The EMBO Journal vol.13 no.22 pp.5361–5369, 1994

RAD26, the functional *S.cerevisiae* homolog of the Cockayne syndrome B gene *ERCC6*

Alain J.van Gool, Richard Verhage[1], Sigrid M.A.Swagemakers, Pieter van de Putte[1], Jaap Brouwer[1], Christine Troelstra, Dirk Bootsma and Jan H.J.Hoeijmakers[2]

MGC Department of Cell Biology and Genetics, Erasmus University Rotterdam, PO Box 1738, 3000 DR, Rotterdam and [1]Laboratory of Molecular Genetics, Leiden Institute of Chemistry, Gorlaeus Laboratories, Leiden University, PO Box 9502, 2300 RA, Leiden, The Netherlands

[2]Corresponding author

Communicated by D.Bootsma

Transcription-coupled repair (TCR) is a universal sub-pathway of the nucleotide excision repair (NER) system that is limited to the transcribed strand of active structural genes. It accomplishes the preferential elimination of transcription-blocking DNA lesions and permits rapid resumption of the vital process of transcription. A defect in TCR is responsible for the rare hereditary disorder Cockayne syndrome (CS). Recently we found that mutations in the *ERCC6* repair gene, encoding a putative helicase, underly the repair defect of CS complementation group B. Here we report the cloning and characterization of the *Saccharomyces cerevisiae* homolog of *CSB/ERCC6*, which we designate *RAD26*. A *rad26* disruption mutant appears viable and grows normally, indicating that the gene does not have an essential function. In analogy with CS, preferential repair of UV-induced cyclobutane pyrimidine dimers in the transcribed strand of the active *RBP2* gene is severely impaired. Surprisingly, in contrast to the human CS mutant, yeast *RAD26* disruption does not induce any UV-, cisPt- or X-ray sensitivity, explaining why it was not isolated as a mutant before. Recovery of growth after UV exposure was somewhat delayed in *rad26*. These findings suggest that TCR in lower eukaryotes is not very important for cell survival and that the global genome repair pathway of NER is the major determinant of cellular resistance to genotoxicity.

Key words: Cockayne syndrome/*ERCC6*/*RAD26*/trancription-coupled repair

Introduction

Nucleotide excision repair (NER) is one of the major pathways by which DNA damage is removed from the genome. This system is able to recognize and repair a remarkably broad spectrum of structurally unrelated lesions in a multistep reaction, first elucidated for the UvrABC repair system in *Escherichia coli* (for comprehensive reviews see Sancar and Sancar, 1988; Van Houten, 1990). A complex of UvrA and UvrB is able to bind to DNA and presumably translocates along the helix. Upon encountering a lesion such as a UV-induced cyclobutane pyrimidine dimer (CPD), a conformational change is induced in the DNA by the stable attachment of UvrB. UvrC binds to this UvrB–DNA pre-incision complex after which an incision is introduced in the damaged strand at the eighth phosphodiester bond 5′ and the fourth or fifth phosphodiester bond 3′ of the damaged nucleotide. UvrD/helicase II removes the oligonucleotide containing the damage and, through gap-filling synthesis by DNA polymerase I and ligase, the integrity of the DNA is restored. The mechanism of NER in eukaryotes may have basic similarities with that in *E.coli*. In mammals, an incision on both sides of the lesion is also found, resulting in the excision of a oligonucleotide of 29 bp (Huang *et al.*, 1992). A sophisticated sub-pathway of NER, transcription-coupled repair, quickly targets the repair machinery to genes that are actively transcribed by RNA polymerase II (Bohr, 1991; Leadon and Lawrence, 1991). This process is responsible for the rapid removal of possible transcription-blocking lesions from the transcribed strand, while the non-transcribed strand is repaired at a slower rate similar to the genome overall (Mellon *et al.*, 1987). Transcription-coupled repair of CPD was found to be conserved from *E.coli* to yeast and mammalian cells (Mellon *et al.*, 1987; Mellon and Hanawalt, 1989; Smerdon and Thoma, 1990). The identification of factors that play a role in transcription-coupled repair is the key to the elucidation of the mechanism of this conserved pathway. In *E.coli* it has been resolved to a considerable detail. A transcription repair coupling factor (TRCF) was isolated that is necessary and sufficient for transcription-coupled repair in a defined *in vitro* system (Selby *et al.*, 1991). TRCF, encoded by the *mfd* gene, is able to recognize and displace a stalled RNA polymerase and lead the UvrABC complex to the site of the lesion presumably via its affinity for the damage recognition subunit UvrA (Selby and Sancar, 1993).

The phenotypic effects of a transcription-coupled repair deficiency in humans are illustrated by the hereditary disorder Cockayne syndrome (CS). CS patients display photosensitivity, impaired physical, sexual and mental development, severe neurological dysfunction, and a wizened appearance. Remarkably, no elevated risk for skin cancer is noted (Nance and Berry, 1992). CS cells still perform normal genome overall repair but they specifically lack the transcription-coupled repair of active genes (Venema *et al.*, 1990; Van Hoffen *et al.*, 1993). Complementation analysis has revealed that at least two genes are involved in the classical form of CS (Tanaka *et al.*, 1981; Lehmann, 1982). Recently we found mutations in the *ERCC6* gene to be responsible for the repair defect of CS complementation group B cells (Troelstra *et al.*, 1992). Thus, CSB/ERCC6 is the first eukaryotic protein identified

© Oxford University Press

that is selectively involved in transcription-coupled repair, and could represent the functional counterpart of the *E.coli* TRCF protein, although significant amino acid sequence homology is absent.

A high degree of conservation of DNA repair genes and repair pathways is found in eukaryotes (reviewed in Hoeijmakers, 1993). However, among the NER-deficient members of the *Saccharomyces cerevisiae* RAD3 epistasis group no mutant has yet been isolated that is selectively disturbed in transcription-coupled repair (Prakash *et al.*, 1993). Here we describe the isolation of the *CSB/ERCC6* homolog of *S.cerevisiae*, designated *RAD26*, and the characterization of the corresponding disruption mutant, that shows a similar defect in transcription-coupled repair to that identified in CS. Surprisingly, the *rad26* mutant displays no increased sensitivity to UV light, cisplatin and X-irradiation, indicating a minor contribution of transcription-coupled repair to survival of yeast cells after genotoxic treatment.

Results

Cloning of the S.cerevisiae homolog of CSB/ERCC6

Southern Zoo blot analysis using genomic DNA from various species, including *S.cerevisiae*, with the human *CSB/ERCC6* cDNA as a probe suggested that this gene is conserved from higher to lower eukaryotes (data not shown). On this basis we decided to attempt cloning of the *S.cerevisiae CSB/ERCC6* homolog using cross-hybridization to the human cDNA probe. We utilized the junction fragment strategy, which is based on the rationale that homology over extended regions is likely to be meaningful. To detect such regions the *CSB/ERCC6* cDNA was divided into three flanking but non-overlapping segments corresponding to the N-terminal, middle and C-terminal parts of the protein (Figure 1A). These probes were hybridized under low stringency conditions to identical Southern blots of *S.cerevisiae* genomic DNA. Autoradiographic exposure of the membrane was extended up to 5 weeks to reveal the weakly hybridizing fragments. Figure 1C indicates that the probe of the middle part hybridizes to a 3.6 kb *Eco*RI and a 4.5 kb *Pst*I fragment (lanes 3 and 9). The latter fragment was also weakly recognized by the N-terminal probe (lane 7), suggesting homology within one *S.cerevisiae* fragment to both the N-terminus and the helicase region of *CSB/ERCC6*. The C-terminal probe did not hybridize to any particular fragment (lanes 5 and 11). These findings suggest that the middle part as well as the N-terminus are sufficiently conserved to recognize a common fragment and that conservation of the C-terminus may be insufficient.

