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EXPRESSION OF *RAD4* GENE OF *SACCHAROMYCES CEREVISIAE* THAT CAN BE PROPAGATED IN *ESCHERICHIA COLI* WITHOUT INACTIVATION

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SUMMARY: The *RAD4* gene of *Saccharomyces cerevisiae*, which is essential for the nucleotide excision repair, was isolated from a yeast genomic library and the expression of this gene has been investigated. *RAD4* mRNA was approximately 2.3 kb and did not contain intervening sequence, as determined by S1 nuclease mapping and Northern blot analysis. Transcription start site was located at 48 bp upstream of the ATG initiation codon. *RAD4* gene is not induced by UV light damages as indicated by the absence of change in mRNA level after UV exposure in wild type yeast cells. The size of Rad4 protein overexpressed in *Escherichia coli* was found to be 89 kDa by SDS-PAGE. This is consistent with the size of the gene's ORF, which encodes 730 amino acids with the calculated molecular weight of 84456. The *RAD4* protein contains many potential kinase dependent phosphorylation sites and its C-terminus is highly acidic like other DNA repair proteins of *S. cerevisiae*.

The *RAD4* gene of *Saccharomyces cerevisiae* is one of the five genes (*RAD1, RAD2, RAD3, RAD4 and RAD10*) absolutely required for the specific incision of damaged DNA during nucleotide excision repair [1]. These five genes were isolated by phenotypic complementation of appropriate rad mutants with a yeast genomic library [2-6]. Fleer *et al.* [7] independently isolated a recombinant plasmid containing *RAD4* gene by gap repair method. These plasmids, however, were observed to be deleted or rearranged when propagated in *Escherichia coli*.

In our previous reports, we have demonstrated that our cloned *RAD4* gene [5] was able to be propagated in *E. coli* without any genetic change and that it has the same restriction map as wild type yeast chromosomal DNA [8]. We also showed that this *RAD4* gene was genetically and physically mapped to the *RAD4* locus on chromosome V [8]. Recently, we determined the

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Abbreviations: ORF, open reading frame; SDS-PAGE, sodium dodesyl sulfate-polyacrylamide gel electrophoresis; IPTG, isopropylthio-β-D-galactoside.

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nucleotide sequence of this gene [9] and also identified *RAD4*-like gene in *Schizosaccharomyces pombe* [10].

At present, however, the extent of regulation of this gene expression and the functional role of its gene product are unknown. As a first step in elucidation of such, we have analyzed *RAD4* mRNA and its UV inducibility in yeast and identified its gene product in *E. coli*.

MATERIALS AND METHODS

Strains and vectors. *S. cerevisiae* wild-type strain LP2693-21B was used for transformation and isolation of total RNA. *E. coli* BL21/DE3 strain was used for expression of the cloned *RAD4* gene. pPC1, the recombinant plasmid harboring the isolated *RAD4* gene [5] and its derivertives [8] were used throughout this investigation.

Transcript mapping. To determine the size of *RAD4* transcript, yeast RNA from LP2693-21B Rad^+ strain was hybridized with the 3.4 kb *Bgl*II DNA fragment of pPC100 containing *RAD4* gene [8]. The hybridized molecules were digested with S1 nuclease (1000 U/ml), electrophoresed and transferred to nitrocellulose paper. The S1 nuclease-protected hybrids were then hybridized again with the same fragment labelled with [γ-^{32}P]ATP (3000Ci/mmole, Amersham Corp.) by T4 polynucleotide kinase (Phamacia). To map the *RAD4* transcription start site, the 1.7 kb *Bgl*II-*Pvu*II fragment [8], which contains the promoter and N-terminal region of the protein, was [γ-^{32}P]ATP-labelled at its 5' end. After DNA-RNA hybridization, the sizes of S1 nuclease-protected DNA fragments were determined on 5% DNA sequencing gel [11].

UV-irradiation. Newly inoculated cells were grown in YPD or selection medium to appropriate densities, irradiated with 50J/m^2 of 254 nm UV-light (1.42 J/m^2/sec) and further incubated for the indicated period time [5].

