THE COCKAYNE SYNDROME B PROTEIN: INVOLVEMENT IN TRANSCRIPTION-COUPLED DNA REPAIR

Alain J. van Gool

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HET COCKAYNE SYNDROOM B EIWIT: RELATIE MET TRANSCRIPTIE-GEKOPPELD DNA HERSTEL

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you can do it any way you want,
just do it by the way.

Phil Collins (Genesis)
(from the album: 'We can't dance')

Voor Karin en Sjors
Voor 's mam en 's pap

Contents:

Chapter 1.	**General introduction**	3
	Scope of this thesis	4
Chapter 2.	**Transcription-coupled DNA repair (TCR)**	7
2.1	TCR: the concept	7
2.2	TCR in prokaryotes	8
	Nucleotide excision repair in *E. coli*	8
	TCR in *E. coli*	10
	TRCF	10
	Model for TCR in *E. coli*	11
2.3	TCR in eukaryotes	12
	Nucleotide excision repair in eukaryotes	12
	TCR in yeast	15
	RAD26	16
	Double mutants in transcription-coupled and global genome repair	16
	TCR in *Drosophila melanogaster*	17
	TCR in man	18
	CSA	19
	CSB	19
	Model for human TCR	20
	Additional roles for CSA and CSB ?	22
Chapter 3.	**Concluding remarks**	27
	Future perspectives	29
References		32
Abbreviations.		42
Appendix I.	***ERCC6*, a member of a novel subfamily of putative helicases, is involved in Cockayne's syndrome and preferential repair of active genes.** Troelstra C., A.J. van Gool, J. de Wit, W. Vermeulen, D. Bootsma and J.H.J. Hoeijmakers. *Cell 71: 939-953, 1992*	45

Appendix II.	***RAD26*, the functional *S. cerevisiae* homolog of the Cockayne syndrome B gene *ERCC6*.** Van Gool A.J., R. Verhage, S.M.A. Swagemakers, P. van de Putte, J. Brouwer, C. Troelstra, D.Bootsma and J.H.J. Hoeijmakers. *EMBO J. 13: 5361-5369, 1994*	63
Appendix III.	**Double mutants of *S. cerevisiae* with alterations in transcription-coupled and global genome repair.** Verhage R.A., A.J. van Gool, N. de Groot, P. van de Putte and J. Brouwer. *Mol. Cell. Biol. 16, 2: 496-502, 1996*	75
Appendix IV.	**Relationship of the cockayne syndrome B protein, involved in transcription-coupled DNA repair, with other repair or transcription.** Van Gool A.J., E. Citterio, S. Rademakers, R. van Os, A. Constantinou, J.-M. Egly, D. Bootsma and J.H.J. Hoeijmakers. *submitted*	85

Summary. 102

Samenvatting. 104

Curriculum vitae. 106

Publications. 107

Nawoord. 108

Chapter 1

General introduction

General introduction

Each organism stores its genetic information in large DNA molecules, present in most cells. DNA is composed of four different nucleotides, abbreviated as G,A,T,and C, which make up the genetic code that is translated into proteins. An intermediate between DNA and protein is the RNA, that is generated by a process called transcription, during which one strand of the double DNA helix serves as a template and is read by a scanning RNA polymerase complex. As a result, a messenger RNA molecule is produced, that in turn forms a template for protein synthesis. It is of major importance that changes (mutations) in the genetic code of the DNA are limited to a minimum. Although mutations form the basis of biological diversity, they can also be the starting point of carcinogenesis in multicellular species.

The genomic DNA is continuously challenged by a variety of damaging agents, interfering with cellular processes that involve DNA metabolism. Of many of those reagents, the nature of the resulting lesion has been established. Various chemical compounds, inducing bulky adducts, and also UV-irradiation cause distortions and bending of the DNA helix. Ionising irradiation and oxygen radicals are known to generate single and double strand breaks in the DNA, while exposure to alkylating agents can lead to various types of modification of single nucleotides. Also, intracellular processes can enhance the intrinsic instability of some of the DNA bonds and can lead to alterations in the DNA, e.g. via hydrolysis and oxidation. Mutations are generated if these modified nucleotides mispair during DNA replication (prior to cell division), which can also happen spontaneously. This will in many cases lead to incorporation of a wrong nucleotide, thus generating a 'mismatch' which, when wrongly processed, can lead to a change of the genetic code.

All organisms have developed an intricate network of DNA repair modes to remove the lesions before they can exert their deleterious effects. Most of the repair pathways are strongly conserved during evolution, from the bacterium *Escherichia coli* to yeast and man (extensively reviewed in 48). The most relevant pathways include: i) immediate reversal of the damage (by single enzymes), ii) base excision repair (in which only the damaged base is replaced), iii) nucleotide excision repair (see below), iv) mismatch repair (repair of misincorporated bases), v) recombination repair (replacement of a damaged region using a homologous sequence) and vi) post-replication 'repair' (a poorly understood damage tolerance mechanism). The different substrate specificities of these pathways ensure that the majority of these lesions is removed from the genome. The best studied and one of the most important DNA repair pathways is nucleotide excision repair (NER). This very conserved repair system is able to recognise and remove a surprisingly wide variety of structurally unrelated damages. The mechanism of nucleotide excision repair has been resolved in considerable detail in the bacterium *E. coli* (see also below), and is already suggested by its name: upon recognition of the lesion, a single-stranded oligonucleotide containing the lesion is excised from the DNA, after which the remaining gap is filled in by a DNA polymerase.

The importance of a functional DNA repair pathway in the prevention of cancer was first illustrated in 1968 by Cleaver (28), who showed that individuals suffering from the cancer-prone hereditary disorder xeroderma pigmentosum (XP), having a high predisposition to developing skin cancer in sun-exposed areas, are defective in nucleotide excision repair of UV-

lesions. Recently, defective repair of mismatched bases, that arise after faulty replication of DNA, was demonstrated in patients suffering from hereditary nonpolyposis colorectal cancer 4 (HNPCC) (113a). Other hereditary cancer-prone disorders for which the relationship with DNA repair has not yet been established, include Fanconi's anaemia (FA), Bloom syndrome (BS), and ataxia telangiectasia (AT). On the other hand, there are hereditary syndromes which are associated with a defect in DNA excision repair, but without increased predisposition for cancer. These include Cockayne syndrome (CS) and trichothiodystrophy (TTD) which are characterised by a wide spectrum of clinical features that does not include the severe pigmentation abnormalities nor the skin carcinogenesis observed with XP.

Due to its size and complexity, the entire DNA molecule obviously can not be repaired at the same time. However, cells have developed an efficient subpathway of NER that focusses on those regions in the DNA that are being transcribed and thus are of great importance for cell function. This transcription-coupled repair pathway preferentially removes transcription-blocking lesions from the transcribed strand of active genes. It thereby allows rapid resumption of the vital process of transcription, while also reducing the occurrence of mutations in active genes. It was found that the DNA repair defect of Cockayne syndrome cells resides in this transcription-coupled repair subpathway, while the slower repair of the global genome still functions as in normal cells (171). The two genes mutated in CS, *CSA* and *CSB* (*ERCC6*), have been isolated recently (58, 159) which allows examination of the function of these gene products and their involvement in transcription-coupled repair.

Scope of this thesis.

In recent years, the interest in transcription-coupled repair has increased in both the transcription as the DNA repair field. This is mainly due to the identification of repair factors in the basal transcription factor TFIIH, providing evidence for a functional link between the two processes. The aim of the experimental work described in this thesis is to gain more insight into the role of the Cockayne syndrome B (CSB) gene in transcription-coupled DNA repair. Two experimental approaches have been followed. Firstly, the yeast *Saccharomyces cerevisiae* homolog of CSB (RAD26) was isolated and the functional consequence of disruption of the gene was analysed in detail, also in combination with other yeast repair mutants (described in appendix II and III). Secondly, after the finding that the previously isolated *ERCC6* gene is involved in CS-B (appendix I), a close examination of stable associations of the human CSB protein with repair and transcription factors was performed (appendix IV). To clarify the current knowledge on transcription-coupled repair, chapter 2 will first discuss the status of transcription-coupled DNA repair in various organisms, the factors that are required for this and recent indications of additional roles that CSA and CSB might have, apart from mediating the coupling between transcription and repair.

Chapter 2

Transcription-coupled DNA repair

2.1 Transcription-coupled DNA repair: the concept.

The discovery of transcription-coupled repair (TCR) was made in 1985 by Phil Hanawalt and coworkers (12). Using an elegant method (Figure 1), Bohr *et al.* showed that repair of UV-induced cyclobutane pyrimidine dimers (CPD) in the active *DHFR* gene in Chinese hamster ovary cells occurred much more efficiently than in the genome overall (12). Subsequently also the active *c-abl* gene was found to be repaired much faster than the inactive *c-mos* gene in mouse 3T3 fibroblasts (96). Initially, it was thought that this was simply due to the degree of compactness of local chromatin in active genes. However, though this parameter indeed influences the rate of repair (17, 142, 154), it is not the principle mechanism of enhanced repair of active genes. This became evident from experiments by Mellon *et al.*, who demonstrated that the preferential repair of active genes is due to faster removal of CPD lesions from the transcribed (template) strand only, whereas the nontranscribed (coding) strand is repaired at a similar rate as the global genome (104).

At least for a number of lesions, the nucleotide excision repair process can thus be dissected into a transcription-coupled and a nontranscription-associated repair pathway. The latter, called global genome repair (GGR), removes lesions from the entire genome, thereby varying in efficiency dependent on factors such as the kind of lesion, or differences in chromatin structure. Several terms have been used to describe these phenomenona. Differential repair indicates the difference in repair efficiencies between two DNA regions, e.g. heterochromatin- versus euchromatin-like DNA, whereas preferential repair reflects the faster repair of active versus inactive genes. Transcription-coupled repair, also referred to as transcription-associated repair, is used to indicate the faster repair rate of the transcribed strand versus the nontranscribed strand of an active gene, and which is dependent on active transcription.

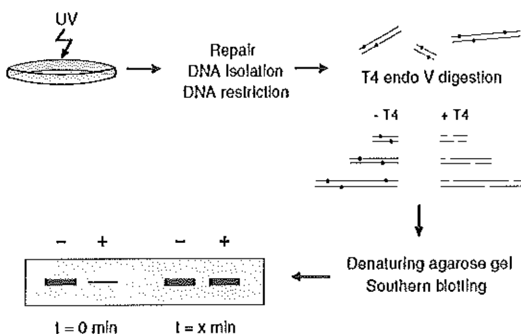


Figure 1. *Schematic representation of the procedure to determine strand-specific DNA repair of a gene.*
Cells are irradiated with a certain dose of UV-C, that is high enough to introduce a sufficient amount of CPDs in the genome, without having immediate effects on cell function. After certain time points to allow repair, genomic DNA is isolated, purified, digested with appropiate restriction enzymes and treated with T4 endonuclease V. The latter enzyme introduces single-stranded incisions in DNA at the site of a pyrimidine dimer. Treated or mock-treated DNA samples are size-fractionated by electrophoresis on denaturing agarose gels, separating the two strands, and transferred to Southern blots. These blots are then hybridised with strand-specific probes of the gene to be studied, visualising repair of the transcribed and of the nontranscribed strand. If dimer repair of the gene is not completed, nicks will be introduced, resulting in lower amounts of full-length fragment. So by comparing the re-appearance of full-length fragment in the treated versus the mock-treated samples in time, one can analyse strand-specific repair of active and inactive genes.

Transcription-coupled repair is likely to be initiated by a stalled RNA polymerase (RNAP) II complex, that is blocked by a lesion in the transcribed strand, and that is signaling the repair machinery to the site of the lesion (57). Several lesions have been shown to form an obstruction for transcription, including UV-CPD (40, 129), cis-platin (31), psoralen monoadducts and interstrand crosslinks (138), dG-C^8-(acetyl)aminofluorene (24, 39), and single strand nicks (194). Also, DNA-bound proteins can block ongoing transcription (114, 123, 190). Enhanced repair of the transcribed strand seems to be restricted to genes that are transcribed by RNAP II, as it has not been found in RNAP I or III genes (26, 85, 148, 179). However, using mutants that are specifically impaired in global genome repair in man and in yeast (see below), it has been shown recently that the transcribed strand of rDNA genes, encoding ribosomal RNA and transcribed by RNAP I, is preferentially repaired, indicative of some form of TCR also of these genes (166, 175). Interestingly, although there is an absolute requirement for active transcription, the rate of TCR does not seem to be correlated to the rate of transcription (7, 170), nor to the transcription-blocking efficiency of the lesion (100).

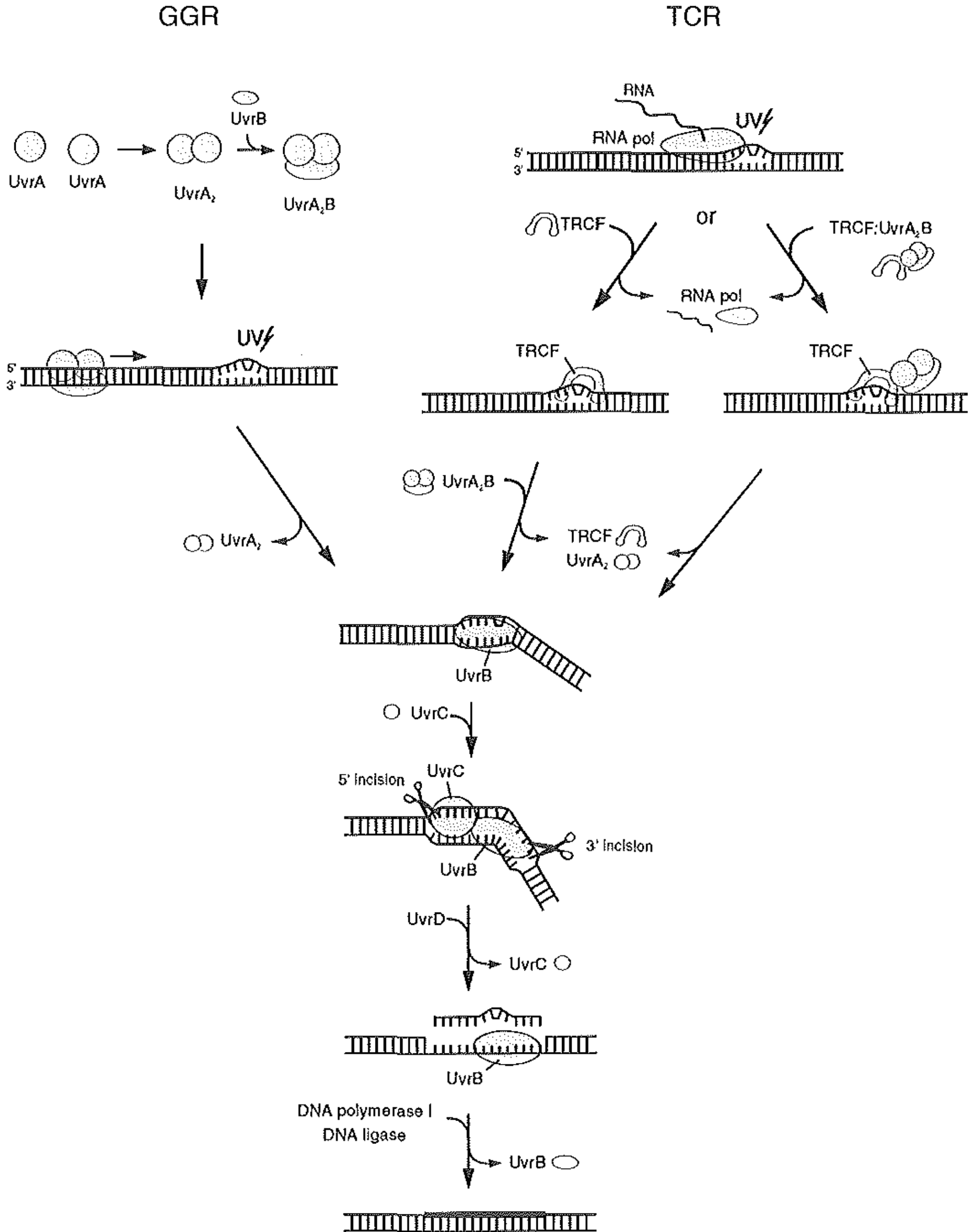
2.2 Transcription-coupled repair in prokaryotes.

Nucleotide excision repair in *E. coli*.

The core mechanism of NER has been elucidated to considerable detail in the bacterium *E. coli*, and forms a paradigm for NER in all organisms studied so far (64). Six proteins, controlling this process, have been identified: UvrA, UvrB, UvrC, UvrD/Helicase II, DNA polymerase I and DNA ligase. The cloning and purification of these repair factors, combined with the generation of *in vitro* damaged DNA templates, has allowed a close examination of every step in the NER pathway (Figure 2) (reviewed in (125, 167, 178)).

E. coli NER is initiated by the formation of a complex of two molecules of UvrA and one of UvrB which together can bind to damaged DNA with high affinity. The broad substrate specificity of the UvrABC system suggests that the $UvrA_2B$ complex more likely recognises the lesion-induced DNA distortion, istead of the lesion itself. Upon binding, UvrB intercalates in the DNA and thus causes a conformational change of the helix. The UvrA dimer dissociates and UvrC binds to the UvrB-DNA preincision complex, after which a dual incision is introduced in the damaged strand at the 8th phosphodiester bond 5′ and the 4th or 5th phosphodiester bond 3′ of the damaged nucleotide. Site specific mutagenesis of *UvrB* and *UvrC* has located the active site for the 5′ incision in UvrC, and the one for the 3′ incision in UvrB (93, 94). However, recent work has indicated that one of the *UvrB* mutants in fact is disturbed in the UvrB-UvrC interaction, thus decreasing the incision efficiency on the 3′ site and raising the question whether UvrB is generating the 3′ cut (105). The concerted action of the helicase UvrD and DNA polymerase I removes the 12-13 mer containing the damage, the UvrB and UvrC proteins and fills the generated gap by DNA synthesis. Finally, DNA ligase seals the remaining nick.

opposite page: **Figure 2.** *Nucleotide excision DNA repair in the bacterium E. coli. Both the global genome repair (GGR) as well as the transcription-coupled repair (TCR) pathways are depicted. See the text above for details.*



GGR
TCR
UvrA
UvrA
UvrA₂
UvrB
UvrA₂B
RNA
RNA pol
UV
5'
3'
TRCF
or
TRCF:UvrA₂B
RNA pol
TRCF
TRCF
UvrA₂
UvrA₂B
TRCF
UvrA₂
UvrB
UvrC
5' incision
UvrC
3' incision
UvrB
UvrD
UvrC
UvrB
DNA polymerase I
DNA ligase
UvrB

Transcription-coupled repair in *E. coli*.

The presence of transcription-coupled repair in *E. coli* was confirmed by repair analysis of the lactose operon under noninduced or induced conditions *in vivo* (102). Only when transcription was induced, the transcribed strand was repaired much faster than the complementary strand (102). When attempts were made to reconstitute transcription-coupled repair *in vitro*, a problem was encountered. RNA polymerase, UvrA, UvrB, UvrC, UvrD, DNA polymerase I and ligase were incubated with a DNA template containing a strong *tac* promoter and defined CPD lesions. Paradoxically, repair of the transcribed strand was inhibited instead of stimulated (129). Apparently a stalled RNAP alone was not sufficient to mediate preferential repair of the transcribed strand *in vitro*. The solution came from the identification and isolation of a *t*ranscription-*r*epair *c*oupling *f*actor (TRCF), that was required and sufficient for transcription-coupled repair *in vitro* (130). TRCF was found to be encoded by the *mfd* gene (131, 135). MFD (*m*utation *f*requency *d*ecline) is the phenomenon that after UV-irradiation, cells rapidly decrease the frequency of revertant mutations in tRNA genes, without loss of viability (186). To be able to observe MFD, cells have to be maintained in medium that allows various energy consuming metabolic processes, like DNA repair, but does not favor protein synthesis (9, 187). Since the revertant phenotype arises from UV-induced mutations in the transcribed strand of the tRNA genes, and *mfd-1* mutant cells can not prevent these mutations to occur, it was predicted that the *mfd* phenotype was caused by a defect in preferential repair of the transcribed strand (10). Indeed, it was shown both *in vitro* (135) and *in vivo* (78, 101) that *mfd-1* cells are impaired in transcription-coupled repair. This defect in extracts from *mfd-1* cells could be corrected by adding purified TRCF protein (131).

TRCF.

The amino acid sequence of TRCF (130 kDa) reveals three interesting motifs, namely a region with considerable homology to UvrB near the N-terminus, seven RecG-like helicase motifs in the central part, and a potential leucine zipper near the C-terminus (131). Using truncated TRCF constructs and affinity chromatography, Selby and Sancar showed that the UvrB homologous region can firmly bind UvrA, and that the complete helicase region of TRCF plus flanking sequences are involved in RNAP binding and displacement (131, 133, 134). The helicase motifs encode a weak ATPase activity, that was not stimulated by DNA nor by stalled RNAP (131). In contrast to the presence of the helicase motifs, TRCF does not display a helicase activity when incubated with various DNA or DNA:RNA hybrid templates (134). However, this region binds DNA in an ATP-dependent fashion, without any preference for DNA structure or the presence of RNA, and footprinting data may indicate a wrapping of the DNA around the protein. TRCF is able to bind both initiation and elongation RNAP complexes, yet only displaces elongating RNAP that is stalled by a lesion, a protein block or by missing-base induced pause sites. This dissociation requires the very C-terminal part and thus possibly the potential leucine zipper, although this motif is not required for RNAP binding and is not conserved in the TRCF homolog of *B. subtillis* (133). The RNAP displacement by TRCF is clearly distinct from the way rho protein terminates transcription by removing elongating RNAP (114, 133). By itself, TRCF seems to stimulate premature termination, but this might be influenced by the *in vitro* conditions.

Model for TCR in *E. coli*.

A model was proposed for transcription-coupled repair in *E. coli*, taken into account the activities of the TRCF and its associations with DNA, UvrA and RNAP (132)(Figure 2). The first step of TCR is formed by stalling of the elongating RNAP complex on a lesion or an other obstruction in the transcribed strand. Such stalled complex is recognised and bound by TRCF, that subsequently may displace RNAP and the nascent RNA chain. TRCF then attracts the $UvrA_2B$ complex via an unknown interaction and via competitive binding at the UvrA-UvrB interface, dissociates the UvrA dimer and stimulates integration of UvrB in the DNA. Alternatively, TRCF may already be associated with the $UvrA_2B$ complex prior to binding to a stalled RNAP. Since, UvrB contains two UvrA binding sites, one of which corresponds to the homologous region with TRCF (133), TRCF may be associated with a UvrA monomer, which in turn is bound to UvrB. It was found *in vitro* that TRCF efficiently disrupts the $UvrA_2B$ complex, but this does not have to occur *in vivo*. In either scenario, TRCF will stimulate the targeting of UvrB to the lesion. The conformational change induced by UvrB-DNA binding may or may not release TRCF, but will stimulate binding by UvrC, and excision of the lesion occurs as described above.

In vivo studies of repair of single nucleotides in the transcribed strand of the active *E. coli* genes *uvrC* (134), *lacZ* and *lacI* (78) and also of the human genes *PGK1* (50) and *JUN* (162) confirms that TCR acts only on an elongating RNAP complex. In fact, these studies indicate inhibition of repair when RNAP was still bound to the initiation site, although *in vitro* TRCF can bind an initiation complex (133). Apparently, the conversion of a RNAP initiation to an elongation form is required for proper transcription-repair coupling. In *E. coli* this would indicate the dissociation of the σ subunit and in man the phosphorylation of the C-terminal domain (CTD) of the large subunit of RNAP II (33, 44).

While *mfd-1* cells characteristically do not show MFD, they do perform partial MFD when transcription initiation is inhibited by rifampicin (91). This could indicate that a part of the MFD is caused by the bulk excision pathway of UvrABC. In the absence of TRCF, transcription complexes are blocked by lesions in the transcribed strand of the tRNA genes and this might inhibit repair, as found *in vitro* (129). When transcription is inhibited, the lesions are accessible and repaired by the UvrABC endonuclease (although not preferentially), resulting in partial MFD. Interestingly, this level of residual MFD is similar to the MFD observed in the mismatch repair mutants *mutS* and *mutL*, indicating that in these mutants TCR might be affected (91). Indeed, *mutS* and *mutL* were found to be deficient in fast repair of the transcribed strand of *lacI*, comparable to the *mfd-1* mutant (101). At present, it is unclear whether the observed TCR defect in mismatch repair mutant cells is a general phenomenon, since it has also been observed in man (103), S.A. Leadon pers. comm.), but not in yeast (150).

2.3 Transcription-coupled repair in eukaryotes.

Nucleotide excision repair in eukaryotes.

The discovery in 1968 that the hereditary disorder xeroderma pigmentosum is caused by a defect in DNA excision repair (28) formed the basis for DNA repair research in eukaryotes. A finding of equal importance was the identification in 1972 of different complementation groups within XP, indicating that XP was a multigenic disorder (36). Complementation analysis is based on the notion that cells carrying mutations in different genes of the same pathway complement each others deficiency when fused. In contrast, cells with mutations in the same gene fail to correct each others defect. Complementation analysis of UV-sensitive Chinese hamster ovary, yeast and human cells has greatly facilitated the cloning and characterisation of the DNA repair genes involved in NER.

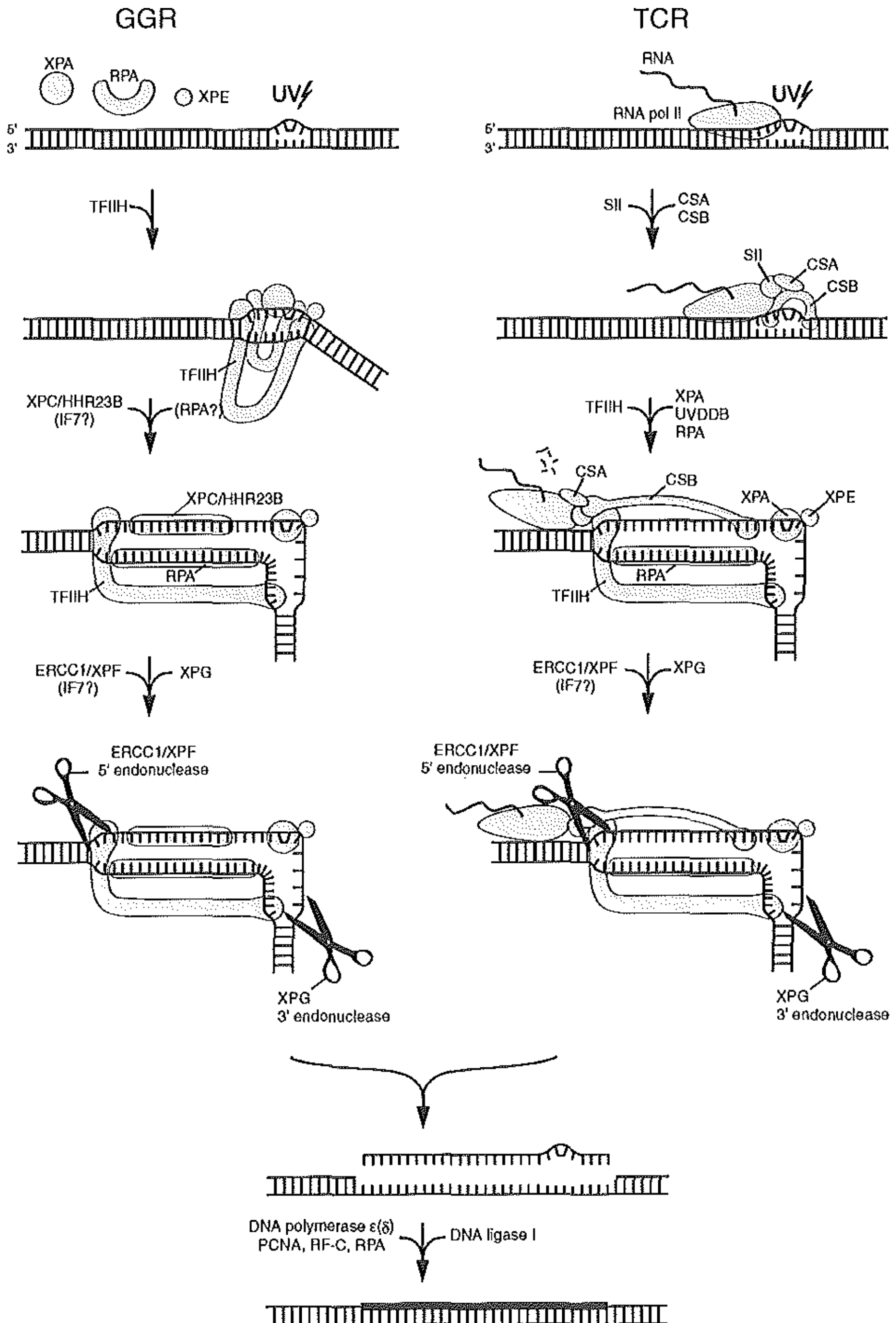
At present, three hereditary human disorders have been associated with a defect in nucleotide excision repair: xeroderma pigmentosum (XP), Cockayne syndrome (CS) and PIBIDS, the photosensitive form of trichothiodystrophy (TTD) (Table 1) (29, 87, 109, 144). Patients suffering from XP display an extreme photosensitivity, pigmentation abnormalities and a strongly enhanced predisposition to develop cancer in sun-exposed areas of the skin. In some cases also neurological degeneration is observed (29). CS and TTD patients also show increased photosensitivity of the skin. However, in contrast to XP, they do not develop skin tumours. Typical clinical features of CS include neurological dysmyelination, immature sexual development, mental retardation, and impaired physical development manifested in cachetic dwarfism, microcephaly, skeletal and retinal abnormalities, and a characteristic 'wizzened' appearance (bird-like face) (87, 109). The most typical TTD features include brittle hair and nails (due to a sulphur-deficiency), ichthyosis (dry skin), and neurological abnormalities resembling those of CS (69, 87). In addition, patients have been described that display combined features of XP/CS and XP/TTD (75).

