynchrony is dependent on the HO cut and can be suppressed by making the zygote homozygous for the rad9 mutation that prevents the checkpoint from activating. This is a major finding and argues that the damage-arrest signal is not diffusible in budding yeast. This contrasts with the way mammalian cells respond when two nuclei are at different stages of DNA replicaiton and also when one nucleus in a heterokaryon is damaged by laser light (though here we don,t know for sure that DNA is the molecule that causes arrest).

8. Adaptation leads to the turning off of the DNA damage checkpoint kinase, Rad53p. In collaboration with Marco Foiani,s lab (Milan) we have examined the Mec1p-dependent phosphorylation of Rad53p and its activation as a protein kinase. In a cell where HO induction induces a switch of mating-type genes by recombination, Rad53p is never activated. But when DNA damage is more permanent, Rad53p is strongly phosphorylated and activated. This activation decreases at the time when cells adapt and resume cell cycle progression, arguing that it is the kinase cascade itself that is turned off.

In adaptation-negative mutations, the checkpoint kinase remains activated for 24 hr at least. rfa1-t11 reduces the intensity of checkpoint activation and causes the losss of kinase activity at the time cells resume growth.

We also have preliminary evidence that the kinetics of activation of Rad52p depends on the rate of 5, to 3, resection of DNA: the faster the resection, the sooner Rad53p kinase activation occurs. But it is clear that Rad53p is activated more rapidly by sub-lethal MMS treatment than by a single (lethal) DSB.

9. Adaptation and recovery are distinct processes.

Because the repair of a DSB by homologous recombination such as MAT switching is not long-lived enough to give detectable Rad53p kinase activation, we decided we needed to create a very slow, but otherwise normal, recombination event in which cells would all arrest at the G2/M checkpoint before completing the repair event. Thus these arrested cells will recover and we can ask if recovery and adaptation are distinct processes or if they both reflect the way cells emerge from a long period of arrest.

To do this we created a single-strand annealing event in which one of the regions of homology flanking the DSB was 25 kb away. With a measured resection rate of 4 kb/hr, it should take 6 hr to make this region single-stranded, and it does. At 6 hr we detect the deletion band expected on a Southern blot. 85% of the cells survive by this treatment versus less than 1% when there is no distant homology.

We then created compared the recovery of the homology-containing strain with the adaptation seen when there is no homology, by following individual cells, in cells carrying the adaptation-defective cdc5-ad mutation. This mutation prevents adaptation but does not prevent recovery. Hence the two processes are distinct.

10. Absence of the DNA damage checkpoint may change the use of different mechanisms of recombination and repair. We then deleted rad9 and/or rad17 from the strain taking 6 hr for repair, to see the consequences of mitosis before repair could be completed. Much to our surprise, repair actually was completed within 3 hr in the rad9 mutant and at 4 hr in rad17 and rad9 rad17.
When we examine the rate of resection of the DSB ends in rad9 and/or rad17, we see no difference in the rate, suggesting that in the absence of the checkpoint there may be a real change in the way DNA repair is occurring. One possibility that we are exploring is that the cells shift from single-strand annealing to break-induced replication. A prediction from this hypothesis is that we would see a product of recombination (though inviable) even if we inverted the orientation of the distant homologous sequence. This is being tested.

11. A possible new DNA damage checkpoint. One of the most potentially interesting discoveries in this work is that there appears to be another DNA daamge checkpoint. When we induce a dingle DSB in a rad9 rad52 strain (or rad9 rad17) where repair of the DSB is prevented by any homologous recombination event, the cells do not arrest, as expected, at pre-anaphase, However, more than 70% of the cells become "hung up" in late anaphase for several hours, though they eventually complete division. We believe tat we have seen the first evidence of a new checkpoint that responds to the presence of one broken chromosome arm by preventing the global completion of mitosis (that is, we would not see the DAPI staining pattern that we do if only one of 16 chromosomes in the haploid were unable to complete the segregation process. We are now doing staining of microbutubles to confirm the DAPI staining. We will also ask if this arrest occurs in a rad52 diploid with an unrepaired DSB on only one chromosome.

Most recent products delivered:

Paques, F. and J.E. Haber (1999) Mechanisms of double-strand break repair in Saccharomyces (review). Mol. Biol. Microbiol. Rev. 63: 349-404.

Colaiacovo, M., F. Paques, and J.E. Haber (1999) Removal of one nonhomologous DNA end during gene conversion by a RAD1-, MSH2-independent pathway. Genetics 151: 1409-1423.

Lee, S.E., F. Paques, J. Sylvan and J.E. Haber (1999) Role of yeast SIR proteins and mating type in directing DNA double-strand breaks to homologous or nonhomologous repair routes. Curr. Genet. 9: 767-770.

Haber, J.E. (2000) Lucky breaks: analysis of recombination in Saccharomyces. Mut. Res. (in press).

Evans, E., N. Sugawara, J.E. Haber and E. Alani (2000) The Saccharomyces cerevisiae Msh2 mismatch repair protein localizes to recombination intermediates in vivo. Mol. Cell. (in press)

Haber, J.E. (2000) Recombination: a frank view of exchanges and vice versa. Curr. Opin. Cell. Biol. (in press)

Haber, J.E. (2000) Partners and Pathways in Repairing a Double-Strand Break. Trends Genet. (in press).

Most recent notes concerning the project:

We have discovered three new proteins involved in DNA damage assessment. Interestingly they are all proteins involved in reombination, but they have very different roles in that process and other proteins that might be expected to be equivalently involved are not. This is developing into a very significant area of research.

The apparent discovery of a new checkpoint in anaphase is certainly of great importance and our efforts right now are to confirm our initial observations.

Equally interesting is the apparent shift in the mechanism of recombination/repair in checkpoint-defective cells.