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TITLE: Genetic Instability and Checkpoint Genes: Insights from a Single Eucaryote (S. Cerevisiae) for Human Breast Cancer

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               Fort Detrick, Frederick, MD  21702-5012

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Genetic Instability and Checkpoint Genes: Insights from a Single Eucaryote (S. Cerevisiae) for Human Breast Cancer

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We study genes involved in cell cycle controls called checkpoints that block eucaryotic cell division after DNA damage. Checkpoint genes are relevant to breast cancer for several reasons, each related to aims of this proposal. Our first aim related to the fact that cancer cells and human checkpoint mutant cells exhibit high levels of genomic instability. We have characterized genomic instability in yeast cells and find an association between checkpoint mutations and chromosome loss and mitotic recombination. More importantly, we have identified and characterized an instance of genome instability (we call "unstable 7") useful in understanding how genomic instability can arise in checkpoint mutants.

Our second goal was to isolate human checkpoint genes, a task we were unsuccessful in probably because checkpoint gene homologs have only low sequence identity across species. We have undertaken a study of how one gene, TEL1, of low sequence homology to MEC1 does suppress mec1 checkpoint mutant defects. We have found suppression of damage-sensitivity, and of defects in transcriptional regulation and in cell cycle arrest. These studies provide a means of determining the order of gene function as well as of developing experimental conditions by human genes may complement yeast checkpoint mutation

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5. Introduction

Checkpoint controls delay the cell cycle at specific cell cycle stages (e.g. G1, G2) after DNA damage (reviewed in Hartwell and Kastan, 1994). The delay provided by checkpoints provide time for DNA repair, and prevent genomic instability that may arise when damaged cells divide unchecked. Checkpoint genes are relevant to cancer in general and to breast cancer in particular for two reasons. First, checkpoint defects appear to contribute to cancer. Cancer progression is generally a multistep process in which cells accumulate mutations, each progressively allowing the cancer cell to grow under conditions where normal cells do not. One source of mutations occurs through genomic instability, a broad term meant to encompass any process which causes a mutant cell to rapidly accrue mutations or rearrangements. When checkpoint controls are defective, the mutant cells do not halt cell division when damaged, and progression with damage leads to further damage and rearrangements of chromosomes. This in turn can result in genetic alternations and progressively more severe cancer cell phenotypes (e.g. metastasis).

That checkpoint mutations coorelate with an acceleration of cancer cell phenotypes gains support from studies of two human genes p53 and recently ATM (Savitsky et al., 1995) that are both checkpoint genes. p53 is a gene long recognized as key in many cancer types. It is mutant in over 50% of all cancers (Hollstein et al., 1991) and plays a prominent role in the G1 checkpoint arrest after damage (Kastan et al., 1991; Kuertitz et al., 1992). ATM, for ataxia telangiectasia mutant, was identified by positional cloning (Savitsky et al 1995), and has a high association with several types of cancer including breast cancer (Swift et al., 1991; see ref. #). ATM proves to be related to our MEC1 gene by both functional and protein sequence criteria (Figure 1, appendix). Both ATM and MEC1 encode putative lipid or protein kinases (Hunter, 1995). Defects in either p53 or ATM, or in the yeast MEC1 gene, lead to genomic instability (Livingston et al., 1992; Yin et al., 1992; Friedberg et al., 1995), a phenotype probably related to the association of p53 and ATM mutations to cancer. In addition, the functional homology between human ATM gene and the yeast MEC1 genes strengthens the view that understanding yeast controls will provide insights into understanding defective controls in cancer cells (Tugendrich et al., 1994).

Our goals in this proposal were as follows. First, we are analyzing the relationship between checkpoint gene functions and four types of genomic instability in Saccharomyces cerevisiae, a simple eucaryotic cell. We have now extended this study to analysis of an instance of genomic instability we call "unstable 7", which has many features suggesting it may be useful in understanding genomic instability in checkpoint mutant cells. Second, we wanted to isolate human checkpoint genes by complementation. Our
efforts have not been successful in this regard. To develop new strategies, we have turned to a detailed analysis of a MEC1-like gene, called TEL1, that can partially suppress mec1 mutant defects. These studies will provide some information on order of gene function in checkpoint pathways. This studies of TEL1 may also identify experimental conditions to identify human checkpoint genes by complementation. We anticipate these studies will further our basic knowledge of checkpoint gene function and enhance our understanding of mechanisms of genomic instability in tumor cells.

