Characterization of the *RAD10* Gene of *Saccharomyces cerevisiae* and Purification of Rad10 Protein†

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ABSTRACT: The *RAD10* gene of *Saccharomyces cerevisiae* is one of at least five genes required for damage-specific incision of DNA during nucleotide excision repair. This gene was previously cloned and sequenced [Weiss, W. A., & Friedberg, E. C. (1985) *EMBO J. 4*, 1575-1582; Reynolds et al. (1985) *EMBO J. 4*, 3549-3552]. In the present studies, we have mapped one major and three minor transcriptional start sites in the *RAD10* gene. The locations of these sites relative to the translational start codon are remarkably similar to those previously identified in the yeast *RAD2* gene [Nicolet et al. (1985) *Gene 36*, 225-234]. The two genes also share common sequences in these regions. However, in contrast to *RAD2* [Robinson et al. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 1842-1846], *RAD10* is not induced following exposure of cells to the DNA-damaging agent 4-nitroquinoline 1-oxide. Native Rad10 protein and also two different Rad10 fusion proteins are rapidly degraded in most *Escherichia coli* strains. However, following overexpression of the cloned *RAD10* gene in yeast, native Rad10 protein was purified to >90% homogeneity. A catalytic function has not been identified for the purified protein. *RAD10* cells (untransformed with the cloned gene) contain fewer than 500 molecules per cell. This is similar to the levels of the UvrA, UvrB, and UvrC nucleotide excision repair proteins in *E. coli*.

The excision of bulky base adducts from DNA is a complex biochemical process referred to as nucleotide excision repair (Friedberg, 1985). At present, the biochemistry of this process is best understood in the prokaryote *Escherichia coli*. In this organism, both genetic and biochemical studies have demonstrated that the products of three genes (*uvrA*, *uvrB*, and *uvrC*) are required for damage-specific recognition and incision of DNA in vitro (Weiss & Grossman, 1987; Sancar & Sancar, 1988). Additionally, the *uvrD* and *polA* genes are required for postincision events that include turnover of the UvrABC protein complex, release of oligonucleotides containing base damage, and repair synthesis (Weiss & Grossman, 1987; Sancar & Sancar, 1988).

In the yeast *Saccharomyces cerevisiae*, at least 10 genes are involved in nucleotide excision repair, 5 of which (*RAD1*, *RAD2*, *RAD3*, *RAD4*, and *RAD10*) are absolutely required for damage-specific incision of DNA (Friedberg, 1988; Friedberg et al., 1990). The *RAD1*, *RAD2*, *RAD3*, and *RAD4* genes are expected to encode polypeptides of calculated molecular weights ranging between 87K and 126K (Friedberg, 1988). The *RAD10* gene is considerably smaller, and the size of the coding region predicts a polypeptide of M_r ~24.1K (Reynolds et al., 1985).

Examination of the predicted amino acid sequence of the cloned *RAD10* gene, as well as searches of several data bases for homology with other proteins, has not suggested a biochemical function for Rad10 protein. Understanding the role of this protein in nucleotide excision repair is therefore predicated on its purification and biochemical characterization. To this end, we have mapped the *RAD10* transcriptional start sites and have tailored the cloned *RAD10* gene into expression vectors for overexpression in *E. coli* and in yeast. Rad10 protein is highly unstable in *E. coli*. However, the protein has been highly purified following overexpression in yeast.

Materials and Methods

E. coli and Yeast Strains and Plasmids. The *E. coli* strains HB101 (*recA*) and TG-1 (*recA*$^+$) are from our laboratory stocks. Strains AR120 (*recA*$^+$) and AR68 (*recA*$^+$ *hptR*) (both carrying phage λ $C1_{857}$) were from Dr. Martin Rosenberg, Smith Kline Beckman. The *E. coli* plasmids pIN-III-ompA-3 (Ghrayeb et al., 1984), pDR540 (de Boer et al., 1983), and pOTS (Rosenberg et al., 1983) were obtained from Dr. M. Inouye, Rutgers University, Pharmacia LKB Biotechnology, and Dr. Martin Rosenberg, respectively.

The yeast strains LP2817-8D (*MATa rad10-1 leu2-3,112 his3-1 trp1-289 ura3-52*), SF657-2D (*MATa pep4-3 leu2-3, 112 his4 ura3-52 gal2*), and BJ2168 (*MATa prc1-407 prb1-1122 pep4-3 leu2 trp1 ura3-52*) were obtained from Dr. Louise Prakash, University of Rochester, Dr. Stan Fields, SUNY at Stonbrook, and the Yeast Genetic Stock Center, Berkeley, CA, respectively. Strain BJ2168ΔRAD10 is a derivative of BJ2168 carrying a deletion of the *RAD10* gene and was constructed as previously described (Weiss & Friedberg, 1985). The yeast shuttle plasmid pG12 (Nicolet & Friedberg, 1987) was obtained from Dr. Andy Buchman, Stanford University.

RAD10 Plasmid Constructions. A 1.1-kb *HpaI*-*PvuII* fragment containing the *RAD10* gene and flanking DNA was cloned into the *SmaI* site of the phage vector M13mp10. RFI DNA was restricted with *EcoRI* (which cleaves the construct 5′ to the *RAD10* insert) and treated with BAL31 nuclease for varying periods. BAL31 digests were blunt-ended by incubation with T4 DNA polymerase in the presence of the 4-deoxyribonucleoside triphosphates. Addition of *HindIII* linkers (5′CAAGCTTG3′; New England Biolabs) followed by

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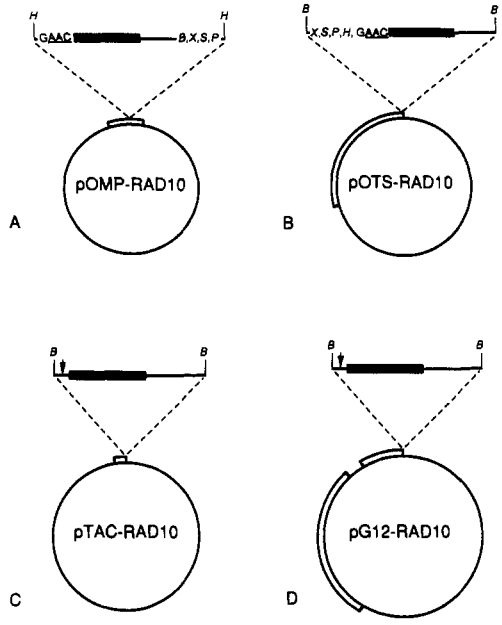