While screening a *S.cerevisiae* genomic phage library using human *CSB/ERCC6* cDNA probes, we discovered using the BLAST computer algorithm (Altschul *et al.*, 1990), a yeast DNA fragment of 177 bp whose translated protein sequence displays a very high degree of homology (94% similarity) to the region of helicase domain V of the human *CSB/ERCC6* protein. This fragment was localized at the 5′ end of a 1779 bp DNA fragment containing the promoter and the coding region of yeast QH2:cytochrome-*c* oxidoreductase subunit II (Oudshoorn *et al.*, 1987). The homology was restricted to the 177 bp DNA fragment since the QH2 gene and flanking sequences showed no similarity to *CSB/ERCC6*. Both ends of the fragment contained *Sau*3A restriction sites, the enzyme used for the construction of the library from which QH2 was isolated. Therefore it is possible that the 177 bp DNA fragment was artificially ligated upstream of the QH2 gene. The homologous fragment was isolated from genomic *S.cerevisiae* DNA via PCR and the resulting 131 bp PCR product was used as a probe to hybridize to an EMBL3 phage library containing *S.cerevisiae* genomic DNA. The DNA inserts of six different phages were isolated and characterized. From hybridizations with the human *CSB/ERCC6* cDNA we deduced that homology was confined to a 5.2 kb *Sal*I–*Hin*dIII fragment, present in four of the isolated phages (Figure 1B). To examine whether this fragment was derived from the region recognized in the yeast genome by the human *CSB/ERCC6* cDNA, fragments of the *S.cerevisiae* genomic insert (Figure 1B) were hybridized to the same blots used for the hybridization with the human probes. The results shown in Figure 1C reveal that the 5′ and middle part of the cloned yeast sequence hybridize to the same *Pst*I fragment as visualized by the human *CSB/ERCC6* 5′ and middle probe (lanes 7 and 8 and 9 and 10). Also a clear correspondence is noted for the 3.6 kb *Eco*RI fragment (lanes 3 and 4). We conclude that the middle portion of the cloned yeast sequence is identical to the main segment cross-hybridizing with human *CSB/ERCC6* cDNA.

Sequence analysis

Sequencing of the 5.2 kb *Sal*I–*Hin*dIII fragment (Figure 1B) revealed an open reading frame (ORF) of 3258 bp, encoding a protein of 1085 amino acids (Figure 2). Two additional ORFs were identified. One represents the *PET191* gene, whose sequence has a stop codon 348 bp upstream of the 3258 bp ORF and shows no homology to *CSB/ERCC6*. One hundred bp downstream of the 3258 bp ORF, the stop codon of an as yet unidentified ORF of at least 1596 bp is located encoded by the opposite strand. Southern blot analysis of a *S.cerevisiae* pulse field electrophoresis gel revealed that the 3258 bp ORF is localized on chromosome X. The predicted amino acid sequence contains several interesting putative domains: an acidic amino acid stretch (amino acids 193–228) found in several nuclear proteins that associate with chromatin or histones (Ptashne, 1988), a sequence matching well with a bipartite nuclear location signal (amino acids 252–273; Robbins *et al.*, 1991) and seven conserved motifs identified in many DNA and RNA helicases (amino acids 317–766; Gorbalenya and Koonin, 1993). Alignment with the human *CSB/ERCC6* protein (Figure 2) shows that the middle part of the protein, containing the putative helicase domains, is highly conserved (71% similarity). The total helicase region is very homologous to comparable parts of functionally different proteins that belong to the rapidly expanding SNF2 sub-family of helicases (Troelstra *et al.*, 1992; Gorbalenya and Koonin, 1993). However, the degree of homology in this part of the yeast and the human *CSB/ERCC6* protein is much higher than the average homology of the CSB/ERCC6 or yeast sequence with other members of the helicase sub-family (Table I). Both the N- and C-terminal parts show less, but still significant, similarity (53% and 64%, respectively, gaps not included). The

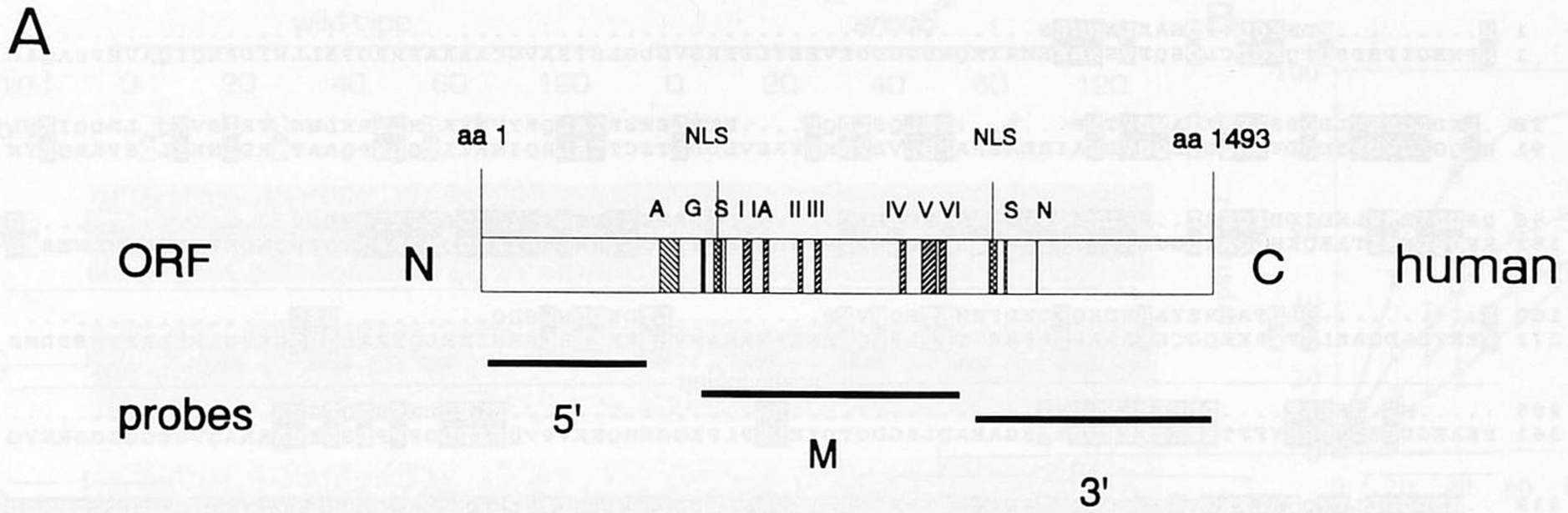


Fig. 1. Detection of cross-hybridizing *ERCC6* fragments in *S.cerevisiae* genomic DNA. (**A**) Schematic representation of the three PCR-generated probes of the human *ERCC6* cDNA used for Southern blot analysis. The probes 5′, M and 3′ encompass the sequence for respectively the N-terminus (amino acids 1–362), the middle part (amino acids 398–977) and the C-terminus (amino acids 998–1493). A, acidic amino acid stretch; G, glycine rich stretch; NLS, putative nuclear location signal; S, putative serine phosphorylation site; I−VI, helicase motifs; N, presumed nucleotide binding fold. (**B**) Map of genomic region of *RAD26*. Restriction map of the 5.2 kb *Sal*I−*Hin*dIII *S.cerevisiae* DNA fragment,containing the complete coding region of *RAD26*. The filled bar indicates the location of the 177 bp fragment used for the isolation of the genomic region. The indicated probes 5′, M and 3′ are restriction fragments that contain the sequences encoding the N-terminus (amino acids 1–402), middle part (amino acids 403–756) and C-terminus (amino acids 871–1085) of RAD26. The predicted functional domains in the gene product are as in (A). H, *Hin*dIII; B, *Bgl*II; S, *Sal*I; P, *Pst*I; E, *Eco*RI. (**C**) Specific genomic restriction fragments hybridize to the human *CSB/ERCC6* probes as well as to similar probes of the isolated yeast gene. Three identical Southern blots of *S.cerevisiae* genomic DNA, digested with *Eco*RI (lanes 1–6) or *Pst*I (lanes 7–12), were hybridized to three non-overlapping human *CSB/ERCC6* cDNA probes (indicated in A) under non-homologous hybridization conditions. Following exposure of the blot to Fuji-RX film for 5 weeks at −80°C with an intensifying screen, the blot was stripped and rehybridized with probes of similar parts of the isolated yeast *RAD26* gene (indicated in B), followed by autoradiography for 16 h. The 6.0 kb *Pst*I fragment in lanes 9 and 10 represents a partial digestion. Lanes a, human *ERCC6* probe; lanes b, *S.cerevisiae ERCC6* probe.