Dot blot analysis. RNA isolated from the Rad^+ yeast strain at various postincubation times after UV-irradiation was denatured and applied to nitrocellulose filter prewetted with 1M ammonium acetate using a 96-well manifold apparatus. The 1.2 kb *Pvu*II fragment which is the internal sequence of the *RAD4* gene [8], was labelled with [α-^{32}P]dCTP (3,000 Ci/mmole) and used as probe with the subsequent DNA-RNA dot blot analysis as described [10].

Construction of *RAD4* gene expression vector. To overproduce the Rad4 protein in *E. coli*, the 2.5 kb fragment of PPC100 [8] was introduced into the overexpression vector pET3a, which contains a T7 promoter ϕ10. Two plasmids constructs (pKC101 and pKC102) were generated with an opposite orientation relative to ϕ10 promotor. *E. coli* BL21/DE3 cells, which provide an inducible T7 RNA polymerase under control of the lac/UV5 promoter were transformed with these recombinant plasmids [12].

Protein electrophoresis. The transformed cells were grown in M9 medium containing 100 μg/ml ampicillin at 37°C. Rad4 protein synthesis was induced in the presence of 0.5 Mm IPTG and the products were separated on SDS-polyacrylamide gels using 6 to 14% discontinuous buffer system as described [13].

RESULTS AND DISCUSSION

Transcript analysis of *RAD4* gene

We have previously shown that the subcloned *RAD4* gene was localized within the 2.5 kb DNA fragment flanked by *Bgl*II and *Bam*HI sites in the recombinant plasmid pPC106 [8].

From the S1 nuclease mapping of *RAD4* transcript, we observed a single RNA transcript of approximately 2.3 kb without any intervening sequence (Fig. 1) and this was also confirmed by Northern blot analysis (data not shown). The *RAD4* transcript size was in good accordance with the size (2190 bp) of the ORF of the cloned *RAD4* gene [9]. The S1-resistant fragment was found to be 760 nucleotides in length, indicating that the transcription start site is located at 48 nucleotides upstream of the first ATG codon within the ORF (Fig. 2).

UV inducibility of *RAD4* gene

Although several DNA damage inducible genes have been isolated from *S. cerevisiae*, it is not known whether *RAD* genes belong to this class [15]. To determine whether the *RAD4* gene is induced by UV light, wild type yeast cells were irradiated with UV light and incubated for up to 4 hours. Total RNA extracted from these cells were blotted on nitrocellulose paper and hybridized with the radiolabelled 1.2 kb *Pvu*II DNA fragment which is the internal sequence of the *RAD4* gene. This slot blot analysis revealed that the transcript levels did not increase as a function of postincubation time after UV irradiation (Fig. 3). This non UV inducibility of *RAD4* gene was further confirmed by *RAD4-lacZ* fusion studies (data not shown).

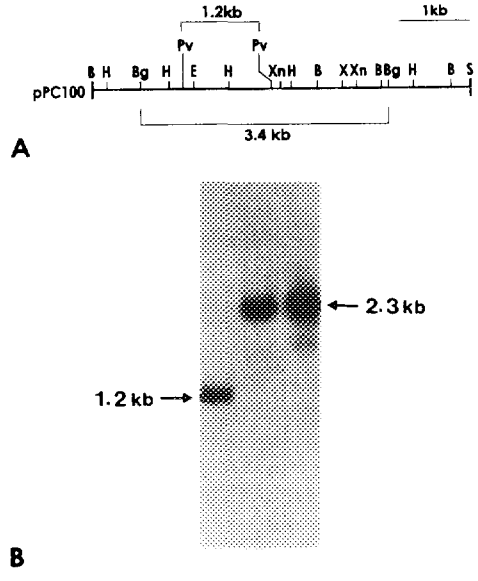

Fig. 1. Determination of the *RAD4* transcript size. Isolated yeast total RNA was hybridized with the 3.4 kb *Bgl*II DNA fragment containing the *RAD4* gene and digested with S1 nuclease (1000 U/ml). The S1-protected hybrids were hybridized with [γ-^{32}P]ATP- labeled 3.4 kb DNA probe. (A) The restriction enzyme sites of pPC100 and the 3.4 kb *Bgl*II fragment used as probe. (B) Lane 1 shows the 1.2 kb band, which is the *Pvu*II DNA fragment hybridized with 3.4kb *Bgl*II DNA probe. Lanes 2 and 3 show the transcript size of the *RAD4* gene determined to be 2.3 kb.