At least eleven genes are involved in these repair syndromes: eight in XP (*XPA-XPG*, and an XP variant *XPV*), two in CS (*CSA* and *CSB*) and one in TTD (*TTDA*), while mutations in *XPB* and *XPD* can lead to a XP, XP/CS or TTD phenotype and mutations in *XPG* result in a XP or XP/CS phenotype (Table 1)(13, 27, 65, 176). In recent years, many of the genes and proteins involved in NER have been identified and isolated (reviewed in 48, 65, 141, 147, 152). Strikingly, there is a very high degree of conservation between the repair genes and pathways of the lower eukaryote *S. cerevisiae* to mammals (65). The analysis of yeast and mammalian repair mutant cells, *in vivo* and *in vitro* protein-protein interactions and characteristics of the proteins and protein complexes involved has allowed close examination of the seperate steps of the eukaryotic NER mechanism and led to a model (1, 48, 106, 107, and references therein). The principle steps of eukaryotic NER are similar to those in *E. coli* (Table 2) and are depicted in Figure 3. For simplicity, I will restrict here to the human NER.

Table 1. *Features of NER-deficient XP, CS and TTD complementation groups.*

compl. group	clinical		repair		NER defect		correcting gene		
	skin cancer	neurological abnormalities[a]	UV sens.	residual UDS (%)[b]	GGR[c]	TCR[d]	human	yeast hom.	Remarks
XP-A	+	+ +[I]	+ + +	< 5	+	+	*XPA*	*RAD14*	Binds UV-damaged DNA
XP-B	+/-	+ +/+[I]	+ +	10-40	+	+	*XPB (ERCC3)*	*RAD25/SSL2*	3′ → 5′ helicase; subunit of TFIIH
XP-C	+	-	+	15-30	-	+	*XPC*	*RAD4*[e]	ssDNA binding; complex with HHR23B
XP-D	+/-	+ +/±[I]	+ +	15-30	+	+	*XPD (ERCC2)*	*RAD3*	5′ → 3′ helicase; subunit of TFIIH
XP-E	+/-	-	±	≥ 50	+	+	?	?	Binds UV-damaged DNA
XP-F	+/-	-/±[I]	+	15-30	+	+	*XPF (ERCC4)*	*RAD1*	5′ incision; in complex with ERCC1 (Rad10p)
XP-G	+/-	+ +[I]	+ +	2-25	+	+	*XPG (ERCC5)*	*RAD2*	3′ incision
CS-A	-	+[II]	+	100	+	-	*CSA (ERCC8)*	*RAD26*	SWI2/SNF2 ATPase/'helicase'
CS-B	-	+[II]	+	100	+	-	*CSB (ERCC6)*	*RAD28*	WD-repeat protein
TTD-A	-	+[II]	+	10%	+	+	?	?	unidentified subunit of TFIIH

For references see text. a) Classified as I: neurological degeneration or II: neurological dysmyelination, b) Unscheduled DNA synthesis (% of wild-type cells), c) Global Genome Repair, d) Transcription-Coupled Repair, e) only sequence homology; functional homologs of *XPC* in yeast are *RAD7* and *RAD16*.


GGR
TCR
XPA
RPA
XPE
UV
5'
3'
RNA
RNA pol II
TFIIH
SII
CSA
CSB
TFIIH
XPC/HHR23B
(IF7?)
(RPA?)
XPA
UVDDB
RPA
XPC/HHR23B
RPA
TFIIH
CSA
CSB
XPA
XPE
ERCC1/XPF
(IF7?)
XPG
ERCC1/XPF
5' endonuclease
XPG
3' endonuclease
DNA polymerase ε(δ)
PCNA, RF-C, RPA
DNA ligase I

opposite page:
Figure 3. *Nucleotide excision repair in man. Both global genome repair (GGR), defective in XP-C, as well as transcription-coupled repair (TCR), impaired in CS-A and CS-B, is schematically depicted. See text below for details (the yeast equivalents are indicated between brackets).*

Damage recognition probably requires the XPA (Rad14p) and XPE proteins, both of which have been shown to have a high affinity for UV-damaged DNA. The region around the lesion is subsequently melted by the combined helicase activities of XPB/ERCC3 (Ssl2p/Rad25p) (3'→5') and XPD/ERCC2 (Rad3p) (5'→3'). These two proteins are subunits of the transcription/repair complex TFIIH, of which also other components are involved in NER (66, 183, 192). The thus generated stretches of single stranded DNA are presumably stabilised by binding of RPA, which is a single strand DNA binding protein and also is involved in DNA replication and homologous recombination (59, 145). Then, the 3' incision is made by XPG (Rad2p) at the 6th phosphodiester bond, while the ERCC1/XPF (Rad1p/Rad10p) complex introduces the 5' incision at the 20-22nd phosphodiester bond resulting in excision of a 28-30 mer containing the damaged base(s). Gap-filling DNA synthesis and ligation are performed by DNA polymerase ϵ and/or σ, PCNA, RPA, RF-C and ligase I. In addition to this simplified model, several gene products, such as the XPC/HHR23B (Rad4p/Rad23p) protein complex (97) and IF7 (1), are required for *in vitro* reconstitution of NER, yet their specific role is unclear.

Table 2. *Proteins involved in each step of nucleotide excision repair in E. coli, yeast and man.*

function	*E. coli*	yeast	human
lesion recognition	$UvrA_2B$	Rad14p/?	XPA/XPE
unwinding damaged region	$UvrA_2B$	TFIIH	TFIIH
3' incision	UvrB/UvrC (?)	Rad2p	XPG
5' incision	UvrC	Rad1p/Rad10p	ERCC1/XPF
excision and repair synthesis	UvrD,DNA pol I	DNA pol II/III	DNA pol δ/ϵ
ligation	DNA ligase	DNA ligase	DNA ligase I

Transcription-coupled repair in yeast.

The first indications of differential repair in the yeast *S. cerevisiae* were provided by repair studies of the two mating type loci *MATα* and *HMLα*, that are identical in sequence. While in yeast *α*-cells the *MATα* locus is actively transcribed, the *HMLα* locus is silenced by the SIR proteins, which results in about 2.5 times more efficient repair of *MATα* (155). This differential repair, however, turned out not to be caused by the transcriptional state of the two loci, but by the difference in local chromatin structure. When the upstream activating sequence (UAS) of the *MATα* locus is mutated, the gene is no longer transcribed, yet repair is still faster than *HMLα*. Also, in a *sir-3* mutant the closed heterochromatin-like structure of *HMLα* is relieved

and *HMLa* repair resembles that of *MATa* (17, 154). Later, true transcription-coupled repair was demonstrated for *URA3* (142), *RPB2* (148), and induced *GAL7* (86). The dependency of fast strand-specific repair on active RNAP II transcription was demonstrated by the absence of TCR in temperature-sensitive RNAP II mutants (86, 148).

RAD26.

At present, only one factor, RAD26, is known to be involved in TCR in yeast. The *RAD26* gene was isolated based on homology with the human *CSB/ERCC6*, one of the two human factors required for TCR (Appendix II, 164). A *rad26* disruption mutant hardly displayed preferential repair of the transcribed strand of the active *RPB2* gene (164) and of the *PHO5 PHO3* locus (unpub. results), indicating that RAD26 is required for TCR in yeast. In contrast to its human equivalent, *RAD26* disruption did not lead to enhanced sensitivity to UV-irradiation. This might be explained by the higher efficiency of global genome repair in yeast, that may compensate for the loss of TCR. Indeed, we recently obtained evidence for an overlap between the two pathways in yeast (175). In a similar way, the *mfd-1* mutation in *E. coli* does not lead to a highly increased UV-sensitivity (51, 131). Interestingly, *RAD26* disruption leads to a slower recovery of growth after UV-irradiation, which might indicate a functional role of TCR in yeast under non-laboratory conditions (164).

Double mutants in transcription-coupled and global genome repair.

Previously it was shown that the yeast genes *RAD7* and *RAD16* are indispensable for global genome repair, i.e. repair of nontranscribed DNA sequences such as the silent *HMLa* locus and the nontranscribed strand of *RPB2* (6, 174). Yeast genetics allowed us to generate isogenic *rad7/16 rad26* double mutants that are disturbed in both transcription-coupled as well as global genome repair (Appendix III, 175). If RAD26 would be the only factor mediating TCR, and if RAD7 and RAD16 were responsible for repair of all nontranscribed DNA, then a *rad7 rad26* or *rad16 rad26* double mutant is expected to have no residual repair at all. However, UV-survival experiments indicated that although there is a significant increase in UV-sensitivity, the double mutant is still capable of removing some lesions from the genome. When strand-specific repair of *RPB2* was studied, it was found that, as expected, the *rad7/16 rad26* mutants did not repair the nontranscribed strand of this active gene. However, the transcribed strand was still repaired, albeit less efficiently than in a *rad26* background. A similar level of residual repair of the transcribed strand of the induced *GAL7* gene was observed in the double mutant. This Rad26p-independent repair of the transcribed strand was clearly dependent on transcription, as it was absent in repressed *GAL7* (175).

These results indicate that there is a considerable overlap between NER subpathways. For instance, Rad26p does contribute to UV-survival, but in a *rad26* null mutant this is compensated for by global genome repair. Also, global genome repair (*RAD7/16*) can operate on the transcribed strand of an active gene, but this is only visualised when transcription-coupled repair (*RAD26*) is impaired. Most importantly, the double mutants revealed a third NER activity that is restricted to the transcribed strand. Therefore, one can distinguish three repair activities within one active gene: Rad26p mediates enhanced repair of the transcribed strand only, Rad7p and Rad16p are required for repair of the nontranscribed strand but can also act on the transcribed strand, while this unknown repair moiety also contributes to transcribed

strand repair. The existence of a second factor coupling transcription and repair can readily explain previous puzzling observations: the persistence of some TCR of *RPB2* in the *rad26* mutant (164), and the finding that *RAD26* disruption had no significant effect on TCR of induced *GAL7*, unless it was combined with *RAD7* or *RAD16* mutations (175). Alternatively, the *GAL7* repair results can also be explained by the fact that under inducing conditions this gene is very heavily transcribed (143), which may alleviate the requirement for a coupling factor, as has been found for the IPTG-induced *lacZ* gene in the *E. coli mfd-1* mutant (78).

There are at least four possible candidates for this second TCR factor. First, a yeast homolog of CSA, the other human factor required for TCR (165, 171), could contribute to TCR similarly to Rad26p. The human *CSA* gene has recently been cloned (58) and a putative *S. cerevisiae* homolog has been identified and isolated (8). Although the two genes share a significant sequence homology, no functional homology has been detected so far. Disruption of this yeast gene, tentatively designated *RAD28*, did not lead to aberrant survival, recovery of growth or strand-specific repair following UV-irradiation, as was observed with the *rad26* mutant. A redundancy of *RAD28* function by *RAD26* was excluded by doing similar repair studies as discussed above in *rad26 rad28*, *rad7/16 rad28*, and *rad7 rad26 rad28* double/triple mutants (8). Second, mismatch repair could be involved in TCR as is suggested from the above mentioned observations in *E. coli* and man. However, various yeast mismatch repair mutants and combinations of them were tested for their ability to perform TCR and no defect was observed. Also, a combination of the *msh2* mutant with *rad7*, *rad16* and *rad26* showed no synergistic effect on transcribed strand repair (150). Third, the transcription elongation factor SII has been shown to stimulate the retractive movement and exonucleolytic RNA cleavage of stalled RNAP II complexes, leading to an increase in by-pass efficiency (40, 72, 76, 79, 121). Since the SII-mediated transcript shortening can form the basis of transcription-coupled repair (57, 164), SII could stimulate TCR independent of Rad26p and be responsible for the residual repair activity observed in the *rad7/16 rad26* mutant. However, *SII* disruption mutants showed no elevated UV-sensitivity nor TCR defect by themselves or in combination with *rad7*, *rad16* or *rad26* (K.S. Sweder, R.A. Verhage, both unpubl. obs.), making it unlikely that SII plays an important role in TCR. Alternatively, there might be more SII-like genes in yeast that provide redundancy (23, 191). Fourth, the stalled transcription machinery itself might stimulate attraction of repair proteins to the site of the lesion, e.g. mediated by the unphosphorylated C-terminal domain (CTD) of the largest subunit of RNAP II (as discussed below).

Transcription-coupled repair in *Drosophila melanogaster*.

In the permanent cell line *Kc*, derived from wild type embryonic *Drosophila melanogaster* cells, no strand-specific transcription-enhanced repair of the active genes *Gart*, *Notch* and *ß$_3$-tubilin* could be demonstrated (34,35). This would suggest that *Drosophila* lacks TCR, which would be highly surprising given the conserved nature of this process. Recent studies on repair activities of mouse embryonic stem (ES) cells might indicate that the observed defect in TCR may be intrinsic to embryonic cells rather than being specific for *Drosophila*. Mouse ES cells are quite UV-sensitive, have a very low overall repair activity, do not show preferential repair of the transcribed strand of *DHFR* (P.van Sloun, H. Vrieling unpubl. obs.). Also, ES cells display a high

level of apoptosis after UV-treatment, which may serve to prevent the fixation of mutations. The altered repair characteristics of ES cells, when compared to mouse fibroblasts, may be linked to the different chromatin structure within these cells, in analogy with differences in chromatin of *Xenopus laevis* early embryonic versus adult cells (38, A.P. Wolffe pers. comm.). Analysis of strand- and gene-specific repair in a differentiated *Drosophila* cell line should clarify the status of TCR in *Drosophila*, but these cells are very difficult to maintain in culture. A recent study of TCR in *Drosophila* using a very large number of isolated brains again did not indicate any evidence for enhanced repair of active genes. However, interpretation of these results might be complicated by the intrinsic variability (especially at early time points) of these complex experiments (P.J.L. van der Helm, J.C.J. Eeken submitted).

Transcription-coupled repair in man.

Most XP genes are required for GGR as well as TCR, with the notable exception of XPC, that is involved in GGR only (Table 1). This is evident from the fact that XP-C cells have functionally lost the ability to repair inactive DNA sequences, including the nontranscribed strand of active genes (77, 172, 173). The transcribed strand of these genes is still repaired as in repair-proficient cells, indicating that TCR can overcome the repair defect imposed by loss of XPC. This is also exemplified by the fact that XP-C cells are fully capable in recovering their RNA synthesis after UV-irradiation, in contrast to other XP and CS cell lines (99). Moreover, it has been shown that the residual repair of XP-C cells is dependent on RNAP II transcription, since it can be completely blocked by specific RNAP II inhibitors (21). The XPC protein was shown to reside in a complex with one of the human homologs of the yeast Rad23p, HHR23B, and in this form displayed a strong single-stranded DNA binding activity (97). Apparently, the XPC/HHR23B complex is required to target the repair machinery to regions in the genome that are not transcribed. Similarly, this complex is absolutely required for *in vitro* (nontranscription-coupled) NER of most damages, but at present its function in NER is unknown (1, 106).

On the other hand, CSA and CSB are essential for TCR. CS-A as well as CS-B cells are deficient in enhanced repair of the transcribed strand of the active genes *ADA* and *DHFR*, while having normal global genome repair activities (165, 171). Moreover, antibodies raised against CSA and CSB can inhibit the residual repair in XP-C fibroblasts, as visualised by UV-induced *u*nscheduled *D*NA *s*ynthesis (UDS) (Appendix IV). When tested in the *in vitro* repair assay (189), cell-free extracts of CS-B cells are as active as repair-proficient cells (E. Citterio, A.J. Van Gool unpublished). Because no transcription takes place under these conditions, this confirms the selective defect of CS-B in transcription-coupled repair.

The recent identification of a dual role in repair and transcription of the basal transcription factor TFIIH (41, 46, 127, 169, 177, 183) provided a plausible link of transcription to TCR, and suggested a central role for TFIIH in this connection. However, it should be noted that mutations in TFIIH subunits, in yeast and in man, lead to defects in both transcription-coupled as well as transciption-independent DNA repair (61, 66, 149, 177, 183, 192). Also, immuno-depletion of repair-competent cell-free extracts using various antibodies raised against subunits of human TFIIH deprived these extracts from *in vitro*, i.e. nontranscription-coupled, repair activity (67, 177). Recently, evidence was presented that TFIIH exists in two forms: as holo-TFIIH for transcription and as part of a nucleotide excision 'repairosome' that participates

in repair of all DNA (146). Although TFIIH might not be the factor determining the strand-biased repair of an active gene, it can certainly contribute to this phenomenon (see below).

CSA.

Both *CSA* and *CSB* genes have been identified and isolated, allowing biochemical analysis of their role in transcription and repair. The Cockayne syndrome A gene was recently isolated by transfections of a cDNA expression library to an immortalised CS-A cell line and selection of UV-resistant colonies (58). *CSA* (which is similar to *ERCC8* (70)) encodes a protein of 44 kDa that contains five WD repeats. These rather loosely defined WD repeats are found in a large family of proteins that have diverse cellular functions, including signal transduction, RNA metabolism, gene regulation, cellular transport, and cell division (reviewed in (110). A common function associated with WD-repeat proteins seems to be a regulatory one, possibly by stimulating the formation of multiprotein complexes. The WD-repeat domain contains certain hydrophobic regions and may fold into a ß-sheet-turn-ß-sheet-turn-ß-sheet structure, which might facilitate interactions with other WD-repeat and additional proteins (110).

CSB.

The Cockayne syndrome B gene was first isolated as *ERCC6* by its ability to correct the UV-sensitivity of the CHO mutant UV61, belonging to complementation group 6 (158). The *ERCC6* gene is localised on chromosome 10q11-q21 (157). Because this region was reported to be deleted in a patient with Cockayne syndrome symptoms (49), transfections of the *ERCC6* cDNA were performed to CS-A and CS-B cells (Appendix I, 159). Transfected CS-B but neither CS-A nor various XP cells showed correction of UV-sensitivity to wild type levels, indicating that *ERCC6* mutations were underlying the repair defect in CS-B. Indeed, severely truncating mutations in *ERCC6* were identified in a CS-B cell line (159), and the gene was renamed *CSB* (88). The *CSB* cDNA encodes a protein of 1493 amino acids (168 kDa), that contains several interesting domains. First, an acidic amino acid stretch is present in the NH_2-terminus (aa 356-

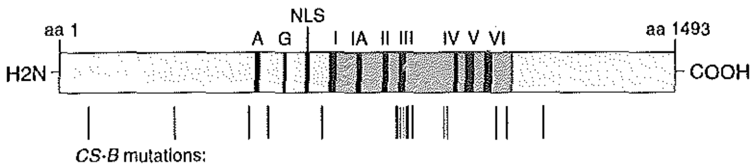


Figure 4. *Schematic representation of the CSB protein.*
The conserved domains are depicted as follows: A, acidic amino acid stretch; G, glycine-rich region, NLS, nuclear localisation signal; I-VI, helicase motifs. The entire middle region (darker box) is strongly conserved (31% identical amino acids) within the SWI2/SNF2 subfamily of ATPases/ putative helicases. Also indicated are CSB mutations identified thus far by Donna Mallery (Dr. A. Lehmann's laboratory), and Bianca Tanganelli (Dr. M. Stefanini's laboratory). Most of these mutations result in nonconservative amino acids changes, frame shifts, or in a premature stop (unpublished).

394), which might be involved in chromatin association (117). This is followed by a short stretch of glycine residues (aa 442-446) and a consensus sequence for a bipartite nuclear localisation signal (aa 466-481) (124). The middle part of the CSB protein (aa 527-950) contains seven motifs which are strongly conserved in many known and putative RNA and DNA helicases (54). Moreover, the complete helicase/ATPase region is strongly conserved to comparable parts of proteins belonging to the still expanding SNF2 subfamily of putative helicases (reviewed in (14, 20, 45). This subfamily encompasses at least 25 proteins of diverse function, such as transcription activation (SWI2/SNF2, ISWI, Brahma, XH2) or repression (STH1, MOT1), various DNA repair pathways (CSB, RAD5, RAD16, RAD54), preservation of chromosome stability (lodestar), and also includes two prokaryotic proteins (45 and references therein, 52, 81, 90).

The function of the conserved helicase/ATPase region is still unclear, but there is increasing evidence that the entire helicase region is required for disruption of DNA-bound protein complexes, simultaneously with ATP hydrolysis (62, 116, 185, 188). The yeast SWI2/-SNF2 protein is part of a large complex of at least 10 proteins (19, 115, 156), which has a DNA-dependent ATPase activity (19, 83a). No standard helicase activity, i.e. displacement of a DNA-bound DNA/RNA primer, could be demonstrated (83a). However, by hydrolysis of ATP the SWI/SNF complex is able to disrupt a nucleosome *in vitro*, thus allowing binding of the GAL4 transcription activator (32, 83), or the TATA-binding protein (TBP) (68). Disruption of nucleosomes might be induced by changes in helical twist that are generated by SWI/SNF binding to promoter DNA (119). Recently, evidence emerged that the SWI/SNF complex is associated with the RNAP II holoenzyme, a megadalton-sized complex containing RNAP II, some basal transcription factors and SRB regulatory proteins (80, 184).

A similar, yet distinct, activity has been reported for the human protein ISWI, being part of the nucleosome remodeling factor (NURF) (161). The NURF complex is also capable of disrupting a nucleosome and thus can stimulate transcription factor binding. The required ATPase activity is not stimulated by naked DNA nor by histone proteins but only by an assembled nucleosome, indicating a target specificity (160).

A third example is provided by the MOT1 protein that is able to disrupt binding of TBP (TATA binding protein) to a promoter sequence, and thus inhibit transcription initiation (5). Disruption required the strong ATPase activity of MOT1, which is not stimulated by DNA (with or without TATA sequences). Interestingly, disruption of TBP binding to TATA-sequences has also been reported for a subunit of TFIIIB, TAF-172, which may be the same protein as MOT1 (151). Immunoblot analysis excluded that TAF-172 is the same protein as CSB (H.Th.M. Timmers, unpublished).

Model for human TCR.

The signal that targets repair to the transcribed strand is likely to be formed by an elongating RNAP II, that is stalled on a lesion (57). Since such a stalled RNAP blocks the lesion from being repaired (40, 129), it has to retract to permit access of repair proteins. The elongation factor SII (TFIIS) has been shown to stimulate this retractive movement that precedes transcriptional read-through (72, 121, 122). The 280 amino acid SII protein contains a RNAP interaction domain and a cryptic Zinc ribbon that is expected to be exposed only when SII is bound to an elongation complex (118, 120). Both of these domains seem to be required

for stimulation of elongation (2, 74). Binding of SII was shown to stimulate the intrinsic RNA cleavage activity of the nascent RNA by the stalled RNAP and the by-pass efficiency of the obstruction (79, 121, 180). This mode of transcription stimulation is clearly distinct from that of TFIIF, elongin (SIII) and the recently identified ELL, that all suppress the time a RNAP pauses during elongation (4, 15, 56, 140). Elongation of RNAP II has been shown to proceed via an 'inchworm' movement, in which the leading part slides along the DNA helix, while the back part synthetises the RNA (3, 22, 111). SII-stimulated nascent RNA cleavage of more than nine nucleotides is associated with upstream movement of the leading part of the elongating RNAP (55, 181), and this may allow access of other proteins to the obstruction. However, it was found *in vitro* that SII-stimulated retraction of RNAP ($\approx$ 10 bp) was insufficient by itself for the relatively small repair enzyme photolyase to repair the positioned UV-CPD (40). This indicates that additional factors are required for increasing the retraction efficiency. Also, besides the clearing of the lesion the repair machinery has to be attracted to the lesion to explain preferential repair of the transcribed strand.

In this perspective, the SWI2/SNF2-like helicase/ATPase region identified in CSB and the WD-repeats in CSA might shed more light on their role in transcription-coupled DNA repair. Analogous to the observations made for other SWI2/SNF2-like helicases, the helicase region in CSB could be involved in a partial disruption of the binding of a stalled RNAP to the DNA. Such disruption is likely to facilitate the SII-stimulated upstream movement of the stalled RNAP, thereby providing the opportunity for repair proteins to recognise and remove the lesion. The WD-repeats in CSA may serve to stabilise interactions between CSB and the stalled transcription complex. The attraction of the repair machinery may involve unidentified domains in CSB, residing in the large N-terminal and C-terminal ends, or may be mediated via the WD-repeats of CSA, interacting with WD-repeat transcription proteins (110). Previously, interactions of CSB with XPB, XPD, XPG (43), and of CSB with TFIIH and XPA (42) have been suggested but not unequivocally established. Using immunoprecipitations of *in vitro* translated proteins an interaction between CSA and CSB, CSA and p44 (a subunit of TFIIH) (58), and between CSB and XPG was found (71), however these interactions could not be demonstrated under more physiological conditions that allow active *in vitro* transcription and repair (163). The absence of associations of CSA and CSB with transcription and repair proteins in active cell-free extracts can be explained by the expected transient character of these interactions, which may only occur when the cell is challenged with damaging agents (163).

A tentative and simplified model for TCR in humans, which takes into account the requirement for CSA and CSB, is shown in Figure 3. An elongating RNA polymerase II complex is blocked by a lesion, present in the transcribed strand of the gene. The stalled RNAP has been has a different conformation (82, 112), which is recognised and bound by SII and CSB. These interactions may be stabilised by CSA. The helicase/ATPase region of CSB then mediates the partial disruption of RNAP-DNA binding, which facilitates the upstream dislocation of the stalled RNAP complex by SII. The repair machinery is attracted to the site of the lesion via transient interactions with CSA and/or CSB, the lesion is repaired and transcription elongation resumes.

Although CSA and CSB are required for TCR in man, the stalled elongation complex itself

may also stimulate repair of blocking lesions. A crucial step in the promoter clearance of many genes seem to be the phosphorylation of the C-terminal domain (CTD) of the largest subunit of RNAP (33). This is thought to be the final step in the multi-step process of transcription initiation (reviewed in (30, 193). CTD phosphorylation is likely to be performed by the CDK-subunits of TFIIH (47, 95, 137, 139), and this may convert the RNAP into the elongating mode. Several studies have indicated that the CTD of an elongating RNAP is again hypophosphorylated (33, 113), which is the state of the CTD when the RNAP becomes blocked by a lesion. The underphosphorylated CTD of a stalled RNAP may form a target for binding of the basal transcription factor TFIIE, that in turn recruits TFIIH, as has been well described for the building up of the transcription initiation complex (53, 98). Together with TFIIH, other repair proteins may become attracted to the site of the lesion, maybe already assembled as a 'repairosome' (46, 146) and the lesion will be removed via NER. After repair is complete, the CTD becomes heavily phosphorylated again by the concerted action of TFIIE and TFIIH (98, 136) and transcription elongation resumes with a fresh start. Such targeting of repair activity by a stalled RNAP may explain the residual repair of the transcribed strand observed in the yeast *rad7/16 rad26* double mutant.

Additional roles of CSA and CSB.

The model for human TCR described above is oversimplified and contains several uncertainties, among which the exact role of CSA and CSB. In addition, several observations, some of which are very recent, indicate that more processes, in particular transcription, are impaired by loss of CSA and CSB in yeast, mouse and man.

Firstly, CS patients display characteristic clinical features that cannot be explained on the sole basis of a repair defect (Table 1). They merely reflect neurological and developmental abnormalities, including growth and mental retardation and impaired sexual development. Death results from progressive neurological degeneration, and in most cases before the age of 20 (reviewed in (87, 109)). Comparable features have also been found in XP-B, XP-D, TTD-A and XP-G patients, of which the first three have been shown to carry mutations in subunits of the transcription/repair factor TFIIH. Previously the origin of these typical features was postulated to reside in a (subtle) defect in transcription rather than in repair, which led to the definition of 'transcription syndromes' (13, 177). Following this reasoning one can imagine that CSA and CSB are involved in the transcription process itself in addition of mediating transcription-repair coupling.

Secondly, recently csb-/- knock-out mice have been generated which were found to display a severe defect in TCR of UV-induced CPD lesions (G.T.J. van der Horst, manus. in prep.), in agreement with the yeast null mutant (*rad26*) (164) and the human CS-B cells (165, 171). Other repair characteristics, such as increased UV-sensitivity of the csb -/- mice as well as of derived embryonal fibroblasts, normal GGR (as measured by UV-induced UDS), and defective recovery of RNA synthesis after UV-irradiation, correspond to those of human CS-B cells as well. The csb -/- mice do not display the characteristic hallmarks of CS as dramatically as the human patients. Nonetheless, some minor growth disturbances and neurological aberrations have been noted (G.T.J. van der Horst, manus. in prep.). Surprisingly, the csb -/- mice efficiently develop skin cancer when treated with the carcinogenic agent DMBA. This is in contrast to human CS patients for who no skin cancer predisposition after UV-exposure has

been reported. This might reflect a different response of skin cells to DMBA as compared to UV. To test this hypothesis, csb -/- mice are currently analysed for carcinogenic response to UV. Alternatively, this discrepancy between man and mice might be explained by the much higher contribution of TCR to total cellular repair in rodents compared to human cells. Nonetheless, these studies reveal a role for CSB in mutagenesis and/or carcinogenesis, which initially was not apparent.