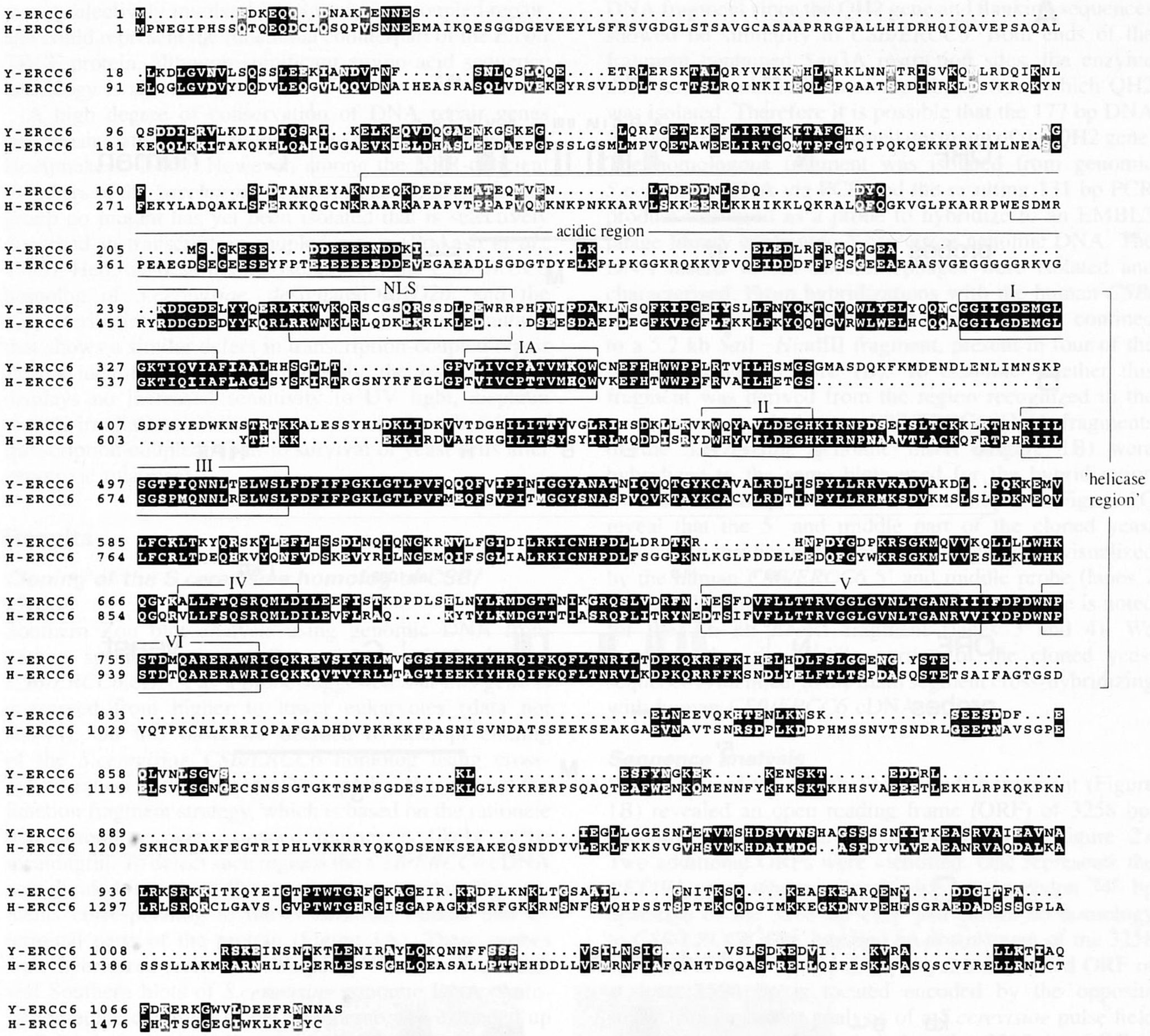


Fig. 2. RAD26 shows a high degree of amino acid homology to CSB/ERCC6, most prominent in the helicase region. A high degree of conservation of amino acids exists between CSB/ERCC6 and RAD26. The MULTATULIN program (Corpet,1988) was used to align the predicted protein sequences of *CSB/ERCC6* and *RAD26* (indicated by H-ERCC6 and Y-ERCC6 respectively). Identical amino acids are indicated by black boxes; shaded boxes represent amino acids of similar character, grouped as Q, N, E, D; G, S, A, P, T; V, I, L, M; H, R, K; W, Y, F. The Genbank accession number of *RAD26* is X81635.

predicted protein sequence is 407 amino acids shorter than the human *CSB/ERCC6* protein although it has an insertion of 43 amino acids between helicase domain Ia and II. It lacks the N-terminal glycine-rich amino acid stretch as well as the C-terminal putative nuclear location signal and potential nucleotide-binding fold. However, the acidic amino acid stretch, the putative nuclear location signal in the N-terminus and the helicase motifs are preserved at similar positions in both genes (Figure 2). The strong overall conservation of amino acids and putative functional domains suggests that the isolated yeast gene encodes the *S.cerevisiae* homolog of *CSB/ERCC6*.

Transcription-coupled repair in the yeast csb/ercc6 disruption mutant

The involvement of the presumed *S.cerevisiae* CSB/ERCC6 homolog in repair was studied to obtain functional evidence for its identity. To this purpose, a disruption mutant was generated by exchanging the N-terminal half of the endogenous gene in the haploid strain W303 for the *HIS3* gene. This mutation had no notable influence on the growth rate or morphology, indicating that the gene, as is the case in CS, has no vital function.

Transcription-coupled repair was analyzed by determining the rate of repair of UV-induced CPD lesions in the *RPB2* gene. Sweder and Hanawalt (1992) demonstrated preferential repair of the transcribed strand (TS) of *RPB2* in repair proficient DB1033 cells, which was dependent on RNA polymerase II activity. The RAD^+ yeast strain W303 used in this study showed a very similar bias in repair of the two strands. In the disruption mutant this bias is strongly reduced, although not completely abolished (Figure 3). Notably, shortly after UV exposure, the NTS seems to be repaired more efficiently in the mutant