Our refined goals are:

Genomic Instability
1) Develop an assay of chromosome loss and mitotic recombination in the presence of checkpoint mutant genes. This assay is based on use of YAC artificial chromosomes, a stable but nonessential chromosome to the cell.
2). Develop an assay of point mutations in presence of checkpoint mutant genes.
3). Develop an assay of translocations between a YAC artificial chromosome and bona fide chromosome.
4) Develop an assay of gene amplification.
5) Analyze an instance of genomic instability- "unstable 7".

Human Checkpoint Genes
6) Study the role of TEL1 and suppression of mec1 mutant defects.
7) Isolate human checkpoint genes by complementation of yeast mutants.

6. Experimental Methods and Preliminary Results:
Our progress to date includes the following, addressing each research objective stated above.

Part1 - Genomic instability
1). Chromosome loss and mitotic recombination assays: We modified the YAC artificial chromosome (Sears et al., 1992) as described in our proposal. This required extensive molecular cloning to insert appropriate selectable markers in the ends of the YAC (Figure 2, appendix). We then transferred these modified YAC into checkpoint mutant strains by karyogamy crosses (mating that allows transfer of only a chromosome into a recipient nucleus).

We found that all our checkpoint mutant strains showed elevated chromosome loss and recombination, especially mec1 mutants. Unfortunately, the YACs were relatively unstable in the original strain, limiting the utility of this system. For example, we were unable to stably
introduce the two YAC chromosomes into the mecl strain; the only clones we could isolate were rearranged by the time we detected chromosome transfer (see Table 1, appendix). Our YAC-based chromosome loss system showed relative high instability (loss rate of 10^{-2} per cell division compared to loss rate of 10^{-5} per cell division for a bonafide chromosomes; (Hartwell and Smith, 1985) even in a checkpoint normal cell (though much less than in a mecl mutant)!

We had proposed two experimental approaches to resolve this issue. We are introducing our checkpoint mutations into a yeast strain in which the YAC's are stable (when those cells have intact checkpoint genes). I have constructed a mecl ts allele linked to a selectable marker (HIS3), and am now introducing this allele into the S288C background (data not shown). Other checkpoint mutations will be introduced into S288C by using null mutations of checkpoint genes we have constructed (Lydall and Weinert, 1995; submitted). If the S288C strain still proves difficult to work with, we still plan to use a second chromosome loss and recombination system based on a haploid strain containing an extra copy of chromosome 7 we used previously to characterize rad9 (Weinert and Hartwell, 1990; and described further below).

Our preliminary results indicate that mecl mutants do show a tremendous instability of YACs, far greater than in our other strains (Table 1, appendix). This is consistent with MEC1 having a key role in DNA replication compared to other checkpoint genes, a conclusion we and others have come to from other lines of research (Kato and Ogawa, 1994; Paulovich and Hartwell, 1995).

2). Point mutation assays:

We have analyze point mutations by two systems; conversion of CAN1S to can1r (selected for by resistance to the drug canavanine conferred by the can1r allele) and conversion of SUP4 to mutant SUP4 (selected by loss of ochre suppression; Pierce et al., 1987). Both assays indicate that our checkpoint mutants do not confer any significant "mutator" phenotype as assayed by point mutation rates, either spontaneous or in the presence of DNA damaging agents (data in Table 2, appendix for can1r system only).

3). Translocation assays:

We attempted to develop an assay of translocations by cloning a yeast gene into the human ALU sequence, to be placed back into the YAC chromosome and assayed for mobility. We completed initially cloning of URA3 yeast gene into an ALU1 sequence. The genomic instability assay in different strain backgrounds has caused us problems as discussed above, and the translocation assay will not be pursued until those other studies are completed.
4). Gene amplification assays:

We were going to generate an ALU::HIS3 clone to being attempts to look at gene amplification. We had difficulty with the basic YAC system, and have therefore delayed development of a gene amplification assay. We have now identified gene amplification in the unstable 7 project described below. Once the region on chromosome 7 that is amplified has been defined, we will introduce a dosage-sensitive gene into that region. We plan to use the HIS3 gene in combination with the drug scheme that selects for cells with a higher HIS3 gene copy number.

