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# INCISION OF UV IRRADIATED DNA IN YEAST



Progress Report

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Louise Prakash

University of Rochester School of Medicine
Rochester, NY 14642

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## ABSTRACT

Our objectives are to determine the details of the incision step of excision repair following exposure to UV light in the eucaryotic organism, the yeast *Saccharomyces cerevisiae*. We have focused on three of the ten genes involved in this process - *RAD1*, *RAD3*, and *RAD10*. We have purified RAD3 protein to near homogeneity, and it possesses single-stranded DNA-dependent ATPase activity and DNA helicase activity which translocates in the 5' -> 3' direction. Site-directed mutagenesis was used to change amino acid residues in a region thought to be required for nucleotide binding and the biological effects of these mutations determined. RAD1 protein is also being purified and RAD10 protein has been purified to near homogeneity. We can now begin to examine RAD10's role in excision repair. A conserved pattern of antisense overlapping transcription has been noted in the yeast *RAD10* gene region and in the homologous human *ERCC-1* gene. Analysis of the overlapping genes in this region has been carried out. The human RAD3 homolog ERCC-2 is also being studied. Since defective DNA repair and enhanced neoplasia characterize several human genetic diseases, a thorough understanding of the molecular mechanisms of DNA repair may provide a better understanding of the causes of carcinogenesis.


Time or effort devoted to project: The principal investigator has devoted 50% of effort on this project since the beginning of the current term of the agreement and will devote 50% of her effort on this project during the remainder of the current term.

Goals of the project: These have not changed since the beginning of the three year project, which deals with the characterization of three *RAD* genes and their encoded proteins involved in the incision step of excision repair following expsoure to UV light in the eucaryotic organism, the yeast *S. cerevisiae*. Specifically, the aims for the three year project period are: **(1) The *RAD3* gene and its protein** - (a) to characterize the activities of the purified RAD3 protein and various rad3 mutant proteins, (b) to determine the essential function of the *RAD3* gene, (c) to use immunoprecipitation, affinity chromatography of proteins binding to a RAD3 column, and the genetic approach of pseudoreversion to identify genes and characterize the encoded proteins which interact with RAD3 **(2) The *RAD10* gene and its protein** - (a) to purify and characterize the RAD10 protein, (b) to raise antibodies to RAD10 protein purified from *E. coli* and use them for regulation and other purposes, (c) to study overlapping transcription in the *RAD10* region, (d) to examine whether *ERCC-1*, the human homolog of the yeast *RAD10* gene, can substitute for *RAD10* in excision repair **(3) The *RAD1* gene and its protein** - (a) to purify and characterize the RAD1 protein of yeast, (b) to raise antibodies to RAD1 protein purified from *E. coli* and use them for various purposes.

A description of the scope of investigations undertaken during the second year of the project and the significant results obtained follows:

### (1) *RAD3* gene and protein

#### (a) Further characterization of RAD3 protein

RAD3 protein contains, near its amino terminus, between amino acids 45 and 49, a glycine rich segment which includes the sequence Gly-X-Gly-Lys-Thr, features that are characteristic of the Walker 'type A' consensus sequence present in various proteins that bind and hydrolyze ATP, including the *E. coli* DNA replication, recombination, and repair proteins DnaA, DnaB, RecA, RecB, UvrA, and UvrD. To examine the functional significance of the invariant lysine and the positive charge of this lysine in ATP binding, ATP hydrolysis, and in DNA helicase activity, we mutated Lys-48 in the RAD3 protein to the similarly charged arginine. We overproduced the protein encoded by the *rad3 Arg-48* mutant gene in yeast and purified it to apparent homogeneity. The biochemical properties of the purified rad3 mutant protein were compared to those of RAD3 wild-type protein. The rad3 Arg-48 protein retained the ability to bind

ATP but lacked ATPase and DNA helicase activities. Yeast strains bearing a genomic *rad3 Arg-48* allele were viable but as sensitive to UV radiation as the well characterized *rad3-1* and *rad3-2* mutants. These observations highlight the involvement of this lysine residue in the hydrolysis of ATP and indicate that the positive charge on arginine can replace that of the lysine residue in the binding of ATP but not in its hydrolysis. The *rad3 Arg-48* mutant is apparently defective in a step subsequent to incision at the damage site in DNA; it can incise UV damaged DNA, but does not remove pyrimidine dimers. The results of these studies have been published in the EMBO J.