Third, an interesting observation was made when human CS cells were exposed to the genotoxic agent N-acetoxy-2-acetylaminofluorene (NA-AAF) (168). This compound is converted in human cells mainly into a dG-C^8-AF adduct, that causes little distortion of the DNA helix (89). dG-C^8-AF lesions have been shown to form a rather inefficient block for an elongating RNAP (24, 39, 100), and they seem not to be subject to preferential repair coupled to transcription in normal cells (153, 168). Remarkably, CS-A and CS-B cells show a 3-fold increased sensitivity and an inability to recover their RNA synthesis after NA-AAF treatment (168). This indicates that the NA-AAF sensitivity of CS cells is not caused by an impaired TCR, but instead may reflect a defect in initiation of transcription after NA-AAF treatment. Impaired transcription of the studied genes would then result in the observed TCR defect. A similar situation could hold for UV lesions, although in normal cells UV damages are subject to TCR. These observations are perhaps indicative of a role for CSA and CSB in initiation of transcription after genotoxic treatment.

Fourth, a recent study indicated a role for CSA and CSB in UV-induced modification of RNA polymerase II (16). After exposure of cells to UV irradiation or cisplatin, the large subunit of RNAP II becomes modified which is apparent by discrete higher molecular weight bands on a protein gel. Using mono-specific antibodies and HA-tagged ubiquitine peptides, it was shown that RNAP was modified by ubiquitination. Ubiquitin conjugation of proteins can have pleiotropic consequences and influences many cellular pathways, including DNA repair, sporulation/ spermatogenesis, cell cycle and transcription (reviewed in (73). Although ubiquitination often precedes proteolytic degradation, it can also influence modification and alternative processing of protein complexes, as has recently been shown in a number of cases (25, 60, 63, 182). Surprisingly, the UV-induced ubiquitination of RNAP II was absent in CS-A and CS-B cells, but could be restored by transfections of the corresponding cDNAs, strongly implying a role for CSA and CSB in this RNAP II modification (16). The RNAP II ubiquitination defect seems to be correlated to absence of CSA or CSB rather than to defective TCR, since wild-type ubiquitination is observed in total repair deficient XP-A, XP-B and XP-D fibroblasts (D.B. Bregman, unpubl. obs.). Interestingly, a low level of ubiquitination of RNAP II is also observed in absence of genotoxic treatment. In absence of CSA and CSB this spontaneous modification of RNAP II could be dysregulated, affecting the turn-over of the enzyme and possibly leading to the subtle transcription defects that are suggested by the clinical features of CS patients.

Chapter 3

Concluding remarks

Concluding remarks

In recent years a vast amount of progress has been made in the eukaryotic DNA excision repair field. While five years ago the main focus was on cloning repair genes and purifying repair activities, it is now possible to completely reconstitute the nucleotide excision repair machinery in a test tube using purified and recombinant factors and damaged DNA templates. Most human repair genes involved in xeroderma pigmentosum and Cockayne syndrome have now been identified and isolated. Moreover, several major findings have been made concerning the overlaps of DNA repair pathways with transcription, cell cycle and recombination and the involvement of various repair genes with tumorigenesis. The human, yeast and other genome sequencing projects greatly facilitate the identification of homologous genes in the computer databases, which in many cases alleviates the time-consuming screening of cDNA libraries. There are still several genes to be identified (e.g. *TTDA*), the identities of some activities have to be established (e.g. IF7), and the requirement for certain factors in (*in vitro*) repair (e.g. the XPC/HHR23B complex and in parallel Rad7p/Rad16p) still needs to be clarified. Nonetheless, modern biochemical techniques increasingly facilitate the elucidation of the precise activity of purified repair proteins (or -complexes), their role in the pathway and the exact protein-protein and protein-DNA interactions that are involved. One can expect this to lead to the complete unraveling of the nucleotide excision repair pathway within a few years, as has recently been achieved for base excision repair of uracil-containing DNA (37).

One of the major topics of interest in the near future will be the cellular processes that are connected with or influenced by nucleotide excision repair. For a long time it is known that when cells are damaged, they arrest at certain phases of the cell cycle (G1/S, G2/M) to allow repair of the lesions. Several protein complexes have now been identified that have dual roles in different metabolic pathways. The basal transcription factor TFIIH is the most striking example: this complex contains at least nine subunits which are involved in transcription, DNA repair, and possibly in cell cycle regulation (66). The ERCC1/XPF (yeast Rad1p/Rad10p) complex is not only involved in NER but also in recombination, since the corresponding CHO mutant cells show increased sensitivity to cross-linking agents (18), while the yeast mutants are defective in mitotic recombination (128). Possibly a similar activity, namely endonucleotic cleavage at the 5' site of respectively unwound or 'flap-like' DNA structures, is required in both pathways (141). The single-stranded DNA binding protein RPA, a heterotrimeric complex, was shown to be required for *in vitro* NER (1), while it is strongly involved in replication and homologous recombination as well (59, 145). As argued above, the CSA and CSB proteins may have a role in transcription-repair coupling as well as in transcription itself, which could correspond to a similar function. In addition, the clinical features of XP-G patients also imply a role for XPG in transcription. It will be of major interest to reveal the connections between DNA repair, transcription regulation, cell cycle progression, replication and recombination as well as the factors that mediate in that regulation.

A second, important goal will be to elucidate the cause of the Cockayne syndrome and trichothiodystropy features observed in CS, combined XP/CS, and TTD patients. As argued above, many of these features likely reflect a subtle defect in transcription, rather than in DNA

repair (13, 177). Moreover, there are also CS and TTD ((I)BIDS) patients without increased photosensitivity, and without obvious NER defect. The combined XP/CS and the TTD features in XP-B, XP-D and TTD-A patients may be explained by assuming that the mutations affect in some unknown manner the transcription function of TFIIH (of which XPB, XPD and TTDA are subunits). Consequently, expression of specific proteins such as myelin basic protein in neuronal cells or cysteine-rich matrix proteins in (brittle) hair is decreased (discussed in 177). However, CS-A, CS-B and some XP-G patients also display typical CS features, while it is not known whether or how these proteins might modulate TFIIH function. Several possibilities can be envisaged.

CS cells were shown to be impaired in TCR of oxidative damage, induced by ionising radiation (84). The mode of repair of these lesions is unclear, since totally NER-deficient XP-A cells are proficient in repair of these lesions. Since oxidative damage can arise from intracellular metabolic processes, it was suggested that accumulation of these lesions leads to the observed clinical features in CS patients (84). Supportive for this idea is the fact that some XP-G patients display similar features, whereas XP-G cells, derived from these patients, are also deficient in repair of oxidative damage, such as thymine glycols (30a).

It has been suggested that CSA and CSB form a 'shuttling vector' that mediates the switch of TFIIH from its 'repair mode' into its 'transcription mode' (168). In absence of CSA or CSB, there will be less TFIIH available to participate in transcription, leading to the observed CS features. However, this assumes that there is a constant level of damage present for which TFIIH is required, which is not very likely. In addition, early onset CS patients already display very severe clinical features (109), while their genome is not expected to contain many damaged nucleotides.

A third, alternative scenario could be that CSA and CSB mutations themselves affect transcription. The observed defect of RNAP II ubiquitination in CS-A and CS-B cells is very provocative in this respect, since such a defect might interfere with RNA polymerase turnover and thus transcription regulation.

A fourth hypothesis is that CSA and CSB function as transcription elongation factors that, similar to TFIIF, elongin (SIII) and ELL, decrease the time an elongating RNA polymerase pauses at a naturally occuring pause site (3, 140). These sites are frequently found in genes and it is suggested that they represent another parameter of gene regulation (22). In absence of CSA/CSB transcription will be less efficient and less RNAP will be available to initiate or complete transcription of particular genes.

Finally, CSB could be involved in the disruption of chromatin, as is suggested by the presence of the SWI2/SNF2-like ATPase domain in its protein sequence. An elongating RNAP molecule also has to pass nucleosomes, present in the active gene, and this disruption and reassembly of the nucleosome might be facilitated by CSB. Mutations of CSB are then also expected to lead to decreased transcription elongation rates. Clearly more experiments have to be done to address this question and to test these or other hypotheses. The various mouse models that are being generated may be very valuable for revealing the origin of CS and TTD features. In addition to the above discussed csb -/- mice, mouse models are being generated that mimick mutations in the *XPD* gene, that have been identified in patients with XP, XP/CS and TTD features (J. de Boer, G. Weeda, pers. comm.). Any observed differences in (neurological) development, mutagenesis and carcinogenesis can then directly be related to the

specific mutation in the same gene.

A third question that needs to be addressed, is how results, obtained *in vitro*, should be correlated to the *in vivo*, cellular situation. Notably, most *in vitro* systems contain damaged DNA templates that lack a proper chromatin structure, which is known to have a major influence on damage induction and repair efficiency. In addition, it seems that most basic DNA-metabolising processes, such as replication, transcription and DNA repair are concentrated at the basis of chromatin loops, near the attachment sites with the nuclear matrix. While this provides a good environment for interactions between these pathways, it is as of yet unclear whether or how this might occur.

Future perspectives.

The experiments described in appendix I to IV did not lead to the complete elucidation of the function of CSB in the cell. The yeast experiments allowed a dissection of repair levels within one active gene, and provided insight into the contributions of global genome and yeast *CSB* (*RAD26*)-dependent transcription-coupled repair pathways in strand-specific repair. The analysis of CSB in repair- and transcription-competent cell-free extracts indicated that the CSB protein resides in a very large molecular weight complex. Additional experiments excluded the possibility that CSB is quantitatively associated with a number of investigated repair and transcription proteins. At the same time that study provided a basis to continue biochemical analysis of the function of CSB (-complex). Below I would like to speculate a little more on what kind of experiments one can imagine to be revealing.

The most direct indication for the function of CSB would be to test for correction in a certain activity assay. However, the defect of CS cells in transcription-coupled repair only makes it very hard to apply the standard assays that have been proven valuable for other repair proteins. When tested in *in vitro* (nontranscription-coupled) repair or transcription assays, CS extracts show wild-type activity. Also *in vivo*, CS-cells have normal transcription and repair (UDS) levels, assayed by incorporation of respectively ^{3}H-Uridine or ^{3}H-Thymidine. The wild-type UDS levels of CS cells are presumably caused by the fact that TCR only enhances repair of a small fraction of the genome, and also because a significant portion of the observed UDS is caused by NER of 6/4 PP lesions, whose repair is so fast that TCR hardly contributes to it (166). CS cells do show a defective recovery of RNA synthesis after 16 hours. It should be possible to correct this DNA repair deficiency by microinjection of *CSB* cDNA constructs or protein preparations, but using *in vivo* RNA synthesis recovery as an assay is difficult due to the intrinsic variability in RNA synthesis levels between individual cells.

The best option would be an *in vitro* TCR assay, in which CS-B cell-free extracts would be deficient of strand-biased repair of a transcribed gene but which can be corrected by the CSB protein (-complex). In this way, one could purify CS-B correcting activity, determine the domains which are required for CSB function and detect the targets for its activity. Unfortunately, it seems that it is very difficult to develop such an assay. Several investigators in independent groups have tried to validate an *in vitro* TCR assay but failed. In most instances damaged DNA templates containing transcription units were mixed with cell-free extracts from

repair-proficient or -deficient cells, and strand-specific repair was analysed. Typically, only a very low, transcription-dependent increase in repair rate of the region just downstream of the promoter was observed. This small increase, however, was not specific for the transcribed strand and was still present in extracts derived from CS-A and CS-B cells.

The generation of functional HA- and His_6-tagged CSB constructs now allows the purification of the CSB protein by subsequent purifications on a HA-affinity column, Ni^{2+}-resin and additional columns (such as heparin or phosphocellulose). CSB can either be purified in a recombinant form from Baculovirus-infected insect cells or from cell-free extracts of transfected CS-B cells. The latter source has the advantage that associating proteins, that might modulate CSB function, are still present in the final preparation. A large scale purification ultimately may allow microsequencing of the copurifying proteins to determine their identity. This might be one of the few reliable ways to identify CSB-associating proteins. The experiments in appendix IV show that although CSB seemed to be present in a large molecular weight complex in cell-free extracts, no associations of CSB with a number of candidate proteins in these extracts could be shown.

Other frequently used methods to detect protein-protein interactions are, in the case of CSB, expected to be sensitive to artifacts. Immunoprecipitation of *in vitro* translated proteins is severly hampered by the large size of CSB (168 kDa). In reticulocyte lysates several shorter, truncated forms of CSB are produced that might encode binding sites for other proteins that under physiological conditions, i.e. within the cell, would not be exposed. Especially the acidic amino acid stretch in the N-terminal part of CSB may contribute to false positive results by aspecific binding to basic proteins. The frequently used two-hybrid analysis suffers from a similar problem. This system should enable one to identify also transient interactions, however no clear candidate proteins have been found (92, A. Yasui pers. comm.). The only positive interaction reported thus far is between CSA and CSB, although interaction of the yeast equivalents could not be demonstrated so far (58, E.C. Friedberg pers. comm.). An alternative is to analyse only parts of the CSB protein in a two-hybrid screen, but this might result in false negative or false positive results. Protein-protein affinity chromatography, e.g. GST-pulldown experiments, or screens of phage expression libraries are also hampered by the large size of CSB. The yeast system often allows a screen for supressor mutations of a certain phenotype, which usually represent mutations in (interacting) proteins in the same pathway. Unfortunately, the yeast *rad26* disruption mutant does not display any sensitivity to damaging agents (164), making selection of suppressors very difficult. In all of these cases it is essential that *in vitro* detected interactions have to be verified under *in vivo*, non-over expression conditions to indicate functional relevance. Alternatively, such interactions gain much more weight if binding is followed by an activity, e.g. increased ATPase activity, disruption of protein complexes, or stimulation of transcription rates.

Once a purified CSB protein (-complex) is available, various biochemical activities can be determined. Probably the most revealing one is to assay whether the ATPase domain of CSB is active and which cofactor stimulates this activity. For other members of the SWI2/SNF2 putative helicase subfamily the latter has shown to correlate well with the observed activities (discussed above). Possible cofactors to be tested, taken into account its required role in TCR,

include (naked) double/single stranded DNA, DNA:RNA hybrids, nucleosomal DNA and a stalled RNA polymerase:DNA:RNA ternary complex. A CSB mutant which is affected in the ATPase or helicase motifs will be very valuable for such assays. Helicase activity of the CSB (-complex) can be determined, although several other members of the SWI2/SNF2 subfamily have been reported not to possess DNA unwinding activity. Affinity of CSB for transcription and/or repair complexes can be determined once it is available in purified form. Addition of CSB (-complex) might stimulate *in vitro* transcription rates, indicative of a role in transcription elongation.

A CSB mutant in the ATPase motif can also be overexpressed in cells to study possible dominant negative effects. If the mutated CSB is incorporated in the CSB complex in a similar manner as the nonmutated form, it might stall the process in which it is involved due to its failure to hydrolyse ATP. In a similar way, mutations in the ATPase motif of XPB/ERCC3 had a dramatic effect on transcription *in vivo* after microinjection (169). Alternatively, an amino acid change of the conserved 'SAT' sequence in helicase motif III has been shown to impair the helicase activity of a splicing factor, without drastically affecting ATPase hydrolysis (116a). A similar mutant in CSB will be very valuable when investigating the function of the helicase motifs.

To be able to identify those amino acids that are required for the specific function of CSB, patient-related mutation data will be very useful. The laboratory of Dr. A. Lehmann and Dr. M. Stefanini have been analysing a large number of Cockayne syndrome B patients and identified many mutations in both alleles of the *CS-B* gene (Figure 4). The majority of the mutations are located in the middle part, containing the helicase/ATPase domain, and few mutations have been found in the N- and C-terminal ends. Most of them are very severe, leading to truncations of a large part of the CSB protein. Some selected mutations are now tested for their correcting activity in cell transfections and UV-survivals. Moreover, these mutations will be tested as soon as an assay for CSB activity becomes available. These experiments, together with the analysis of the csb -/- mice, might ultimately lead to the complete elucidation of the role of the Cockayne syndrome B protein.

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Abbreviations

General

ATP	adenosine triphosphate
cDNA	complementary DNA
CHO	Chinese Hamster Ovary
CPD	cyclobutane pyrimidine dimer
CTD	C-terminal domain of largest subunit of RNA polymerase II
DMBA	7,12-di-methylbenz[a]anthracene
DNA	deoxyribonucleic acid
dsDNA	double-stranded DNA
Drosophila	*Drosophila melanogaster* (fruitfly)
E. coli	*Escherichia coli* (bacterium)
ERCC (ERCC)	(human) excision repair cross complementing gene (protein)
ES	embryonic stem cell
GGR	global genome DNA repair
kDa	kiloDalton
MFD	mutation frequency decline
NA-AAF	N-acetoxy-2-acetylaminofluorene
NER	nucleotide excision repair
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
6-4 PP	(6-4) pyrimidine-pyrimidone photoproduct
RAD	radiation sensitive
RAD (Rad..p)	*S. cerevisiae* repair gene (protein)
(m)RNA	(messenger) ribonucleic acid
S. cerevisiae	*Saccharomyces cerevisiae* (baker's yeast)
SDS	sodium dodecyl sulphate
ssDNA	single-stranded DNA
SV40	Simian virus 40
TCR	transcription-coupled DNA repair
UDS	unscheduled DNA synthesis
UV	ultraviolet light
Uvr	ultraviolet resistance
Uvr (Uvr)	*E. coli* repair gene (protein)

Genetic disorders

AT	ataxia telangiectasia
BS	Bloom syndrome
CS	Cockayne syndrome
FA	Fanconi anemia
HNPCC	herediatry nonpolyposis colorectal cancer
TTD	trichothiodystrophy
XP	xeroderma pigmentosum

Protein names

CSA/CSB	Cockayne syndrome A/B protein
DNA pol δ/ϵ	DNA polymerase δ/ϵ
HHR23B	human homolog of RAD23
IF7	initiation factor 7
ISWI	imitation switch
MOT1	modifier of transcription
NURF	nucleosome remodeling factor
PCNA	proliferating cell nuclear antigen
RF-C	replication factor C
RPA	replication protein A (human ssDNA binding protein)
RNAP II	RNA polymerase II
SWI	refers to *S. cerevisiae* mutants, defective in mating type switching
SNF	refers to *S. cerevisiae* sucrose non-fermenting mutants
TBP	TATA binding protein
TFIIH	transcription factor IIH
TRCF	transcription repair coupling factor
XPA(-G)	xeroderma pigmentosum group A (-G) protein

Appendix I

ERCC6
,a member of a subfamily of putative helicases, is involved in Cockayne's syndrome and preferential repair of active genes.

ERCC6, a Member of a Subfamily of Putative Helicases, Is Involved in Cockayne's Syndrome and Preferential Repair of Active Genes

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Summary

Cells from patients with the UV-sensitive nucleotide excision repair disorder Cockayne's syndrome (CS) have a specific defect in preferential repair of lesions from the transcribed strand of active genes. This system permits quick resumption of transcription after UV exposure. Here we report the characterization of *ERCC6*, a gene involved in preferential repair in eukaryotes. *ERCC6* corrects the repair defect of CS complementation group B (CS-B). It encodes a protein of 1493 amino acids, containing seven consecutive domains conserved between DNA and RNA helicases. The entire helicase region bears striking homology to segments in recently discovered proteins involved in transcription regulation, chromosome stability, and DNA repair. Mutation analysis of a CS-B patient indicates that the gene is not essential for cell viability and is specific for preferential repair of transcribed sequences.

Introduction

The genetic information of an organism is continually subjected to the damaging effects of environmental and internal genotoxic agents, chemical instability, and the inherent imperfection of DNA metabolizing processes. If unrepaired, such DNA injury will lead to mutations, carcinogenesis, general deterioration of cell functioning, and cell death. To counteract these deleterious consequences, an intricate network of DNA repair systems has evolved (for review see Friedberg, 1985). Among the most important and best-studied repair mechanisms, both in prokaryotes and eukaryotes, is the nucleotide excision repair (NER) pathway. This system eliminates a remarkably broad spectrum of structurally unrelated lesions, such as ultraviolet (UV)-induced cyclobutane pyrimidine dimers (CPD) and (6-4) photoproducts, bulky chemical adducts, and DNA cross-links. Removal of these lesions proceeds via a multistep reaction, which is resolved to considerable detail in the bacterium Escherichia coli (van Houten, 1990): a $UvrA_2B$ helicase complex scans the DNA for structural distortions by locally unwinding the two strands (Oh and Grossman, 1987) and delivers the UvrB subunit to the lesion (Orren and Sancar, 1989). UvrC interacts with the damage-specific UvrB–DNA complex and catalyzes incision of the damaged strand on both sides of the injury. Release of the lesion-containing 12–13 nt oligomer, as well as the bound UvrC, is performed by UvrD (helicase II), after which DNA polymerase I turns over UvrB and fills the resulting single-stranded gap (Orren et al., 1992). Finally, the newly synthesized repair patch is ligated to the parental DNA. Knowledge of the molecular mechanism of NER in eukaryotes is rapidly accumulating. Recently, a dual incision has been demonstrated (Huang et al., 1992), and several proteins required for pre- or postincision steps were identified (Shivji et al., 1992).

The prototype repair disorder xeroderma pigmentosum (XP) illustrates the phenotypic consequences of defective NER. Patients suffering from this rare recessive inherited disease characteristically exhibit extreme sun (UV) sensitivity, pigmentation abnormalities, predisposition to skin cancer, and frequently progressive neurological degeneration (Cleaver and Kraemer, 1989). Extensive genetic heterogeneity was disclosed by cell hybridization with the identification of at least seven excision-deficient complementation groups (XP-A to XP-G) (de Weerd-Kastelein et al., 1972; Vermeulen et al., 1991). The biochemical basis for the XP NER defect probably resides in undefined early steps of the pathway preceding incision and repair synthesis (Shivji et al., 1992).

An immediate and urgent effect of the presence of lesions in DNA is interruption of the vital process of transcription (Mayne and Lehmann, 1982). To release this otherwise lethal block, a sophisticated NER subpathway appears to operate that primarily focuses on the rapid and preferential removal of certain lesions (from the transcribed strand of) active genes. This NER process was first discovered in mammalian cells (Bohr et al., 1985) and found to explain the apparent paradox that rodent cells perform only a fraction of CPD removal compared with human cell lines, yet they exhibit the same degree of UV resistance (Zelle et al., 1980; van Zeeland et al., 1981). It appeared that rodent lines mainly rely on the strand-selective repair of active genes and are largely deficient in the NER subpathway that is responsible for the slower and incomplete removal of CPD from the genome overall (including the nontranscribed strand) (Bohr et al., 1985; Mellon et al., 1987). Human cells are proficient for both processes (Mellon et al., 1986, 1987; Venema et al., 1990b). This demonstrates the importance for cell survival of preferential repair of the small but vital active compartment of the genome. Recent analysis of mutations in various genes has provided evidence that strand-selective repair also contributes significantly to prevention of mutagenesis originating from this strand in cultured cells (Vrieling et al., 1991). The universality of preferential repair is demonstrated by the recent discovery that in yeast and E. coli also the transcribed strand is more rapidly cleared from CPD lesions than the nontranscribed strand (Mellon and Hanawalt, 1989; Smerdon and Thoma, 1990). These observations suggest that a direct coupling or a feedback mechanism exists between transcription and NER.

The notion that this important process constitutes a dis-

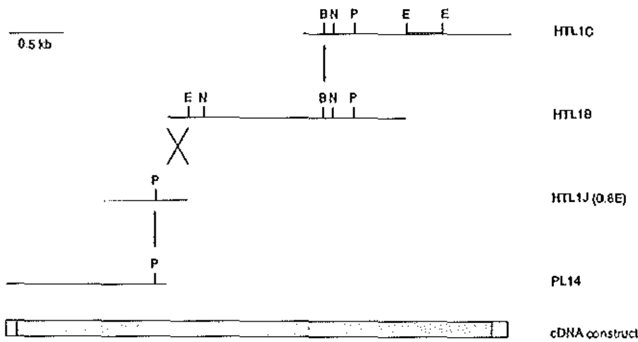


Figure 1. Construction of a Functional *ERCC6* cDNA

The inserts of HTL1B and HTL1C (human testis cDNA library) were isolated from the phage arms via PCR and joined by ligation (vertical arrows) at the unique BglII site (B). The fragment HTL1J(0.8E) is added through homologous recombination (X), and finally PL14 (human placenta cDNA library) is added to the resulting molecule by ligation at the PflMI (P) site. The parts obtained via PCR or homologous recombination were checked by sequence analysis. The thick line in HTL1C corresponds to the probe used for Southern blot analysis in Figure 6B. Other symbols: E, EcoRI; N, NsiI; dotted line, sequence not representing *ERCC6* (artificially ligated to *ERCC6*); stippled region, the ORF. See Experimental Procedures for details.

tinct subpathway of NER came from the finding that patients suffering from Cockayne's syndrome (CS) carry specific defects in this system. CS is a rare genetic disorder that manifests UV hypersensitivity (but no other cutaneous abnormalities), retarded growth, and severe progressive neurological degeneration. Remarkably, in striking contrast with XP, no significant increase in skin cancer is noted (Lehmann, 1987; Nance and Berry, 1992). Cell fusion experiments have revealed the presence of at least two complementation groups, group A (CS-A) and group B (CS-B), within the classical form of the disease (Tanaka et al., 1981; Lehmann, 1982). A characteristic hallmark of the disease is the observation that CS fibroblasts perform rates of repair synthesis in the normal range yet, in contrast with repair-competent cells, are unable to recover their RNA synthesis after UV irradiation (Mayne and Lehmann, 1982). The early suggestion put forward by Mayne and Lehmann (1982) that the CS defect resides in the repair of transcribed genes was only borne out by the recent finding of Venema et al. (1990a) that CS fibroblasts have lost the preferential repair of active genes but are proficient in overall genome repair. For CS-A, it was demonstrated that the absence of preferential repair correlates with a complete deficiency of selective repair of the transcribed strand (Venema, 1991).

Here we report a key step toward defining this system at the molecular level by the analysis of a gene implicated in the preferential repair pathway. This human gene, termed *ERCC6*, was cloned by virtue of its ability to correct the NER defect of a UV-sensitive Chinese hamster ovary (CHO) mutant (Troelstra et al., 1990). The mutant, UV61, is a representative of complementation group 6 of the laboratory-generated, excision-deficient rodent cell lines (Busch et al., 1989). We show that the *ERCC6* gene at the same time corrects the repair defect of CS-B, the most common form of the disease, and that the gene encodes a protein with a presumed DNA unwinding function. The helicase segment bears striking resemblance to similar domains in transcription regulators and proteins involved in various repair processes and chromosome stability recently identified in yeast and Drosophila.

Results

Isolation of the *ERCC6* cDNA

The human excision repair gene *ERCC6* was recently isolated, after large-scale transfection experiments of human (HeLa) genomic DNA to UV-sensitive CHO mutant UV61 (rodent complementation group 6 [CG-6]) and rescue of the correcting human sequences from a secondary transformant (Troelstra et al., 1990). The gene appeared to have a respectable size (for transfection cloning) of close to 100 kb. Unique conserved genomic *ERCC6* sequences were identified and used as probes to hybridize to a human testis cDNA library. This resulted in the isolation of a number of overlapping cDNA clones, together having an insert size of 3 kb with a polyadenylation signal 20 nt from the 3′ end. Northern blot analysis with part of this 3 kb cDNA as a probe revealed the presence of two messenger RNAs (mRNAs) of about 5 kb and 7–7.5 kb in length. Sequence analysis and restriction mapping indicated that the difference in size is due to alternative polyadenylation (unpublished data). Both mRNAs are very lowly expressed, an observation upheld by the recovery of very low numbers of *ERCC6* cDNA clones from various cDNA libraries. After screening of several cDNA libraries, an almost full-length molecule of 4.7 kb could be constructed (see Figure 1 for details).

Correction of UV61 by the *ERCC6* cDNA

The 4.7 kb cDNA molecule was inserted in mammalian expression vector pSLM (see Experimental Procedures) under the control of the SV40 late promoter. Cotransfection of this plasmid with pSV2neo to UV61 cells yielded UV-resistant clones with very high efficiency (transfection frequency of UV and G418 resistance was similar to that of G418 resistance alone). UV survival of these transformants appeared within the wild-type range (Figure 2A).

To verify whether other DNA repair endpoints were corrected by *ERCC6*, we examined the recovery of RNA synthesis after UV exposure. In normal cells, transcription drops rapidly after UV irradiation (owing to the appearance of blocking lesions in the DNA template) but resumes

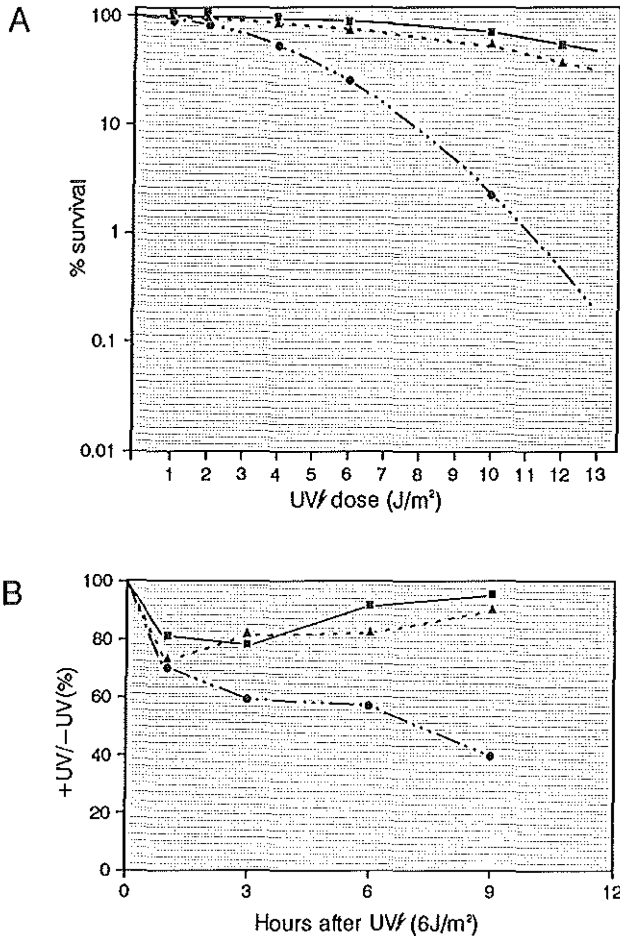


Figure 2. Correction of the UV61 Repair Defect by *ERCC6* cDNA

(A) UV survival curves of wild-type CHO cell line AA8 (closed boxes), mutant UV61 (closed circles), and *ERCC6* cDNA transformant 61E6a (closed triangles).