FIGURE 1: Diagrammatic representation of plasmids used for overexpression of the *RAD10* gene in *E. coli* (A–C) and yeast (D). Plasmids were constructed as described under Materials and Methods. The letters in italics represent M13 polylinker restriction sites: B, *Bam*HI; X, *Xba*I; S, *Sal*I; P, *Pst*I; H, *Hin*dIII. The solid bars represent the entire 210-codon *RAD10* coding region (C and D) or the carboxy-terminal 208 codons (A and B). In (A) and (B), the second (AAC) codon of the *RAD10* coding region is shown (underlined), as is the guanine (G) nucleotide immediately 5′, introduced with the *Hin*dIII linker. The arrows in (C) and (D) indicate the position of the major *RAD10* transcription start site shown in Figure 4. Open bars represent *ompA* gene sequences (A), λ sequences (B), the *tac* promoter (C), the *GAL1* promoter [shorter bar in (D)], and the *GAL4* gene [longer bar in (D)].

cleavage with *Hin*dIII generated a series of *RAD10* 5′ deletion constructs containing the M13mp10 polylinkers at the 3′ end of the inserts. These inserts were then cloned into the *Hin*dIII site of the vector M13mp11, thereby generating *RAD10* 5′ deletion constructs in which the M13 polylinker was reiterated at both ends of the gene. Various 5′ deletion end points were identified by DNA sequencing as previously described (Weiss & Friedberg, 1985).

The vectors pBa1+4 and pBa1-44, containing deletions which end at positions +4 and −44 relative to the *RAD10* translation start site, were used for tailoring the *RAD10* gene adjacent to selected heterologous promoters. The deletion in pBal+4 removes the first ATG codon and replaces it with an 8 bp *Hin*dIII linker. The deletion in pBal-44 generates a *Hin*dIII site 5′ to the transcriptional start sites shown in Figure 4.

Plasmid pOMP-RAD10 (Figure 1A) contains the *Hin*dIII fragment of pBal+4 cloned into the *Hin*dIII site of pIN-III-ompA-3. The resulting plasmid contains the *ompA* promoter, translational start codon, and signal sequence fused to the terminal 209 codons of the 210-codon *RAD10* open-reading frame. Plasmid pOTS-RAD10 (Figure 1B) contains the *Bam*HI fragment of pBal+4 cloned into the *Bam*HI site of pOTS. mRNA produced from this plasmid is expected to initiate translation from the λ *cII* start codon and continue through 11 in-frame sense codons from the M13mp11 vector polylinker and through the 209 terminal *RAD10* codons. It is expected to express a fusion protein slightly larger than native Rad10 protein.

Plasmid pTAC-RAD10 (Figure 1C) contains the *Bam*HI fragment of pBal-44 cloned into the *Bam*HI site of pDR540. mRNA produced from this plasmid is expected to bind to the *E. coli* ribosome using the Shine–Delgarno-like sequence which begins at position −19 in the *RAD10* 5′ untranslated region (Weiss & Friedberg, 1985). Plasmid pG12-RAD10 (Figure 1D) contains the *Bam*HI fragment of pBal-44 cloned into the *Bam*HI site of pG12. Plasmid pGem3-RAD10 (not shown) contains the *Sal*I fragment of pBal-44 cloned into the *Sal*I site of pGem3 (Promega), in the orientation such that the sense strand is transcribed from the SP6 promoter.

Growth of E. coli and Yeast Cells. *E. coli* strains AR120 and AR68 transformed with plasmid pOTS-RAD10 were grown to OD_{600} = 1 at 30 °C in L broth plus ampicillin (50 μg/mL), and expression of the *RAD10* gene was induced by adding an equal volume of L broth preheated to 65 °C. The cultures were then incubated at 42 °C for varying periods of time, and cells were harvested. Induction of the plasmids pOMP-RAD10 and pTAC-RAD10 using the lactose analogue isopropyl thiogalactoside (IPTG) was as described (Nicolet & Friedberg, 1987). Expression of *RAD10* from the *GAL1* promoter of the plasmid pG12-RAD10 is constitutive because this plasmid also contains the *GAL4* gene (Nicolet & Friedberg, 1987).

For large-scale growth of yeast cells for protein purification, strain SF657-2D transformed with plasmid pG12-RAD10 was grown at 30 °C in 1-L starter cultures containing yeast nitrogen base (0.17%) with added ammonium sulfate (0.5%), dextrose (4%), histidine (20 μg/mL), and uracil (40 μg/mL). The starter cultures were inoculated into 10 L of this medium in a Chemap AG fermenter and grown at 30 °C to OD_{600} = 2. Cultures grown under these experimental conditions typically yielded 30–50 g of yeast paste.

Transcriptional Analysis. To examine induction of the *RAD10* gene, cells were treated with the DNA-damaging agent 4NQO as previously described (Robinson et al., 1986), and *RAD10* transcripts were measured at 0, 30, and 60 min after treatment. Extraction of total RNA from uninduced and induced cells and S1 nuclease mapping of *RAD10* transcriptional start sites were carried out as previously described (Robinson et al., 1986). The DNA probe used was a 0.7-kb *Pvu*II–*Ava*II fragment containing the 5′ end of the cloned *RAD10* gene (Weiss & Friedberg, 1985) and was radiolabeled at the *Ava*II site with alkaline phosphatase and T4 polynucleotide kinase as described (Cobianchi & Wilson, 1987). Hybridization of the probe was carried out with 100 μg of total RNA. The optimal hybridization temperature (43 °C) and S1 nuclease concentration (1500 units/mL) were determined empirically.

Gel Electrophoresis and Measurement of Proteins. SDS–polyacrylamide gel electrophoresis of proteins was as previously described (Laemmli, 1970). Gels were stained with Coomassie blue (Sigma technical bulletin MWS-877PSB) or silver nitrate (Merril et al., 1983). Protein determinations were performed by the method of Bradford (1976) using bovine serum albumin as a standard.

