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TITLE: Alternative DNA Damage Checkpoint Pathways in Eukaryotes

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### REPORT DOCUMENTATION PAGE

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**Sponsoring Agency:** U.S. Army Medical Research and Materiel Command, Fort Detrick, Maryland 21702-5012

**Abstract:**

The goals of this grant are to perform a mutagenesis screen to identify the genes in the alternative DNA damage checkpoint pathway in yeast, to characterize and clone these genes, and to isolate and analyze the human counterparts of these genes. During the first year of this grant, we have constructed a cdc9-8, rad9Δ double mutant strain SCP2, which is both temperature-sensitive at 30°C and UV-sensitive, for the mutagenesis screen. Approximately 220,000 colonies of SCP2 have been mutagenized by EMS and screened for temperature-sensitivity at 30°C. This primary screen yields three temperature-sensitive mutants, *chb13*, *chb16*, and *chb57*. A secondary UV sensitivity screen was also performed on these three *chb* mutants. In summary, *chb13* is a strong mutant that can no longer be rescued by *CHESI* in both temperature- and UV-sensitivity. The other two mutants have only one strong phenotype. Specifically, *chb16* is highly temperature-sensitive but only moderately UV-sensitive, whereas *chb57* is very sensitive to UV but not as sensitive to high temperature. Our next goal is cloning the genes mutated in these three strains.
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[Signature]  15 Apr 99
PI - Signature  Date
Progress Report (March 1998 to March 1999)

Technical Objective 1-1
Perform a comprehensive mutagenesis screen in S. cerevisiae to isolate yeast mutant strains which have defective checkpoint bypass genes

Our working hypothesis is that CHESI suppresses the radiation sensitivity of mecl-1 and rad9Δ by activation of an alternative DNA damage-induced G2 checkpoint pathway in S. cerevisiae. The genes in the pathway can be identified genetically. The ultimate objectives of this grant proposal are to identify, characterize, and clone the genes in this alternative pathway, and to isolate the human homologs of these genes and analyze their structure and expression in human breast cancer derived cell lines and human breast tumor samples. The goals of the first year of this grant are to develop a temperature sensitive strain, to optimize mutagenesis conditions, and to accomplish a mutagenesis screen to identify mutant strains that can no longer be rescued by CHESI in the absence of wild type RAD9.

The method proposed in this project utilizes a temperature sensitive strain that does not grow at 30°C and is sensitive to UV irradiation. This requires the combination of cdc9-8 and rad9Δ mutations in the same genome. The rad9Δ not only lowers the permissive temperature of cdc9-8 from 30°C to 23°C, but also provides the UV sensitive phenotype. The cdc9-8 gene was gap repaired out from the 9085-1-10-4 strain and put onto an integration vector with URA3 marker. The plasmid was then introduced into a rad9Δ strain (Y438 from the Elledge’s laboratory) and plated onto URA deficient- and 5-FOA media sequentially. This allowed isolation of a strain where the cdc9-8 allele replaces the wild type CDC9 to generate the double mutant strain SCP2.

Different amount of EMS, ranging from 0 to 50 μl, was tested to assay for optimal mutagenesis. We chose 5 μl for it gave approximately 50% killing in SCP2. The mutagenesis screen was carried out by a slightly modified method to the one originally described in the application. Specifically, SCP2 was transformed with two plasmids, each carrying CHESI or RAD9 genes, before EMS mutagenesis. After exposure to EMS, the cells were washed and plated onto selective medium at 30°C. Since the cells still contain wild type RAD9 at this point, they are able to grow at 30°C. The mutants were replica-plated to 5-FOA to select against the RAD9 plasmid and screened for growth at 23°C but not at 30°C. The tentative name assigned to the qualified mutants is chb for checkpoint bypass. The colonies identified on this first pass were then patched and replica-plated to test for temperature sensitivity more carefully at 37°C, 32°C, 30°C, and room temperature. The clones, which no longer grew at 30°C from the second temperature sensitivity test, were subject to a secondary screen to determine if they have also lost the response of CHESI after UV irradiation. Our model predicts that checkpoint bypass gene mutants should be UV sensitive even when CHESI is present in this rad9Δ strain. The UV sensitivity screen was performed by quantifying the survival rate after exposure to 10 J/m² of UV radiation, in comparison to the survival rate of the parental strain transformed with either empty vector or CHESI-containing plasmid.