(B) Recovery of RNA synthesis in wild-type cell line AA8, mutant UV61, and *ERCC6* cDNA transformant 61E6a. Cells unirradiated or UV irradiated with 6 J/m^2 were pulse labeled with [^{3}H]uridine at different times after irradiation; the incorporated radioactivity was determined by liquid scintillation counting (see Experimental Procedures for details on the procedure). For symbols see (A). The standard error of the mean (three duplicate plates) falls within the size of the symbols.

within several hours as the result of preferential (strand-specific) repair. In excision-deficient cells such as XP and CS, recovery of RNA synthesis does not occur (Mayne and Lehmann, 1982). Since this repair parameter has never been investigated for rodent mutants, we decided to assess the behavior of RNA synthesis in CHO wild-type AA8, mutant UV61, and cDNA transformant 61E6a upon UV irradiation. As shown in Figure 2B, RNA synthesis (expressed as the ratio of [^{3}H]uridine incorporation in UV-irradiated cells to that in unirradiated cells) initially decreases within 1 hr to 70%–80% but quickly resumes in AA8, whereas it remains low in UV61. Recovery of RNA synthesis, as in wild type, is observed in *ERCC6* transformant 61E6a, indicating that the human gene also corrects this repair endpoint.

To investigate the specificity of correction by *ERCC6*, the gene was transfected to mutants from rodent CG-4, CG-5, and CG-7 and to two other members of group 6 (CG-1, CG-2, and CG-3 were not tested since the correcting genes for these mutants have been cloned [van Duin et al., 1986; Weber et al., 1990; Weeda et al., 1990a, 1990b]). No UV-resistant transformants were obtained in the heterologous CG, whereas numerous UV-surviving colonies were observed in the other representatives of CHO group 6, demonstrating the specificity of *ERCC6* correction and validating the complementation studies.

Nucleotide and Amino Acid Sequence of *ERCC6* cDNA

The nucleotide sequence of the *ERCC6* cDNA is shown in Figure 3A. The first ATG codon (nucleotide 80), preceded by two in-frame stop codons, starts an open reading frame (ORF) of 4479 bp. The purine often found at the important −3 position before a start codon (Kozak, 1991) is present, as is the G at +4. The other nucleotides within the start site, however, deviate from the optimal initiation start site GCC(A/G)CCATGG (Kozak, 1991). All other ORFs present encode much smaller polypeptides. Therefore, we con-

A

B

wt N aa 1 aa 1493 C
A G S I IA III IV V VI S N

CS1AN
N aa 1 aa 336 C
N aa 1 aa 657 C
A G S I IA III
100 aa

Figure 3. Nucleotide and Predicted Amino Acid Sequence of the *ERCC6* cDNA

(A) *ERCC6* nucleotide and predicted amino acid sequence. The sequence of 4714 nt has been obtained from the cDNA fragments, as depicted in Figure 1, together with a cDNA clone from an adenovirus-transformed retina cell library (the first 16 nt). Putative functional units are overlined: CKII phos, casein kinase II phosphorylation site; NLS, nuclear location signal (basic regions are double overlined); NTB, nucleotide binding fold; numbers I, IA, and II–VI refer to the corresponding helicase motifs. The AATTAAA polyadenylation signal is underlined. Arrows indicate the mutations in patient CS1AN; CSD means the cryptic splice donor, while putat. SA indicates the putative splice acceptor; the putative branch point, the pyrimidine stretch, and the AG dinucleotides are underlined; the third AG represents the normal exon 13 border.

(B) Schematical representation of predicted functional domains in the ERCC6 protein. The truncated ERCC6 proteins encoded by the CS1AN alleles are indicated. A, acidic amino acid stretch; G, glycine-rich region; S, serine phosphorylation site preceded by a nuclear location signal; N, nucleotide binding fold; arrow, nonsense sequence due to a frameshift in allele b of patient CS1AN.

clude that the ORF of 4479 bp, depicted in Figure 3A, defines the ERCC6 protein.

The deduced amino acid sequence specifies a protein of 1493 amino acids (see Figures 3A and 3B), with a predicted molecular size of 168 kd. Although a systematic computer search did not identify any protein with overall homology to ERCC6, a close inspection of the sequence did reveal several regions of interest and similarity to other polypeptides.

First, there was an acidic part from residue 356–394 harboring close to 60% glutamic or aspartic acids including a stretch of 10 acidic residues. Acidic regions have been found in a number of nuclear proteins that associate with chromatin or histones (Lapeyre et al., 1987; Ptashne, 1988; Sung et al., 1988; Wen et al., 1989). This part is followed by a stretch of glycin residues (amino acids 442–446) and a very hydrophilic segment (from residue 447–495) containing >60% charged amino acids.

Second, two regions with the hallmarks of a bipartite nuclear location signal (amino acids 466–481 and 1038–1055; Robbins et al., 1991) were found in close vicinity to potential casein kinase II phosphorylation sites (Kemp and Pearson, 1990, amino acids 483–490 and 1068–1074). Recently, it was noticed that putative casein kinase II phosphorylation sites are located nearby many nuclear location signal sequences (Rihs et al., 1991).

Third, the region from amino acid 527 to amino acid 950 contains seven sequences (starting with a nucleotide binding fold) matching seven consecutive motifs recognized by several investigators to be conserved between two superfamilies of RNA and DNA helicases (Gorbalenya et al., 1989). This region (amino acids 527–1007) exhibits, in addition, a very high overall homology with parts of eight other proteins containing the same seven motifs: RAD16 (Bang et al., 1992; Schild et al., 1992), RAD54, SNF2

(SWI2), STH1 (Laurent et al., 1992), MOT1 (Davis et al., 1992), FUN30 (Clark et al., 1992), brm (Tamkun et al., 1992), and lodestar (Girdham and Glover, 1991) (Figure 4B). The similarity is confined to this particular segment (Figure 4A) and is far higher than the motif-restricted homology normally detected between different helicases (see Figure 4B for the consensus sequence of the seven helicase motifs).

Fourth, a second putative nucleotide binding domain (Saraste et al., 1990) is found in the C terminus (amino acids 1134–1138).

Involvement of *ERCC6* in CS

The *ERCC6* gene, correcting a CHO mutant, could possibly also be implicated in one of the forms of XP or CS. The first clue concerning this possibility was presented by a patient with symptoms of late onset CS, being heterozygous for an interstitial 10q211 deletion in all cells (Fryns et al., 1991), i.e., a chromosomal region to which we have assigned *ERCC6* (Troelstra et al., 1992). Southern blot analysis of DNA from the patient suggested that *ERCC6* resides within the deleted segment (data not shown). Since no cells of this patient were available and the complementation group to which he belonged was unknown, we decided to transfect the *ERCC6* cDNA expression plasmid together with the dominant selectable marker pSV2neo into CS3BE.S3.G1 and CS1AN.S3.G2, immortalized cell lines representing CS CG-A and CG-B, respectively (Mayne et al., 1986). G418-resistant transformants of each cell line were grown in mass culture. In view of the poor transfectability of many SV40-immortalized human fibroblasts, Southern blot analysis was performed to verify whether a significant fraction of the G418-resistant transformants had indeed incorporated the cotransfected *ERCC6* cDNA. For CS3BE.S3.G1, this fraction was very low, but detectable, on Southern blot (average incorporation of <0.1 intact *ERCC6* cDNA copy per cell). CS1AN.S3.G2 cells had an average integration of one to two copies per transformant (data not shown). To test whether *ERCC6* restores the DNA repair capacity of one of the CS complementation groups, the G418-resistant mass populations (consisting of ± 100 and 800–1000 independent clones for CG-A and CG-B, respectively) were subjected to a UV dose lethal to the parental CS cells (8 or 6 J/m^2, which gives a 0.25% survival for CS-A and CS-B, respectively, and 15% or 25% survival for wild-type cells; the treatment was repeated after 48 hr). For CS3BE.S3.G1, none of the G418-resistant cells survived the UV selection, and neither did the transfected mass population show enhanced survival (at different doses). This suggests that *ERCC6* is unable to correct the repair defect of CS-A (although it should be kept in mind that less than 10% of the transformants had incorporated an intact copy of the *ERCC6* cDNA). *ERCC6* did confer UV resistance to CS1AN.S3.G2 cells; the transfected mass population efficiently survived the UV selection. The UV survival of two independent transformants (E61ANa and E61ANd) is depicted in Figure 5A and shows that both have regained a wild-type UV resistance. To establish the specificity of correction further, *ERCC6* cDNA was introduced (either by DNA transfection or by microinjection) into representative cell lines of XP-C and XP-E to XP-G; the correcting genes for XP-A (Tanaka et al., 1990), XP-B (Weeda et al., 1990b), and XP-D (Flejter et al., 1992) have already been isolated and are different from *ERCC6*. Because XP-E cells are only slightly UV sensitive, it was not possible to derive definite conclusions from the transfection experiments to this complementation group. The *ERCC6* gene was unable to correct the repair defect of the other XP groups tested. These results confirm the complementation data and demonstrate that no overlap exists between CS-B and any of the known XP groups (with the possible exception of XP-E), in contrast with CS-C, which is identical to XP-B.

RNA Synthesis Recovery in *ERCC6* cDNA Transformants of CS1AN.S3.G2

As mentioned above, CS cells are, in contrast with repair-proficient human cells, unable to recover their RNA synthesis after UV exposure (Mayne and Lehmann, 1982). To see whether *ERCC6* also complements this CS-typical repair parameter, RNA synthesis was measured after UV irradiation in SV40-transformed normal fibroblasts, CS1AN.S3.G2, and cDNA transformants E61ANa and E61ANd. As shown in Figure 5B, transcription initially drops to ≈60% within 1 hr after irradiation. The RNA synthesis quickly recovers in normal cells, whereas it remains repressed in CS1AN.S3.G2 cells. The cDNA transformant E61ANd resumes transcription with almost wild-type kinetics; E61ANa, on the other hand, recovers more slowly, being retarded at early times after UV but reaching wild-type levels at 16 hr. In Figure 5C the level of transcription 16 hr after UV irradiation is tested at different UV fluences. In both cDNA transformants, RNA synthesis is similar to that in repair-proficient cells for all UV doses, whereas transcription in CS1AN.S3.G2 is consistently lower. The slow (but after 16 hr complete) recovery of E61ANa, in contrast with the fast recovery of E61ANd (Figure 5B), might be attributed to the slow growth rate of E61ANa. Alternatively, expression levels of the cDNA, influenced by, e.g., the integration site, could have an effect on the kinetics.

To demonstrate the universality of CS-B correction, primary fibroblasts of another CS-B patient, CS1BE, were microinjected with *ERCC6*. Recovery of UV-induced transcription inhibition was, as in the CS1AN cDNA transformants, restored to levels within wild-type range (data not shown). Preliminary data suggesting that microinjection of *ERCC6* into primary fibroblasts of CS-A did not restore RNA synthesis recovery after UV irradiation confirm the complementation data.

The *ERCC6* Mutation in CS1AN Cells

The previous experiments showed that *ERCC6* specifically corrects the UV survival and RNA synthesis recovery after UV exposure of CS-B. To obtain direct proof that *ERCC6* is responsible for the CS-B repair defect, we searched for mutations in the gene. The polymerase chain reaction (PCR) was used to amplify *ERCC6* cDNA selectively from RNA of the SV40-transformed CS-B cell line CS1AN.-S3.G2. For both cDNA synthesis and PCR, the *ERCC6*

A

'helicase' domains

SNF2 1 1703

Lodestar 1 1051

RAD16 1 790

RAD54 1 898

ERCC6 1 I IA II III IV V VI 1493

B

Figure 4. Homology of the ERCC6 Helicase Region to the Similar Region of RAD54, RAD16, lodestar, SNF2, STH1, brm, MOT1, and FUN30

(A) Schematical representation of ERCC6, RAD54, RAD16, lodestar, and SNF2 to indicate the location of the homologous segment in the proteins. Stippled boxes represent homology; helicase motifs are indicated by closed boxes.

(B) Alignment of conserved regions of the ERCC6, RAD54, RAD16, lodestar, SNF2, STH1, brm, MOT1, and FUN30 amino acid sequences. Sequences that correspond to the seven consensus motifs identified among two superfamilies of DNA and RNA helicases are overlined. The consensus (consens) sequences given below the corresponding domains are from Gorbalenya et al. (1989); hydrophobic (I, L, V, M, F, Y, W) and charged or polar residues (S, T, D, E, N, Q, K, R) are indicated by plus and 0, respectively. Shaded amino acids are similar in at least five of the proteins compared; residues belonging to one of the following groups are considered to be similar: L, V, I, M; F, Y, W; S, T, A, P, G; K, R, H; and E, D, Q, N. The RAD54 sequence is taken from Emery et al. (1991) and the SNF2 sequence from Laurent et al. (1991); other references are given in the text. A small change has been introduced in the published sequence of the *lodestar* gene (Girdham and Glover, 1991), since we noticed that this results in the presence of all seven helicase domains, instead of the reported three. By introducing a loop of 61 amino acids between domains III and IV in the FUN30 amino acid sequence, we were able to perfectly align domains I–III, in addition to domains IV–VI that were identified by Clark et al. (1992).

A

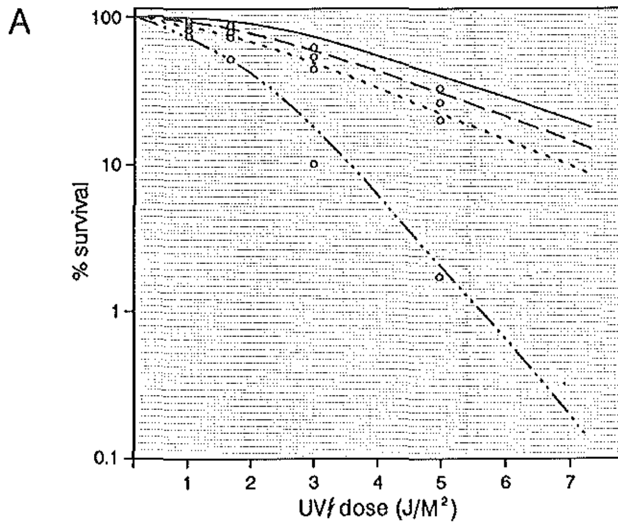


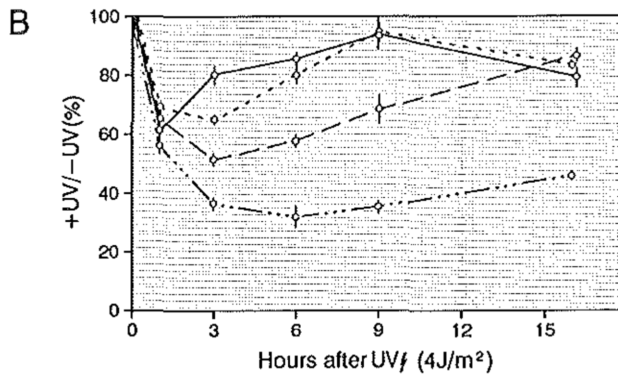
Figure 5. Correction of the CS1AN.S3.G2 Repair Defect by *ERCC6* cDNA

(A) UV survival curves of an SV40-transformed wild-type cell line (solid line), mutant CS1AN.-S3.G2 (dashed and dotted line), and *ERCC6* cDNA transformants E61ANa (dashed line) and E61ANd (dotted line).

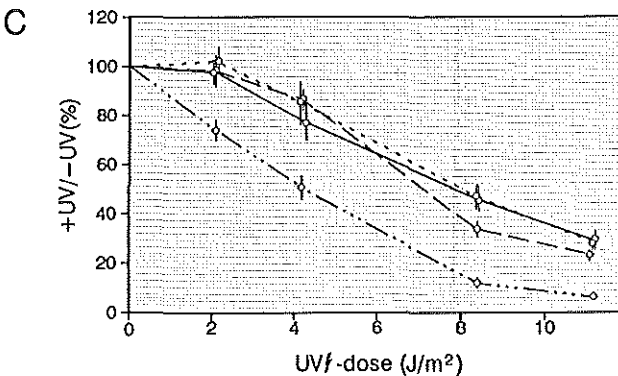
(B) Recovery of RNA synthesis at different times after UV irradiation. Cells unirradiated or UV irradiated with 4 J/m² were pulse labeled with [³H]uridine at different times after irradiation; the incorporated radioactivity was determined by liquid scintillation counting. For symbols see (A); the average (open circle) and the standard error of the mean (vertical line) are given (within one experiment).

(C) RNA synthesis 16 hr after UV irradiation with increasing dose. Cells unirradiated or UV irradiated with different dose were pulse labeled with [³H]uridine 16 hr after irradiation; the incorporated radioactivity was determined by liquid scintillation counting. For symbols see (A) and (B).

B



C



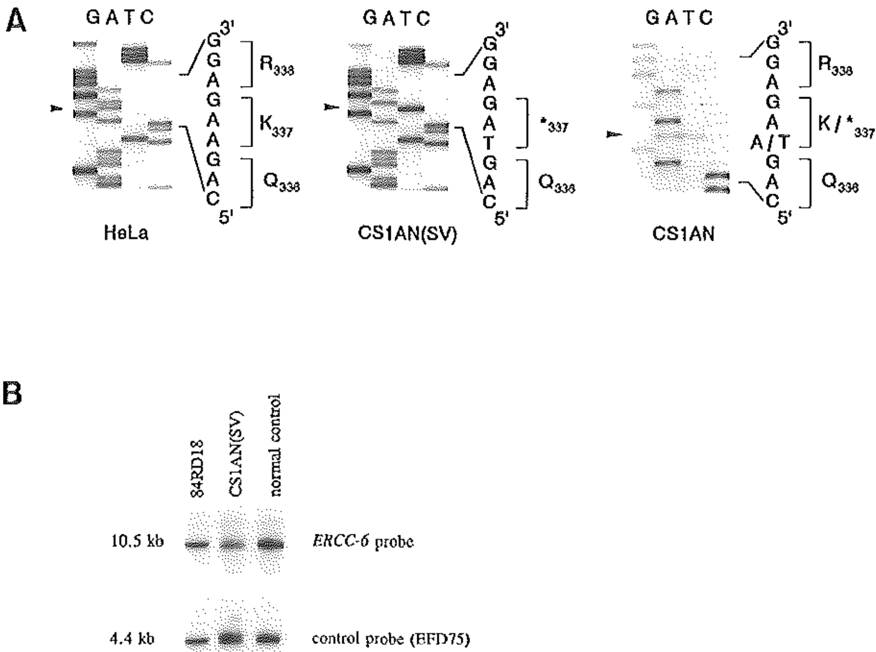


Figure 6. *ERCC6* Mutation in CS1AN.S3.G2 and Allele a of Patient CS1AN

(A) Part of the *ERCC6* nucleotide sequence of total PCR product (from cDNA) from HeLa, CS1AN.S3.G2 (CS1AN(SV)), and the primary fibroblasts of patient CS1AN. The A to T transversion, resulting in a premature stop codon (the mutation in allele a), is indicated by an arrowhead.

(B) Southern blot analysis of CS1AN.S3.G2 and two control cell lines. The same Southern blot was hybridized with two probes. The *ERCC6* probe is a 350 bp EcoRI cDNA fragment (see Figure 1), and EFD75 is a control probe derived from the telomeric end of chromosome 10q (10q26) (Simpson and Cann, 1990). The control probe recognizes two polymorphic bands in DNA of CS1AN.S3.G2, each having an intensity half of that from the single band of both controls. This indicates that at least this telomeric part of chromosome 10 is diploid in CS1AN.S3.G2. For the two controls, the *ERCC6* signal is comparable with that of EFD75. In CS1AN.S3.G2, however, the intensity of the *ERCC6* signal is similar to that of only one of the two polymorphic EFD75 bands, suggesting that only one copy of *ERCC6* is present.

mRNA was divided into six overlapping segments of approximately 1 kb each. Single-stranded DNA for direct sequence analysis of the PCR product was prepared by asymmetric PCR, using amplified cDNA as a template. Sequencing revealed an A to T transversion at nucleotide 1088, resulting in a premature stop at amino acid position 337 (Figure 6A). Only one type of allele was detected, both at the level of RNA and in genomic DNA, implying that CS1AN.S3.G2 is either homozygous or hemizygous for the A^{1088} to T transversion. In the family history of patient CS1AN, consanguinity has not been reported. Southern blot analysis of DNA from CS1AN.S3.G2 confirmed that at least part of one *ERCC6* allele was deleted (Figure 6B).

Since SV40-transformed fibroblasts are chromosomally unstable, sequence analysis of the *ERCC6* gene and cDNA was verified in the primary fibroblasts of patient CS1AN, GM00739. The A to T transversion at nucleotide 1088 was confirmed, but unexpectedly the primary line appeared to possess a second allele with the wild-type A residue at nucleotide position 1088 (Figure 6A). Further sequence analysis of the total PCR product derived from RNA of GM00739 revealed the appearance of a double sequence ladder when entering from exon 14 backward into exon 13 (Figure 7A). When the normal nucleotide sequence of exon 13 is subtracted, a perfect match is found with a sequence 100 bp upstream in exon 13. The resulting 100 bp deletion is not present in RNA from the SV40-transformed CS1AN.S3.G2 cell line (Figure 7A), thus proving that it is derived from the second allele (allele b) of GM00739. The 3' end of the deletion coincides exactly with the end of exon 13, whereas the 5' end occurs in a sequence (GG/GTACT; see Figure 3A, nucleotides 2576–2582) matching the splice donor consensus (AG/GTPuAG; Pu = A or G) (Senapathy et al., 1990). This suggests that, in allele b, a cryptic donor is utilized instead of the normal exon 13 splice donor. Therefore, a mutation in the splice donor of exon 13 of allele b was expected. At the level of DNA, however, neither the normal nor the cryptic splice donor was mutated (Figure 7B). The only detectable mutation was a C^{2648} to T transition 30 nt upstream of the normal

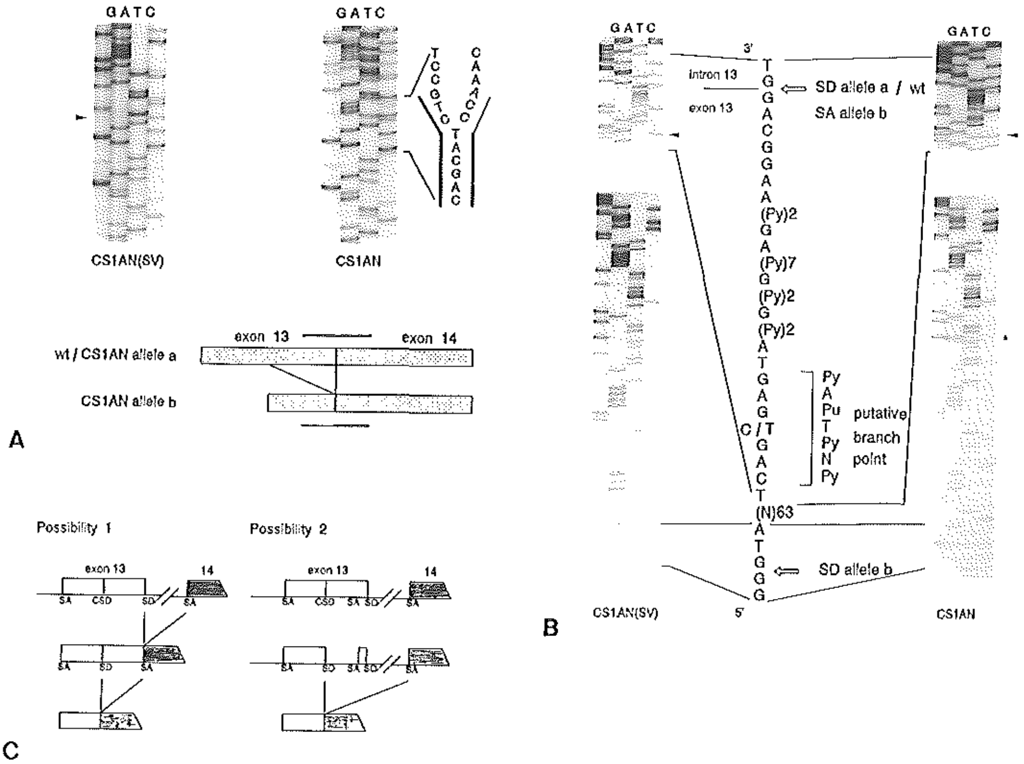


Figure 7. *ERCC6* Mutation in Allele b of Patient CS1AN

(A) *ERCC6* nucleotide sequence from the exon 14/exon 13 border of both CS1AN.S3.G2 (CS1AN(SV)) and the primary fibroblasts CS1AN. Sequence analysis was performed on total PCR product obtained after amplification of RNA. The start of the double sequence ladder in CS1AN, resulting from a deletion of 100 bp (allele b, schematically depicted underneath the sequences), is indicated by an arrowhead. The sequence obtained with CS1AN.S3.G2 is identical to that using HeLa RNA.

(B) Nucleotide sequence of part of exon 13 at the level of the genome of CS1AN.S3.G2 (CS1AN(SV)) and the primary fibroblasts CS1AN. The C to T transition in CS1AN, responsible for the 100 nt deletion at the mRNA level, is indicated by an arrowhead. The asterisk next to the CS1AN sequence indicates a sequence artifact that was also detected in CS1AN(SV) (and HeLa) on longer exposures.

(C) Schematical representation of the two possible splicing reactions that could result in the 100 nt deletion in the mRNA of CS1AN.

splice donor of exon 13. How could a single point mutation at this position have such a profound influence on the apparent functioning of the still normal splice donor sequence 30 nt downstream? A search for dramatic changes in secondary structure caused by the C to T transition failed to reveal any clues to explain this effect. Close inspection of the sequence revealed that the C to T transition might create a putative branch point sequence (Ruskin et al., 1984; Senapathy et al., 1990) at position 2645–2651, which, together with a coincidently present pyrimidine stretch and one of the three subsequent AG dinucleotides, completes a possible splice acceptor site (see Figures 3A and 7B). This newly created acceptor, together with the cryptic splice donor in exon 13, may generate a new intron. The 100 nt deletion could thus be the result of an extra splice event if the third (C)AG is used. The normal splice donor site of exon 13 should then have a dual role: splice donor in the normal splicing of intron 13 and splice acceptor in a subsequent erroneous splice event, removing the last 100 nt of exon 13 (see Possibility 1 in Figure 7C). Alternatively, use of the first AG dinucleotide, which in in vitro and in vivo studies is demonstrated to be strongly preferred above any second AG (Smith et al., 1989; Weeda et al., 1990a), would define a very small exon of 9 nt at the end of exon 13. It is known from studies of several investigators that such small exons may be skipped (both in vitro and in vivo) when the surrounding splice sites are nonoptimal (Black, 1991; Dominski and Kole, 1991, 1992). This would result in the ligation of the cryptic splice donor within exon 13 to exon 14 (see Possibility 2 in Figure 7C). For the ERCC6 gene product, the resulting 100 nt deletion has profound consequences. The protein will only be 857 amino acids long and consequently lacks half of the helicase region (see Figure 3B). Presumably, as with allele a,

Table 1. Comparison of Repair Characteristics of UV61 and CS Cells

Repair Characteristics	UV61[a]	CS
UV sensitivity (at D_{10})	≈2.7 x[b]	≈3–3.5 x[c]
RNA synthesis recovery after UV	–[d]	–[e]
Repair synthesis (percentage of wild type)	≈70%[f]	Wild-type range[g]
Preferential repair of CPDs in active genes	37%[h]	–[i]
Overall genome repair	–[b,j]	+[g]
Repair of (6–4) photoproducts	+[b]	+[k]
UV-induced mutagenesis	Elevated[b]	Elevated[l,m]

[a] Mutant, probably leaky (Troelstra et al., 1990).
[b] Thompson et al. (1988a).
[c] CS1AN.S3.G2 (unpublished data).
[d] This study.
[e] Mayne and Lehmann (1982).
[f] Unpublished data.
[g] Andrews et al. (1978).
[h] Lommel and Hanawalt (1991).
[i] Venema et al. (1990a).
[j] Characteristic of rodent cells.
[k] Barrett et al. (1991).
[l] Henderson and Long (1981).
[m] Arlett and Harcourt (1982).

allele b in CS1AN cells will produce a nonfunctional ERCC6 protein.