Western Blotting. Western blotting was carried out as previously described (Nicolet et al., 1985). After reaction with antisera, staining was performed using alkaline phosphatase or horseradish peroxidase coupled secondary antibodies and appropriate substrates (Vector Systems, Burlingame, CA). Immunodot blot assays were carried out in the same way except that samples were loaded directly onto nitrocellulose paper (Schleicher & Schuell) using a Micro-Sample filtration manifold (Schleicher & Schuell).

Production and Affinity Purification of Rad10 Antisera. *E. coli* AR68 cells transformed with plasmid pOTS-RAD10 were disrupted by sonication in 67 mM sodium phosphate

buffer (pH 7.5). The lysate was centrifuged, and the cell pellet was resuspended in 0.2% sarkosyl and recentrifuged. The insoluble fraction was solubilized in 1% sarkosyl and electrophoresed through a 12% SDS–polyacrylamide gel. Rad10 protein was identified by Coomassie blue staining as a prominent band at M_r ~25K. This band was cut out of the gel as a slice, homogenized in phosphate-buffered saline, and injected into rabbits after harvesting preimmune serum. The rabbits were boosted after 4 weeks, and immune sera were collected 10–14 days later.

Sera were cleared by precipitation in 40% ammonium sulfate followed by resuspension in phosphate-buffered saline and dialysis. To remove nonspecific antibodies, this fraction was serially incubated with extracts of untransformed *E. coli* AR68 cells and extracts of a yeast *rad10* deletion mutant, each coupled to cyanogen bromide activated Sepharose. The unbound fraction was then affinity-purified by incubation with a Sepharose-coupled extract of *E. coli* cells transformed with the overexpressing plasmid pOTS-RAD10. The Sepharose matrix was harvested by low-speed centrifugation and was incubated in 0.2 M glycine, pH 2.5, for 5 min. Following further low-speed centrifugation, the supernatant was neutralized with 1 M potassium phosphate, pH 9.0, and dialyzed against phosphate-buffered saline.

In Vitro Transcription and Translation of Rad10 Protein. In vitro transcription of pGem3-RAD10 and in vitro translation of the resulting RNA were performed with reagents from Promega according to the manufacturer's recommendations.

Immunoprecipitation of Rad10 Protein Translated in Vitro. Immunoprecipitation of Rad10 protein prepared by in vitro transcription/translation of the cloned *RAD10* gene was performed essentially as described (Anderson & Blobel, 1983), except that anti-IgG–agarose (Sigma) was used as the precipitant. For competition experiments, a 100-fold excess of unlabeled purified Rad10 protein (fraction IV) was added.

Purification of Rad10 Protein. (A) Crude Extracts. Yeast cells (100 g) transformed with plasmid pG12-RAD10 were suspended in 100 mL of buffer A (10 mM Hepes buffer, pH 7.5, 10 mM sodium bisulfite, 1 mM EDTA, 0.1% β-mercaptoethanol, and 10% glycerol) containing 0.3 M NaCl, 1 mM PMSF, 2 mM benzamidine, 40 μM leupeptin, and 12 μM pepstatin A. Cells were disrupted with a BioSpec bead beater, using 10 pulses of 30 s and a 2-min interval between each pulse to allow cooling. The resulting extract was centrifuged at high speed (40 000 rpm for 1 h) in a Beckman L8 ultracentrifuge using a 60Ti rotor. The supernatant is designated fraction I.

(B) Hydroxyapatite Chromatography. Typically, 100 mL of fraction I was adjusted to a conductivity equal to that of buffer A containing 0.3 M NaCl and pumped onto a hydroxyapatite column (20 × 2.5 cm) via a small G25 Sephadex column (5 × 1.5 cm). The latter served to remove particulate material not sedimented by ultracentrifugation. The hydroxyapatite column was equilibrated in buffer A containing 0.3 M NaCl and was washed with the same buffer at a flow rate of 15 cm/h. Under these conditions, Rad10 protein was recovered in the flow-through volume. This material was dialyzed overnight against 5 volumes of buffer A (fraction II).

(C) Phosphocellulose Chromatography. Fraction II was applied to a phosphocellulose column (12 × 2.5 cm) equilibrated in buffer A with 0.05 M NaCl. After the column was wahsed with 3 bed volumes of this buffer, a linear NaCl gradient (0.05–0.5 M) was applied in 200 mL of buffer A at a flow rate of 15 cm/h. Rad10 protein was detected with Rad10 antisera on dot blots (Figure 2) or by silver staining

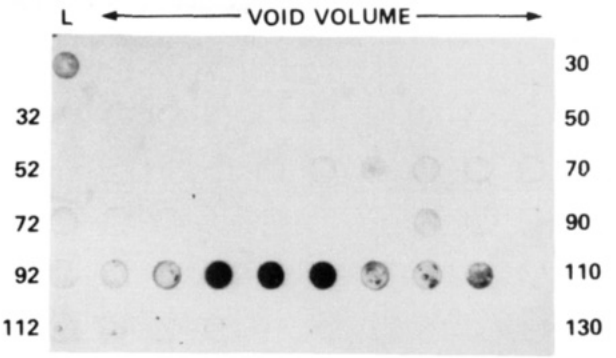

FIGURE 2: Identification of Rad10 protein by Western dot-blot analysis following phosphocellulose chromatography. Fraction II (25 mg of protein) was loaded onto a phosphocellulose column and eluted as described under Materials and Methods. Aliquots (10 μL) of individual fractions were tested against affinity-purified anti-Rad10 antibodies as described in the text. L = sample of the loaded fraction II. The first 30 fractions represent the void volume. Fractions 30–130 represent elution of the phosphocellulose column. Rad10 protein was detected in fractions 96–108.