5). The unstable chromosome 7 project

We have identified an instance of chromosome instability in rad9 mutants we think will be very informative for genomic instability. A chromosome 7 disome (Figure 3, appendix; strain described in Weinert and Hartwell, 1990) allows us to select for loss of a marker at the end of the chromosome. This system was designed initially to identify chromosome loss and mitotic recombination events. It now proves to be an exceptionally useful tool to study other forms of genomic instability. I describe our current results next.

A. Genetic analysis:

We noticed a chromosomal event that shows hallmarks of a breakage-fusion-bridge cycle (McClintock, 1984). The genetic identification of this form of genomic instability occurs as follows: The initial strain used a haploid rad9 strain with 2 copies of chromosome 7 (Figure 3A, appendix; Genotype of initial cell). One homolog (denoted "B" for bottom) has a dominant selectable gene CAN1s gene which we can select for the loss of by plating cells on medium containing canavanine. The other homolog (denoted "T" for top) does not have the CAN1s gene. The initial cell with an intact CAN1s gene dies when plated on media containing canavanine. The few cells that lose CAN1s survive (frequency of survivors; 10^-4 in rad9- cells and 10^-5 in RAD+ cells). Most can1r cells have undergone chromosome loss, an equivalent number undergo the event described below, and some have undergone mitotic recombination.

Among the cells that are resistant to canavanine were cells that gave rise to colonies with extensive sectoring ("nibbled" or "sectored" colonies; Figure 3B, appendix. Sectors is probably due to cell death). For two reasons we believed that the sectored colonies had an event that was related to DNA damage checkpoint controls. First, the sectored colonies occurred spontaneously at about 10x higher rate in rad9- than RAD9+ cells. Second, these sectoring colonies were also inducible by x-irradiation (not shown). We therefore set out to determine the source of the chromosomal event.
B. Sectoring colonies show extreme secondary instability

We first characterized the genotype of the sectoring colonies and found, remarkably, that >90% had the same genotype, denoted in Figure 3C, appendix. They appeared to have lost all DNA distal to the TRP5 locus (shown as a dotted line). We thought initial that they were mitotic recombinants homozygous for the left arm of the chromosome. Subsequent analysis indicated otherwise.

The sectored colonies proved to be themselves extremely unstable genetically. This was identified by propagating the cells further on unselected medium. We surmised the following: consider an initial cell that has 2 chromosome 7's(T,B; Figure 3A, appendix). By selection, CAN1s on the B homolog is lost. The sectored colonies contain T and a version of B we call here B*; they are T,B* (Figure 3B, appendix). When propagated these sectored cells with a "T,B*" genotype apparently undergo further events where either the T or B* chromosome is lost (frequency of secondary events in this experiment was: 18% lose T; 11% lose B*; 71% retain T, B*; Figure 3D, appendix). Therefore, TB* cells undergo subsequent events at 1 in 3 cell divisions.

We have isolated 100's of TB* strains de novo from the initial disomic strain. The TB* cells always have the same characteristics with respect to genetic structure and instability (as shown in Figure 3C and D, appendix). We have not detected TB* strains arising from RAD+ strains; the few sectored colonies we detected had different genetic composition and did not give rise to subsequent instability as did the TB* in rad9 mutants (data not shown).