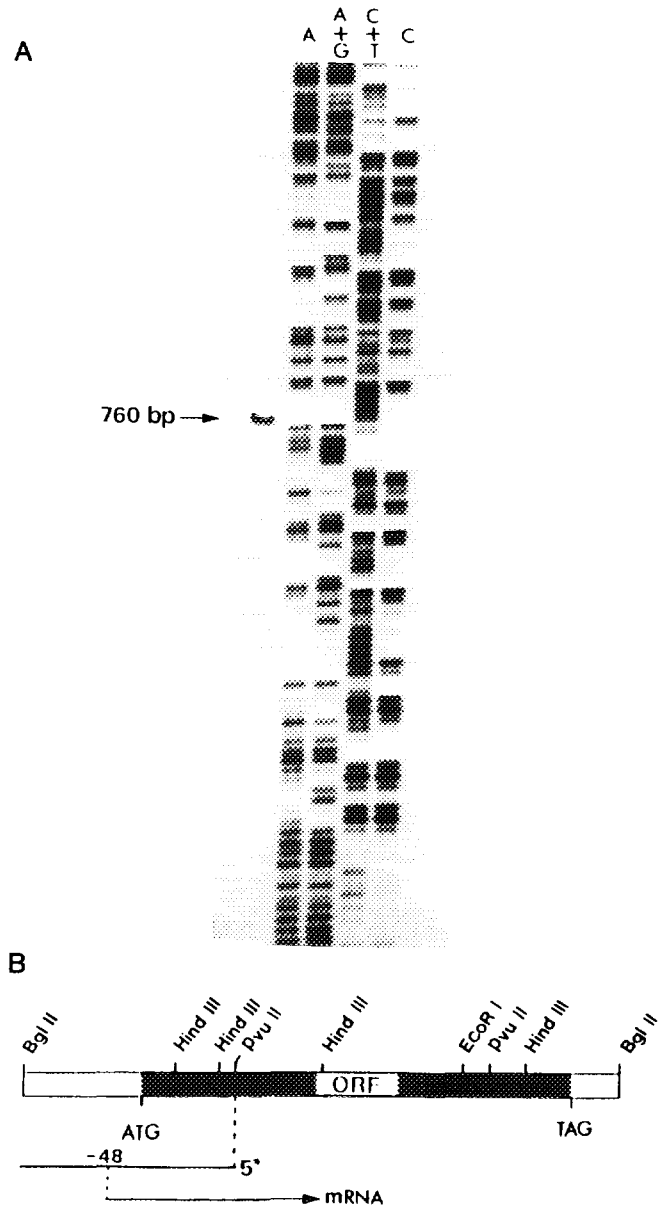

Fig. 2. Determination of the 5' terminus of the *RAD4* transcript. (A) The 5' end of *RAD4* transcript was mapped by S1 nuclease protection analysis using the 1.7 kb *Bgl*II-*Pvu*II 5' end-labelled fragment. The length of the protected fragment was determined by polyacrylamide gel electrophoresis. The position of the protected DNA fragment is indicated by the arrow. (B) The initiation and termination codons of the ORF are shown on the restriction map. The 5' end-labelled 1.7 kb *Bgl*II-*Pvu*II fragment used as probe and transcription initiation site are shown in the lower part.

Among the repair-related genes, the transcript levels of *CDC9* and *RAD2* were elevated after UV irradiation [16-17]. Our present results indicate that the *RAD4* gene is not UV inducible as the other genes in *RAD3* epistatic group except for *RAD2* gene.