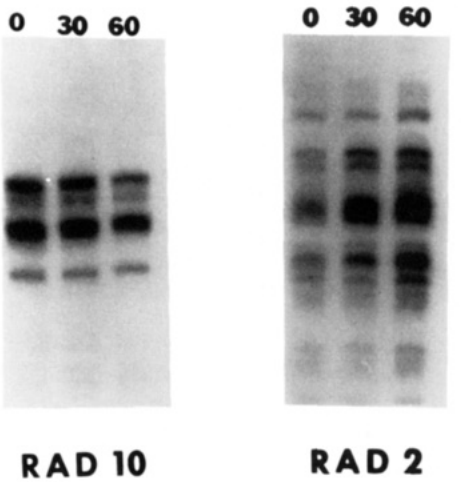

FIGURE 3: *RAD10* gene has multiple transcriptional start sites and is not DNA damage-inducible. Cells either were untreated (0 min) or were treated with 0.25 μg/mL 4NQO for 30 or 60 min as described under Materials and Methods. Isolation of total RNA and S1 nuclease mapping of *RAD10* transcripts were as described previously (Robinson et al., 1986). The *RAD10* DNA probe used was a 0.7-kb *Pvu*II–*Ava*II fragment containing the 5′ end of the *RAD10* gene. One major and three minor *RAD10* transcripts are detected. The steady-state level of *RAD10* transcripts is unaffected in cells exposed to 4NQO, in contrast to that of *RAD2* transcripts.

of SDS–polyacrylamide gels after electrophoresis of selected fractions. Rad10 protein consistently eluted with a sharp peak at 0.13 M NaCl. Peak fractions were pooled (fraction III).

(D) Blue Sepharose Chromatography. Fraction III was adjusted to a conductivity equal to buffer A containing 0.4 M NaCl and loaded onto a blue Sepharose column (8.0 × 0.9 cm) equilibrated in buffer A containing 0.4 M NaCl. The column was washed with 8 bed volumes of the same buffer. A linear NaCl gradient (0.4–1.0 M) was applied in 40 mL of buffer A at a flow rate of 5 cm/h. Rad10 protein eluted in a broad peak centered at 0.75 M NaCl. Peak fractions were pooled (fraction IV).

Results

Transcriptional Analysis of the RAD10 Gene. RAD10 transcriptional start sites were mapped by S1 nuclease protection analysis in untransformed cells. *RAD10* utilizes one major and three minor transcriptional start sites (Figures 3 and 4). The observation that all transcriptional start sites

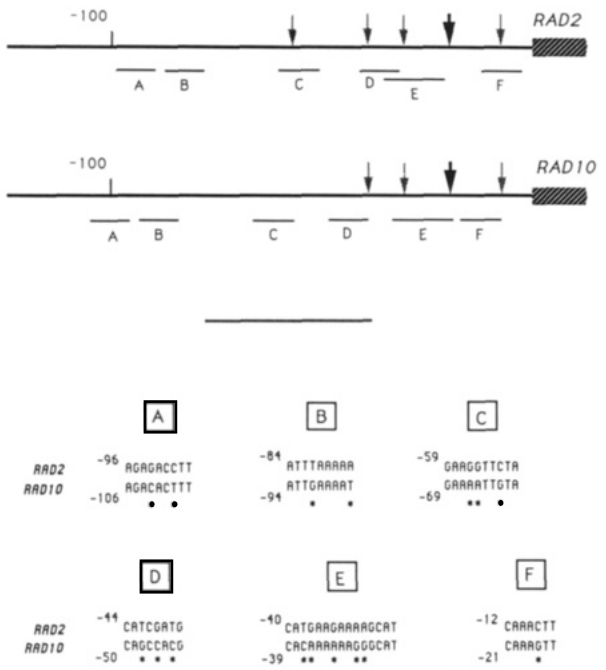

FIGURE 4: Comparison of the *RAD2* and *RAD10* transcriptional start sites. The upstream regions of the *RAD10* and *RAD2* genes are diagrammatically represented. The hatched areas represent the *RAD2* and *RAD10* coding regions and the bold lines the respective upstream noncoding regions. The bold arrows indicate the positions of major transcriptional start sites. The lighter arrows show the mapped positions of minor transcriptional start sites. The lettered bars represent regions of sequence homology between the two genes, which are shown in detail in the lower half of the figure.

are located upstream of the first in-frame ATG codon in the *RAD10* open-reading frame confirms the identification of this triplet as the translational start codon. Additionally, the observed pattern of transcriptional start sites indicates that the *RAD10* promoter extends at least 45 bp 5′ to the initiating ATG codon.

The relative locations of these sites are remarkably similar to those previously mapped in the *RAD2* gene (Figure 4). Additionally, the 5′ noncoding regions of the *RAD10* and *RAD2* genes contain clusters of related sequences situated at comparable distances from the start of the coding regions, in which overall ~72% of the nucleotides compared are identical (Figure 4). However, in contrast to the *RAD2* gene which shows an increase in the steady-state level of mRNA when cells are exposed to a variety of DNA-damaging agents (Madura & Prakash, 1986; Robinson et al., 1986; Siede et al., 1989), treatment of cells with the DNA-damaging agent 4NQO did not result in quantitative or qualitative changes in *RAD10* transcripts 30 or 60 min after treatment (Figure 3).

Overexpression of Rad10 Protein in E. coli. Initial experiments were directed at overexpressing Rad10 protein in *E. coli*, with a view to using this material as an immunogen for raising specific polyclonal antisera in rabbits, and potentially for preparative purification of the protein. A series of 5′ deletions was generated in the cloned *RAD10* gene to remove the endogenous promoter, and selected *RAD10*-deletion constructs were fused with three different heterologous promoters (*ompA*, *tac*, and λ P_L; see Materials and Methods for details).