C. Molecular analysis of unstable 7 (figures 4 and 5)

We wished to determine the molecular structure of B*. There are three key observations in deduction of the structure of B*. First, in strains undergoing the instability, we identified a chromosome that is physically larger than the initial chromosome 7 (Figure 4, appendix; note lane 4 in particular). We identified the larger chromosome using pulsefield gel electrophoresis that separates chromosomes by size on agarose gel matrix (Schwartz and Cantor, 1984). Most sectored colonies actually have the gel pattern shown in lane 4 (>90%). (The six cell preparations in Figure 4 derive from a non-random group of sectored colonies that had different genetic properties.) The starred bands represent extra bands corresponding to an unstable 7. That these extra bands are from chromosome 7 was demonstrated by hybridization of radio-labeled molecular probes from chromosome 7 (probe C, right panel). Furthermore, molecular analysis of the B* chromosomes verified that DNA genetically undetectable from the chromosome (see Figure 3C) was in fact not present on the unstable B* chromosomes. For example, the B* chromosome in
Figure 4, lane 4 hybridizes to a probe containing sequence C, so it contains the right arm of chromosome 7, but it does not hybridize to probe A, so it does not contain the left arm of chromosome 7. The shorter B* chromosomes in Figure 4 also have the right arm but not the left arm of chromosome 7. (The band of lower molecular weight that hybridizes in the center and right panels is on chromosome 3, a control detected by using a probe, the LEU2 gene, specific for chromosome 3.)

It thus appears that the B* chromosome has physically lost DNA. Yet, the truncated chromosome usually has the mobility of a larger chromosome by pulsedfield gel electrophoresis.

We have identified molecularly the region of the breakpoints of the unstable chromosome, narrowed to a region of 10-50kb (not shown). This was accomplished using yeast DNA cloned into a bank of lambda phages (Riles et al., 1993.). Most unstable 7 chromosomes have similar though not identical breakpoints (judging from the intensity of the hybridization signals). The breakpoints appear heterogeneous but cluster in this one region of the chromosome. This site appears, therefore, to be some sort of stabilization site and/or selectively broken site. We do not now know which interpretation is correct.

D. Defining the structure of B*: Use of "Stretch DNA" technology.

We needed a cytogenetic test of yeast chromosome structure. Yeast chromosomes in metaphase are unfortunately not visible like yeast chromosome structure is not visible, though a different technique to probe structure was required. During a recent Gordon Conference I had the good fortune to learn of a new technique to visualize chromosomes and specific sequences by a method termed "stretch DNA". Such a test is now possible, in collaboration with Ulli Weier at UC Berkeley. He has a method to lay out (stretch) intact chromosomes and identify the relative distances between sequences using fluorescence probes (FISH; Weier et al., 1995). His method allowed us to test the physical structure of the B* chromosome.

We sent him gel-isolated (see Figure 4) unstable and stable chromosome 7. He prepared "stretched chromosomes" and studied their structures using fluorescently labeling DNA probes (FISH). To prepare "stretched chromosomes" he applies the isolated chromosomes to a glass slide, and upon dehydration the DNA molecule stretches across the slide, remaining intact. These "stretched chromosomes" are subjected to FISH hybridization using probes to specific regions of chromosome 7 (obtained from the yeast lambda clones; Riles et al., 1993).

We have received data on structure of the stretched chromosomes. The B* chromosomes structures show three unexpected features (Figure 5, appendix). First, they do contain two right chromosome ends (identified by FISH probe to a right-end sequence; Figure 5, righthand image). B*
therefore appears to be a head to head fusion of chromosome 7. Second, they identified only a single centromeric sequence (Figure 5, lefthand image). The \text{B}^* \text{ structure is, surprisingly, not a dicentric. Third, another surprise; some 100-200 kb DNA circles were found as well. These circles of DNA do contain chromosome 7 sequences, in fact from the right arm (region C). The fiber analysis, with 50-100 fibers analyzed in each sample was quantitative; all \text{B}^* \text{ chromosomes had 2 signals with a right end probe, each near the fiber ends as expected from the physical distance of the sequence to the chromosome end. Each normal chromosome had one signal near an end, and both samples showed only a single signal to a centromeric probe.}

**E. Model**

A model to explain these observations is shown in Figure 6 (appendix). We hypothesized that an initial rare event lead to a chromosome break. The checkpoint-defective cells continue through the cell cycle, rereplicate the broken chromosome, generating two truncated sisters (with sequence B near break points). These can then fuse, recombine with other homolog (not shown) be propagated as is or be lost. If the two fuse, this structure can undergo breakage-fusion-bridge cycles and rearrangements, generating a structure shown in Figure 6 (McClintock, 1984).