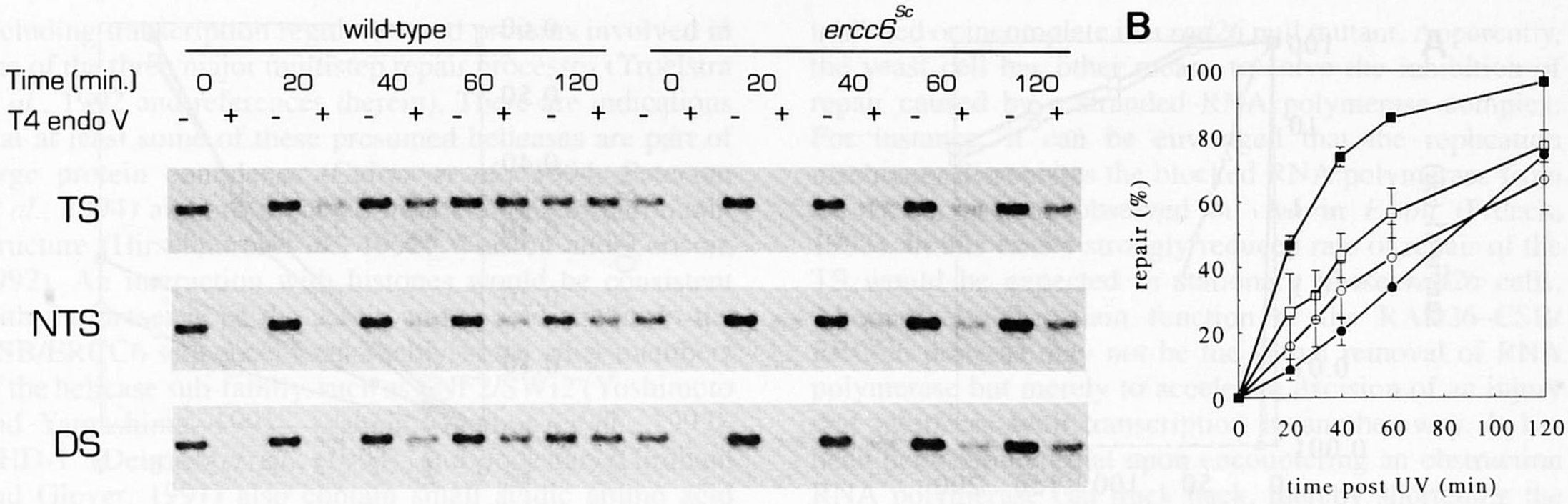


Fig. 3. Transcription-coupled repair of *RPB2* is impaired in *rad26* null mutant. Genomic DNA was isolated from cells that were UV irradiated (70 J/m^2) and allowed to repair for the indicated time. Following digestion by *Pvu*I and *Pvu*II, the DNA was mock-treated or treated with T4 endo V, indicated by − or +, respectively. Samples were electrophoresed on a denaturing gel and Southern blot analysis was performed using probes that hybridize to the transcribed strand (TS), the non-transcribed strand (NTS) or both strands (DS) of *RPB2*. (**A**) Autoradiogram showing removal of CPD from the *RPB2 Pvu*I−*Pvu*II fragment. The amount of radioactivity of the 5.2 kb DNA fragment in each lane was quantified, the percentages of repair were calculated for each time point using the Poisson expression, and are graphically presented in (**B**). Curves represent the average ± SEM of two independent UV irradiation experiments, for which *RPB2* repair was determined using six or four Southern blots respectively. ■, TS W303; ●, NTS W303; □, TS *rad26*; ○, NTS *rad26*.

compared to the parental strain. It is evident however that disruption of the isolated yeast gene leads to an impairment of transcription-coupled repair of *RPB2*. We conclude that the gene is the functional homolog of *CSB/ERCC6* and propose to designate it *RAD26* consistent with the nomenclature of the yeast *RAD3* epistasis group genes.

Genome overall repair in rad26 disruption mutant

The absence of an active transcription-coupled repair pathway in humans does not lead to a substantial decrease in overall repair activity, as was shown previously for CS cells (Mayne *et al.*, 1982). To investigate this for yeast, the effect of disruption of *RAD26* on genome overall repair was analyzed using specific antibodies. Figure 4 shows that there is a small but reproducible decrease in rate of CPD removal in mutant cells. The magnitude of this effect is compatible with the reduced CPD removal in the *RPB2* gene. The removal of 6/4 photoproducts occurs at an equal rate in both cell types. This is expected because repair of these lesions by the global genome repair pathway is already so fast (Mullenders *et al.*, 1993), that the transcription-coupled repair process, if operational for this type of damage, is overruled.

Effect of defective transcription-coupled repair on survival in yeast

Human CS-B cells as well as the rodent cell line UV61, all carrying a mutation in *ERCC6*, are highly sensitive to UV irradiation (Troelstra *et al.*, 1992). Since the yeast genome is much more compact than the mammalian genome, a relatively larger part is transcriptionally active. Thus, inactivating transcription-coupled repair by disrupting *RAD26* is expected to have a more dramatic effect on survival after exposure to UV than is seen in CS. Surprisingly, disruption of *RAD26* conferred no significant UV sensitivity compared with the parental strain W303 (Figure 5A). In addition, *rad26* mutant cells showed no sensitivity to the genotoxic agent cisplatin, which mainly introduces inter- and intrastrand crosslinks that are repaired via NER and other pathways (data not shown). Recently,

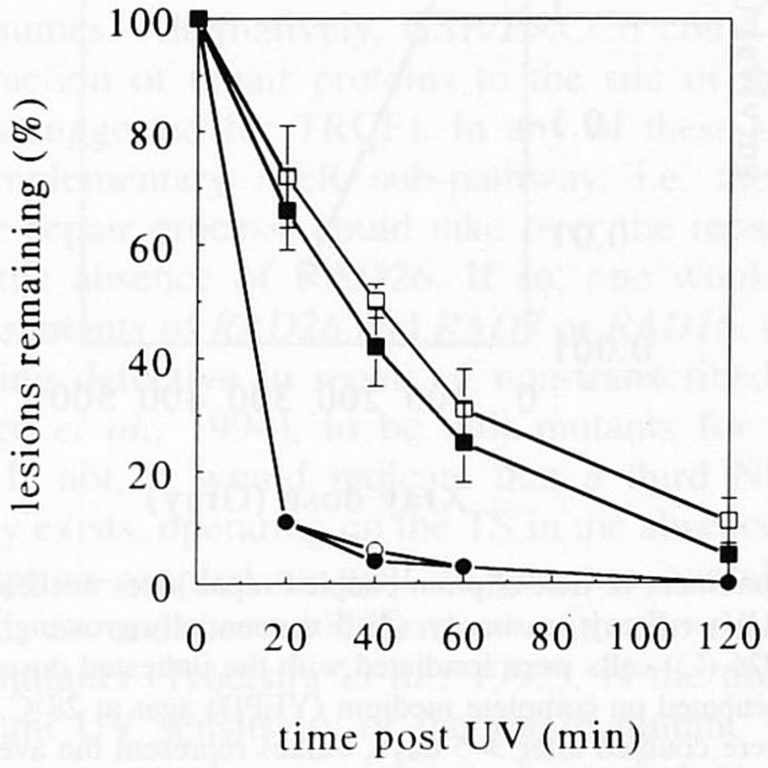
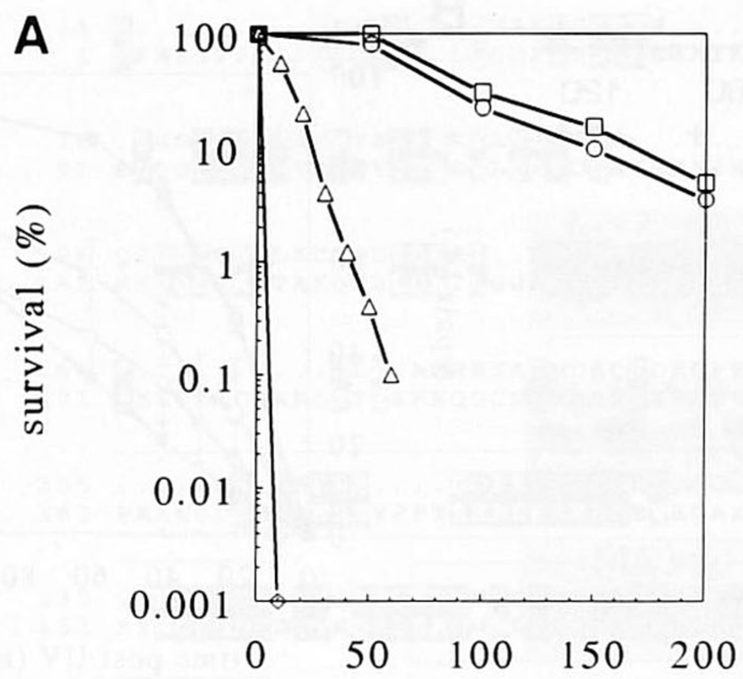