We have mutagenized and screened approximately 220,000 clones and 310 putative temperature sensitive clones were picked originally. The majority of clones
failed on the second temperature sensitivity test, leaving three strong candidates that are truly temperature sensitive in the presence of CHES1 when the RAD9 plasmid was lost. Among those, chb13 and chb16 are highly temperature sensitive whereas chb57 is a weaker allele. With regard to UV sensitivity, chb13 and chb57 are also sensitive to UV radiation but the effect of chb16 is intermediate when compared to the controls (figure 1). The growth of chb16 is slower than the other two chb strains, therefore, chb16 is a relatively unhealthy mutant in general. Overall, chb13 appears to be a very strong mutant that has lost all response to CHES1 by our assays. Both chb16 and chb57 are moderate mutants that have one strong phenotype. The results of this mutagenesis screen are summarized in table 1. Construction of a cdc9-8 and rad9Δ double mutant strain of the opposite mating type is underway in order to perform complementation analysis to determine whether the mutants are dominant or recessive. Once defined, we will clone the genes that were recessively mutated in the chb strains by introducing a S. cerevisiae genomic library into the mutant strain and selecting for growth at 30°C in the presence of CHES1.

Our previous results showed that a chkl mutant is at least partially defective in the response to CHES1 (D. Pati, unpublished data). This suggests that CHK1 maybe a candidate gene in the proposed alternative pathway. We will test all three chb mutants for the complementation by CHK1. Disruption of CHK1 in the parental strain SCP2 is also underway for the comparison of the phenotypes with these chb mutants.

The analysis of these mutants has also allowed us to confirm that the CHES1-dependent pathway is partially parallel to the RAD9-dependent pathway. Our previous data showed that CHES1 could either act on a gene product downstream of DUN1 in the primary MEC1-dependent pathway or, more likely, activate an alternative pathway (Pati et al., 1997). Consistent with the parallel pathway model, introduction of a wild type RAD9 gene restores UV resistance to the SCP2 strain and all three mutants. Since the chb mutants are UV resistant when RAD9 is present on the episome, they are apparently not simply downstream of RAD9. This favors the alternative parallel pathway model. Our current model for the CHES1 activated checkpoint is demonstrated in figure 2.

In the “Statement of Work” attached to the original proposal, the task for the first year was to finish both the primary temperature sensitivity screen and the secondary UV sensitivity screen. These have been completed.

Reference

Figure 1 – Results of secondary assay of three chb mutants identified in the large-scale mutagenesis screen. The percent survival after exposure to 10 J/m² is shown for the parental rad9Δ strain with a control vector or CHES1 and the three chb mutant strains with CHES1.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Growth at 30°C</th>
<th>Survival after 10 J/m² UV irradiation</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \text{rad9Δ, cdc9-8 + Vector} )</td>
<td>-</td>
<td>7.0%</td>
</tr>
<tr>
<td>( \text{rad9Δ, cdc9-8 + CHES1} )</td>
<td>++</td>
<td>20.5%</td>
</tr>
<tr>
<td>( \text{rad9Δ, cdc9-8, chb13 + CHES1} )</td>
<td>-</td>
<td>8.9%</td>
</tr>
<tr>
<td>( \text{rad9Δ, cdc9-8, chb57 + CHES1} )</td>
<td>+</td>
<td>6.7%</td>
</tr>
<tr>
<td>( \text{rad9Δ, cdc9-8, chb16 + CHES1} )</td>
<td>-</td>
<td>15.1%</td>
</tr>
</tbody>
</table>

Table 1 – Summary of both primary and secondary screens for mutants obtained in the mutagenesis screen.
Figure 2 – Current Model of CHES1 Dependent Checkpoint Pathway.