Discussion

A Rodent Equivalent of CS-B

The *ERCC6* gene was previously isolated by virtue of its ability to correct the repair deficiency of UV61, a NER-deficient rodent mutant cell line from complementation group 6 (Troelstra et al., 1990). In this paper, we report the isolation of a functional *ERCC6* cDNA molecule that efficiently and specifically corrects both the UV sensitivity of UV61 (and other representatives of rodent group 6) and its inability to restore RNA synthesis after UV exposure. Introduction of this cDNA into cells of several XP, CS, and rodent complementation groups by transfection or microneedle injection demonstrates a specific correction of the repair deficiency of CS-B fibroblasts: both UV survival as well as RNA synthesis recovery are restored to a level within wild-type range. This result implies UV61 and other members of rodent complementation group 6 (Thompson et al., 1988b; Busch et al., 1989) to be the rodent equivalents of the human repair disorder CS-B, the most common form of the disease. In retrospect we note that the repair phenotypes of UV61 and CS-B can be reconciled with each other (Table 1), taking into account the fact that rodent and human excision repair display some principal differences (Zelle et al., 1980; van Zeeland et al., 1981; Mellon et al., 1987) and the notion that UV61 is very likely a leaky mutant (Troelstra et al., 1990).

The *ERCC6* Mutations in Patient CS1AN

Sequence analysis of the *ERCC6* gene in patient CS1AN revealed deleterious mutations in both alleles. Allele a, which is the only allele present in CS1AN.S3.G2 cells (see below), carries an A^{1088} to T transversion leading to a premature stop at amino acid 337. The encoded protein of 336 amino acids lacks all postulated domains (Figure 3B). Allele b contains a C^{2648} to T transition that triggers an erroneous splice event and thereby results in a frameshift. As depicted in Figure 7C, two splice mechanisms can be envisaged that could give rise to the deletion of 100 bp. Although undetected in CS1AN mRNA, we cannot exclude the presence of trace amounts of the partially spliced intermediate depicted in Figure 7C (Possibility 1). Similarly, undetectable amounts of mRNA including the newly defined 9 nt exon (depicted in Figure 7C, Possibility 2) might be produced. However, none of these transcripts will encode a protein longer then 860 amino acids. Presumably, these severely truncated ERCC6 proteins will be nonfunctional. Nevertheless, cells from patient CS1AN seem to be affected in preferential DNA repair only, implying that ERCC6 is specific for this NER subpathway. In addition, these findings suggest ERCC6 to be a nonvital protein, in contrast with the ERCC2 and ERCC3 gene products, of which at least the yeast homologs RAD3 and $ERCC3^{Sc}$ (SSL2) are shown to be indispensable for cell survival (Higgins et al., 1983; Naumovski and Friedberg, 1983; Gulyas and Donahue, 1992; J. H. J. H., S. Prakash, and L. Prakash, unpublished data).

The immortalized CS1AN derivative CS1AN.S3.G2 only retained allele a and lacked allele b. Presumably, this is not due to loss of one total copy of chromosome 10, since CS1AN.S3.G2 is polymorphic for at least one locus on 10q26 (EFD75; see Figure 6B). A similar case was reported recently with another repair disorder resulting from mutations in the ligase I gene. The primary fibroblasts of the patient, designated 46BR, carried different mutations in each ligase I gene copy, whereas the SV40-transformed derivative 46BR.1G1 only retained one of the two (mutated) alleles (Barnes et al., 1992). Although the genomic instability of SV40-transformed cell lines is generally known, these findings further emphasize the importance of including primary cell lines as controls in this type of study.

ERCC6, Member of a Recently Identified Family of Putative Helicases

The predicted amino acid sequence suggests the ERCC6 gene product to be a nuclear DNA (or RNA) unwinding protein. *ERCC2* and *ERCC3* have also been postulated to encode DNA helicases, as deduced from their amino acid sequence (Weber et al., 1990; Weeda et al., 1990b), and for ERCC2, in addition, based on the demonstrated DNA helicase activity of its yeast homolog RAD3 (Sung et al., 1987). Therefore, the *ERCC6* gene may encode the third DNA unwinding function in mammalian NER. In E. coli both the $UvrA_2B$ complex and the UvrD protein have been shown to exhibit DNA unwinding activity (Oh and Grossman, 1987; Orren et al., 1992, and references therein). Similarly, several DNA helicases might be expected to function in mammalian excision repair. The gene product of *ERCC6*, however, certainly differs from the E. coli

$UvrA_2B$ and UvrD proteins in that it is specific for preferential repair of active genes.

The entire helicase region of the ERCC6 protein appears to be highly homologous to a similar region in proteins from a recently identified, rapidly expanding family of postulated helicases (Bang et al., 1992; Clark et al., 1992; Davis et al., 1992; Johnson et al., 1992; Laurent et al., 1992; Schild et al., 1992; Tamkun et al., 1992). This novel subgroup encompasses proteins implicated in transcription regulation (SNF2, MOT1, and brm), preservation of chromosome stability (lodestar), and DNA repair (RAD16, RAD54, and the very recently identified RAD5 protein). The group also includes two proteins of unknown function (STH1 and FUN30). The degree of homology (around 30% identity and up to 57% similarity) by far exceeds the resemblance normally present among helicases. This suggests a specific type of helicase function common to all members of this gene family.

The transcription regulators SNF2 (Laurent et al., 1991) and brm (Tamkun et al., 1992) are thought to be engaged in transcription activation, SNF2 as a component of a larger functional complex involving SWI1, SWI3, SNF5, and SNF6 (Laurent et al., 1991; Peterson and Herskowitz, 1992). The gene product MOT1, on the other hand, is supposed to be a negative regulator of transcription (Davis et al., 1992). One of the proposed activities for the MOT1 protein is that it strips resident DNA-binding proteins (which would otherwise activate transcription) from their cognate binding sites by melting the DNA helix (Davis et al., 1992).

Mutations in the maternal-effect gene *lodestar* result in chromatin bridges at anaphase. The precise molecular pathway in which this protein functions is unknown, but one of the processes suggested is completion of incomplete DNA repair or replication prior to mitosis (Girdham and Glover, 1991).

It is of interest to note that the three major DNA repair pathways in yeast are all equipped with at least one member of this subfamily: RAD16 in NER of the inactive HMLα locus, i.e., opposite from the function of ERCC6 (Terleth et al., 1990); RAD54 in recombination repair (Saeki et al., 1981; Budd and Mortimer, 1982); and RAD5 in postreplication repair (Johnson et al., 1992). Our results demonstrate that preferential repair of active genes also involves a member of this helicase subfamily (*ERCC6*). Common denominators of all proteins comprising this gene family could be complex formation (as described for SNF2 [Laurent et al., 1991; Peterson and Herskowitz, 1992]), complex delivery at the desired site in DNA, and release of DNA-bound proteins (as proposed for MOT1 [Davis et al., 1992]), although other options can be envisaged as well.

Hypothetical Models for ERCC6 Function

The predicted amino acid sequence of ERCC6 advocates the protein to be a nuclear DNA helicase, with strong sequence homology to the subfamily described above. The ability of the *ERCC6* cDNA to correct the repair defect of CS-B fibroblasts and the severe mutations in CS1AN cells provides evidence for a specific role of ERCC6 in preferential repair of active genes. Preferential repair has been documented in mammals (Bohr et al., 1985; Mellon et al., 1986, 1987), yeast (Terleth et al., 1989; Smerdon and Thoma, 1990), and E. coli (Mellon and Hanawalt, 1989). The molecular mechanism of the process itself is, nevertheless, completely unknown. A major step toward defining this NER subpathway in E. coli is the recent purification of the transcription repair coupling factor (TRCF) (Selby and Sancar, 1991). This polypeptide, identified as the *mfd* gene product (Selby et al., 1991), specifically enhances repair of the template strand of an actively transcribed gene in an in vitro repair system. It is speculated that the protein recognizes a RNA polymerase complex that is stalled at a DNA lesion and forms together with this complex a high affinity binding site for other repair proteins (the UvrABC complex) (Selby et al., 1991). With respect to the biochemical reaction that the ERCC6 protein might catalyze within the mammalian preferential repair process, we can envisage several models. First, a transcription complex stalled at a lesion will probably sterically hinder access to the damage by the NER machinery and should somehow be displaced. A possibility would be that the presumed helicase activity of ERCC6 serves to dissociate the RNA polymerase complex from the DNA, e.g., by locally melting the nascent RNA–DNA hybrid region, or that the protein forces the polymerase complex either past the injury or backward (see MOT1 [Davis et al., 1992]). Extrapolating from this idea, it may be that an important function of this subfamily of putative helicases is to strip bound proteins from specific DNA regions either in the context of repair or in transcription regulation. Dissociation of protein complexes from nucleic acids by helicases has already been suggested in the process of splicing (Ruby and Abelson, 1991). Second, or in addition, the ERCC6 helicase could, as part of a repair complex, be involved in scanning the transcribed strand of active genes for a stalled RNA polymerase, thus efficiently guiding the NER machinery to lesions that thwart transcription (see TRCF [Selby et al., 1991]). Which of the presented or other possible hypotheses concerning ERCC6 function corresponds to the actual role of the protein in the preferential repair subpathway remains to be elucidated.

Experimental Procedures

General Procedures

Purification of nucleic acids, restriction enzyme digests, gel electrophoresis, DNA ligation, synthesis of radiolabeled probes using random oligonucleotide primers, and filter hybridization were performed according to established procedures (Sambrook et al., 1989).

Isolation of cDNA Clones and Sequence Analysis

Unique genomic fragments were used as a probe to hybridize a λgt11 human testis cDNA library (Clonetech). cDNA fragments isolated were subsequently used as probes to screen cDNA libraries from human testis, placenta (Ye et al., 1987), brain (cortex), amygdala, ovary (Clonetech), adenovirus-transformed retina cells, or pre-B cell line Nalm-6 (provided by Dr. A. Bernards), and a cDNA library provided by Dr. H. Okayama. Sequence analysis was performed on denatured double-stranded plasmid by the dideoxy chain termination method (Sanger et al., 1977) using T7 DNA polymerase (Pharmacia). Sequences were determined on both strands either by construction of plasmids containing progressive unidirectional deletions with the Erase-a-Base System (Promega) or by sequence-specific primers. Se-

quences have been compared with the protein and nucleotide data bases using TFASTA and BLAST (Altschul et al., 1990).

Plasmids

Construction of the cDNA expression plasmid was done as follows (see Figure 1): HTL1B and HTL1C (nucleotides 1462–3746 and ±2700–4714), containing several internal EcoRI sites, were isolated from the λ vector (cloning site EcoRI) via PCR and joined at the unique BglII site (nucleotide 2991). The 3.2 kb cDNA thus obtained was cloned into the SalI site of pTZ19R containing a more 5′ but overlapping fragment (HTL1J(0.8E)) of 700 bp, with 205 bp overlap in the following orientation: EcoRI–700 bp fragment 5′ to 3′–EcoRI–SalI). Transformation into E. coli strain DH5αF′ resulted in a plasmid that had recombined the two *ERCC6* fragments somewhere in the 205 bp overlapping region. The *ERCC6* sequence of this plasmid was verified, and one mistake originating from the PCR was removed by exchanging the NsiI fragment containing the error with the original NsiI fragment. The total ORF was then obtained by ligation of the most 5′ fragment to the PflMI site at position 1361. This almost full-length cDNA molecule was then ligated into a modified pSVL eukaryotic expression vector (Pharmacia; M. van Duin, unpublished data), yielding pSLME6(+).

Cell Lines, Transfection Experiments, UV Selection, and UV Survival

The CHO cell lines used were wild-type AA8, mutants UV41, UV135, UV61, and VB11 from CG-4, CG-5, CG-6, and CG-7, respectively (Zdzienicka et al., 1988; Busch et al., 1989), and two new and unpublished mutants from group 6 provided by Dr. D. Busch. The immortalized human fibroblasts are SV40-A'dam (wild type), CW12 (XP-C from XP7CA), CW3 (XP-E from XP2RO) (Wood et al., 1987), XP2YOSV (XP-F) (Yagi and Takebe, 1983), CS3BE.S3.G1 (CS-A) (Mayne et al., 1986), and CS1AN.S3.G2 (CS-B) (Mayne et al., 1986). Primary fibroblasts used are XP1BI (XP-G) (Keijzer et al., 1979), CS3BE (CS-A), CS1AN (CS-B), and CS1BE (CS-B) (Lehmann, 1982). All CHO cells and immortalized human fibroblasts used were grown in 1:1 F10–Dulbecco's minimal essential medium supplemented with antibiotics and 8%–10% fetal calf serum (F10–DMEM). Primary fibroblasts were cultured in F10 supplemented with antibiotics and 10%–15% fetal calf serum. Transformed cells harboring the E. coli neo dominant marker gene (plasmid pSV2neo) were selected and cultured in medium containing G418 (concentration 300–850 μg/ml, depending on the cell line).

Transfection of CHO cells and immortalized fibroblasts was performed using lipofectin (Bethesda Research Laboratories), essentially as described by the manufacturer. All cells were transfected at approximately 80% confluence (on 60 mm petri dishes), with 2 μg of pSV2neo and 5 μg of pSLME6(+). DNA (a total of 7 μg) and 14 μg of lipofectin were separately added to Opti-MEM (Bethesda Research Laboratories); thereafter, the two were mixed and left at room temperature for 15 min. Cells were washed with phosphate-buffered saline (PBS) before addition of the Opti-MEM–DNA–lipofectin mixture. CHO cells were incubated for 6–7 hr; thereafter 10% fetal calf serum was added. Medium was refreshed with F10–DMEM after approximately 16 hr. Immortalized human fibroblasts were incubated for a similar period, but medium was subsequently refreshed with F10–DMEM. Cells were trypsinized (and, depending on cell density, divided over more dishes) and placed on selection medium 48 hr after transfection. Selection for repair-proficient transformants was started after the appearance of G418-resistant colonies. Cells were trypsinized, and UV selection was started 16–20 hr after trypsinization for CHO cells, 20–48 hr after trypsinization for immortalized human fibroblasts, cells were irradiated three times, CHO cells at 1 day intervals and the SV40-immortalized fibroblasts at 2 day intervals. The UV dose given (Philips TUV low pressure mercury tube, 15 W, 0.45 $J/m^2/s$; predominantly 254 nm) varied with the cell line: 2 J/m^2 (UV135); 4 J/m^2 (UV41, XP2YOSV); 6 J/m^2 (CW12, CS1AN.S3.G2); 8 J/m^2 (CS3BE.S3.G1); 8.4 J/m^2 (UV61); and 11 J/m^2 (VB11).

For determination of UV sensitivity of G418-resistant mass populations (CS3BE.S3.G1 and CS1AN.S3.G2 transformants), G418-resistant plus UV-resistant mass populations (61E6a), or isolated UV-resistant colonies (E61ANa and E61ANd), cells were plated at densities varying from 2×10^2 to 5×10^5 cells per 60 mm petri dish, depending on cell line and UV dose. Cells were rinsed with PBS and exposed to UV light about 1 day after plating. A series of dishes was irradiated for each cell line, each receiving a single UV dose (three dishes per UV dose). Clones were fixed and stained with Coomassie brilliant blue 6–10 days after UV irradiation, and relative cloning efficiencies were determined.

RNA Synthesis Recovery

The method used was as described by Mayne and Lehmann (1982), with several modifications. Cells were seeded in 30 mm petri dishes in 1.5 ml of medium. One day after seeding, the medium was changed for F10, supplemented with antibiotics, 15% fetal calf serum, 20 mM HEPES, and 0.025 μCi/ml [^{14}C]thymidine (50 mCi/mmol). The cells were grown for 16 hr, and then the medium was removed; cells were rinsed with PBS and UV irradiated (at 254 nm, fluence rate 0.7 $J/m^2/s$, three petri dishes for each time point or UV dose). At various times after UV irradiation, the medium was replaced by medium containing 8 μCi/ml [^{3}H]uridine (45 Ci/mmol), and the cells were incubated for 1 hr. At the end of the labeling period, cells were washed once with ice-cold PBS containing >1 mM uridine and once with ice-cold PBS. Cells were scraped from the dishes and spotted onto GF/C Whatman glass filters soaked in 10% trichloroacetic acid. These were then washed once in 10% trichloroacetic acid and 96% ethanol, dried, and counted in a liquid scintillation counter. ^{14}C counts served as an internal control for the number of cells present.

Microinjection

ERCC6 construct pSLME6(+) was injected into nuclei of XP or CS homopolykaryons as described by van Duin et al. (1989). Cells were incubated 24–48 hr to allow expression of the injected DNA. To determine the influence of the *ERCC6* gene on DNA repair, injected XP cells were subjected to UV-induced repair synthesis (unscheduled DNA synthesis), which was measured by autoradiography as described previously (Vermeulen et al., 1986). Injected CS fibroblasts were UV irradiated and subsequently incubated with [^{3}H]uridine to measure recovery from UV-induced RNA synthesis inhibition.

DNA Amplification and Mutation Determination

Total RNA (10 μg) was used for preparation of cDNA with *ERCC6*-specific primers. RNA was dissolved in 5 μl of water, heated for 3 min at 85°C, and cooled on ice. Subsequently, the RNA was added to 15 μl containing 50 pmol of 3′ primer, 18 U of RNAguard (Boehringer), 12.5 U of avian myeloblastosis virus reverse transcriptase (Boehringer), 1 mM dNTPs, 50 mM KCl, 20 mM Tris HCl (pH 8.4 at 20°C), 2.5 mM $MgCl_2$, and 100 μg/ml bovine serum albumin; incubation was for 1 hr at 37°C. The cDNA (4 μl of the above reaction mixture) was added to 96 μl containing 10 pmol of 5′ primer, 0.2 mM dNTPs, 50 mM KCl, 20 mM Tris HCl (pH 8.4 at 20°C), 2.5 mM $MgCl_2$, 100 μg/ml BSA, and 2.5 U of Taq polymerase (Bethesda Research Laboratories); the mixture was overlayed with oil. In some experiments, Taq polymerase was added only after the samples reached the denaturing temperature of 94°C (hot start). Amplification was done in 35 cycles: 1 (or 2) cycles of 5 min denaturation at 94°C, 5 min annealing (temperature depending on primer combination used), and 5 min extension at 72°C, followed by 34 (or 33) cycles of 2 min at 94°C, 2 min annealing, and 3 min at 72°C. Chromosomal DNA was amplified as above, starting from 200 ng, in 25 cycles. Asymmetric PCR was performed on 1 μl of PCR product, using the conditions mentioned above (35 cycles), with a primer ratio of 1:100 (0.5 pmol of one and 50 pmol of the other primer). If available, internal primers were used. Products were extracted once with phenol–chloroform–isoamylalcohol and once with chloroform, separated from oligonucleotides and dNTPs on a Centricon-100 column (Amicon), and dried under vacuum in a SpeedVac concentrator (New Brunswick Scientific). Pellets were dissolved in 15 μl, 7 μl of which was used for each sequencing reaction.

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GenBank Accession Number

The accession number for the sequence reported in this paper is L04791.

Appendix II

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

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

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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

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

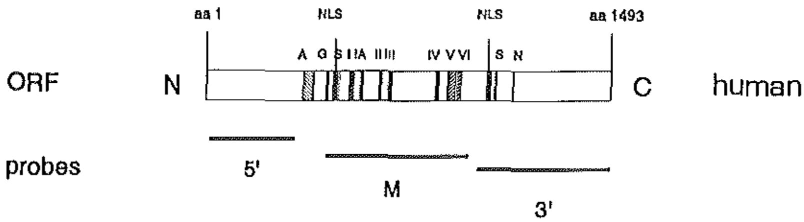
Southern Zoo blot analysis using genomic DNA from various species, including *S.cerevisiae*, with the human *CSB/ERCC6* cDNA as a probe suggested that this gene is conserved from higher to lower eukaryotes (data not shown). On this basis we decided to attempt cloning of the *S.cerevisiae CSB/ERCC6* homolog 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

A



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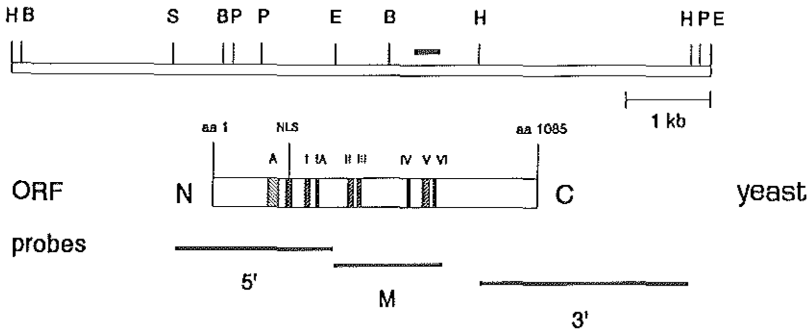

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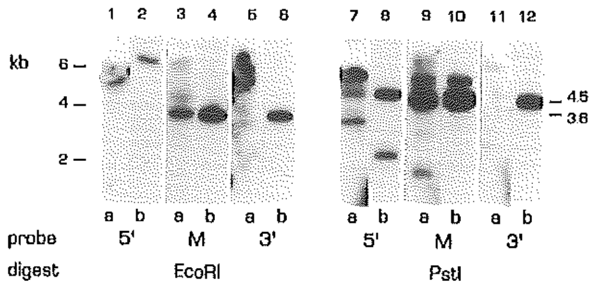

Fig. 1. Detection of cross-hybridizing *ERCC6* fragments in *S.cerevisiae* genomic DNA. (A) Schematic representation of the three PCR-generated probes of the human *ERCC6* cDNA used for Southern blot analysis. The probes 5', M and 3' encompass the sequence for respectively the N-terminus (amino acids 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 *SalI*–*HindIII* *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, *HindIII*; B, *BglII*; S, *SalI*; P, *PstI*; E, *EcoRI*. (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 *EcoRI* (lanes 1–6) or *PstI* (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 *PstI* 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

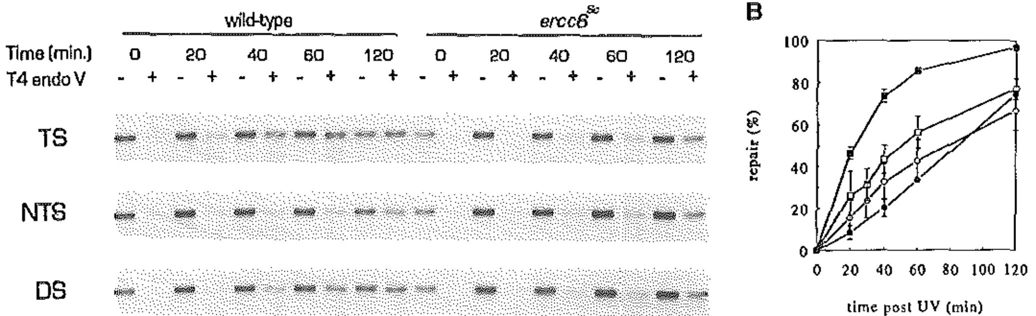

Fig. 3. Transcription-coupled repair of *RPB2* is impaired in *rad26* null mutant. Genomic DNA was isolated from cells that were UV irradiated (70 J/m²) and allowed to repair for the indicated time. Following digestion by *PvuI* and *PvuII*, the DNA was mock-treated or treated with T4 endo V, indicated 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 PvuI–PvuII* 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*; O, 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,

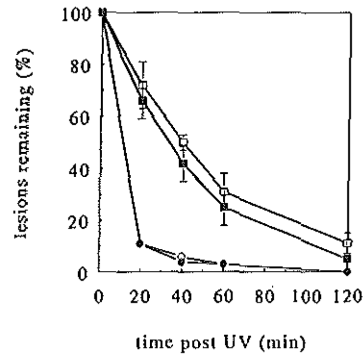

Fig. 4. *Rad26* null mutant cells possess a wild type overall repair capacity. Exponentially growing cells were UV irradiated with 70 J/m² and allowed to repair for the indicated periods of time. Genomic DNA was isolated and assayed for the presence of cyclobutane pyrimidine dimers (CPD) and 6/4 photoproducts (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; O, 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/m²

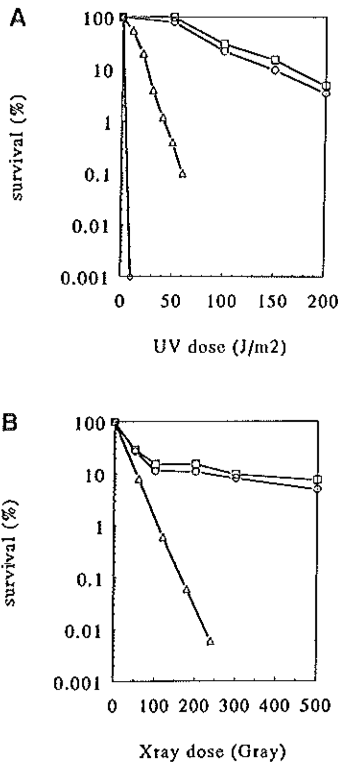

Fig. 5. Impairment of transcription-coupled repair does not lead to increased UV or X-ray sensitivity. (A) Exponentially growing W303 (□) or *rad26* (O) cells were irradiated with the indicated doses of 254 nm UV, incubated on complete medium (YEPD) agar at 28°C, and colonies were counted after 3–5 days. Values represent the average of two independent experiments, each performed in duplicate. For comparison, survival of the *rad16* [(Δ), 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).

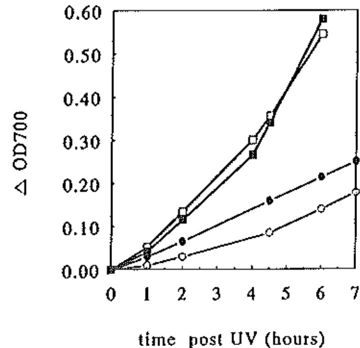

Fig. 6. *Rad26* cells show a slower recovery of growth following UV irradiation. Exponentially growing W303 and *rad26* cells were unirradiated or irradiated with 70 J/m² of 254 nm UV. Cells were diluted in YEPD medium and allowed to grow at 28°C. Growth was monitored by measuring the OD_{700} at the indicated time-points. ■, W303, unirradiated; □, *rad26*, unirradiated; ●, W303, 70 J/m²; and O, *rad26*, 70 J/m².

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.

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,

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 (*MATα, 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.

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Appendix III

Double mutants of Saccharomyces cerevisiae with alterations in global genome and transcription-coupled repair.

Double Mutants of *Saccharomyces cerevisiae* with Alterations in Global Genome and Transcription-Coupled Repair

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The nucleotide excision repair (NER) pathway is thought to consist of two subpathways: transcription-coupled repair, limited to the transcribed strand of active genes, and global genome repair for nontranscribed DNA strands. Recently we cloned the *RAD26* gene, the *Saccharomyces cerevisiae* homolog of human *CSB/ERCC6*, a gene involved in transcription-coupled repair and the disorder Cockayne syndrome. This paper describes the analysis of yeast double mutants selectively affected in each NER subpathway. Although *rad26* disruption mutants are defective in transcription-coupled repair, they are not UV sensitive. However, double mutants of *RAD26* with the global genome repair determinants *RAD7* and *RAD16* appeared more UV sensitive than the single *rad7* or *rad16* mutants but not as sensitive as completely NER-deficient mutants. These findings unmask a role of *RAD26* and transcription-coupled repair in UV survival, indicate that transcription-coupled repair and global genome repair are partially overlapping, and provide evidence for a residual NER modality in the double mutants. Analysis of dimer removal from the active *RPB2* gene in the *rad7/16 rad26* double mutants revealed (i) a contribution of the global genome repair factors Rad7p and Rad16p to repair of the transcribed strand, confirming the partial overlap between both NER subpathways, and (ii) residual repair specifically of the transcribed strand. To investigate the transcription dependence of this repair activity, strand-specific repair of the inducible *GAL7* gene was investigated. The template strand of this gene was repaired only under induced conditions, pointing to a role for transcription in the residual repair in the double mutants and suggesting that transcription-coupled repair can to some extent operate independently from Rad26p. Our findings also indicate locus heterogeneity for the dependence of transcription-coupled repair on *RAD26*.

The molecular details of the versatile process of nucleotide excision repair (NER) are becoming increasingly clear as more of the proteins involved are purified and biochemically analyzed (1, 8, 17; for reviews about NER, see references 7 and 12). However, the process of differential repair, the difference in rate of removal of cyclobutane pyrimidine dimers from different parts of the genome (for reviews, see references 9 and 32), is not yet fully understood at the molecular level. Although the actual process of dimer removal is likely to be performed by the same repair enzymes in the same molecular way for the whole genome, one of the first steps of NER, DNA damage recognition in chromatin, might differ for lesions in specific regions. This notion has led to the idea of two subpathways of NER: (i) a process called global genome repair that is essential for removal of damage from nontranscribed DNA sequences and (ii) a system known as transcription-coupled repair that is involved in the specific fast and efficient repair of damage from the transcribed strand of active genes. Transcription-coupled repair has been found to occur in mammalian cells (16), *Escherichia coli* (15), and the yeast *Saccharomyces cerevisiae* (14, 27, 30), and it causes preferential repair of the transcribed strand over the nontranscribed strand and other inactive DNA.

Several genes have been shown to be specifically involved in each of the subpathways in mammals and in *S. cerevisiae*. In humans, the *XPC* gene is implicated in global genome repair, since cell lines with a mutation in this gene repair only dimers in transcribed strands of active genes (37). In *S. cerevisiae*, *rad7* or *rad16* disruption mutants are completely deficient in repair of the silent mating-type loci (2, 31) and the nontranscribed strand of an active gene (38). Repair of the transcribed strand is not affected in these mutants, suggesting that at least when the template strand is transcribed, the function of the global repair proteins Rad7p and Rad16p (and in human cells XPC) is restricted to repair of the nontranscribed strand.