Fig. 4. *Rad26* null mutant cells possess a wild type overall repair capacity. Exponentially growing cells were UV irradiated with 70 J/m^2 and allowed to repair for the indicated periods of time. Genomic DNA was isolated and assayed for the presence of cyclobutane pyrimidine dimers (CPD) and 6/4 photoproducts (6/4 PP) using specific antibodies as described (Roza *et al.*, 1988). Curves representing CPD removal show the average of an ELISA and slot blot analysis,while removal of 6/4 PP was determined via slot blot analysis. ■, CPD W303; □, CPD *rad26*; ● 6/4PP W303; ○, 6/4PP *rad26*.

Leadon and Cooper (1993) described a mild sensitivity of CS-A and CS-B cells to X-ray irradiation. Exposure to X-rays induces single and double breaks in the DNA, which are generally removed by ligase and recombination repair (Price, 1993). However, Figure 5B clearly shows that *rad26* cells display no significant X-ray sensitivity. Although the transcription-coupled repair pathway, as well as the factors involved, are preserved in yeast and mammalian cells, the contribution to survival after genotoxic treatment is clearly different.

Although no effect on colony forming ability after exposure to UV is observed, we examined whether disruption of *RAD26* affects the recovery of growth after UV treatment. Figure 6 shows that resumption of growth was delayed in *rad26* cells following irradiation with 70 J/m^2



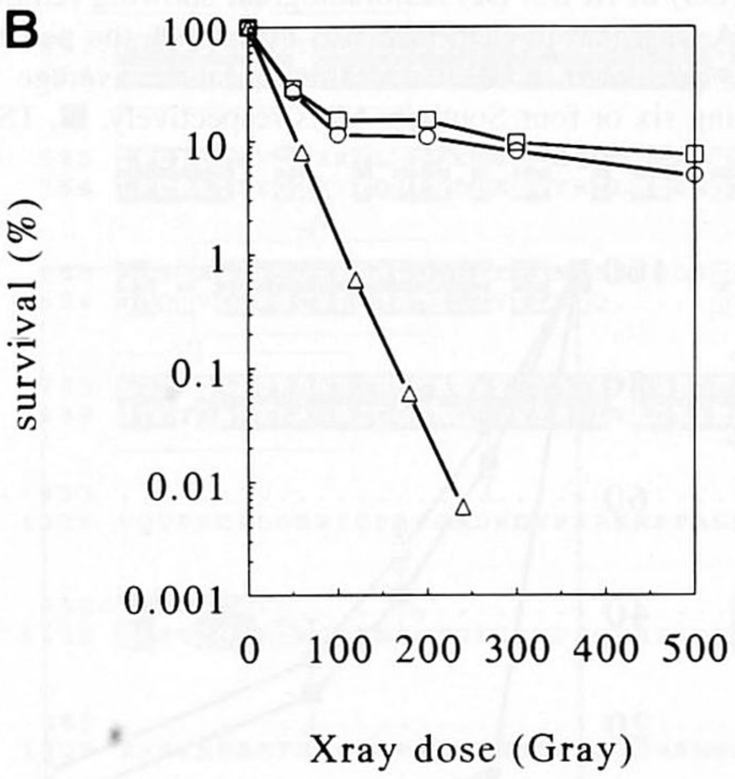


Fig. 5. Impairment of transcription-coupled repair does not lead to increased UV or X-ray sensitivity. (**A**) Exponentially growing W303 (□) or *rad26* (○) cells were irradiated with the indicated doses of 254 nm UV, incubated on complete medium (YEPD) agar at 28°C, and colonies were counted after 3–5 days. Values represent the average of two independent experiments, each performed in duplicate. For comparison, survival of the *rad16* [(△), deduced from Bang *et al.*, 1992, and *rad3* (◇), Higgins *et al.*, 1983] NER mutants after UV irradiation are also indicated. (**B**) For X-ray survival a similar procedure was followed to that described in (A), but cells were irradiated with various doses of X-rays at a dose rate of 30 Gy/min. The survival of the X-ray sensitive *rad52* mutant (Δ) is also indicated for comparison (data taken from Game and Mortimer, 1974).

of UV. When the cells are unirradiated, no effect on growth rate is seen, indicating that the altered recovery of growth is not due to the mutation itself but only becomes manifest after UV irradiation. The altered ability to recover quickly from a genotoxic treatment could be indicative of a biological importance of RAD26 and transcription-coupled repair for yeast cells, as discussed below.

Discussion

Sequence conservation and significance of functional domains in ERCC6

An important step towards elucidation of the mechanism of transcription-coupled repair is the identification of the factors involved. We report here the isolation and characterization of the *S.cerevisiae* homolog of *CSB/ERCC6*, designated *RAD26*. While this manuscript was in preparation a sequence with significant homology to *CSB/ERCC6* was identified in the framework of the yeast genome sequencing project (Huang *et al.*, 1994). Comparison revealed that it is identical with the sequence of *RAD26*. The conservation of the acidic amino acid stretch, the putative nuclear location signal and in particular the region containing the helicase motifs point to a functional significance of these presumed domains. The complete helicase region is preserved to a considerable extent in proteins that belong to the SNF2 helicase sub-family (Troelstra *et al.*, 1992; Gorbalenya and Koonin, 1993). However, the degree of homology of this segment in the human and yeast *CSB/ERCC6* amino acid sequence is much higher (71% similarity) than the average conservation of the region in the helicase sub-family (53%; Table I). A similar homology (77% similarity) is found when comparing the helicase region of the *Drosophila melanogaster* and human *brahma* gene product (Tamkun *et al.*, 1992; Muchardt and Yaniv, 1993). The SNF2 sub-family comprises at least 17 gene products of different origin,

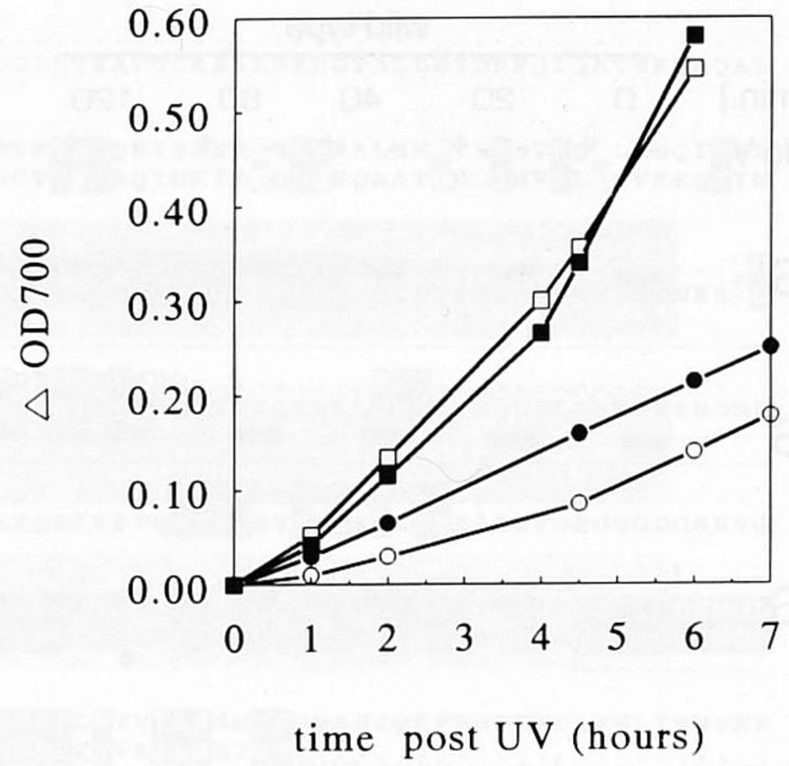


Fig. 6. *Rad26* cells show a slower recovery of growth following UV irradiation. Exponentially growing W303 and *rad26* cells were unirradiated or irradiated with 70 J/m^2 of 254 nm UV. Cells were diluted in YEPD medium and allowed to grow at 28°C. Growth was monitored by measuring the OD_{700} at the indicated time-points. ■, W303, unirradiated; □, *rad26*, unirradiated; ●, W303, 70 J/m^2; and ○, *rad26*, 70 J/m^2.