Plasmid pOMP-RAD10 was transformed into *E. coli* HB101 cells, and cultures were induced with IPTG for varying periods of time. The optical density of cultures of induced cells declined rapidly, whereas uninduced cultures transformed with this plasmid, and induced cultures transformed with the vector

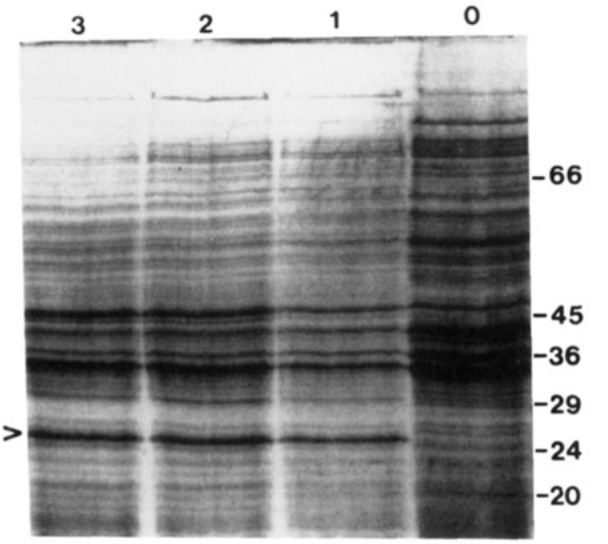

FIGURE 5: Expression of Rad10 protein in *E. coli*. *E. coli* strain AR68 was transformed with plasmid pOTS-RAD10, and cells were thermally induced for 0, 1, 2, or 3 h (numbers over the lanes). Cells were harvested, and the insoluble fraction of crude lysates was extracted with 1% sarkosyl. These extracts were run on a 12% SDS–polyacrylamide gel, and the gel was stained with Coomassie blue. Rad10 protein (arrow) is present in all extracts in which expression of the *RAD10* gene was induced.

alone, grew normally. Additionally, no novel protein species was detected by PAGE of extracts of induced cells. These observations suggest that the OmpA-Rad10 fusion protein is toxic when overexpressed in *E. coli*.

We attempted to overexpress native Rad10 protein from a different construct carrying the regulatable *tac* promoter (pTAC-RAD10). Cytotoxicity was not detected with cells transformed with this plasmid. However, examination of cell extracts by PAGE again failed to reveal detectable Rad10 protein under various conditions of cell growth and induction. Similar results were obtained following examination of extracts of induced cells pulse-labeled with [^{35}S]methionine. Collectively, these experiments suggest that multiple different forms of Rad10 protein are rapidly degraded in *E. coli*.

Phage λ cII protein is also extremely labile when overexpressed in *E. coli* and can only be detected in certain protease-defective strains, including one designated AR68 (M. Rosenberg, personal communication). This strain is temperature-sensitive for protease activity and also carries a prophage with a temperature-sensitive mutation in the λ repressor.

E. coli strain AR68 was transformed with plasmid pOTS-RAD10. As indicated above (see Materials and Methods), this plasmid is expected to encode a fusion protein slightly larger than native Rad10. A novel protein species of the expected molecular weight (~25.4K) was readily observed in extracts of thermally induced cells (Figure 5). This protein was detectable by PAGE within 1 h after induction, at levels representing a few percent of the total cell protein. However, the protein was present exclusively in the insoluble fraction of crude extracts and required extraction with 1.0% sarkosyl for solubilization.

Despite the apparent stability of this Rad10 fusion protein in strain AR68, when plasmid pTAC-RAD10 (containing the complete *RAD10* coding region) was transformed into this strain, native Rad10 protein was not detected in either soluble or insoluble fractions under any conditions tested. Hence, the use of a fusion protein was necessary to achieve Rad10 overexpression in *E. coli*, and we have been unable to overexpress native Rad10 protein in this organism. The inability to over-

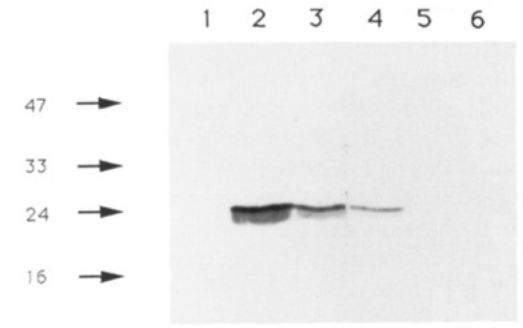

FIGURE 6: Sensitivity and specificity of anti-Rad10 antibodies. Western analysis of samples separated on a 12% SDS–polyacrylamide gel. Lane 1, 2.5 μL of nuclease-treated rabbit reticulocyte lysate. Lane 2, 2.5 μL of reticulocyte lysate programmed with 10 μg/mL in vitro transcribed *RAD10* RNA. Lane 3, 20 ng of purified Rad10 (fraction IV). Lane 4, 40 ng of fraction II from yeast cells carrying the plasmid pG12-RAD10 (~2 ng of Rad10 protein). Lane 5, 20 μg of fraction II from yeast cells expressing normal levels of Rad10 protein. On the original blot, a faint band is visible in this lane. Lane 6, 20 μg of fraction II from a RAD10 deletion strain carrying the pG12 plasmid vector. The arrows show the electrophoretic position of prestained polypeptide standards from Bio-Rad (lysozyme, 16 kDa; soybean trypsin inhibitor, 24 kDa; carbonic anhydrase, 33 kDa; ovalbumin, 47 kDa).

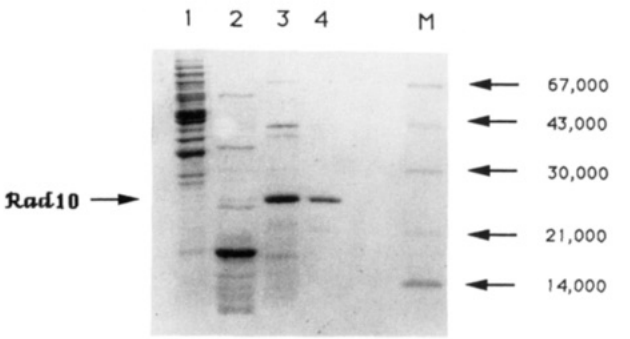

FIGURE 7: Electrophoretic analysis of Rad10 purification. Ten micrograms each of fractions I–IV (lanes 1–4) was precipitated in 5% trichloroacetic acid, run on a 12% SDS–polyacrylamide gel, and stained with Coomassie blue dye. Molecular weight markers are indicated on the right. The position of Rad10 protein is indicated by the arrow.

express small foreign proteins in *E. coli* except as fusion proteins has been observed by others (Wetzel & Goeddel, 1983).