Many features of our data are consistent with this model; rare initial event inducible by radiation; high frequency secondary chromosome loss events; high frequency in a checkpoint mutant cell; structures shown in Figure 5.

**F. Future direction:**

1. The instability of \text{B}^* \text{, which is a moncentric chromosome, is yet to be explained. Instability may be related to the circles of DNA found co-migrating with \text{B}^*. These circles remain enigmatic.}

The entire sequence of chromosome 7 is now in the GCG database. We will perform DNA sequence analysis to determine the following:

- Are there sequence features near the breakpoint that account for why these structures are preferentially recovered? Are there inverted repeat sequences or telomere-like sequences in this region?

- Are there sequences near chromosome ends that can explain the generation of circles- for example are their direct repeats in region C?

2. We have also identified a 1400kb \text{B}^* \text{ chromosome that we will analyze by stretch DNA methodology. This chromosome has the correct size to be a complete dicentric, which can be tested directly by FISH probes to centromere DNA.
3. We have identified sequences proximal to the breakpoint that are amplified (determined by quantitative southern analysis; data not shown). We will insert a dosage-sensitive gene, \textit{HIS3}, into this region and determine if we can use this as a system to genetically detect gene amplification.

4. We have contracted \textit{rad9ts} chromosome 7 disomic strains to ask if reactivation of the checkpoint will limit propagation of the B*-containing cells. This experiment tests if the checkpoint controls can limit these types of events once the abnormal B* structure is generated.

\textbf{Part 2: Isolation of Human checkpoint genes.}

The idea that checkpoint genes are conserved in humans and yeast has been verified - the ATM gene from humans is very similar functionally and by protein sequence (though at the low end- 20% identical, 50% similar; Savitsky, et al. 1995) to that of our \textit{MEC1} gene (see Figure 1). Others who have the ATM gene are attempting complementation of \textit{mecl} mutants or test of dominant negative activity of \textit{MEC1} in human cells (Steve Friend, Seattle, Wa; Mike Kastan, Baltimore, Md; personal communications). Therefore, the existence of human counterparts to at least some yeast checkpoint genes seems highly likely.

Complementation of yeast checkpoint mutants by human cDNAs has not, in our hands, been a successful strategy. Others have tried and failed as well (see Plon et al, 1993). The reason for these failures may be in that homologs bear the low level sequence homology. this is true for both human ATM and yeast \textit{MEC1} genes, as well as for four pairs of homologous genes between budding and fission yeasts (@20% identity; 45% similarity; see Lydall and Weinert, 1995, 1996). Though functions may be conserved, molecular contacts with other proteins may have diverged significantly to make cross function unlikely. The only strategy we will now pursue is through the use of homology identities between our yeast genes and cDNA sequences from random human cDNA sequences, called EST sequences. Any putative match will be followed up by collaboration with those who identified the putative matching EST sequence.

\textbf{Part 3: Analysis of \textit{TEL1}, a yeast \textit{MEC1}-like and ATM like gene.}

In the interim, we have initiated a project to characterize one gene that bears low level homology with \textit{MEC1}, a yeast gene called \textit{TEL1} (Greenwell et al., 1995; Morrow et al., 1995; Sanchez et al., 1996). The analysis described below has two goals. The primary goal is to determine the order of gene function, a goal in which we have made significant progress. A secondary goal is to identify a complementation strategy, a checkpoint-related phenotype, that may detect genes with lower amount of function.
We have undertaken a study of the role of TEL1 in the cells responses to DNA damage. The principle questions and preliminary findings are the following: To what extent can TEL1 substitute for MEC1? The answer is, a little. Second, does TEL1 provide a useful genetic tool to order gene functions in the checkpoint pathway? The answer appears to be "yes". Third, by learning how TEL1 can substitute for MEC1 can we develop a sensitive strategy to identify other human genes that have low level of homology to our checkpoint genes? We do not yet know the answer to this question.