Preferential repair of the transcribed strand over the nontranscribed strand was shown to be dependent on transcription (14, 30), indicating a role for the transcription process in efficient recognition of damage in transcribed DNA. In *E. coli*, a factor coupling the DNA repair machinery to transcription has been found (25). TRCF (transcription-repair coupling factor, the product of the *mfd* gene [24]) recognizes RNA polymerase stalled at a lesion, and through affinity for UvrA, it directs the repair enzymes to the damage; upon release of the polymerase-RNA complex, the lesion is repaired (25). Transcription-coupled repair has also been found to occur in higher eukaryotes, and it has been shown that this process requires transcription and additional proteins. One such protein is the *CSB* gene product, which complements the UV sensitivity and the deficiency in recovery of RNA synthesis after UV irradiation in Cockayne syndrome (CS) group B (CS-B) cells (33). CS-B cell lines have been shown to lack efficient repair of active DNA (35, 36), implying a role for the complementing gene *CSB* in transcription-coupled repair. We have cloned the yeast homolog of *CSB* and designated this gene *RAD26* (34). Disruption of this gene indeed leads to a defect in transcription-coupled repair of the *RPB2* gene in yeast cells (34). Remarkably,

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TABLE 1. *S. cerevisiae* strains used

Strain	Genotype	Reference
W303-1B	*MATα ho can1-100 ade2-1 trp1-1 leu2-3,112 his3-11,15 ura3-1*	21
W303236	*rad16Δ::URA3*[a]	38
MGSC97	*rad7Δ::URA3*[a]	38
MGSC102	*rad26Δ::HIS3*[a]	34
MGSC104	*rad7Δ::LEU2*[a]	38
MGSC106	*rad7Δ::LEU2 rad26Δ::HIS3*[a]	This study[b]
MGSC107	*rad16Δ::LEU2 rad26Δ::HIS3*[a]	This study[b]
MGSC108	*rad7Δ::LEU2 rad16Δ::URA3 rad26Δ::HIS3*[a]	This study[b]
MGSC126	*rad16Δ::LEU2*[a]	This study[b]
MGSC139	*rad14Δ::LEU2*[a]	This study[b]
MGSC140	*rad14Δ::LEU2 rad26Δ::HIS3*[a]	This study[b]

[a] The remainder of the genotype is that of W303-1B.
[b] Constructed as described in Materials and Methods.

a *rad26* disruption mutant is not more UV sensitive than a RAD^+ strain, in contrast to human CS-B cell lines.

It is possible that the defect in transcription-coupled repair in a *rad26* mutant is partly compensated for by global genome repair, explaining the absence of UV sensitivity in such a mutant. To examine this possibility, survival after UV irradiation of *rad7 rad26* and *rad16 rad26* double mutants was determined and compared with survival of various single mutants. Analysis of these mutants also allowed testing of the idea that NER is accomplished by the additive contribution of transcription-coupled repair and global genome repair. Removal of dimers from both individual strands of the active *RPB2* gene and the induced or repressed *GAL7* gene in the various mutants was determined to answer this question. The results provide more insight into the relationship between transcription-coupled and global genome repair, have implications for the role of the *RAD26* gene in transcription-coupled repair in *S. cerevisiae*, and may have implications for the molecular defect in CS.

MATERIALS AND METHODS

General procedures. All general procedures, including DNA purification, restriction enzyme digestion, cloning, PCR, and gel electrophoresis, were performed according to standard procedures (22). Plasmids were propagated in *E. coli* JM101 under appropriate antibiotic selection.

Yeast strains and media. The yeast strains used for this study are listed in Table 1. All strains were kept on selective YNB (0.67% yeast nitrogen base, 2% glucose, 2% Bacto Agar) supplemented with the appropriate markers. Cells were grown in complete medium (YEPD) (1% yeast extract, 2% Bacto Peptone, 2% glucose) at 28°C under vigorous shaking conditions. For induction of *GAL7*, glucose was replaced by galactose (28). Induction of *GAL7* on galactose-containing medium was confirmed by Northern (RNA) blot analysis.

Construction of disruption mutants. Yeast cells were transformed by electroporation (2.250 V/cm, 250 μF, 200 Ω). Cells were plated on YNB with the necessary amino acids and incubated at 28°C for 2 to 5 days. Successful disruption (21) was confirmed by Southern analysis.

Strain MGSC102 (*rad26Δ::HIS3* [34]) was transformed with *Bgl*I-linearized plasmid pRAD7Δ::LEU2 (38) to obtain strain MGSC106 (*rad7 rad26*).

Strains W303-1B and MGSC102 (*rad26Δ::HIS3*) were transformed with *Pvu*I-linearized plasmid pUB33 (gift of D. D. Bang), which contains the *LEU2* gene inserted in place of the *Hin*dIII fragment of *RAD16* (2), to obtain strains MGSC126 (*rad16*) and MGSC107 (*rad16 rad26*), respectively. These strains carry the same *rad16* deletion as strain W303236 (38), but with *LEU2* as a selectable marker instead of *URA3*.

MGSC105 (*rad7Δ::LEU2 rad16Δ::URA3* [38]) was transformed with linearized pPTZSHE6ScΔ::HIS3 (34) to generate strain MGSC108 (*rad7 rad16 rad26*).

W303-1B and MGSC101 (*rad26Δ::HIS3*) were transformed with *Sac*I-*Nco*I-digested pBM190 (gift of L. Prakash [3]) to generate strains MGSC139 (*rad14*) and MGSC140 (*rad14 rad26*), respectively.

UV survival curves. Yeast cells were grown in YEPD to an optical density of 0.6, diluted in water, and irradiated with the indicated UV doses, and dilutions were plated on YEPD. After 3 days of incubation at 28°C in the dark, colonies were counted and survival was calculated.

UV irradiation and DNA isolation. Yeast cells diluted in chilled phosphate-buffered saline were irradiated with 254-nm UV light (Philips T UV 30W) at a rate of 3.5 $J/m^2/s$. Cells were collected by centrifugation, resuspended in growth medium, and incubated for various times in the dark at 28°C prior to DNA isolation (26). DNA was purified on CsCl gradients (22).

Specific probes. Construction and isolation of single-stranded M13-derived probes recognizing the *RPB2* gene were performed as described before (38).

To construct strand-specific probes recognizing the *GAL7* gene, oligonucleotides 5′GGTTTTGCAATCGAGCCTGGTAG3′ and 5′GGCCAGATGGCCGAGTATG3′ were synthesized and PCR was performed on yeast strain W303-1B chromosomal DNA with these primers (35 cycles, annealing temperature of 55°C), generating a 1.6-kb fragment. This fragment was digested with *Acc*I, the site was filled with Klenow enzyme to generate a blunt end, and the fragment was digested with *Bgl*II. The resulting 1.1-kb blunt-*Bgl*II fragment was cloned in both orientations in M13 digested with *Hinc*II and *Bam*HI (M13mp18 and M13mp19).

Single-stranded DNA was isolated as described by Sambrook et al. (22) and used for primer extension to generate ^{32}P-labeled strand-specific probes as described earlier (34, 38).

Gene-specific repair assay. Genomic DNA was cut with restriction endonucleases *Pvu*I and *Pvu*II, generating a 5.2-kb *RPB2* fragment (30) and a 4.7-kb *GAL7* fragment. DNA samples were divided in two equal parts. One was incubated with T4 endonuclease V (isolated as described in reference 18), the other was mock treated, and both were loaded on denaturing agarose gels as described by Bohr et al. (4). After electrophoresis, the DNA was transferred to Hybond N+ (Amersham) and hybridized to strand-specific probes. After hybridization and data analysis, the probe was removed by alkaline washing, and subsequently the blot was hybridized to another probe. In this way, it was possible to determine dimer removal from both strands of the *RPB2* fragment and the *GAL7* fragment on every blot (four probes).

The amount of hybridized labeled probe in each band on the Southern blots was quantified with a Betascope 603 blot analyzer (Betagen) and used to calculate the amount of dimers per fragment according to the Poisson distribution as described previously (4). After being scanned in the blot analyzer, autoradiographs were prepared from the Southern blots.

RESULTS

Survival of double mutants disturbed in transcription-coupled as well as global genome repair. To investigate whether the lack of UV sensitivity of a *rad26* mutant is due to compensation of the NER defect by global genome repair, we studied mutants that lack both *RAD26* and factors essential for global genome repair. Isogenic *rad26*, *rad7*, *rad16*, *rad7 rad26*, *rad16 rad26*, and *rad7 rad16 rad26* disruption mutants were constructed from the repair-proficient (RAD^+) strain W303-1B (see Materials and Methods and Table 1). Isogenic strains totally deficient in NER were constructed by disruption of the *RAD14* gene (3). The survival of the strains after irradiation with UV was measured.

From the results in Fig. 1, it is clear that although a single *rad26* mutant is not more UV sensitive than a RAD^+ strain (34), the *rad7 rad26* and *rad16 rad26* double mutants are more UV sensitive than single *rad7* or *rad16* mutants. In contrast, the survival of a *rad14 rad26* double mutant is identical to that of a completely NER-deficient *rad14* single mutant, indicating that no repair systems other than NER are impaired by a *rad26* mutation. Since *rad7*, *rad16*, and *rad14* mutants are in the same (*rad3*) epistasis group, the greater UV sensitivity of *rad7 rad26* and *rad16 rad26* mutants than of single *rad7* or *rad16* mutants is due to the combination of defects in global genome and transcription-coupled repair.

Strikingly, although an additive effect from disturbing both transcription-coupled and global genome repair is observed, the *rad7 rad26* and *rad16 rad26* double mutants are clearly not as UV sensitive as a completely NER-deficient *rad14* strain. This result indicates that there must be residual repair activity left in the double mutants.

Analysis of dimer removal from both strands of the *RPB2* gene. To examine the nature of the remaining repair activity in the double mutants, we analyzed dimer removal from the individual strands of an active gene with the method described by Bohr et al. (4). The results of the repair experiments using the *RPB2* gene are shown in Fig. 2.

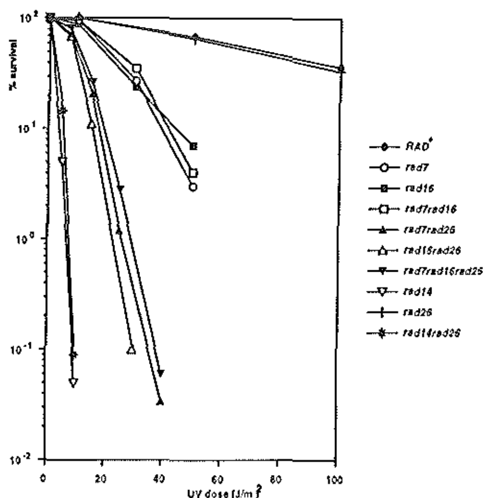

FIG. 1. UV survival of double mutants disturbed in global genome and transcription-coupled repair. The survival of strains W303-1B (*RAD*$^+$), MGSC102 (*rad26*), MGSC104 (*rad7*), W303236 (*rad16*), MGSC105 (*rad7 rad16*), MGSC106 (*rad7 rad26*), MGSC107 (*rad16 rad26*), MGSC108 (*rad7 rad16 rad26*), MGSC139 (*rad14*), and MGSC140 (*rad14 rad26*) after UV irradiation is depicted.

As we described before (34, 38), *rad7* or *rad16* single mutants are defective in repair of the nontranscribed strand of *RPB2*, while *RAD26* disruption leads to a strong decrease of the preferential repair of the *RPB2* transcribed strand (Fig. 2B).

Survival experiments suggested that global repair can contribute to removal of dimers from the transcribed strand when Rad26p is absent (see above). This was tested more directly by analyzing repair of *RPB2* in the *rad7 rad26* and *rad16 rad26* double mutants. Repair of the transcribed strand in these double mutants is less efficient than in the single *rad26* mutant (Fig. 2C), suggesting that *RAD7* and *RAD16* contribute to repair of the transcribed strand when transcription-coupled repair is hampered. Since the *rad7 rad26* and *rad16 rad26* mutants are not as UV sensitive as *rad14* strains, they should be able to repair at least part of their DNA. Figures 2A and C show that the transcribed strand and not the nontranscribed strand of *RPB2* is still repaired to a considerable extent in the *rad7 rad26* and *rad16 rad26* double mutants, suggesting that the residual repair activity in these mutants is transcription coupled.

Repair of the *GAL7* gene under induced and repressed conditions. To determine whether the residual repair activity in the *rad7 rad26* and *rad16 rad26* double mutants is indeed dependent on transcription, we analyzed strand-specific repair of the *GAL7* gene. This gene is repressed in medium containing glucose, whereas it is strongly induced in medium containing galactose (28). We studied repair of the *GAL7* gene in a *Pvu*I-*Pvu*II fragment that is comparable in size to the *RPB2* *Pvu*I-*Pvu*II fragment, enabling direct comparison of the rates of dimer removal from the *GAL7* and *RPB2* genes on the same blot, thus providing an internal control to exclude possible medium effects.

Figure 3A illustrates that in the *RAD*$^+$ strain, the template strand (the strand that is transcribed under induced conditions) is repaired faster than the nontranscribed strand when the gene is induced and that this difference is almost absent when the gene is repressed, which is consistent with earlier observations by Leadon and Lawrence (14).

Repair of the nontranscribed strand of *GAL7* is fully dependent on *RAD7* and *RAD16* (Fig. 3B), as expected and in agreement with the results obtained with *RPB2*. Also, the repair of the template strand confirms the results obtained with *RPB2*: under induced conditions with transcription-coupled repair active, *RAD7* or *RAD16* does not contribute to repair of this strand (Fig. 3B). Under repressed conditions, however, global genome repair does contribute to repair of the template strand, since in the *rad7* and *rad16* mutants, the repair of this strand is strongly inhibited (Fig. 3B). Under these conditions, there is still some residual repair of the template strand of the *GAL7* gene in the *rad7* and *rad16* mutants. This may be attributable to transcription-coupled repair as a result of some residual transcription, especially since it appears that this repair is dependent on *RAD26* (see below).

When the effect of *RAD26* disruption on the repair of the transcribed strand of *GAL7* was measured, a rather surprising result was obtained (Fig. 3C). In contrast to *RPB2*, for which a strong reduction in the repair rate of the transcribed strand is found (analyzed by using the same DNA on the same blot), the repair of the template strand of *GAL7* under induced conditions is nearly the same as in *RAD*$^+$ cells. Apparently the contribution of Rad26p to transcription-coupled repair can vary for different loci and is much more apparent for the *RPB2* gene than for *GAL7* under induced conditions. Under repressed conditions, both strands of the *GAL7* gene are repaired at the same rate in the *rad26* mutant (Fig. 3C), a result that was expected since under the same conditions almost no difference is observed between repair of both strands of *GAL7* in *RAD*$^+$ cells (Fig. 3A). This finding is consistent with the idea that in the absence of transcription, removal of dimers from both strands is performed by global genome repair that is independent of Rad26p.

When the global genome repair pathway is also impaired (in the *rad7 rad26* and *rad16 rad26* double mutants), the nontranscribed strand of *GAL7* is not repaired (Fig. 3D), as expected. Notably, the template strand of *GAL7* is still repaired under induced conditions, but no dimers are removed from this strand under repressed conditions (Fig. 3D). This finding demonstrates that repair of the *GAL7* template strand in the double mutants is dependent on transcription and moreover suggests that transcription per se can accomplish transcription-coupled repair independent of Rad26p. The residual repair of the template strand that was observed under repressed conditions in the *rad7* and *rad16* mutants (Fig. 3B) requires a functional *RAD26* gene, implying that Rad26p contributes to transcription-coupled repair of *GAL7* under repressed conditions.

Taken together, these results point to the existence of a Rad26p-independent mode of transcription-coupled repair and to locus heterogeneity with regard to the influence of Rad26p on transcription-coupled repair.

DISCUSSION

***RAD26* and transcription-coupled repair.** *rad26* disruption mutants are as UV resistant as *RAD*$^+$ strains. Possibly global genome repair can compensate for the loss of *RAD26* by ensuring repair of transcribed DNA (34). Here we show that this is indeed the case because there is a more than additive effect of mutations in *RAD26* combined with mutations in the *RAD7* and *RAD16* genes involved in global genome repair (38), implying that global genome repair factors are responsible for the

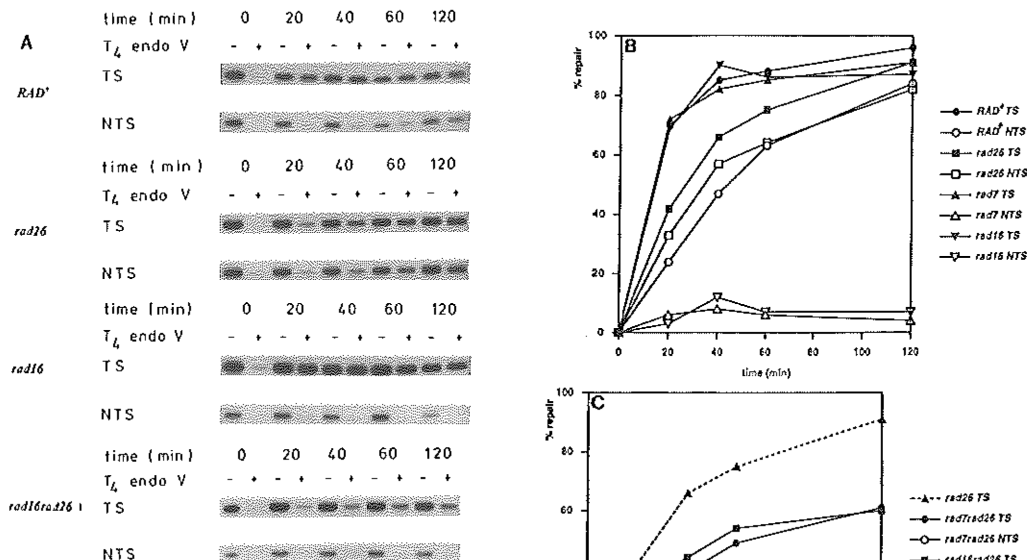

FIG. 2. Residual repair of the transcribed strand of *RPB2* in *rad7 rad26* and *rad16 rad26* cells. (A) Representative Southern blots showing the removal of T4 endonuclease V (endo V)-sensitive sites from *RPB2* in W303-1B (*RAD*+), MGSC102 (*rad26*), W303236 (*rad16*), and MGSC107 (*rad16 rad26*). Time points after UV irradiation are indicated; samples were mock treated (−) or treated with T4 endonuclease V (+). TS, transcribed strand; NTS, nontranscribed strand. (B and C) Graphical presentation of the percent repair at the different time points as calculated according to the Poisson distribution; each point is the average of six to nine experiments, and the average standard error is 7%. (B) Repair of both strands of *RPB2* in *RAD*+, *rad26*, *rad7*, and *rad16* cells confirms our earlier results (34, 38). (C) Repair of *RPB2* in *rad7 rad26* and *rad16 rad26* double mutants. For comparison, data for repair of the transcribed strand in a *rad26* mutant are also depicted (dashed line). The degree of repair of both strands in the *rad7 rad16 rad26* triple mutant is identical to the degree of repair in the *rad7 rad26* and *rad16 rad26* double mutants.

lack of UV sensitivity of a *rad26* mutant. Human CS-B cells, in contrast, are markedly UV sensitive. A possible explanation for this difference is that the global genome repair process may be more efficient in *S. cerevisiae* than in higher eukaryotic species with a more complex genome. The contribution of Rad26p to survival after UV exposure that is revealed in the double mutants stresses the involvement of the *RAD26* gene in general transcription-coupled repair and unmasks the contribution of this process to cellular UV resistance. Therefore, these data strengthen the correspondence between *RAD26* and *CSB*.

Although the foregoing and previous findings unequivocally establish the involvement of Rad26p in transcription-coupled repair, double mutants lacking *RAD26* and global genome repair are significantly less UV sensitive than completely NER-deficient mutants. Furthermore, analysis of gene- and strand-specific repair in the *RPB2* and *GAL7* genes reveals that these mutants are, to a variable extent, still capable of repairing the transcribed strand only. The experiments with the inducible *GAL7* gene strongly suggest that this repair is transcription dependent. It has already been demonstrated that strand-specific repair of *RPB2* and *GAL7* requires functional RNA polymerase II (14, 30), pointing to a direct role for the transcription machinery in transcription-coupled repair. Since strains that lack Rad26p and global genome repair display residual repair of the transcribed strand selectively, this repair must be dependent on transcription. We conclude that part of the transcription-coupled repair is Rad26p independent. Incomplete inactivation of transcription-coupled repair also explains the slight but reproducible preferential repair of the transcribed strand over the nontranscribed strand of the *RPB2* gene in the *rad26* mutant (34) (Fig. 2B).

The effect of *RAD26* disruption on repair of the template strand of *GAL7* under conditions such that the transcription rate is high (28) is very small or absent (Fig. 3C), in contrast to the clear and significant effect on transcription-coupled repair of *RPB2* (Fig. 2B) and the *PHO5 PHO3* locus (unpublished results). Apparently, Rad26p-independent transcription-coupled repair is more efficient for induced *GAL7* than for the *RPB2* gene. Since deletion of *RAD26* has some effect on *GAL7* repair under repressed conditions (Fig. 3B and D), it might be possible that the high efficiency of *RAD26*-independent transcription-coupled repair in *GAL7* is related to a high transcription rate of the *GAL7* gene under induced conditions. Although without further experimentation this hypothesis remains merely speculative, it is interesting that a similar observation has been reported for *E. coli*: *mfd* mutants (lacking TRCF [24]) are still able to preferentially repair the transcribed strand of the *lacZ* gene in vivo when this gene is induced with isopropylthiogalactopyranoside (IPTG) but not when the gene is transcribed at a low rate (13).

The observation of Rad26p-independent transcription-cou-

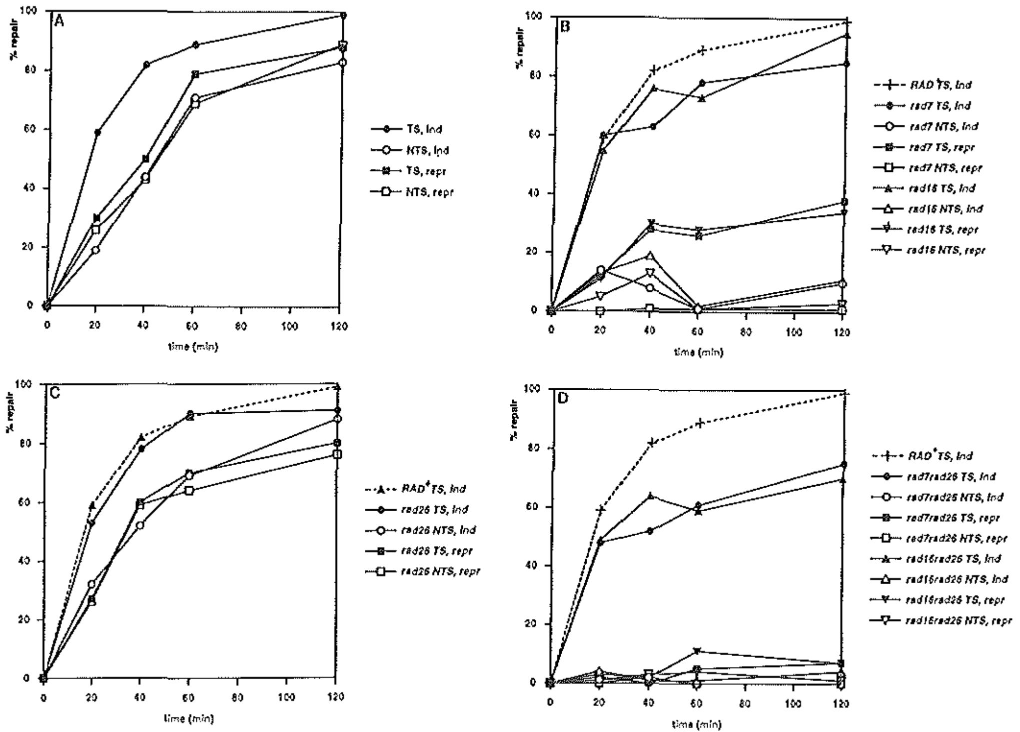

FIG. 3. *GAL7* is repaired only under induced conditions in *rad7 rad26* and *rad16 rad26* mutants. Repair of the *GAL7* gene was calculated according to the Poisson distribution for each time point; the data are from three to four experiments, and the average standard error is 6%. TS, transcribed (template) strand; NTS, nontranscribed (nontemplate) strand; ind, induced conditions (galactose); repr, repressed conditions (glucose). (A) Repair of *GAL7* in RAD^+ cells. (B) Repair of *GAL7* in *rad7* and *rad16* cells. For comparison, data for the transcribed strand under induced conditions in RAD^+ cells are also depicted (dashed line). (C) Repair of *GAL7* in *rad26* cells. For comparison, data for the transcribed strand under induced conditions in RAD^+ cells are also depicted (dashed line). (D) Repair of *GAL7* in *rad7 rad26* and *rad16 rad26* cells. For comparison, data for the transcribed strand of RAD^+ cells under induced conditions are also depicted (dashed line). No difference in repair of the constitutively expressed *RPB2* gene was observed for the same strain on medium containing glucose compared with medium with galactose. Therefore, all differences observed in repair of *GAL7* in cells grown in both media are specifically due to the induced and repressed states of the *GAL7* gene in these cells and not due to general repair differences as a consequence of the different media.

pled repair suggests that Rad26p has an auxiliary function important for the efficiency of transcription-coupled repair but is not essential for this process. It is therefore not likely that Rad26p is the yeast counterpart of the *E. coli* TRCF, a protein that couples repair to transcription by specifically targeting repair enzymes to lesions that obstruct RNA polymerase (25). If Rad26p is a transcription-repair coupling factor, then either such a coupling factor is not essential in yeast cells or a protein other than Rad26p can independently perform transcription-repair coupling. A possible candidate for such a redundant factor is the yeast homolog of CSA, although in human cell lines defects in either *CSA* or *CSB* lead to abolishment of transcription-coupled repair (11, 35). Transcription-repair coupling may have different molecular backgrounds in prokaryotes and eukaryotes (9). The necessity for a TRCF as found in *E. coli* (25) might in eukaryotes be obviated by the intimate association of several NER enzymes with basal transcription factors (6, 23, 29). If backtracking of a blocked RNA polymerase is a prerequisite for eukaryotic transcription-coupled repair (5), Rad26p is not essential for this process. The recent notion that CS may be caused by defects in the transcription process, indirectly leading to defects in transcription-coupled repair (11), should also be considered. Transcriptional defects in *rad26* cells could account for the defect in transcription-coupled repair that we observe. However, we did not detect defects in growth rates (34) or transcription of *RPB2* and *GAL7* in our *rad26* mutants (unpublished data), although subtle transcriptional defects cannot be excluded.

Global genome repair. *RAD7* and *RAD16* are essential for global genome repair in yeast cells. Silenced DNA and the nontranscribed strands of the *RPB2* and *GAL7* genes are not repaired in *rad7* and *rad16* mutants, but the transcribed strand is repaired with the same efficiency as in repair-proficient cells. However, inactivating global genome repair leads to a repair defect for the template strand of *GAL7* when transcription is nearly absent (Fig. 3B), proving that global genome repair is capable of functioning on a strand that is a template for transcription-coupled repair under induced conditions. The diminished repair rate of the transcribed strand in *rad7 rad26* and *rad16 rad26* double mutants compared with *rad26* single mu-

tants probably also reflects a contribution of *RAD7* and *RAD16* to repair of the transcribed strand. Therefore, the term global genome repair is really warranted for this system, which was previously implicated only in repair of nontranscribed DNA (38).

How the proteins involved in global genome repair act at the molecular level is still unknown. One could envisage that the chromatin context of the DNA damage in eukaryotes necessitates such factors to make the damaged DNA a substrate for the actual incision enzymes. Interestingly, Rad16p shares functional domains with Swi2p/Snf2p (2), a factor that may be involved in suppression of chromatin-mediated repression of transcription (20), while Rad7p has been shown to interact with Sir3p (19), a putative component of silent yeast chromatin (10). Rad7p and Rad16p seem dispensable for a reconstituted NER reaction on naked plasmid DNA (8), corroborating a role for these proteins in vivo on DNA packed into chromatin. However, our *rad7* and *rad16* mutants are completely defective for NER in a cell-free system that is also devoid of transcriptional activity (39).

The reconstitution of in vitro NER reactions (1, 8, 17) is highly informative with regard to the biochemistry of NER. Nevertheless, our results underscore the importance of analyzing dimer removal in vivo to be able to appreciate the complexity of NER and identify components that play a role in the organization of NER. Since the incision and subsequent steps of NER are probably not different for the two strands (1, 8, 17), we hypothesize that dimers can be removed only when they are made accessible for repair enzymes either by transcription or by the global genome repair proteins. Absence of both transcription and one of the global repair proteins, Rad7p or Rad16p, leads to complete inactivation of NER in vivo, as a mutation in one of the core components of NER does.

In summary, we show in this report that transcription-coupled repair and global genome repair are partially overlapping subpathways of NER, we demonstrate a role for the *RAD26* gene in UV survival, and we infer the existence of Rad26p-independent transcription-coupled repair.

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Appendix IV

Relationship of the Cockayne syndrome B protein ,involved in transcription-coupled DNA repair, with other repair or transcription factors.

Relationship of the Cockayne syndrome B protein, involved in transcription-coupled DNA repair, with other repair or transcription factors.