Antibodies to Rad10 Protein. The Rad10 fusion protein expressed from plasmid pOTS-RAD10 was isolated from the insoluble fraction of *E. coli* AR68 extracts and was used as an immunogen to raise polyclonal antisera in rabbits (see Materials and Methods for details). The sensitivity and specificity of the resulting affinity-purified (see Materials and Methods) antiserum were demonstrated in several experiments. First, the antiserum did not react with any proteins in extracts of a yeast strain deleted of the *RAD10* gene and carrying the plasmid vector used for overexpression (Figure 6, lane 6), nor with any proteins in uninduced *E. coli* AR68 cells carrying the pOTS-RAD10 plasmid (data not shown). Second, the affinity-purified antiserum reacted specifically with nanogram amounts of Rad10 protein prepared by in vitro transcription/translation from a plasmid carrying only the cloned *RAD10* gene under control of the SP6 promoter. This immunoreactivity was demonstrated both by immunoblotting (Figure 6, lanes 1 and 2) and by immunoprecipitation (data not shown) of rabbit reticulocyte extracts with and without translated Rad10 protein. Rad10 antisera also did not cross-react with Rad1, Rad2, or Rad3 proteins overexpressed in yeast, nor with UvrA, UvrB, UvrC, or UvrD excision repair proteins overexpressed in *E. coli*.

Overexpression of Rad10 Protein in Yeast. To overexpress Rad10 protein in yeast, the *RAD10* gene was cloned into a yeast plasmid vector containing a 2-μm circle replication origin, the yeast *GAL1* promoter, the *GAL4* gene, and the *leu2d* selectable marker (see Materials and Methods). This construct (pG12-RAD10) was shown to complement the UV sensitivity of a mutant *rad10* strain, indicating that it expresses functional Rad10 protein in yeast. Following transformation of the protease-defective strain SF657-2D, both soluble and insoluble fractions of disrupted cells contained a protein of M_r ~24K which reacted with affinity-purified anti-Rad10 antibodies (data not shown).

Purification of Rad10 Protein. No catalytic activity has yet been associated with Rad10 protein. Hence, a functional assay could not be used for fractionation of yeast cell extracts. In initial experiments, we used a dot-blot immunoassay in which fractions were tested for reactivity with affinity-purified antiserum containing anti-Rad10 antibodies (Figure 2). We subsequently established that during later stages of purification Rad10 protein can be readily identified by its molecular weight (24K) in SDS gels, and by its distinctive orange color when stained with silver nitrate.

Table I: Rad10 Purification

fraction	purification step	total protein mg	total protein %
I	crude extract	1134.0	100.0
II	hydroxyapatite chromatography	23.4	2.1
III	phosphocellulose chromatography	0.78	0.07
IV	blue Sepharose chromatography	0.23	0.02

SDS–PAGE of fraction IV shows a single major band of calculated M_r ~24K (Figure 7). This material has the identical electrophoretic mobility as a protein generated by in vitro translation of RNA transcribed from a vector carrying only the cloned *RAD10* gene. Additionally, both polypeptides react specifically with affinity-purified antiserum containing antibodies specific for Rad10 protein (see above; Figure 6). Finally, the protein in fraction IV competes with radiolabeled Rad10 protein generated by in vitro transcription/translation for binding to anti-Rad10 antibodies. On the basis of these results, we conclude that the purified material in fraction IV is yeast Rad10 protein. Staining of gels with Coomassie blue or silver nitrate indicates that Rad10 protein is >90% pure.

There is considerably more Rad10 protein in 40 ng of fraction II from the Rad10-overexpressing strain than in 20 μg of fraction II from an isogenic untransformed strain expressing normal levels of Rad10 (Figure 6, lane 4 vs lane 5). Hence, the level of overexpression achieved by the plasmid pG12-RAD10 is ~1000-fold at a minimum estimate.

The amount of receovered protein in fraction IV represents ~0.02% of the starting amount of total cell protein (Table I). From a series of Western blots using 2-fold serial dilutions of all fractions, we estimate our recovery of Rad10 protein during the purification to be ~2.5% of the starting amount. Hence, despite at least 1000-fold overexpression, Rad10 protein represents only ~0.8% of the total cellular protein. This quantitative result is consistent with that obtained following overexpression of other cloned *RAD* genes in yeast, including *RAD1* (A. J. Cooper and E. C. Friedberg, unpublished data) and *RAD3* (I. Harosh, L. Naumovski, and E. C. Friedberg, unpublished data), and constrasts with the high protein yields obtained following overexpression of the *uvrA*, *uvrB*, and *uvrC* genes of *E. coli* (Yoakum et al., 1982; Yeung et al., 1983; Thomas et al., 1985).

Rad10 protein has weak nonspecific DNA binding affinity and can be eluted from either single- or double-stranded DNA–cellulose with 0.05 M NaCl. However, the purified protein, alone or in combination with purified Rad3 protein, does not bind preferentially to UV-irradiated DNA as determined by gel shift assays previously described (Chu & Chang, 1988). Purified Rad10 protein has no detectable DNA-dependent ATPase activity nor does it stimulate the ATPase or DNA helicase activities of purified Rad3 protein (Harosh et al., 1989). These assays were performed with partially purified Rad10 protein (fraction III), because this fraction is obtained relatively quickly, thus minimizing the likelihood of loss of activity during purification.

Preparations of partially purified Rad10 protein (fraction III) contain an endonuclease activity that converts supercoiled (form I) plasmid DNA to a relaxed (form II) configuration in the presence of 10 mM Mg^{2+}. The endonuclease activity is stimulated in the presence of 10 mM Mn^{2+} or Co^{2+} and is inhibited by NaCl (data not shown). These features differentiate it from previously described yeast endonucleases (Burbee et al., 1988; von Tigerstrom, 1982; Bryant & Haynes, 1978; Pinon & Leney, 1975; Pinon, 1970). The endonuclease can be distinguished from Rad10 protein by several criteria. It is not inhibited by anti-Rad10 antibodies. Furthermore, the activity is present in preparations purified from yeast cells deleted of the chromosomal *RAD10* gene. Finally, the activity elutes from blue Sepharose earlier than Rad10 protein and can be readily separated from Rad10 protein during this purification step.