(b) Studies with *ERCC-2*, the human homolog of *RAD3*

We have begun a collaborative study with Dr. Larry Thompson, Lawrence Livermore Laboratory, Livermore, CA, on the human homolog of *RAD3*, the *ERCC-2* gene. The protein encoded by the *ERCC-2* gene shows over 50% identity with the yeast RAD3 protein (Thompson, personal communication). When conserved residues are taken into account, there is over 70% homology between the two proteins. This high degree of conservation during evolution indicates that DNA repair proteins play a significant role in the cell. Dr. Thompson has provided us with a cDNA clone of *ERCC-2* lacking the first two amino acids residues and containing a 5' intron, since that was the largest cDNA clone obtained by them. We have almost completed the construction of a fusion of the promoter of the highly expressed, constitutive alcohol dehydrogenase I gene of yeast, *ADC1*, and the open reading frame (ORF) of the *ERCC-2* gene. Since it was necessary to reconstruct the 5' terminus of the ORF, many steps have been required to generate the final product.

**(2) *RAD10* gene and protein**

(a) Purification and characterization of RAD10 protein

We had overproduced RAD10 protein in yeast by placing the *RAD10* open reading frame under the control of the yeast *ADC1* promoter, the strong promoter used routinely in our laboratory for overproducing yeast proteins. Antisera raised against a ρ-RAD10 hybrid protein were used in immunoblot analyses for identification of the RAD10 protein in yeast extracts and for monitoring RAD10 protein during its purification on various chromatographic matrices. RAD10 protein was very difficult to purify because of its instability. However, Dr. Patrick Sung in the group, who successfully purified and characterized RAD3 protein, was finally able to purify RAD10 protein to near homogeneity. The yield of RAD10 is lower than the yield of RAD3; however, we can now begin to examine the role of RAD10 in excision repair.

(b) Analysis of overlapping transcription in *RAD10* region DNA

Last year, we summarized results from our laboratory concerning overlapping transcription in *RAD10* region DNA. We had found that the 3' ends of a 1.9 kb antisense transcript overlap the 3' ends of the 3 *RAD10* encoded transcripts (whose sizes are 1.0, 1.5, and 1.8 kb) by over several hundred nucleotides. The human *ERCC-1* gene, whose encoded protein shows homology with the yeast *RAD10* gene, also shows a similar pattern of overlapping transcription. Since naturally occurring antisense trnascription is rare in *S. cerevisiae* and humans (this is the first exmaple in human cells), these findings indicate that antisense transcription in the *ERCC-1-RAD10* gene regions represents an evolutionarily conserved feature. A manuscript synthesizing these findings has been published in Molecular and Cellular Biology in collaboration with the laboratory of Dr. Dirk Bootsma of Erasmus University in Rotterdam, the Netherlands.

During the past year, we have continued our analysis of the overlapping 3' and 5' antisense genes, both of which were sequenced. The overlapping 3' antisense gene has a 1575 base open reading frame encoding a protein of 525 amino acids with a calculated molecular weight

of 59,505. The *RAD10* open reading frame and the 3' antisense gene open reading frame overlap by one codon. Sequence analyses of the protein encoded by the overlapping 3' antisense gene show no homology with any proteins in the data bank. We have constructed genomic deletions of both the overlapping 3' and 5' genes as single mutations and find that the deletion haploids are no more sensitive to UV, ionizing radiation, or methyl methanesulfonate (MMS) than the wild type. A double deletion combination of both the overlapping 5' and 3' genes may show sensitivity to UV, ionizing radiation, or MMS.

(c) Studies with *ERCC-1*, the human homolog of *RAD10*

We have constructed fusions between the *S. cerevisiae ADC1* promoter and the *ERCC-1* open reading frame. These fusions will be tested for expression of ERCC-1 protein in yeast by using anti-ERCC-1 antibodies.