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The Cockayne syndrome B protein (CSB) is, together with CSA, required for transcription-coupled DNA repair (TCR) in man. This subpathway of nucleotide excision repair (NER) is responsible for the preferential removal of lesions from the transcribed strand of active genes, thereby permitting resumption of blocked transcription. Here we demonstrate by microinjection of antibodies against CSB and CSA into cultured primary fibroblasts, that both proteins are required for TCR as well as recovery of RNA synthesis after UV-irradiation *in vivo*. In contrast, these microinjections had no notable effect on transcription of unirradiated cells, indicating that CSB and CSA are not essential for this process *in vivo*. Immunofluoresce localises CSB in the nucleus, while in metaphase cells the protein colocalises with the microtubules of the mitotic spindle. The central role of CSB in the coupling between transcription and DNA repair suggests that CSB is interacting with other proteins involved in these two processes. Here we analysed whether CSB is associated with such factors in repair- and transcription-competent Manley-type whole cell extracts. Size fractionations indicate that CSB resides in a large (>700 Kda) molecular weight complex, whereas CSA is part of a distinct (420 Kda) complex. Column fractionations of HeLa extracts excluded copurification of CSB with a number of basal transcription and repair proteins. Functional HA-, His_6-double tagged CSB permitted isolation of the CSB complex under physiological conditions, but no association was found of CSB with several candidate repair and transcription proteins, among which CSA, XPG, TFIIH, TFIIF and RNA pol II. CSB-immunodepletion of active HeLa whole cell extracts had no effect on *in vitro* repair and transcription, indicating that neither CSB nor the CSB-associating proteins are required for these activities *in vitro*. We conclude that CSB resides in a large molecular weight TCR complex that does not include core NER or transcription components.

Nucleotide excision repair (NER) is a universal DNA repair pathway that is capable of removing a large variety of types of damage from the genome. NER entails a multistep reaction in which a lesion is recognised and bound by the repair machinery, followed by a local unwinding step, preceding a dual single-strand incision, one at each side of the lesion. Subsequently an oligonucleotide containing the damaged base(s) is excised and via gap-filling DNA synthesis and ligation the integrity of the DNA is restored (reviewed in (21, 63). Although in principle NER acts on the entire genome, several findings indicate that there is a profound heterogeneity in the efficiency by which lesions are removed in different parts of the genome. Apart from a strong

influence of local chromatin structure on accessibility of the DNA for repair proteins (7, 44), a clear link exists between transcription and repair efficiency (5). Bohr *et al.* (6) were the first to show that active, RNA polymerase II transcribed genes are repaired, at least for a number of lesions, with a higher efficiency than the genome overall. Subsequently, it turned out that this transcription-coupled DNA repair (TCR) pathway enhances repair of the transcribed strand of the active gene only, while the nontranscribed strand is repaired at a slower rate similar to that of the global genome (30). The recent finding that several components of the multisubunit transcription factor TFIIH are encoded by repair proteins offered an attractive explanation for enhanced repair of the transcribed strand during transcription-coupled repair (14, 37). However, mutations in the human and yeast genes that encode TFIIH subunits, lead to a DNA repair defect of both transcribed as well as nontranscribed DNA sequences (45, 61, 62). So although TFIIH can play an active role in TCR, it is clearly not specific for it and it is likely that other factors mediate the coupling between transcription and repair.

Evidence for this came from the observation that cells derived from Cockayne syndrome (CS) patients display a defect in the transcription-coupled repair pathway only, while global genome repair is unaffected (52, 58). It was shown that CS cells carry mutations in either the *CSA* or the *CSB* (*ERCC6*) gene (20, 47), and the cloned genes correct the UV-sensitivity and the impaired recovery of RNA synthesis after UV irradiation. These findings strongly suggest that CSA and CSB are required for transcription-coupled DNA repair. In support of this concept we found that disruption of the *S. cerevisiae* homolog of *CSB*, *RAD26*, results in a specific defect in TCR (51). In addition, analysis of knock-out CS-B mice indicated a total impairment of TCR (Van der Horst *et al.*, manus. in prep.). A model for the role of CSA and CSB in TCR has been suggested before (18, 51). Coupling between transcription and DNA repair is presumably mediated by an elongating RNA polymerase II complex that is stalled at a lesion. During TCR this lesion is recognised and repaired with high efficiency, enabling transcription to resume. The role of CSA/CSB could be to enhance this repair by stimulating retraction of the RNA polymerase, thus providing access for the repair complex, and/or by attracting repair proteins to the site of the lesion. The presence of the SWI2/SNF2-like 'helicase/ATPase' domain in CSB is intriguing in this respect, since other members of the SWI2/SNF2 subfamily have been shown to be able to disrupt protein-DNA interactions (4, 11, 48).

While the defect in transcription-coupled DNA repair of CS cells has been well documented, the clinical data suggest that more processes are affected in CS patients, because a number of CS features cannot be attributed to a repair defect only. The consequence of a total NER defect is illustrated by xeroderma pigmentosum group A (XP-A) patients, who show extreme sensitivity to sun (UV) light, pigmentation abnormalities and a high predisposition to develop skin cancer in sun-exposed areas. In addition, they suffer from accelerated neurodegeneration. CS patients on the other hand are also sensitive to UV light, but typically display impaired physical and sexual development, a wizened senile-like appearance, and various neurological abnormalities such as mental retardation, spasticity, deafness and patchy demyelination of neurons. In most cases CS patients die before the age of 20 (25, 32). Comparable features have also been found in XP-B, XP-D, TTD-A (thrichothiodystrophy group A) and XP-G patients, of which the first three have been shown to possess a defective TFIIH complex (61). The origin of these typical features was postulated to reside in a (subtle) defect in transcription rather than in repair, which led to the

definition of 'transcription syndromes' (61).

Following this reasoning one can imagine that CSA and CSB fulfil an auxiliary, non-essential role in the transcription process itself in addition of mediating in transcription-repair coupling. Here we partially characterised the function of CSB in TCR and in transcription. Antisera raised against CSB and CSA were microinjected into cultured fibroblasts, which severely inhibited TCR and the recovery of RNA synthesis after UV-irradiation *in vivo*, but had no apparent effect on basal transcription levels. Native gelfiltrations, column fractionations, and immuno- precipitations of (tagged) CSB from repair and transcription competent Manley-type whole cell extracts indicated that CSB resides in a large molecular weight complex that does not include CSA nor any of the other investigated repair or transcription factors.

MATERIALS AND METHODS

Cell lines and extracts.

The immortalized cell lines used in this study were HeLa, VH10-Sv (wild type), CS1AN-Sv (CS-B), CS3BE-Sv (CS-A), CW12 (XP-A), XPCS1BA-Sv (XP-B), XP4PA-Sv (XP-C), HD2 (XP-D), XP2YO-Sv (XP-F), XP3BR-Sv (XP-G) and TTD1BR-Sv (TTD-A). The fibroblasts were cultured in a 1:1 mixture of Ham's F10 and DMEM, supplemented with antibiotics and 8-10% fetal calfs serum. Whole cell extracts (WCE) were prepared according to Manley (27) as modified by Wood (27), dialysed against buffer A containing 25 mM HEPES/KOH pH 7.8, 100 mM KCl, 12 mM $MgCl_2$, 1 Mm EDTA, 2 mM DTT and 10 or 17% (v/v) glycerol, and stored at -80°C. The protein concentration of these WCE was 15-20 mg/ml.

Antibodies and immunoblot procedures.

Anti-CSB antibodies were raised against the C-terminal 158 amino acids of CSB. A 640 bp *Eco*RI fragment of pSLME6(+) (47) was overproduced as a protA fusion product (using vector pRIT2T, Pharmacia) in the *E. coli* strain pop2136, purified using an IgG column, and used for rabbit immunisation (nrs. 242 and 243). A highly purified GST fusion protein containing the same 158 C-terminal amino acids of CSB, was obtained after overexpression in *E. coli* DH5α using the GST fusion overexpression system (Pharmacia). In the competition experiment, 25 μg of the GST fusion protein was mixed with 200 μl of sonicated DH5α cell extract and incubated with 10 μl crude anti-CSB antiserum for 1 hour at room temperature. After centrifugation, the supernatant was directly incubated with the immunoblot. For affinity purification, the GST-CSB fusion protein was immobilised on immunoblot and incubated overnight with crude anti-CSB antiserum at 4°C. High affinity anti-CSB antibodies were eluted from the blot strips using a KSCN buffer (0.1 M KPi pH 7.0, 3 M KSCN, 1 mg/ml BSA) and desalted using G50 columns. Monoclonal anti-HA antibodies were isolated from the hybridoma cell line 12CA5 and purified as described (19). Anti-CSA antibodies were raised against the C-terminal half of the CSA protein. The CSA cDNA was isolated via RT-PCR from human granulocyte RNA using SuperRT and SuperTaq enzymes (HT Biotechnology Ltd), the sense primer 5'GCTAGAATTCTAATGCTGGGGT TTTTGTCC3' and the antisense primer 5'CCAAGAATTCTCATCCTCCTTCATCACT3', which were based on the published *CSA* sequence (20) and contained *Eco*RI restriction sites (underlined). The C-terminal half of CSA (a 670 bp *Bam*HI*Eco*RI fragment encoding amino acids 176-396) was overproduced as a GST fusion product (using vector pGEX2T, Pharmacia) in DH5α at 37°C, purified from inclusion bodies using prep cell electrophoresis, and used for rabbit immunisation (nrs. 1881 and 1882). Affinity purified anti-CSA antibodies were obtained by incubation of the crude serum with immunoblot strips containing the GST-CSA fusion protein, followed by elution with acidic glycine buffer (0.1 M glycine Ph 1.0, 0.5M NaCl, 0.5 mg/ml BSA) and immediate neutralisation in 10x KPi buffer. The polyclonal anti-ERCC1 (56), anti-XPG (33) and monoclonal anti-p89/XPB (37) antibodies are described before.

To be able to visualise large molecular weight proteins such as CSB (168 kDa) on immunoblot, a slightly modified blotting procedure was developed. Following electrophoresis on 8% SDS-PAGE, the gel was incubated for 5-10 min in blotting buffer without methanol (25 Mm Tris-HCl pH 8.3, 0.2 M glycine). Proteins were transferred to PVDF or nitrocellulose membrane by blotting for 2-3 hours at 4°C in blotbuffer with the addition of SDS to 0.01%. Aspecific sites were blocked by incubating the membrane in low-fat milk containing 0.1% Tween-20 and 0.02% sodium

azide for 1 hour. To visualise CSB, the blot was incubated overnight with crude (dilution 1:500) or affinity-purified (1:400) anti-CSB serum, followed by nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) or chemiluminescence detection, depending on the secondary antibody used. For smaller proteins, the standard procedure for immunoblotting was followed.

Immunofluorescence.

HeLa and CS1AN-Sv cells were grown on slides, washed with PBS, and fixed by incubation in 2% paraformaldehyde-PBS for 10 min and in methanol for 20 min. Slides were washed three times in PBS^+ (PBS, 0.15% glycine, 0.5% BSA) and incubated with affinity purified anti-CSB (1:5 dilution) for 1.5 hours in a moist chamber. After washing in PBS^+, slides were incubated with fluorescein isothiocyanate (FITC)-conjugated goat-anti-rabbit antiserum at a 1:80 dilution for 1.5 hours. Slides were washed and embedded in vectashield mounting medium (Brunschwig) that contained 4'-6-diamino-2-phenylindole (DAPI). DAPI-stained DNA and FITC-labeled CSB was visualised using fluorescence microscopy.

Microneedle injection of antisera and analysis of repair and transcription levels.

Microinjection of immunesera into cultured fibroblasts was performed as described previously (57). The anti-CSA and anti-CSB antisera were microinjected into the cytoplasm of wild-type (C5RO) or XP-C (XP21RO) fibroblasts. After microinjection, cells were further incubated for 24 hours at 37°C in standard medium to allow antibody-antigen reaction. The effect on NER activity by microinjection of the antisera in XP-C fibroblasts (represented by UV-induced unscheduled DNA synthesis) was determined by UV-irradiation of the cells (254 nm; 15 J/m^2), pulse-labelling for 2 hours using $[^3H]$thymidine (30 μCi/ml, s.a. 50 Ci/mmol), fixation and autoradiography. Grains above the nuclei of injected (polykaryon) and noninjected (monokaryon) cells were counted and compared. Levels of RNA synthesis after microinjection in wild-type cells were analysed by pulse-labelling with $[^3H]$uridine (10 μCi/ml; s.a. 50 Ci/mmol) for 1 hour in standard medium, and further processing as mentioned above. The recovery of RNA synthesis post-UV was assayed by microinjecting the antisera in wild-type cells, followed by a further incubation for 8 hours at 37°C. Then the cells were UV-irradiated (254 nm; 10 J/m_2) and 24 hours later the RNA was pulse-labelled as described above.

Size fractionations.

HeLa or CS1AN-Sv WCE (1 mg) were loaded on a Superdex-200 column (SMART system, Pharmacia) that was first calibrated using the molecular markers thyroglobulin (669 Kda), ferritin (440 Kda), catalase (240 Kda) and albumin (67 Kda). Chromatography was performed in buffer A to allow direct comparison between size fractionations, *in vitro* activity assays and (co)immunodepletions. Fractions were collected and tested on immunoblots. DNAse pretreatment of the HeLa WCE was performed by incubating 1 mg WCE with 10 μl (10 μg/μl) DNAse for 10 minutes at 37°C. Complete digestion of all DNA was verified by agarose gel electrophoresis.

Column fractionations.

Fractions of the TFII purification scheme were obtained as previously described (15). In short, HeLa WCE was loaded on a Heparin-Ultrogel column in buffer A and eluted with 0.22, 0.4 and 1.0 M KCl, while the Heparin 0.4 M KCl fraction was further fractionated on a DEAE-Spherodex column by elution with 0.2 and 0.35 M KCl. Phosphocellulose column chromatography was performed as described (40) by loading HeLa WCE on a Phosphocellulose column in buffer A, supplemented with Kcl to 0.15 M. The bound proteins were eluted in buffer A containing 1.0 M Kcl. Fractions were analysed on immunoblot as described above.

Generation of tagged CSB constructs.

N-terminal hemagglutinin (HA) and C-terminal histidine (His_6) tagged CSB constructs were generated to facilitate immunoprecipitation and allow isolation of CSB-associating proteins. The N-terminal HA-epitope was introduced via PCR using the *CSB* CDNA (in pSLME6(+), (47), the sense primer 5' CATCGAGCTCATGTACCC ATACGATGTTCCAGATTACGCTAGCCCAAAT GAGGGAATCCCC 3' (encoding a *Sac*I restriction site (underlined), start codon, HA-epitope (double underlined), and *CSB* CDNA bp 4-21) and the antisense primer EC179-2 5' CTCTGGCCTCATGTCTGACTCCCA 3' (*CSB* CDNA bp 1062-1085). The generated PCR products were cloned in the pCRII vector (Invitrogen) and checked for possible sequence errors introduced by PCR. A 741 bp *Sac*I fragment containing the HA-tagged N-terminus of CSB was isolated from correct clones and exchanged with the corresponding *Sac*I fragment in the *CSB* CDNA from pSLME6(+).

In a similar way, a stretch of six histidines was linked to the C-terminal end of CSB. For PCR amplification the sense primer EC179-5 5'

GTGAAACAAGAGTGAGGCCAA GG 3′ (*CSB* cDNA bp 3705-3729) and the antisense primer 5′ CTGGGGCCCTTAGTGATGGTGATGGTGGTG ACGACCTTCGATGCAGTATTCTGGCTTGAG3′ (encoding *CSB* cDNA bp 4460-4477, a factor Xa cleavage site, the His_6 stretch (double underlined), ochre stop codon and an *Apa*I restriction site (underlined) were used. The His_6-tagged CSB C-terminus was isolated as a 254 bp *Apa*I fragment and exchanged with the corresponding region in pSLME6(+).

DNA transfections and UV-survival.

CS1AN-Sv fibroblasts were transfected with pSLME6(-) (anti-sense *CSB*), pSLME6(+) (sense *CSB*) or pSLM2tE6 (*HA-CSB-His₆*), together with the selectable marker pSV2-neo using a modification of the calcium phosphate precipitation method (17). Following G418 selection, cells were split and selected for UV-resistance by three daily irradiations with 4 J/m^2 UV-C (254 nm). UV-selected mass populations of CS1AN-Sv+pSLME6(+) and CS1AN-Sv+pSLM2tE6 and non UV-selected mass populations of CS1AN-Sv, CS1AN-Sv+pSLME6(-) and VH10-Sv cells were further characterised by UV survival. For this, cells were plated ($2x10^5$ per 3 cm dish, 2-4 dishes per dose) and exposed to 0, 2, 4 or 7 J/m^2 UV 1 day after plating. Survival was determined after 4-6 days incubation at 37°C by ^3H-Thymidine pulse labelling as described elsewhere (42).

***In vitro* translation and immunoprecipitations.**

In vitro translated CSB and CSA protein was synthesised using the TNT™ T7 Coupled Reticulocyte Lysate System (Promega) as described by the manufacturer, in a total volume of 50 µl. The *CSB* cDNA was encoded by the 4.7 kb *Sal*I fragment of pSLME6(+), which was subcloned into pBluescript KS^+, while the *CSA* cDNA was cloned in pcDNA3 (Invitrogen) as a 1.2 kb *Eco*RI fragment.

Immunoprecipitation of *in vitro* translated CSB protein was achieved by incubating CSB protein with crude anti-CSB serum for 1 h at 4°C in NETT buffer (100 mM NaCl, 5 mM EDTA, 50 Mm Tris-HCl Ph 7.5, 0.5% Triton X-100), after which protein-A Sepharose beads (Pharmacia) were added and incubation was continued for 1 h at 4°C. Beads were washed 4 times with NETT buffer, and the bound proteins were subjected to SDS-PAGE after addition of sample buffer and boiling. Immuno precipitation of endogenous CSB or CSA from HeLa whole-cell extracts was achieved by first incubating crude anti-CSB serum with protA beads in PBS-Tween (0.5%) for 1-2 hours at 4°C, followed by extensive washing (2 times with PBS-Tween and 4 times with buffer A) and a further incubation of the anti-CSB coated protA beads with 1 mg HeLa WCE for 5 hours at 4°C. The CSB-depleted HeLa extract was recovered from the beads by spinning and analysed on immunoblot, together with the proteins bound to the beads. Immunoprecipitation of HA-CSB-His_6 using the Mab anti-HA was done by incubating the Mab 12CA5 overnight at 4°C with the WCE of the CS1AN-Sv cells transfected with the double-tagged CSB construct, followed by addition of protein-G Sepharose beads (Pharmacia) and further incubation for 5 hours at 4°C. Depleted extract and bound proteins were analysed by SDS-PAGE and immunoblotting.

***In vitro* repair and transcription assays.**

Analysis of *in vitro* repair activity was performed as described in detail before (64), by mixing 100 µg of (depleted) cell-free extract with a mixture of AAF-modified and nondamaged plasmids (56). Repair activity, i.e. incorporation of [α-^{32}P]dATP into the damaged plasmid, was visualised by autoradiography.

In vitro transcription activity was assayed as described before (15), by incubating 100 µg (depleted) cell-free extract with an Ad2MLP promoter containing template, together with the required nucleotides. The 309 nt [α-^{32}P]CTP labelled run-off transcripts were visualised using autoradiography.

RESULTS.

Characterisation of polyclonal anti-CSB and anti-CSA antibodies.

The crude anti-CSB serum stained several proteins in immunoblot analysis of a HeLa whole cell extract (WCE), among which a 168 Kda protein that corresponds to the expected size of CSB (47) and to the size of the *in vitro* translated CSB protein (Fig 1 lanes 1 and 5). The 168 Kda protein likely represents CSB, since its staining could be specifically competed for by pre-incubating the crude antiserum with a GST-CSB fusion protein (Fig 1 lane 2), while the band is missing in a WCE derived from CS1AN-Sv cells that lack the C-terminal part (aa 337-1493) of the CSB protein, containing the determinant against which

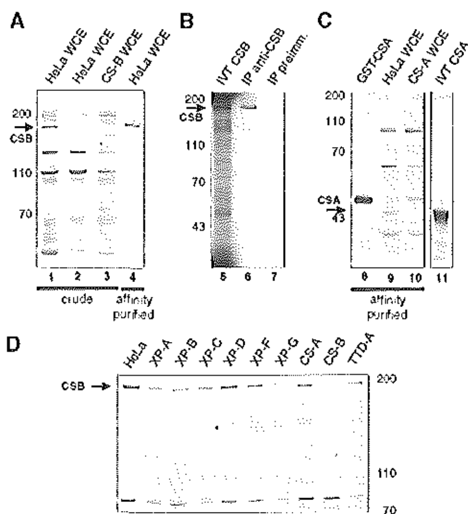


Figure 1. Characterisation of anti-CSB and anti-CSA antibodies.
A. Specificity of the anti-CSB serum. Immunoblots of HeLa or CS1AN-Sv (CS-B) WCE were incubated with: crude anti-CSB serum (lane 1 and 3), crude anti-CSB serum preincubated with GST-CSB fusion protein (lane 2), and affinity-purified anti-CSB serum (lane 4). Immunoblot procedures were adapted to visualise CSB as explained in Material and Methods. The molecular weight of prestained marker proteins is indicated. ***B.*** *The anti-CSB antiserum immunoprecipitates the in vitro translated CSB protein. In vitro translated CSB (lane 5) was incubated with either crude anti-CSB serum (lane 6) or preimmune serum (lane 7) in NETT buffer and binding to protA beads was analysed on SDS-PAGE.* ***C.*** *Specificity of the anti-CSA antiserum. An immunoblot of 2 ng of the GST-CSA fusion protein (lane 8), and 10 µg of HeLa (lane 9) or CS3BE (lane 10) WCE was incubated with affinity-purified anti-CSA antiserum. On the same gel in vitro translated CSA protein was analysed (lane 11). Note that the serum cross-reacts with other cellular proteins.* ***D.*** *CSB is specifically absent in CS-B WCE, but not in other repair-deficient extracts. Equal amounts (10 µg) of the indicated WCE were analysed on immunoblot for the presence of CSB. The 80 kDa cross-reacting band provides an internal control on protein loading differences.*

the antiserum was raised (47) (Fig 1 lane 3). Finally, the anti-CSB serum was able to immunoprecipitate the *in vitro* translated CSB protein (fig 1 lane 5-7). Following affinity purification, the serum strongly stained the 168 Kda CSB band (fig 1 lane 4), while occasionally a 80 Kda protein is also recognised (fig 1D).

The affinity-purified anti-CSA antiserum strongly reacted with a very low amount (2 ng) of GST-CSA fusion protein on immunoblot (Fig 1 lane 8), and recognised multiple proteins in a HeLa WCE among which one of 44 Kda (lane 9). This band is absent in an extract of CS3BE (CS-A) cells (lane 10), in which a large part of *CSA* may be deleted (E.C. Friedberg, pers. comm.), while the size matches with the *in vitro* translated CSA protein (lane 11), indicating that the 44 kDa protein is CSA.

Intracellular localisation of CSB.

The presence of a consensus sequence for a nuclear location signal in CSB and its central role in transcription-coupled DNA repair predict that CSB is located in the nucleus. This was confirmed by immunofluorescence studies (fig 2). Although the gene is very lowly expressed (46), specific CSB staining was observed in the nuclei, but not the cytoplasm, of HeLa cells (fig 2A). Notably, the metaphase cell in figure 2A indicates that CSB, while normally present in the nucleus, is not associated with the condensed chromosomes. Instead, in the majority of metaphase cells CSB colocalises with the microtubules of the mitotic spindle, which mediate the segregation of the chromosomes to the spindle poles (23). The significance of this finding is unknown at this moment. Also during other phases of mitosis no colocalisation of CSB with chromatin was found (fig 2B). This is in contrast to the anaphase/telophase-specific association with chromatin of the XPC/HHR23B complex (50). The CS1AN-Sv cells show no staining of CSB at all, confirming the immunoblot results shown above, and indicating that the increased staining of the spindle apparatus in some metaphase cells is specific for CSB (fig 2A).

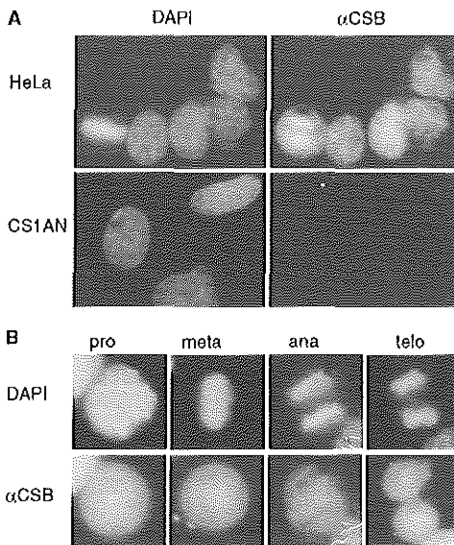


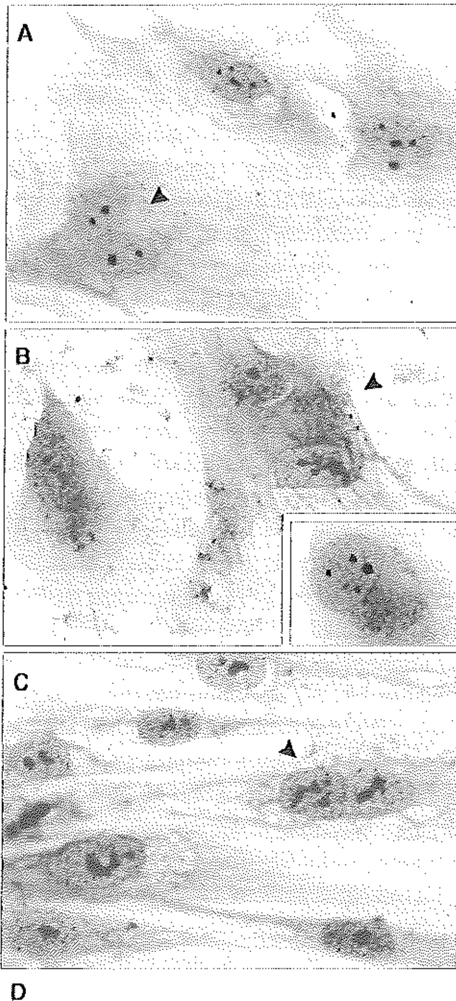
Figure 2. **The CSB protein is localised in the nucleus.**
*A. The affinity-purified anti-CSB antibody was used to stain the endogenous CSB protein in HeLa or CS-B (CS1AN-Sv) cells. The left panel displays the DAPI-stained chromosomal DNA, while the right panel depicts CSB staining, visualised by FITC-conjugated secondary antibodies. **B.** CSB does not colocalise with chromatin during various stages of mitosis. Indicated are prophase, metaphase, anaphase and telophase (from left to right).*

Effect of microinjection of anti-CSB and anti-CSA antibodies *in vivo*.

To gain more insight into the function of CSA and CSB *in vivo*, we microinjected the specific antisera into cultured fibroblasts and analysed the effects on repair and transcription. Repair activity is reflected by the level of UV-induced unscheduled DNA-synthesis (UDS), determined by [^{3}H]thymidine incorporation, whereas transcription levels are quantitated by pulse-labelling with [^{3}H]uridine (57, 61). Labelling was visualised by *in situ* autoradiography and quantified by counting of silver grains above the nuclei. Previously, it was shown that CS-A and CS-B cells are specifically deficient in transcription-coupled repair of UV-induced CPD (52). The contribution of TCR to UDS of wild-type cells shortly after UV-irradiation is not expected to be very high, since TCR only enhances repair of a relatively small fraction of the genome, and also because a significant part of the observed UDS is derived from repair of 6/4 photoproducts, which are removed so fast that TCR hardly contributes to this (51). This provides a likely explanation for the wild-type UDS levels in CS cells. For this reason we microinjected the antisera into XP-C fibroblasts, that are defective in global genome repair and only possess TCR (59). Previously it has been shown that the residual UDS in XP-C cells reflects TCR, since it could completely be blocked by addition of a specific RNA polymerase II inhibitor (9). As shown in figure 3, the two CSA antisera inhibited the residual UDS of XP-C fibroblasts by a factor 2 to 2.5, while the pre-immune serum had no effect. More dramatically, microinjection of two anti-CSB antisera reduced the residual UDS of injected XP-C cells to 15-22% of the levels in uninjected cells.

A hallmark of CS cells is the failure to recover RNA synthesis after UV-irradiation, which may be the consequence of the defect in TCR (29, 47). Figure 3 shows that microinjection of the anti-CSA and anti-CSB (but not the preimmune) antisera into repair-proficient fibroblasts significantly inhibits the recovery of RNA synthesis. This indicates that both antisera are capable of inhibiting the function of CSA and CSB *in vivo*, and provides direct evidence for their involvement in the TCR pathway.

In a very similar way, both antisera were injected into wild-type fibroblasts to assay inhibition of overall RNA synthesis. However, no significant difference was



D

Injected antiserum	% residual UDS XP-C[a]	% recovery of RNA synthesis[b]	% transcription
none	100	100	100
preimmune	100	95	-[c]
anti-CSA (1881)	47	-	109
anti-CSA (1882)	40	65	104
anti-CSB (242)	22	-	105
anti-CSB (243)	15	61	99

Figure 3. **Microinjection of anti-CSB and anti-CSA antisera inhibits TCR and recovery of RNA synthesis post-UV, but not transcription, *in vivo*.**
Independently raised anti-CSB and antiCSA antisera were microinjected into XP-C (XP21RO) fibroblasts to assay the effect on TCR (visualised by unscheduled DNA synthesis)(A), or in wild-type (C5RO) fibroblasts to determine the effect on transcription levels (C). The latter was also done in wild-type cells, that were UV-irradiated 8 hours after microinjection, to analyse recovery of RNA synthesis (B). ***A-C.*** *Micrographs of anti-CSB antiserum injected cells (indicated by an arrow) and uninjected (surrounding mononuclear) cells.* ***D.*** *Quantification of the microinjection results. Percentages are calculated by comparing injected versus uninjected cells on the same slide, with a typical standard error of the mean of 5%. [a] 100% corresponds to 19 grains, which represents 10% of the UDS observed in wild-type cells that were treated simultaneously in a similar way. [b] Recovery of RNA synthesis is defined by the ratio of transcription levels in UV-irradiated wild-type cells relative to unirràdiated cells. 100% corresponds to a recovery of 70% after 24 hours in wild-type cells. CS1AN-Sv (CS-B) cells that were included in the experiment (insert in B), displayed a recovery of 13%, relative to wild-type values. [c] -: not determined.*

observed between injected versus noninjected cells (fig 3), suggesting that neither CSA nor CSB has a major contribution to transcription of undamaged cells *in vivo*.