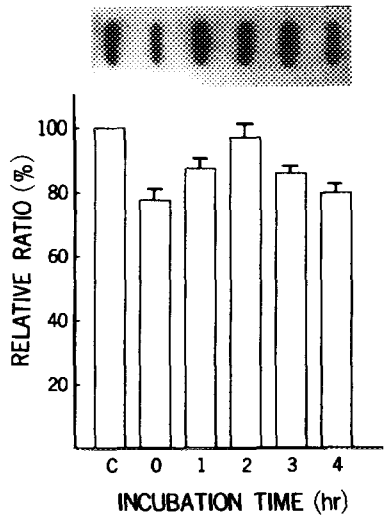

Fig. 3. Time course of *RAD4* transcript levels after UV-irradiation. RNA samples were prepared at various postincubation times after UV-irradiation. Slot blots of RNA from UV-irradiated and nonirradiated control cells were hybridized with an [α-^{32}P]dCTP-labelled *RAD4* probe. The relative ratio is the densitometric scanning value in UV-treated cells divided by the corresponding value in untreated control cells.

Expression of *RAD4* gene

In the *RAD3* epistatic group, only the biochemical activity of *RAD3* gene has been identified. The Rad3 protein, with a size of 89.7 kDa, exhibits 5' to 3' DNA helicase and single-stranded DNA-dependent ATPase activities [6, 18, 19] and has considerable homology with a human repair gene, ERCC2 [20].

An 89 kDa protein band was observed only in cells harboring pKC101 plasmid with the *RAD4* ORF in correct orientation (Fig. 4). This size is consistent with the calculated molecular weight of 84456 based on the ORF of the gene which encodes 730 amino acids [9].

Choi *et al.* [9] have shown that the Rad4 protein has a high content of basic residues, containing 15.9% basic, 9.4% acidic, 31.6% polar, and 43.1% nonpolar amino acid residues. The Rad4 protein contains many potential protein kinase phosphorylation sites as screened by the computer PC gene program (Intelligenetics). These potential phosphorylation sites are mainly localized in amino acid position at 17-26 and 609-615 residues. These results suggest that the Rad4 protein might play a role in modulating the main repair enzyme activity by kinase dependent phosphorylation-dephosphorylation.

With such results, the biochemical activities and functional role of *RAD4* gene are beginning to be elucidated in our laboratory by purification of Rad4 protein and complementation studies with other repair deficient mutants.