The model of gene interactions we are currently testing is shown in Figure 7A. There is considerable evidence in favor of this model, including genetic, molecular and biochemical data. For example, MEC1 and TEL1 share sequence similarity (Greenwell et al., 1995; Morrow et al., 1995; Sanchez et al., 1996); TEL1 can restore some responses to DNA damage in mecl mutants (Morrow et al., 1995; Sanchez et al., 1996; see below); RAD53 may act downstream of MEC1 and TEL1 because Rad53p is phosphorylated in vivo after damage in a MEC1 or TEL1-dependent manner (Sanchex et al., 1996; Sun et al., 1996); DUN1 acts specifically in transcriptional regulation (Zhou and Elledge, 1993); PDS1 acts specifically in G2/M arrest (Yamamoto et al., 1996).

Our current strategy is to evaluate how TEL1 restores activity in mecl mutants. Restoration of activity requires that we overexpress TEL1 from a high copy plasmid and monitor each of four responses: restoration of DNA damage resistance, restoration of G2/M arrest, restoration of transcriptional regulation of damage-inducible genes, and restoration of viability to mecl null mutants. The restoration of damage sensitivity is significant, and may be useful in some form in screens of human checkpoint genes (Morrow et al., 1995; Sanchez et al., 1996; data Figure 7B). We have recently shown that TEL1 can restore transcriptional induction after damage by the alkylating agent MMS (Figure 7C), and that TEL1 can restore partially cell cycle arrest (Figure 7D).

A key question is whether any of these TEL1-mediated responses occur by activation of checkpoint pathways, or do they occur by some unknown and therefore uninterpretable pathway activated by overexpression. Gratifyingly, we found that transcriptional induction by TEL1 requires DUN1, suggesting this response proceeds by the defined pathway (Figure 7C). Second, suppression of lethality is DUN1 dependent (data not shown). Third, overexpression of TEL1 causes cell cycle arrest that requires PDS1 (data not shown). Interestingly, overexpression of TEL1 does not require damage nor RAD9, suggesting that overexpression causes a constitutive activation of the checkpoint pathway leading to arrest. We are in the process of analyzing this network of genes in more detail to test the hypothetical pathway. Overexpression of RAD53, PDS1,
and DUN1 all cause phenotypes potentially useful in genetic epistasis experiments done in combination with loss of function alleles in any of the five genes shown.

7. Conclusions

First, analysis of genomic instability in checkpoint mutant cells remains an incomplete task. Our initial tests of mecl suggest it leads to high rates of mitotic recombination, and we will pursue this observation using the YAC recombination system and meclts allele we have constructed. The point mutation frequencies do not seem to be elevated in our mutants. We will try to generate a system to identify gene amplification using the sequences on chromosome 7 that can be amplified.

Our most significant progress remains with the genomic instability of chromosome 7, a phenomenon that we now call unstable 7. This event may provide us the opportunity to study genomic instability occurring by the breakage-fusion-bridge cycle as proposed by McClintock (1984). Further analysis of the unstable 7 chromosome should provide additional insights on the role of checkpoint genes in genomic stability.

We are still uncertain as to whether human checkpoint genes can be identified by complementation of yeast mutants. Detailed analysis of TEL1 may provide tests of order of gene function as well as clues to sensitive assays that may be useful to identify human checkpoint genes.

8. References:


Figure 1 Similarity of ATM, MEC1 and other PI3 kinases

An alignment of genes in the PI3 kinase family. From Meyn, S 1995. Cancer Research 55, 5991-6001. Ataxia-telangiectasia and Cellular Responses to DNA Damage. Shown are the ATM gene; DNA-PK gene that encodes a mammalian protein kinase activated by DNA damage; MEI41, the functional Drosophila homolog of the yeast MEC1; the yeast Tel1p; RAD3, the fission yeast homolog of MEC1; and MEC1. The identity in the solid box is 60-70% similarity, in the shaded box is 40-50% similarity, and the rest of the sequences have less than 20% similarity.
Figure 2, Yac chromosomes constructed to detect chromosome loss and recombination. These YACs were transferred into a ura3, trp1 lys2 cyhr strain. The loss of the dominant sensitivity allele CYH-S, that confirms sensitivity to cycloheximide allows selection of chromosomal events. Loss generates cells that are cyhr ura- Lys+ Trp+, and recombinants are cyh2r, Ura+ Lys+ Trp+.