Table I. Percentages of homology between CSB/ERCC6 and RAD26

	Identical (%)	Similar (%)
N-terminal region	31	53
Helicase region	57	71
Average of helicase sub-family	31	53
C-terminal region	38	64
Total coding region	44	64

For the calculation of the degree of homology the following regions were considered: N-terminal region: CSB/ERCC6, amino acids 1–493; RAD26, amino acids 1–283; helicase region: CSB/ERCC6, amino acids 494–1008; RAD26, amino acids 284–824; C-terminal region: CSB/ERCC6, amino acids 1009–1493; RAD26, amino acids 825–1085. Similar amino acids are defined as in Figure 3. The average amino acid conservation of the SNF2 helicase sub-family was calculated for the following proteins: RAD5, RAD16, RAD54, CSB/ERCC6, SWI2/SNF2, BRM, MOT1, LDS, fun30 and STH1 (Troelstra *et al.*, 1992 and references therein). Gaps that had to be introduced for proper alignment were not included.

including transcription regulators and proteins involved in one of the three major multistep repair processes (Troelstra *et al.*, 1992 and references therein). There are indications that at least some of these presumed helicases are part of large protein complexes (Cairns *et al.*, 1994; Peterson *et al.*, 1994) and are involved in alterations of chromatin structure (Hirschhorn *et al.*, 1992; Winston and Carlson, 1992). An interaction with histones would be consistent with the presence of the acidic amino acid stretch in the CSB/ERCC6 sequence. Remarkably, some other members of the helicase sub-family, such as SNF2/SWI2 (Yoshimoto and Yamashima, 1991), brahma (Tamkun *et al.*, 1992), CHD-1 (Delmas *et al.*, 1993) and lodestar (Girdham and Glover, 1991) also contain small acidic amino acid stretches N-terminal of the helicase region, suggesting that these residues might contribute to the activity of this domain. Until now, no DNA unwinding activity has been shown for any member of the helicase sub-family. However, a DNA-stimulated ATPase activity has been reported for SNF2/SWI2, which is impaired by mutations of the nucleotide-binding fold in the helicase motif I (Laurent *et al.*, 1993). Notably, the *E.coli* TRCF protein, involved like CSB/ERCC6 and RAD26 in transcription-coupled repair, displays a weak ATPase but no helicase activity, although all postulated helicase motifs are present (Selby and Sancar, 1993). An attractive hypothesis is that a cryptic helicase activity is involved, which is utilized to dissociate DNA-bound protein complexes from the template by local denaturation of the two strands.

The phenotype of the rad26 disruption mutant: functional implications

To reveal the biological role of RAD26 a disruption mutant was generated by exchanging the N-terminal half of the endogenous *RAD26* gene for the selectable marker *HIS3*. The observed phenotype of this null mutant has several important implications for the biological role of RAD26 and transcription-coupled repair in yeast.

First, *RAD26* disruption is not lethal to yeast cells. This is in agreement with the mutations of *CSB/ERCC6* found in CS (Troelstra *et al.*, 1992). Moreover, this finding excludes a model in which RAD26, and by inference CSB/ERCC6, has an essential role in transcription and as a secondary function provides the coupling with repair. In contrast, several of the recently identified proteins of BTF2/TFIIH, and its yeast counterpart factors, have a functional overlap in transcription and repair and have essential roles in both of these processes (Feaver *et al.*, 1993; Schaeffer *et al.*, 1993, 1994). Nevertheless, an auxiliary function of RAD26 or CSB/ERCC6 in transcription is still possible. This could readily explain the typical clinical features of CS patients that cannot be rationalized by an impaired NER, but instead more likely coincide with a defect in transcription (Vermeulen *et al.*, 1994).

Second, the *rad26* mutant is impaired in transcription-coupled repair and thus forms a valid yeast model for the human repair disorder Cockayne's syndrome. An interesting observation made here is the persistence of significant repair of the TS in the absence of a functional *RAD26* gene product. When RAD26 is involved in the removal or displacement of a blocked RNA polymerase, to give the NER machinery access to the lesion, it is surprising that repair of the TS is not more strongly inhibited or incomplete in a *rad26* null mutant. Apparently, the yeast cell has other means to solve the inhibition of repair caused by a stranded RNA polymerase complex. For instance, it can be envisaged that the replication machinery dissociates the blocked RNA polymerase from the DNA, as was observed *in vivo* in *E.coli* (French, 1992). In this case a strongly reduced rate of repair of the TS would be expected in stationary phase *rad26* cells. Alternatively, the main function of the RAD26–CSB/ERCC6 proteins may not be the actual removal of RNA polymerase but merely to accelerate excision of an injury that interferes with transcription in another way. It has been demonstrated that upon encountering an obstruction RNA polymerase can track back, thereby shortening the already synthesized RNA, and resume RNA synthesis in an effort to pass the pause site (Kassavetis and Geiduschek, 1993 and references therein). The elongation factor TFIIS (SII) has been shown to stimulate this anti-terminating activity (Reines *et al.*, 1989; Izban and Luse, 1992). Transcript shortening probably forms the basis of transcription-coupled repair (Hanawalt, 1992; Hanawalt and Mellon, 1993). CSB/ERCC6 could coordinate the retraction process, so the lesion can be repaired before transcription resumes. Alternatively, CSB/ERCC6 could mediate the attraction of repair proteins to the site of the lesion (as was suggested for TRCF). In any of these scenarios the complementary NER sub-pathway, i.e. the overall genome repair process, could take over the repair of the TS in the absence of RAD26. If so, one would expect double mutants of *RAD26* and *RAD7* or *RAD16*, the latter two being defective in repair of non-transcribed strands (Verhage *et al.*, 1994), to be null mutants for excision repair. If not, it would indicate that a third NER sub-pathway exists, operating on the TS in the absence of both transcription-coupled as well as genome overall repair.