**(3) *RAD1* gene and protein**

The purification of RAD1 protein from yeast is currently being carried out by standard chromatographic techniques. Antibodies generated against a $\lambda P_L$ promoter - ρ protein - RAD1 protein fusion expressed in *E. coli* are being used to verify that the correct band as detected on SDS-polyacrylamide gels is being purified from yeast extracts prepared from strains carrying a RAD1 overexpressing plasmid. Once RAD1 protein has been purified, we will examine its biochemical properties.

**Description of plans for the coming year:**

**(1) *RAD3* gene and protein and its human homolog, *ERCC-2***

Biochemical studies of the yeast RAD3 and mutant rad3 proteins will be carried out to further our understanding of RAD3's role in excision repair.

We have fused the open reading frame of the human *ERCC-2* gene to the promoter of the highly expressed, constitutive alcohol dehydrogenase gene *ADC1* of *S. cerevisiae*. The ERCC-2 protein will be purified from yeast cells carrying the ERCC-2 overproducing plasmid, and characterized for its biochemical properties. This should enable us to compare the properties of the RAD3 and ERCC-2 proteins. We will also determine whether the *ERCC-2* gene complements the excision repair and viability defects of *rad3* excision defective and *rad3* deletion mutants, respectively.

We will raise antibodies against the ERCC-2 protein by fusing *ERCC-2* to the $\lambda P_L$ promoter and gel purifying the overexpressed protein from *E. coli*. The antibodies will be used to examine the expression of ERCC-2 in yeast cells and for identifying ERCC-2 protein during its purification from yeast cells.

**(2) *RAD10* gene and protein**

The yeast RAD10 protein will be characterized biochemically. We will examine its role in excision repair by determining whether RAD10 protein binds to DNA, whether it shows specificity for UV irradiated DNA, whether it has ATPase activity, exonuclease activity, endonuclease activity, etc.

Further analysis of the overlapping genes located 5' and 3' to *RAD10* will be carried out. A genomic deletion of the 5' overlapping gene coupled with the 3' overlapping gene will be

constructed and the properties of the double mutant determined. Response of the double mutant to UV, ionizing radiation, and MMS will be examined.

We will determine whether the *ERCC-1* gene can complement the excision repair defect of the *rad10* mutation. *rad10* mutants will be transformed with *ADC1::ERCC-1* fusion plasmids and examined for UV sensitivity and excision repair.

### (3) *RAD1* gene and protein

RAD1 protein will be purified from yeast and its biochemical properties determined, as described above for RAD10 protein. Combinations of RAD1, RAD3, and RAD10 proteins will be tested for their ability to incise UV irradiated DNA. If no activity is found, the proteins encoded by the other genes involved in excision repair will be added, as we purify them in our laboratory.

### (4) Other genes involved in excision repair in yeast

Thus far, our laboratory has cloned and characterized to different extents 7 of the 10 genes involved in excision repair in yeast. During the coming year, we will concentrate on cloning the three remaining genes - *RAD14, RAD16,* and *MMS19*. It is important for us to have all the genes involved in excision repair and their encoded proteins if we are to successfully reconstitute the incision activity *in vitro*.

**PUBLICATIONS** (since the last continuation application was submitted in June, 1988)

Sung, P., D. Higgins, L. Prakash, and S. Prakash (1988) Mutation of lysine-48 to arginine in the yeast RAD3 protein abolishes its ATPase and DNA helicase activities but not the ability to bind ATP. EMBO J. 7: 3263-3269.

van Duin, M., J. van den Tol, J. H. J. Hoeijmakers, D. Bootsma, I. P. Rupp, P. Reynolds, L. Prakash, and S. Prakash (1989) Conserved pattern of antisense overlapping transcription in the homologous human *ERCC-1* and yeast *RAD10* DNA repair gene regions. Mol. Cell. Biol. 9: 1794-1798.

Prakash, L. and S. Prakash (1989) Excision repair genes of *Saccharomyces cerevisiae*. In Cellular Responses to DNA Damage. Ann. Ist. Super. Sanità. 25:99-114.

Copies of the above articles are included with the Progress Report.