Discussion

Genetic analysis indicates that nucleotide excision repair in the yeast *S. cerevisiae* is biochemically complex (Friedberg, 1988; Friedberg et al., 1990). At least 10 genes are involved in this process (Friedberg, 1988; Friedberg et al., 1990). Mutagenic inactivation of any of five of these genes (*RAD1*, *RAD2*, *RAD3*, *RAD4*, and *RAD10*) confers a phenotype of defective damage-specific incision of DNA and defective excision of bulky base adducts such as pyrimidine dimers (Friedberg, 1988; Friedberg et al., 1990). These phenotypes are comparable to those of the *uvrA*, *uvrB*, and *uvrC* mutants of *E. coli* (Weiss & Grossman, 1987; Sancar & Sancar, 1988) and suggest that like the UvrABC complex the proteins encoded by these yeast *RAD* genes assemble as a multiprotein complex required for specific recognition and endonucleolytic cleavage of damaged DNA.

Genetic complexity for nucleotide excision repair is evident from studies with other eukaryotes, including mammalian cells. Chinese hamster ovary (CHO) mutants defective in this DNA repair mode fall into eight distinct genetic complementation groups (Thompson et al., 1988), and the hereditary human repair-defective disease xeroderma pigmentosum is also represented by at least eight genetic complementation groups (Cleaver et al., 1983). The human excision repair genes *ERCC1* and *ERCC2*, which complement CHO mutants from genetic complementation groups 1 and 2, respectively, show amino acid sequence similarities with the yeast *RAD10* (van Duin et al., 1986) and *RAD3* (C. A. Weber and L. H. Thompson, personal communication) genes, suggesting that eukaryotic genes for nucleotide excision repair are conserved and that yeast is an informative eukaryotic paradigm for studying this aspect of DNA metabolism.

The *RAD1*, *RAD2*, *RAD3*, *RAD4*, and *RAD10* genes have been isolated by molecular cloning (Friedberg, 1988). Consideration of codon bias and measurement of steady-state mRNA levels and of the levels of several Rad/β-galactosidase fusion proteins indicate that these genes are weakly expressed (Friedberg, 1988). This conclusion is supported by the results of Western analysis with extracts of untransformed cells probed with antibodies to various Rad proteins (Nicolet & Friedberg, 1987; A. J. Cooper and E. C. Friedberg, unpublished observations), including Rad10. In order to approach the biochemistry of damage-specific recognition and incision of DNA, we have tailored the cloned yeast *RAD* genes into expression vectors designed for overexpression of Rad proteins in *E. coli* and in yeast.

The relative ease of growth and disruption of large quantities of *E. coli* make this organism an attractive vehicle for overexpression of yeast Rad proteins. However, expression of native Rad10 protein in *E. coli* was limited by extreme lability, despite the use of a protease-defective strain. We were able to stably express a Rad10 fusion protein in which the first 10 amino acids are derived from the phage M13mp11 polylinker. This construct replaced three rare codons in this region of the *RAD10* gene with codons abundantly used in *E. coli*. The substitution of rare codons with abundant ones has been observed by others to enhance expression of heterologous genes in *E. coli* (M. Rosenberg, personal communication). Thus, the combination of a protease-defective host and of sequences containing frequently used codons may be important for expression of Rad10 protein in *E. coli*.

Antisera raised against the Rad10 fusion protein reacted with Rad10 protein overexpressed in yeast. The absolute immunospecificity of the antisera is supported by the failure to detect any cross-reacing material in extracts of cells deleted of the *RAD10* gene but carrying the plasmid vector used for overexpression, as well as the demonstration of a positive signal (by immunoblotting) with protein expressed from the cloned *RAD10* gene by transcription/translation in vitro.

These antisera did not cross-react with Uvr proteins involved in nucleotide excision repair in *E. coli* (data not shown). This result is consistent with the lack of amino acid sequence similarity between the *RAD10* and *E. coli* genes and with the failure to observe complementation of *rad10* mutants with cloned *E. coli uvr* genes (Friedberg, 1988).

Overexpression of Rad10 protein in yeast was facilitated by the use of a plasmid construct carrying the yeast *GAL1* promoter under positive regulatory control of the *GAL4* gene. To further enhance expression, we used a multicopy plasmid containing the *leu2d* allele, which results in amplification of plasmid copy number when the growth medium is limited for leucine (Erhart & Hollenberg, 1983). We estimate the extent of overexpression to be greater than 1000-fold, yet we did not observe remarkable differences in the growth of cells transformed with the *RAD10*-containing expression vector and those carrying the vector alone, indicating that overexpression of Rad10 protein is not overtly toxic to yeast cells. Extracts of yeast cells containing overexpressed Rad10 protein yielded a strong Western signal at the molecular weight expected for Rad10 protein. This immunoreactivity provided a convenient assay for fractionation of the protein and facilitated purification of Rad10 protein to >90% purity.

Identification of the purified material as yeast Rad10 protein is confirmed by the demonstration of identical electrophoretic mobility and specific immunoreactivity (both by Western analysis and by immunoprecipitation) with a polypeptide generated by transcription of the cloned *RAD10* gene in vitro and translation of the transcript using a well-characterized rabbit reticulocyte system.

Overexpressed Rad10 protein represents ~0.8% of the total cell protein. The extent of overexpression is >1000-fold. Thus, Rad10 constitutes <0.001% of the total cell protein when expressed at wild-type levels. This corresponds to less than 500 molecules per cell. Assuming that each damage-specific recognition/incision complex formed in yeast contains a single molecule of Rad10 protein, each complex must patrol ~30 kb of the yeast genome.

The number of UvrA, UvrB, and UvrC molecules per *E. coli* cell have been estimated at 20, 200, and 10, respectively (Sancar, 1987). As the size of the *E. coli* genome is about one-fifth that of yeast, both organisms face patrolling problems of a similar magnitude. This may provide one explanation for the possible linkage of nucleotide excision repair to transcription in both prokaryotes and eukaryotes (Bohr et al., 1985; Mellon & Hanawalt, 1989). Transcriptional linkage could reduce the search space for the nucleotide excison repair complex and might provide a directed one-dimensional search mechanism for locating sites for DNA damage processing.