Third, an unexpected difference with the mammalian *ercc6* mutants (Troelstra *et al.*, 1992), is the absence of significant UV sensitivity of the *rad26* mutant. Survival following exposure to cisplatin or X-irradiation was also not influenced by disruption of *RAD26*. When transcription-coupled repair was first discovered for the amplified *DHFR* gene in rodent cells (Bohr *et al.*, 1985), it provided a plausible explanation for the rodent/human repair paradox. Rodent cells repair CPD lesions in only a small fraction of their genome (the transcribed strand of active genes) but nevertheless display a similar UV survival to human cells, which possess a much higher repair activity (Mellon *et al.*, 1987). Extrapolation of this functional significance of transcription-coupled repair to yeast apparently does not hold, at least for the mutagens and the conditions tested here. It is still possible that the relative importance of this conserved pathway in preventing cell killing and mutagenesis is different in multicellular and unicellular organisms. In agreement with this, the *E.coli mfd* mutant also shows only a very mild UV sensitivity but displays a highly elevated mutation induction of the transcribed strand (Oller *et al.*, 1992). In the absence of any effect on survival the *raison d'être* of transcription-coupled repair could be to permit a rapid resumption of growth after genotoxic treatment, as is apparent from Figure 6. Its primary role would then be to increase the efficiency of displacement of a stalled RNA polymerase II complex and to facilitate repair, thus shortening damage-induced

cell cycle arrest. As a separate consequence it would stimulate cell survival of multicellular organisms with a more complex genome. Following this reasoning, it is even possible that the CSB/ERCC6-RAD26 protein acts as a general displacement factor for RNA polymerase II when this is blocked for any reason. Obviously, the absence of UV sensitivity explains why a *CSB/ERCC6* analogous mutant has not been isolated in yeast before. At the same time this notion opens the possibility that additional factors selectively involved in transcription-coupled repair, such as the CSA gene, are hidden in the yeast genome. Finally, these findings underline the importance of the global genome repair pathway for cellular resistance to genotoxins in yeast.

Materials and methods

General procedures

DNA purification, restriction enzyme digestion, DNA ligation, PCR, gel electrophoresis, ^{32}P-labeling of probes using random oligonucleotide primers and filter hybridizations were performed according to standard procedures (Sambrook *et al.*, 1989), unless stated otherwise. Alkaline DNA transfer to Zetaprobe (Bio-Rad, Richmond, California) or Hybond N^+ (Amersham) blotting membranes was performed as described by the manufacturer. Sequence analysis was performed by dideoxy chain termination using a combination of sub-clones, exonuclease III deletions (Pharmacia) and synthetic primers.

Construction of the rad26 disruption mutant

For gene disruption of *RAD26* the plasmid PTZSHE6Sc, which contains the complete coding region on a 5.2 kb *Sal*I−*Hin*dIII fragment, was used (Figure 1B). The N-terminal 1.6 kb *Bgl*II fragment was substituted for a 1.8 kb *Bam*HI−*HIS3* fragment, leaving 0.5 kb homologous DNA 5′ and 3.0 kb 3′ of the *HIS3* insert. This construct was linearized and transformed to the haploid yeast strain W303 (*MAT*α, *ho, ade2, trp1, leu2, can1, his3, ura3*) generating strain MGSC102, hereafter referred to as *rad26*. Disruption of the endogenous *RAD26* through homologous recombination was confirmed by Southern blot analysis using probes that hybridize either 5′ or 3′ of the 5.2 kb *Sal*I−*Hin*dIII fragment.

Repair analysis of UV-induced CPD

Determination of transcription-coupled repair of UV-induced CPD in *RPB2* was performed basically as described by Bohr *et al.* (1985). Exponentially growing cells (OD_{700} = 1.2) were collected by centrifugation, diluted in cold PBS and UV-irradiated with 70 J/m^2 at a rate of 3.5 J/m^2/s. Following centrifugation and resuspension in YEPD medium, cells were incubated for various periods of time at 28°C in the dark to allow repair. Genomic DNA was isolated, purified on CsCl gradients and digested with *Pvu*I and *Pvu*II to give a 5.2 kb *RPB2* fragment. DNA digests were divided in two equal parts and treated or mock-treated with T4 endonuclease V. Following overnight electrophoresis on a denaturing agarose gel with recirculating buffer. DNA was transferred to Hybond N^+. Repair of *RPB2* was visualized by hybridizations with strand-specific probes. A PCR-generated *RPB2* fragment was digested with *Eco*RI and *Xho*I and the resulting 1 kb fragment was cloned in M13 in both orientations. Single-stranded M13 molecules were isolated and radiolabeled by primer extension of the M13 hybridization primer (Pharmacia). First, annealing of the primer was accomplished by incubating 500 ng of ssM13 with 2 pmol M13 hybridization primer in 10 ml of 10 mM Tris−HCl (pH 7.5), 0.1 mM EDTA, 100 mM NaCl for 2 min at 100°C and subsequently for 30 min at 37°C. Then 10 ml of 2 mM Tris−HCl (pH 7.5), 2 mM $MgCl_2$, 0.2 mM DTE, 400 mM (dGTP, dCTP, dTTP), 4 mM dATP, 0.4 mM [^{32}P]dATP (3000 Ci/mmol) and 2 U Klenow polymerase (Boehringer Mannheim) were added and the mixture was incubated for 60 min at room temperature. The unincorporated fraction of [^{32}P]dATP was removed using Sephadex G50 columns (Pharmacia) and the probes were directly used for hybridizations. The ^{32}P-labeled *RPB2* PCR product was used as probe to determine repair of both strands. Following hybridization of the blot with the probe of one strand, the radioactive signal was thoroughly removed by incubating the blot 5 min at 100°C in 10 mM Tris−HCl, 1 mM EDTA, 1% SDS and the blot was rehybridized with a probe directed to the opposite strand or to both strands. The membranes were scanned using a PhosphorImager (Molecular Dynamics). Repair of CPD was calculated by comparing the amount of radioactivity in the mock-treated and T4 endonuclease-treated *RPB2* fragment using the Poisson expression (Bohr *et al.*, 1985).

Analysis of genome overall repair was performed as described in detail previously (Verhage *et al.*, 1994). Removal of cyclobutane pyrimidine dimers was detected using specific antibodies (Roza *et al.*, 1988) in an ELISA or slot-blot analysis, while repair of 6/4 photoproducts could only be detected via slot-blotting.

Survival experiments

For determination of survival after UV irradiation, exponentially growing cells were collected by centrifugation and diluted in cold PBS. Small aliquots were irradiated with 0–200 J/m^2 254 nm UV (Philips T UV 30 W) at a rate of 1 or 1.5 J/m^2/s. Survival after exposure to cisplatin was measured in cells collected, resuspended in cold water, exposed to 0–35 mg/ml cisplatin for 2 h at 28°C and washed twice. For determination of survival after exposure to X-rays, cells were irradiated with 0–500 Gy X-rays at a dose rate of 30 Gy/min. Appropriate dilutions were plated on complete medium (YEPD) agar and colonies were counted after 3–5 days of incubation at 28°C.

Acknowledgements

We thank Dr A.Verkerk for providing the Southern blot of the yeast genomic pulse field gel, I.van der Velde for technical assistance in the yeast experiments, D.F.R.Murris for his skilled help in the X-irradiations, Dr L.Roza and J.Bergen-Henegouwen for providing the facilities, CPD antibodies and technical assistance in the determination of the overall repair, Professor O.Nikaido for providing the 6/4PP antibodies, Dr N.G.J.Jaspers for his help in data analysis and M.Kuit for photography. This research was supported by the Commission of European Community (contract number BJ6-141-NL) and by the Dutch Scientific Organization through the Foundation of Medical Scientific Research (contract number 900-501-093).

References

Altschul,S.F., Gish,W.,Miller,W., Myers,E.W. and Lipman,D.J. (1990) *J. Mol. Biol.*, **215**, 403–410.

Bang,D.D., Verhage,R., Goosen,N., Brouwer,J. and v.d. Putte,P. (1992) *Nucleic Acids Res.*, **20**, 3925–3931.

Bohr,V.A. (1991) *Carcinogenesis*, **12**, 1983–1992.

Bohr,V.A., Smith,C.A., Okumoto,D.S. and Hanawalt,P.C. (1985) *Cell*, **40**, 359–369.

Cairns,B.R., Kim,Y.-J., Sayre,M.H., Laurent,B.C. and Kornberg,R.D. (1994) *Proc. Natl Acad. Sci. USA*, **91**, 1950–1954.

Corpet,F. (1988) *Nucleic Acids Res.*, **16**, 10881–10890.