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Table I Chromosome Loss and Recombination of YAC chromosomes in checkpoint mutants.

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<th>Recombination Frequency(x10^-2)</th>
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<td>cyhr Ura- Lys+ Trp+</td>
<td>cyhr Ura+ Lys+ Trp+</td>
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<td>MEC+(trial1)</td>
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<td>0.3 +/- 0.1</td>
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<tr>
<td>(trial 2)</td>
<td>0.13 +/- 0.17</td>
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<tr>
<td>mecl</td>
<td>not determinable</td>
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<tr>
<td>mec2 (rad53)</td>
<td>23.4 +/- 17.2</td>
<td>10.2 +/- 13.2</td>
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<td>rad9 (trail 2)</td>
<td>0.71 +/- 0.76</td>
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Table 2 Point Mutation Frequency in Checkpoint mutant strains.

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<th>Strain</th>
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Figure 3. Unstable 7

A. Genotype of initial cell

T
B
CAN1s

---
lys-  cyhr  trp5-leu1+  ADE3+  ade6-
---
chromosome 7 disome with marked homologs
-rad9-

B. Selection for T,B* unstable cells

select loss of CAN1s

colony morphologies on agar plate

○ loss or recombinants

❖ sectored events

C. Genotype of T,B* unstable cell

T
B*

---
lys-  cyhr  trp5-leu1+  ADE3+  ade6-
---

D. T,B* is extremely unstable

TB* - 71%
T - 18%
B* - 11%

* Note: this event is not detected in RAD+ strains beyond the generation of sectored colonies. Upon propagation the cells acquire stable genotypes, and we have not detected extra chromosomes by pulsefield gel analysis in RAD+ cells.
Figure 4- Molecular analysis of B*

- Prepare chromosome DNA from many TB* colonies
- Separate by pulsefield gel electrophoresis

Ethidium Stain

Probe A

Probe C

Normal

Control
Figure 5. Structures of stretched B* chromosomes.
left- hybridized with centromere probe- note white arrow.
right panel- hybridized with right-arm probe- note two black arrows.
Figure 6. Model of Unstable 7

1. DNA replication
2. DNA break (spontaneous or x-ray)
3. rad9
4. RAD9+
5. ARREST
6. segregate chromosomes in mitosis
7. replicate
8. fuse truncated sisters (unstable chromosome shown only for simplicity)
9. fusion
10. segregation at mitosis
11. breakage, fusion, bridge chromosome cycles
12. lose a centromere
13. circular DNAs

STILL VERY UNSTABLE
A. **Hypothesis on Order of Gene Function**

![Diagram of gene function](image)

**Rad9** activates **Rad53** and then **Mec1** and **Tel1**. These pathways lead to **Dun1** and **Pds1**, which are essential for transcriptional induction and G2/M arrest, respectively. Solid lines represent the normal pathway, dotted lines the pathway achieved upon overexpression.

**Figure 7. Analysis of Order of Gene Function**

A. Model. DNA damage (top) activates **Rad9** and then subsequent genes. Solid lines represent the normal pathway, dotted lines the pathway achieved upon overexpression.

B. **Suppression of UV sensitivity of mec1 mutants by overexpression of Tel1.**
- □ wildtype
- △ mec1 + vector
- ○ mec1 + Tel1 (1 copy)
- Δ mec1 + Tel1 (high copy)

C. **Tel1 restores transcriptional induction of a damage-inducible gene (Rnr3) in mec1**
- Relative levels of RNR3, determined by northern analysis, with and without DNA damage from 0.01% MMS, 4 hours. Induction is **Dun1** dependent.

D. **Tel1 restores partial cell cycle arrest to mec1 mutants.**
- Cells were synchronized in G1 with mating pheromone, released into the cycle at the restrictive temperature (36°C) for **cdc13** after washing out mating pheromone. **cdc13** mutation generates damage during S phase. Cells were fixed and stained for nuclear morphology. **cdc13** cells arrest to determine arrest. **cdc13** cells arrest, **cdc13 mec1** mutants do not, and **Tel1** partially restores arrest.
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