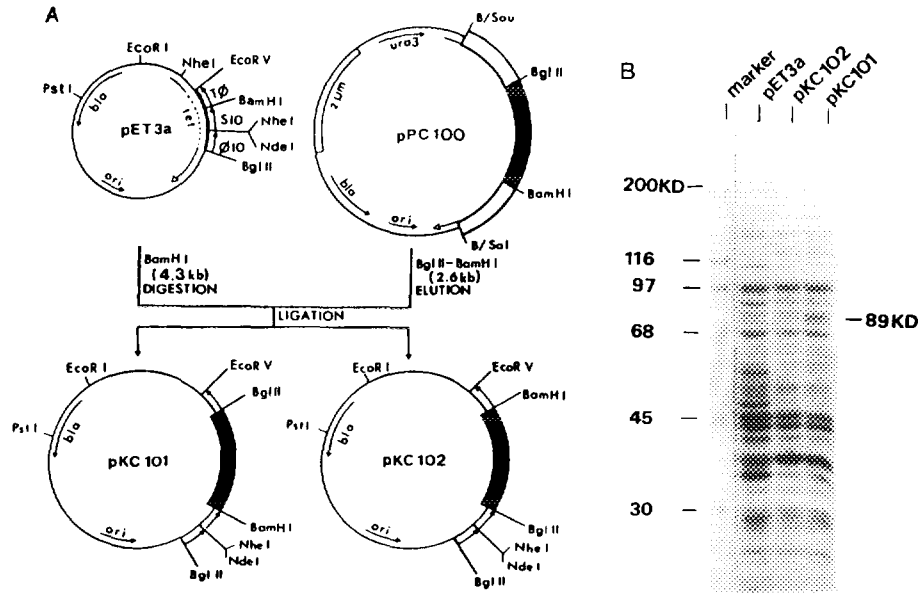

Fig. 4. Expression of Rad4 protein in *E. coli* BL21/DE3 cell. (A) The 2.5 kb *Bgl*II-*Bam*HI DNA fragment of *RAD4* gene was introduced into the *Bam*HI site of pET3a vector under the control of ϕ10 promoter for T7 RNA polymerase and designated as pKC101. Plasmid pKC102 was constructed by the integration of entire *RAD4* DNA with reverse orientation. (B) Host *E. coli* BL21/DE3 cells transformed with pET3a, pKC101 and pKC102 were grown in the presence of IPTG. Crude extracts of each samples were subjected to 6 to 14% SDS-PAGE and staining with Coomassie blue. The arrow indicates the position of newly synthesized 89 kDa Rad4 protein (Lane 4). Lane 1, molecular weight size marker (kDa); Lane 2, crude extracts of cells harboring plasmid pET3a; Lane 3, pKC102; Lane 4, pKC101.

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REFERENCES

1. Wilcox, D. R., and Prakash, L. (1981) J. Bacteriol. 148, 618-623.
2. Higgins, D. R., Prakash, S., Reynolds, P., and Prakash, L. (1983) Gene 26, 119-126.
3. Higgins, D. R., Prakash, L., Reynolds, P., and Prakash, S. (1984) Gene 30, 121-128.
4. Weiss, W.A., and Friedberg, E. C. (1985) EMBO J. 4, 1575-1582.
5. Yoon, J. K., Choi, I. S., Huh, S. O., Choe, S. Y., and Park, S. D. (1985) Korean J. Genet. 7-3, 97-104.
6. Reynolds, P., Higgins, D. R., Prakash, L., and Prakash, S. (1985) Nucl. Acids Res. 13, 2357-2372.
7. Fleer, R. C., Nicolet, C. M., Pure, G. A., and Friedberg, E. C. (1987) Mol. Cell. Biol. 7, 1180-1192.
8. Choi, I. S., Kim, J. B., Lee, K. N., and Park, S. D. (1990a) Photochem. Photobiol. 5, 395-400.
9. Choi, I. S., Kim, J. B., and Park, S. D. (1990b) Nucl. Acids Res. 18, 7137.
10. Choi, I. S., Kim, J. B., Hong, S. H., and Park, S. D. (1991) FEMS Microbiol. Lett. 77, 97-100.
11. Sharp, P. A., Berk, A. T., and Berget, S. M. (1980) Meth. Enzymol. 65, 750-768.

12. Rosenberg, A. M., Lade, B. N., Chui, D., Lin, S., Dunn, J. J., and Studier, W. (1987) Gene 56, 125-135.
13. Laemmli, U. K. (1970) Nature 227, 680-685.
14. Studier, F. W., and Moffatt, B. A. (1986) J. Mol. Biol. 189, 113-130.
15. McClanahan, T., and McEntee, K. (1986) Mol. Cell. Biol. 4, 2356-2363.
16. Peterson, T. A., Prakash, L., Prakash, S., Osley, M. A., and Reed, S. I. (1985) Mol. Cell. Biol. 5, 226-235.
17. Madura, K., and Prakash, S. (1986) J. Bacteriol. 166, 914-923.
18. Sung, P., Prakash, L., Weber S., and Prakash, S. (1987a) Proc. Natl. Acad. Sci. U.S.A. 84, 6045-6049.
19. Sung, P., Prakash, L., Matson, S. W., and Prakash, S. (1987b) Proc. Natl. Acad. Sci. U.S.A. 84, 8951-8955.
20. Weber, C. A., Salazar, E. P., Stewart, S. A., and Thompson, L. H. (1990) EMBO J. 9, 1437-1447.