Delmas,V., Stokes,D.G. and Perry,R.P. (1993) *Proc. Natl Acad. Sci. USA*, **90**, 2414–2418.

Feaver,W.J., Svejstrup,J.Q., Bardwell,L., Bardwell,A.J., Buratowski,S., Gulyas,K.D., Donahue,T.F., Friedberg,E.C. and Kornberg,R.D. (1993) *Cell*, **75**, 1379–1387.

French,S. (1992) *Science*, **258**, 1362–1364.

Game,J.C. and Mortimer,R.K. (1974) *Mutat. Res.*, **24**, 281–292.

Girdham,C.H. and Glover,D.M. (1991) *Genes Dev.*, **5**, 1786–1799.

Gorbalenya,A.E. and Koonin,E.V. (1993) *Curr. Opin. Struct. Biol.*, **3**, 419–429.

Hanawalt,P.C. (1992) In Bohr,V., Wasserman,K. and Kreamer.K.H. (eds), *DNA Repair Mechanisms.* Alfred Benzon Symposium 35. Munksgaard, Copenhagen, pp.231–241.

Hanawalt,P.C., Donahue,B.A. and Sweder, K.S. (1994) *Curr. Biol.*, **4**, 518–521.

Higgins,D.R., Prakash,S., Reynolds,P., Polakowska,R., Weber,S. and Prakash,L. (1983) *Proc. Natl Acad. Sci. USA*, **80**, 5680–5684.

Hirschhorn,J.N., Brown,S.A., Clark,C.D. and Winston,F. (1992) *Genes Dev.*, **6**, 2288–2298.

Hoeijmakers,J.H.J. (1993) *Trends Genet.*, **9**, 211–217.

Huang,J.C., Svoboda,D.L., Reardon,J.T. and Sancar,A. (1992) *Proc. Natl Acad. Sci. USA.*, **89**, 3664–3668.

Huang,M.-E., Chuat,J.-C. and Galibert, F. (1994) *Biochem. Biophys. Res. Commun.*, **201**, 310–317.

Izban,M.G. and Luse,D.S. (1992) *Genes Dev.*, **6**, 1342–1356.

Kanawalt,P.C. and Mellon,I. (1993) *Curr. Biol.*, **3**, 67–69.

Kassavetis,G.A. and Geiduschek,E.P. (1993) *Science*, **259**, 944–945.

Laurent,B.C., Treich,I. and Carlson,M. (1993) *Genes Dev.*, **7**, 583–591.

Leadon,S.A. and Cooper,P.K. (1993) *Proc. Natl Acad. Sci. USA*, **90**, 10499–10503.
Leadon,S.A. and Lawrence,D.A. (1991) *Mutat. Res.*, **255**, 67–78.
Lehmann,A.R. (1982) *Mutat. Res.*, **106**, 347–356.
Mayne,L.V., Lehmann,A.R. and Waters,R. (1982) *Mutat. Res.*, **106**, 179–189.
Mellon,I. and Hanawalt,P.C. (1989) *Nature*, **342**, 95–98.
Mellon,I., Spivak,G. and Hanawalt,P.C. (1987) *Cell*, **51**, 241–249.
Muchardt,C. and Yaniv,M. (1993) *EMBO J.*, **12**, 4279–4290.
Mullenders,L.H.F., Dokkum,A.-M.H.-v., Kalle,W.H.J., Vrieling,H., Zdzienicka,M.Z. and Zeeland,A.A.v. (1993) *Mutat. Res.*, **299**, 271–276.
Nance,M.A. and Berry,S.A. (1992) *Am. J. Med. Genet.*, **42**, 68–84.
Oller,A.R., Fijalkowska,I.J., Dunn,R.L. and Schaaper,R.M. (1992) *Proc. Natl Acad. Sci. USA*, **89**, 11036–11040.
Oudshoorn,P., Steeg,H.v., Swinkels,B.W., Schoppink,P. and Grivell, L.A. (1987) *Eur. J. Biochem.*, **163**, 97–103.
Peterson,C.L., Dingwall,A. and Scott,M.P. (1994) *Proc. Natl Acad. Sci. USA*, **91**, 2905–2908.
Prakash,S., Sung,P. and Prakash,L. (1993) *Annu. Rev. Genet.*, **27**, 33–70.
Price,A. (1993) *Cancer Biol.*, **4**, 61–71.
Ptashne,M. (1988) *Nature*, **335**, 683–689.
Reines,D., Chamberlin,M.J. and Kane,C.M. (1989) *J. Biol. Chem.*, **264**, 10799–10809.
Robbins,J., Dilworth,S.M., Laskey,R.A. and Dingwall,C. (1991) *Cell*, **64**, 615–623.
Roza,L., v.d.Wulp,K.J.M., MacFarlane,S.J., Lohman,P.H.M. and Baan,R.A. (1988) *Photochem. Photobiol.*, **46**,627–634.
Sambrook,J., Fritsch,E.F. and Maniatis, T. (1989). *Molecular Cloning: A Laboratory Manual.* 2nd Edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
Sancar,A. and Sancar,G.B. (1988) *Annu. Rev. Biochem.*, **57**, 29–67.
Schaeffer,L., Moncollin,V., Roy,R., Staub,A., Mezzina,M., Sarasin,A.., Weeda,G., Hoeijmakers,J.H.J. and Egly,J.M. (1994) *EMBO J.*, **13**, 2388–2392.
Schaeffer,L., Roy,R., Humbert,S., Moncollin,V., Vermeulen,W., Hoeijmakers,J.H.J., Chambon,P. and Egly,J.-M. (1993) *Science*, **260**, 58–63.
Selby,C.P. and Sancar,A. (1993) *Science*, **260**, 53–58.
Selby,C.P., Witkin,E.M. and Sancar,A. (1991) *Proc. Natl Acad. Sci. USA*, **88**,11574–11578.
Smerdon,M.J. and Thoma,F. (1990) *Cell*, **61**, 675–684.
Sweder,K.S. and Hanawalt,P.C. (1992) *Proc. Natl Acad. Sci. USA*, **89**, 10696–10700.
Tamkun,J.W., Deuring,R., Scott,M.P., Kissinger,M., Pattatucci,A.M., Kaufman,T.C. and Kennison,J.A. (1992) *Cell*, **68**, 561–572.
Tanaka,K., Kawai,K., Kumahara,Y., Ikenaga,M. and Okada,Y. (1981) *Somat. Cell Genet.*, **7**, 445–455.
Troelstra,C., van Gool,A., de Wit,J., Vermeulen,W., Bootsma,D. and Hoeijmakers,J.H.J. (1992) *Cell*, **71**, 939–953.
Van Houten,B. (1990) *Microbiol. Rev.*, **54**, 18–51.
Van Hoffen,A., Natarajan,A.T., Mayne,L.V., v.Zeeland,A.A., Mullenders,L.H.F. and Venema,J. (1993) *Nucleic Acids Res.*, **21**, 5890–5895.
Venema,J., Mullenders,L.H.F., Natarajan,A.T., Van Zeeland,A.A. and Mayne,L.V. (1990) *Proc. Natl Acad. Sci. USA*, **87**, 4707–4711.
Verhage,R., Zeeman,A.-M., d.Groot,N., Gleig,F., Bang,D.D., v.d.Putte,P. and Brouwer,J. (1994) *Mol. Cell Biol.*, **14**, 6135–6142.
Vermeulen,W.*et al.* (1994) *Cold Spring Harbor Symp. Quant. Biol.*, **59**, in press.
Winston,F. and Carlson,M. (1992) *Trends Genet.*, **8**, 387–391.
Yoshimoto,H. and Yamashima,I. (1991) *Mol. Gen. Genet.*, **228**, 270–280.

Received on July 20, 1994; revised on August 29, 1994