At present, no biochemical function has been identified for Rad10 protein. This result is not totally unexpected. Purified *E. coli* UvrB and Uvr C proteins have no catalytic activity, and UvrB protein does not bind to damaged DNA in isolation (Weiss & Grossman, 1987; Sancar & Sancar, 1988). However, this protein has a high affinity for purified UvrA protein (Weiss & Grossman, 1987; Sancar & Sancar, 1988), and it has recently been suggested that when bound to DNA a UvrBC protein complex constitutes a catalytically active endonuclease (Orren & Sancar, 1989).

In yeast, specific recognition of bulky base damage and specific incision of DNA at or near sites of such damage are transacted by the Rad1, Rad2, Rad3, Rad4, and Rad 10 proteins (Friedberg, 1988; Friedberg et al., 1990). To date, only the Rad3 and Rad10 proteins have been extensively purified (Sung et al., 1987a; Harosh et al., 1989). Rad3 protein is known to be an ATPase/DNA helicase (Sung et al., 1987b; Harosh et al., 1989), although the role of this helicase in nucleotide excision repair is unclear. The present studies provide no indication for specific interactions between Rad3 and Rad10 proteins. Conceivably, Rad10 protein interacts with one or more of the other Rad proteins referred to above. Under any circumstances, the elucidation of the precise role of this protein in nucleotide excision repair in yeast must await the purification and characterization of all five Rad proteins of interest.

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Substrate Specificity of Recombinant Human Renal Renin: Effect of Histidine in the P_2 Subsite on pH Dependence

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ABSTRACT: Steady-state kinetic analysis of human renin demonstrates the histidine proximal to the substrate scissile peptide bond contributes to the unique specificity and pH dependence of this aspartyl protease. Recombinant human renal renin purified from mammalian cell culture appears to be indistinguishable from renin isolated from human kidney with respect to specific activity (1000 Goldblatt units/mg). Recombinant renin contains carbohydrate covalently attached to asparagines at positions 5 and 75 (renin numbering) and disulfide linkages at Cys-51/Cys-58, Cys-217/Cys-221, and Cys-259/Cys-296. Renin pH dependence was evaluated between pH 4.0 and 8.0 by using a synthetic substrate identical with the amino terminus of porcine angiotensinogen (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-*His*-Leu*Leu-Val-Tyr-Ser, where the asterisk indicates the scissile peptide bond and the proximal histidine is in italics) and an analogous tetradecapeptide where the proximal histidine was substituted with glutamine. Comparison of the pH profiles shows the catalytic efficiency (V/K_m) and maximal velocity (V) of renin are greater above pH 6.5 with the substrate containing histidine proximal to the scissile peptide bond, but below pH 5.0 these parameters are greater with the glutamine substrate analogue. Solvent isotope effects show that proton transfer contributes to the rate-limiting step in catalysis with both substrates and that the proximal histidine does not serve as a base in the catalytic mechanism. Molecular modeling indicates the substrate histidine could hydrogen bond to Asp-226 of the enzyme (renin numbering), thus perturbing the ionization of the catalytic aspartyl groups (Asp-38 and Asp-226). This enzyme-substrate complex would enable the proximal histidine to "direct" catalysis and account for the activity of renin at physiological pH, which is uncommon among structurally homologous cellular aspartyl proteases. Thus, interactions in the renin-substrate complex rather than amino acid substitutions between renin and cellular aspartyl proteases appear to account for renin activity at physiological pH.

Renin (EC 3.4.23.15) is a plasma aspartyl protease that regulates the initial step in the production of the potent pressor octapeptide angiotensin II (Ondetti & Cushman, 1982). Renin selectively cleaves the decapeptide angiotensin I from the amino terminus of the α_2-globulin angiotensinogen. The angiotensinogen specificity and strategic role of renin in the regulation of blood pressure make renin an attractive target for inhibitors that could be powerful antihypertensive agents.

Despite the interest in renin inhibitors, little is known about the catalytic mechanism of renin relative to other proteases. Pepstatin inhibition (Aoyagi et al., 1972) and specific modification with diazoacetyl-D,L-norleucine methyl ester (Inagami et al., 1974; McKown & Gregerman, 1975) and 1,2-epoxy-3-(*p*-nitrophenoxy)propane (Misono & Inagami, 1980) have established that renin is functionally homologous to aspartyl proteases. Cloning of the human renin gene (Imai et al., 1983) and its expression in mammalian cell culture (Carilli et al., 1988) have permitted X-ray diffraction studies of the enzyme (Sielecki et al., 1989). The crystal structure demonstrates human renal renin is structurally homologous to cellular aspartyl proteases. However, the structure-function relationships that account for renin's restricted substrate specificity and preference for neutral pH relative to cellular aspartyl proteases remain undefined.

The pH dependence of recombinant human renal renin has been evaluated with a synthetic tetradecapeptide identical with the amino terminus of porcine angiotensinogen (PTDP,[1] Table I) and an analogous substrate (HP2Q, Table I), where glut-

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[1] Abbreviations: PTDP, porcine tetradecapeptide (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu-Leu-Val-Tyr-Ser); HP2Q, tetradecapeptide of sequence Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-Gln-Leu-Leu-Val-Tyr-Ser; P_2-His, histidine in the P_2 position of PTDP or angiotensinogen; K_m Michaelis constant; V, maximal velocity; V/K_m, catalytic efficiency; PMSF, phenylmethanesulfonyl fluoride; SBTI, soybean trypsin inhibitor; BSA, bovine serum albumin; TFA, trifluoroacetic acid; HPLC, high-pressure liquid chromatography; MES, 2-(*N*-morpholine)ethanesulfonic acid; v, initial rate of product formation; S, substrate concentration; γ, kinetic parameter (V/K_m or V); c, pH-independent value of the kinetic parameter; K_a, dissociation constant for a group that ionizes at low pH; K_b, dissociation constant for a group that ionizes at high pH; CHO, Chinese hamster ovary; $^D(V/K_m)$, ratio of V/K_m determined in H_2O to V/K_m determined in D_2O; $^D(V)$, ratio v determined in H_2O to V determined in D_2O.

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