Analysis of the CSB protein in other NER-deficient complementation groups.

Transfections of the *CSB* CDNA, under transcriptional control of the strong SV40 promoter, does not lead to apparent overproduction of CSB protein (compare fig 6B with fig 1A). Similar observations have previously been made with overexpressed ERCC1, that forms an heterodimeric complex with XP-F (34, 43), and which is rapidly degraded when it is in a free form (56). These findings are compatible with the idea that CSB is part of a complex as well. Previously it has been shown for the ERCC1/XPF (55) and XRCC1/DNA ligase III (8) complexes, that mutations in one subunit cause instability and degradation of the other, non-mutated subunit. To see whether this is the case for CSB we performed immunoblot analysis of the amount of CSB in WCE derived from XP-A to XP-G, CS-A and TTD-A cells. Figure 1D

indicates roughly equal levels of CSB in all groups, suggesting that these factors are not complexed with CSB in a similar manner as ERCC1/XPF (fig 1D). Moreover, the amount of CSA is also not reduced in a CS-B WCE (fig 4).

Size fractionation of HeLa whole cell extract.

The mono-specific anti-CSB antiserum was utilised to investigate whether CSB is complexed with other proteins or exists as a free molecule within the cell. The native state of CSB was determined by size fractionation of HeLa WCE, fully competent in repair and transcription, followed by immunoblot analysis (fig 4). Gel filtration was performed under physiological conditions, identical to those in which *in vitro* repair and transcription assays are conducted. Although denatured CSB has a molecular weight of 168 Kda, it chromatographed in an estimated native size of more than 700 Kda. A similar native size of CSB was also found on Sephacryl S-300 and S-500 columns (data not shown). The native elution profile of other protein complexes, such as ERCC1/XPF (280 kDa) and XPB (TFIIH) (500 kDa), and also HHR23A (70 kDa) and HHR23B (140 kDa) (data not shown), were as found before (50, 55), making aggregation of proteins in the extract unlikely. These results could indicate that CSB is associated with other proteins in HeLa cells.

Surprisingly, CSA eluted at a native molecular weight of 420 kDa, which is quite different from the one of CSB. Since the two elution peaks overlap, it remained possible that part of CSA is complexed with CSB. However, in the CS1AN extract (lacking CSB) the same native size of CSA was found (Fig 4B), strongly supporting the idea that CSB does not reside in the CSA complex.

To investigate the stability of the CSB complex and to obtain information on its composition we performed size fractionation under different salt conditions.

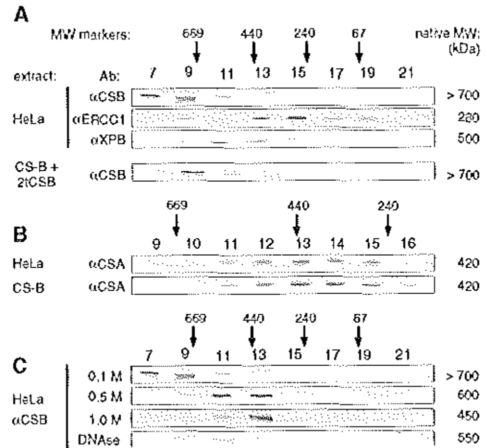


Figure 4. **CSB resides in a very large molecular weight complex, that does not include CSA.** *A. Various whole cell extracts were size fractionated on a Superdex-200 column, loaded in the Manley-type buffer A, containing 0.1 M Kcl. Elution fractions were tested by immunoblotting using the indicated antisera. The native sizes of molecular weight marker proteins, as well as the estimated sizes of the proteins tested are indicated. The WCE notated CS-B + 2tCSB is derived from CS1AN-Sv fibroblasts transfected with HA- and His_6-tagged CSB (see also figure 6).* ***B.*** *The native size of CSA is unchanged by severe CS-B truncation. Elution fractions of the Superdex-200 column, loaded with either HeLa or CS1AN-Sv (CS-B) WCE were analysed using the anti-CSA serum. The CSB gene in CS1AN-Sv cells contains a premature stop mutation, leading to truncation of amino acid 337 to 1493 of the CSB protein (47).* ***C.*** *The native size of CSB is dependent on salt concentration and DNAse pretreatment. Equal amounts of HeLa WCE were loaded on the Superdex-200 column in buffer A containing respectively 0.1, 0.5 and 1.0 M KCl, or first treated with DNAse and then loaded on the column. Fractions were tested on immunoblot using affinity-purified anti-CSB antiserum. Elution of the molecular weight marker proteins was hardly affected by the altered salt conditions.*

When the salt concentration was increased from 0.1 to 0.5 and 1.0 M KCl, the native size of the CSB complex decreased from >700 kDa to 500 and 450 kDa respectively (fig 4C), which is still

considerably larger than the single CSB protein. Notably, no free CSB protein is detected at high salt, making it unlikely that multimerisation of CSB causes the large native size at 0.1 M salt. A pretreatment of the HeLa WCE with DNAse resulted in a native size of CSB of about 550 kDa (fig 4C), implying that at low salt conditions the CSB complex may still contain some DNA, which is dissociated from the complex at higher salt.

Identification of CSB-copurifying proteins.

To determine the identity of the CSB-associated proteins we first analysed whether there are known repair and transcription factors that copurify with CSB. For this we assayed fractions of the TFII purification scheme that has been used to isolate basal transcription factors of RNA polymerase II (RNA pol II) (15). This scheme has proven to be valuable in the purification of large protein complexes such as TFIIH and RNA pol II. On the first column of the TFII purification scheme, Heparin Sepharose, CSB eluted at 1.0 M KCl (fig 5A). This already excluded copurification of CSB with TFIIA, TFIIB, TFIID, TFIIH, and the ERCC1/XPF complex (15, 55). In addition, the XPA and XPG proteins elute in the Heparin 0.4 M and subsequently in the DEAE 0.2 and 0.35 M KCl fraction respectively, as tested by immunoblot (Fig 5A) and correction of *in vitro* and *in vivo* repair defect (A.J. van Vuuren, W. Vermeulen unpubl. obs.). When these fractions were tested for the presence of CSA, we found elution in the Heparin 0.4 M fraction (and a trace at 0.22 M), followed by elution in the DEAE 0.35 M KCl fraction, clearly distinct from the CSB elution (fig 5A). Interestingly, the Heparin 1.0 M fraction also contains TFIIF and RNA pol II (15), both of which are factors involved in transcription elongation (2), as well as the XPC/HHR23B complex (50). However, RNA pol II and CSB fractionate differently on a DEAE-5PW column, that was loaded with a Heparin 0.6M fraction that also contains

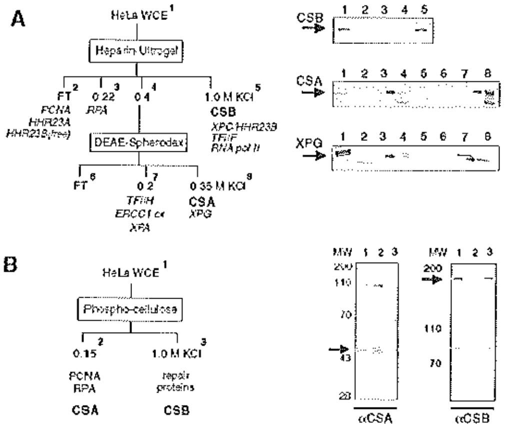


Figure 5. **CSB does not copurify with the majority of the tested repair and transcription proteins.**
A. Elution fractions of the TFII purification scheme were tested on immunoblot for the presence of CSB, CSA and XPG. The presence of TFIIH, ERCC1, XPA, XPG and XPC correcting activities was tested on immunoblot and confirmed by microinjection and in vitro complementation (55, 57), A. Eker, W. Vermeulen, pers. comm.). The purification of TFIIF, RNA polymerase II and other transcription proteins was described previously (15). B. HeLa WCE was fractionated on a phosphocellulose column by loading in buffer A containing 0.15 M KCl, and eluting in buffer A supplemented with KCL to 1.0 M. Elution fractions were tested on immunoblot for the presence of CSA and CSB, while the fractionation of the other proteins was described earlier (40).

some CSB (data not shown). In addition, RNA pol II, TFIIF and XPC/HHR23B do not coimmunoprecipitate with CSB (see below), making a stable association unlikely.

Secondly, phosphocellulose column chromatography was recently used to fractionate WCE from HeLa cells that ultimately led to the reconstitution of NER *in vitro* (1). The fraction that is not bound to the column at low salt (CFI) contains RPA and PCNA, while the bound fraction (CFII) contains all other proteins required for NER *in vitro* (40). Remarkably, CSB is present exclusively in CFII, while CSA is present in CFI (Fig. 5B). This again indicates that CSA and CSB are not stably

associated. More specifically, CSB elutes between 0.4 and 0.6 M KCl (fraction FIII (1)), again excluding copurification with RPA, PCNA, XPG, XPA, and ERCC1/XPF (data not shown).

In conclusion, there is no copurification of CSB with CSA nor with the investigated repair and basal transcription proteins. However, the gel filtration studies described above imply that the composition of the CSB complex changes upon increasing the salt concentration. Thus, the absence of copurifying proteins does not exclude weak interactions that may be disrupted due to the harsh, high salt conditions that are used for elution.

Construction and immunoprecipitation of HA-, His_6-double tagged CSB.

To further investigate protein-protein interactions and to allow isolation of CSB-associating proteins under physiological conditions, we generated CSB constructs containing a N-terminal hemagglutinin antigen (HA) epitope as well as a C-terminal histidine (His_6) tag (see Material and Methods) and used monoclonal anti-HA antibodies for immunoprecipitation.

To verify that the addition of tags did not interfere with CSB function, we transfected CS1AN-Sv cells with the double tagged CSB construct. Clearly, the cells transfected with the HA-CSB-His_6 construct showed a correction of UV-sensitivity to wild-type level, identical to the nontagged version (fig 6A). Also, the native size of tagged CSB in a cell-free extract of these transfected cells is again very large (>700 kDa) (fig 4A), indicating that the tagged protein is assembled in the CSB complex in a similar way as the nontagged version. Using the N-terminal HA-epitope we could completely immunoprecipitate the CSB protein by binding to protein-G beads that were coated with monoclonal HA antibody (Fig 6B). Since this was done under identical buffer conditions as the Superdex-200 chromatography above, we assume

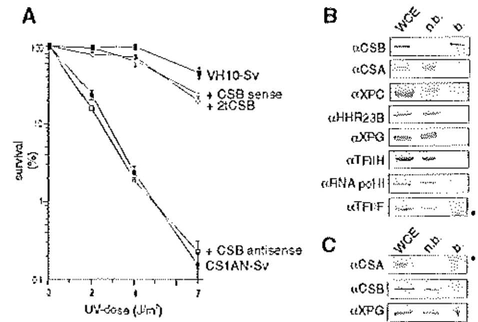


Figure 6. Function and immunoprecipitation of tagged CSB.
A. Tagging of CSB does not interfere with its cellular function. CS-B fibroblasts were transfected with the indicated constructs, and the UV-sensitivity of mass populations was determined by UV-irradiation and pulse labelling with ^{3}H-TdR. □: CS1AN-Sv (CS-B), ●: CS1AN-Sv + antisense CSB, ○: CS1AN-Sv + sense CSB, ◇: CS1AN-Sv + HA-CSB-His_6 (2tCSB), ■: VH10-Sv (wild-type). B. None of the tested candidate proteins coimmunoprecipitates with tagged CSB. HA-, His_6-double tagged CSB was immunoprecipitated in buffer A from a WCE of UV-resistant CS1AN-Sv transformants using anti-HA antibody-coated protG beads. The WCE, non-bound (n.b.) and bound (b.) proteins were analysed on immunoblots with antisera specific for the indicated factors. Anti-XPB antibodies were used as representative for the TFIIH complex; antisera against other components gave similar results (not shown). The RAP30 subunit of TFIIF gave the same result as the RAP74 subunit (shown here). The asterisk indicates IgG bands. C. CSB nor XPG coimmunoprecipitates with CSA. The endogenous CSA protein from HeLa WCE was immunoprecipitated in buffer A using crude anti-CSA antiserum coupled to protA beads. The WCE, non-bound (n.b.) and bound (b.) proteins were tested on immunoblot using the indicated antisera.

that the entire CSB complex is bound to the beads. Equal amounts of the original extract, the depleted extract and an aliquot of the beads were tested on immunoblot and probed with various antibodies (Fig 6B). Clearly, CSA did not coimmunoprecipitate with CSB nor did XPC, HHR23B, XPG, several subunits of TFIIH, RNA pol II nor both subunits of TFIIF. Similar results were

obtained when CSB was immunoprecipitated using the crude anti-CSB serum (data not shown). Since the latter serum is raised against the C-terminus while the HA-epitope is linked to the N-terminus of CSB, it is unlikely that binding of the antibody disrupts the interaction of CSB with the proteins analysed.

To independently investigate whether CSA and CSB are associated with each other, the CSA protein was immunoprecipitated from HeLa cell-free extracts using the anti-CSA antiserum (Fig. 6C). Also now, CSB was not coimmunoprecipitated. In addition, the XPG protein, that copurifies with CSA in the TFII purification scheme, was not coimmunoprecipitated with CSA, indicating that also CSA and XPG are not stably associated.

In conclusion, under physiological conditions in repair and transcription competent Manley whole cell extracts CSB seems to reside in a very large molecular weight complex (>700 kDa), but it is not stably associated with detectable amounts of TFIIA, TFIIB, TFIID, TFIIF, TFIIH, RNA pol II, PCNA, RPA, XPA, XPB and XPD (TFIIH), XPC/HHR23B, ERCC1/XPF, XPG or CSA.

Effect of CSB-immunodepletion on *in vitro* repair and transcription.

To test at the functional level whether CSB and the CSB-associated proteins are required for repair and transcription *in vitro*, we conducted immunodepletion experiments. The crude anti-CSB serum completely immunoprecipi-tated the *in vitro* translated 168 kDa CSB protein (fig 1B), as well as the endogenous CSB protein from an active HeLa WCE (fig 7). Clearly, depletion of CSB, performed under low stringency conditions, had no significant effect on *in vitro* repair or transcription activities (fig 7). This finding confirms the absence of basal transcription and repair factors in the CSB complex, shown above, and implies that the CSB complex is not essential for these activities *in vitro*.

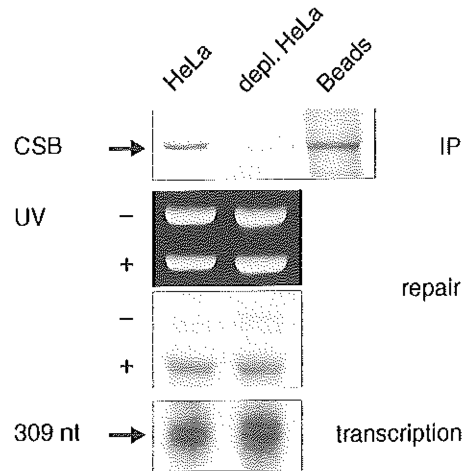


Figure 7. **Complete CSB-immunodepletion has no significant effect on *in vitro* repair and transcription.**
A repair-proficient HeLa WCE was immunodepleted using crude anti-CSB serum, that was coupled to protA beads, under low stringent (buffer A) conditions. Complete CSB depletion from the extract, and binding to the beads was verified by immunoblotting of 10 µg of (non-)depleted extract (top panel). Exactly equal amounts (100 µg) of non-depleted and depleted HeLa extract were tested in the in vitro repair (middle panel), and in in vitro transcription (bottom panel) assays. Immuno-depletion using the preimmune serum did not precipitate the CSB protein and consequently, this had no effect on the in vitro repair activity (not shown).

DISCUSSION

Complex cellular pathways can only be understood in detail when all participating components have been identified, their function elucidated and their interactions unravelled. Most of the factors required for nucleotide excision repair are now isolated, which recently resulted in the reconstitution of the core of the NER reaction *in vitro*, using damaged naked DNA as substrates (1, 31). Similarly, the combined efforts of numerous laboratories has culminated in the *in vitro* reconstitution of the basal RNA polymerase II transcription

apparatus using defined promoters (10, 65). However, little is known about how these processes operate in the context of chromatin and about the machinery that is invoked to resolve the problem when an elongating RNA polymerase II complex encounters a lesion in the transcribed strand. The only case in which the transcription-coupled repair reaction is elucidated in considerable detail is in the prokaryote *Escherichia coli* (39). Using an elegant *in vitro* assay, a factor was purified that was required and sufficient to mediate TCR. This transcription-repair-coupling-factor (TRCF) was shown to be able to bind and displace a stalled RNA polymerase (38). Since TRCF displays an affinity for the damage recognition protein UvrA, it was suggested to play an active role in the subsequent attraction of the UvrABC endonuclease to the site of the lesion, and thus stimulate repair (38). In eukaryotes, the TCR reaction is probably much more complex. At least 11 proteins (complexes) are involved in the core NER reaction (1), while the composition of the transcription elongation complex is still largely unknown. Previously, cellular studies have suggested a central role for the Cockayne syndrome A and B proteins in transcription-coupled repair (52, 58). Moreover, the clinical hallmarks displayed by CS patients suggest that also the transcription process itself is affected (61). Here we partially characterised the function of CSB by *in vivo* microinjection of antisera, and by *in vitro* analysis of protein-protein interactions involving CSB.

The study of protein-protein interactions is a delicate matter. Frequently used methods encompass immunoprecipitation of proteins, that are *in vitro* synthesised or purified from overexpression systems. One of the caveats in these studies is the fact that the specific protein is studied outside of its natural context. Particularly, when the protein *in vivo* resides in a large complex with multiple interaction domains, it may exhibit artificial association behaviour when examined in isolation. Another complication may arise when proteins are overproduced in heterologous systems, leading to incomplete synthesis of a fraction of the molecules, improper folding or lack of post-translational modification. Similar potential complications can arise when proteins are overproduced during a two-hybrid screen in yeast. Therefore, interactions identified in these systems have to be verified *in vivo* under physiological conditions or by valid genetic means.

To examine protein associations of intact CSB in a cellular context in a natural non-overproduced state, we utilised Manley-type whole cell extracts and functional tagged CSB protein. These extracts are frequently used as starting material for purification of protein (complexes) and are active in *in vitro* repair, transcription and splicing assays (15, 64). The fact that these intricate multi-step pathways can function efficiently indicates that within the extracts multi-subunit protein complexes are stable and can easily reassemble. Indeed we found that CSB resides in a large molecular weight complex in the nucleus. In an attempt to identify the activity of the CSB complex under the same (Manley-) buffer conditions, we found no effect of complete CSB immunodepletion on repair or on basal transcription, indicating that the CSB complex does not contribute significantly to these activities *in vitro*. However, microinjection of antisera clearly indicated a contribution of CSB (and CSA) to transcription-coupled repair *in vivo*. Moreover, the microinjection experiments showed that CSA and CSB are not required for basal transcription *in vivo*. An auxiliary role for CSA and CSB in this process is however certainly not excluded at this moment. The identity of the CSB-associating proteins was investigated by analysis of column fractionations and of immunoprecipitations of functional tagged CSB, performed under the same, low stringent buffer conditions.

During transcription-coupled repair the stalled RNA polymerase II complex presumably forms the signal that ultimately leads to the attraction of the repair machinery to the site of the lesion. In man the CSA and CSB proteins are required for this coupling and as such were expected to be associated with each other. Our findings suggest that this may not be the case. In the size fractionation experiment CSA and CSB have a different native size while the native size of CSA is not affected by severe CSB truncation. CSA and CSB fractionate differently at low salt concentrations on both the Heparin and the phosphocellulose column. When immunoprecipitating CSA or CSB from different extracts using various antibodies, no stable association of the two proteins is detected. Obviously, our studies do not exclude transient interactions that may occur in the course of the TCR reaction or very fragile complexes that are disrupted during the preparation of the Manley-type extracts. Also, our extracts are made from undamaged cells, which are therefore not expected to perform high rates of NER. Preliminary studies with extracts of UV-irradiated cells did not reveal substantial differences in the interaction of CSB with other NER components (unpubl. res.), but it is not excluded that minor changes occur. Indeed, binding of *in vitro* translated CSA and CSB proteins to each other was recently found (20) and also an interaction in the two-hybrid system was observed (E.C. Friedberg, pers. comm.), indicating that under certain conditions these proteins are able to interact.

Interactions between CSB and subunits of TFIIH have been suggested before (13, 52). The TFIIH complex is required for transcription initiation of many promoters, presumably by local melting of the promoter region and phosphorylation of the C-terminal domain of the largest subunit of RNA polymerase II, resulting in promoter clearance (16, 22, 26). Several subunits of TFIIH, and possibly the entire TFIIH complex, are also required for NER (12, 61, 62) and this dual role led to the suggestion that TFIIH also plays a central role in TCR. In addition, since patients carrying mutations in the *CS* genes and in the *XPB* and *XPD* subunits of TFIIH display comparable clinical features, it has been suggested that the *CSA* and *CSB* mutations interfere with the transcription mode of TFIIH (13, 52, 61). In the analysis presented here we fail to detect any association of CSB with TFIIH subunits in column fractionations and immunoprecipitations, performed under conditions that leave the TFIIH complex intact. In a reciprocal experiment, in which the TFIIH complex was immunoprecipitated using a HA-tagged XPB subunit from Manley-type WCE as well as from nuclear extracts, also no indication for association with CSB was found (B. Winkler, G. Weeda pers. comm.).

In addition to XP-B and XP-D, also XP-G patients display characteristic CS features (60), perhaps reflecting a disturbed XPG-CSB interaction. To investigate this, we previously tried to detect interactions between *in vitro* translated, full-length XPG and CSB proteins by performing coimmunoprecipitations with anti-CSB or anti-XPG antisera under various buffer conditions, but failed to detect any (dat not shown). However, recently binding of an *in vitro* translated XPG protein to unlabelled, *in vitro* translated CSB was observed (24). In contrast, the analysis in cell-free extracts presented here does not indicate any (stable) association between XPG and CSB: i) XPG fractionates differently on a Heparin column (Fig 5A), ii) XPG does not coimmunoprecipitate with CSB using the anti-HA monoclonal antibody (Fig 6B) or using the crude anti-CSB serum (not shown), and iii) no coimmunoprecipitation of CSB with XPG is observed when using a crude anti-XPG serum (not shown).

The Heparin column chromatography (Fig 5A) indicated co-fractionation of CSB with the XPC/HHR23B complex, involved in global genome repair (28, 59). Since CSB and XPC are involved in complementary

repair pathways it seems unlikely that they are associated and indeed, no indication for this was found (Fig 6B). The transcription initiation/elongation factor TFIIF and the RNA polymerase II complex also fractionate in the Heparin 1.0 M fraction, but again, no stable associations can be found in further column fractionations or immunoprecipitations (Fig 6B).

In conclusion, we have shown that in Manley-type whole cell extracts CSB exists in a very large molecular weight complex, which is distinct from the one of CSA. Analysis of column fractionations and CSB-immunoprecipi-tations of tagged CSB excluded a number of candidate proteins to be associated to CSB in these extracts. Analogous to these results is the recent identification of two nucleosome remodelling factors, that include other members of the SWI2/SNF2 subfamily of 'helicases'/ATPases (35, 49). These factors, NURF and SWI-SNF, constitute large protein complexes which are able to disrupt a nucleosome ternary structure in a promoter region, thus stimulating transcription efficiency (11, 48). Many of the components of the NURF and SWI-SNF complexes have been identified, but no basal transcription factors have been found, similar to our analysis of the CSB complex. The *in vivo* microinjection experiments suggest a requirement for CSB in TCR but not in basal transcription. Nonetheless the CSB complex could modulate the efficiency of transcription *in vivo* in a subtle manner, as suggested by the clinical features of CS patients, and as a secondary function mediate in TCR. In such manner, CSB may be associated to transcription elongation factors, such as SII, elongin/SIII or ELL (3, 36, 40). Further identification of the CSB-associating proteins has to await the purification of components of the CSB complex which is greatly facilitated by the generation of transformants stably expressing functional double-tagged CSB protein.

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Summary

Organisms have developed an intricate network of repair pathways to protect their genome from the occurrence of mutations. These alterations of the genetic code can be the consequence of the intrinsic instability of the DNA molecule itself, replication errors, or DNA lesions that are introduced by genotoxic agents. Persistent DNA damage can interfere with various basic cellular processes, such as RNA synthesis (transcription) and DNA replication. This could ultimately lead to premature cell death or, conversely, to uncontrolled cell growth resulting in tumor formation. One of the most important repair pathways in the cell is nucleotide excision repair (NER), that is capable of removing a large variety of damages from the genome by replacing a small piece of DNA that contains the damaged nucleotide(s) by a new undamaged copy. The importance of a functional NER in man is illustrated by the UV-sensitive, genetic disorders xeroderma pigmentosum (XP), Cockayne syndrome (CS) and trichothiodystrophy (TTD). Patients suffering from these disorders display various common features, such as skin abnormalities and increased photosensitivity. Remarkably, only XP patients have a highly elevated risk of developing skin tumors. On the other hand, the genetic defect in CS and TTD seems to affect also other processes, since these patients display typical neurological and developmental abnormalities that can not easily be rationalised by a NER defect alone.

Previously it has been shown that repair of the DNA genome does not occur in a uniform manner. Genes that are being transcribed at the time of damage induction, are preferentially repaired. More specifically, this so-called transcription-coupled DNA repair (TCR) is restricted to the transcribed strand of these genes. This DNA strand is used by the elongating RNA polymerase molecule as a template to synthetise the messenger RNA, that is translated to protein elsewhere in the cell. Chapter 2 deals with the current knowledge on TCR in various organisms and discusses the factors that are involved in this pathway. Genetic and biochemical studies have indicated that mutations in most XP genes lead to a total DNA repair defect, i.e. both the fast transcription-coupled as well as the slower global genome repair pathway are impaired. In contrast, CS cells only possess a defect in TCR. This directly implicates that the products of the two genes mutated in Cockayne syndrome, *CSA* and *CSB*, mediate the coupling between transcription and DNA repair. The work presented in this thesis was aimed to increase our knowledge on the function of the Cockayne syndrome B protein in transcription-coupled repair and other processes within the cell.

This study started with the finding that mutations in the previously isolated, human DNA repair gene *ERCC6* are responsible for Cockayne syndrome group B (appendix I). The *ERCC6* (*E*xcision *R*epair *C*ross *C*omplementing) gene was originally isolated by its ability to correct the UV-sensitivity of a rodent repair mutant of complementation group 6. By transfecting the *ERCC6* cDNA to cells of all known XP and CS complementation groups, specific correction of the repair defect of CS group B cells was found, after which the gene was renamed *CSB*. The function of CSB was subsequently investigated in yeast, mice and human cells. Yeast is a very suitable organism for analysing the effect of gene mutations on a cellular level, and it is relatively easy to combine disruptions in several genes, that are involved in different pathways. We isolated the yeast *Saccharomyces cerevisiae* homolog of *CSB*, designated *RAD26*, and generated a *rad26* disruption mutant (appendix II). We were able to show that also the yeast

homolog of CSB is required for TCR. Surprisingly, impairment of this function did not lead to increased sensitivity to genotoxic agents in contrast to the human equivalent. By generating double yeast mutants of *rad26* with *rad7* and *rad16*, the latter two being required for repair of nontranscribed DNA, it became possible to dissect the different repair levels within one active gene (appendix III). This provided evidence for an overlap between transcription-coupled and global genome repair, and revealed an as of yet unknown repair activity that is specific for the transcribed strand. The possible identity of this residual repair moiety is discussed in chapter 2.

Due to its central role in TCR and its possible involvement in transcription itself, the CSB protein is expected to interact, at least transiently, with other repair and transcription proteins. In addition, since mutations in *CSA* lead to a similar defect in man, CSB could also interact with CSA. Appendix IV discusses biochemical experiments that were performed to identify such CSB-associated proteins. Antibodies were raised against human CSB, and were shown to inhibit TCR *in vivo* when microneedle injected into cultured cells. CSB interactions were assayed under near-physiological conditions, i.e. in whole cell extracts that were prepared according to Manley and which are active in *in vitro* repair and transcription assays. These extracts were fractionated on several columns or used as a source to immunoprecipitate the CSB protein with associating proteins using CSB-specific antibodies. We found indications that CSB resides in a very large molecular weight protein complex, but which does not include CSA. In addition, a number of candidate transcription and repair proteins were also excluded from being part of the CSB-complex. Nonetheless, these proteins can still transiently interact with CSB. Further purification of the CSB-complex is expected to lead to the identification of the CSB-associating proteins. This will presumably provide indications on how CSB mediates the coupling between transcription and repair, and may also reveal whether CSB has an additional function in the cell.

Chapter 3 addresses several items in the DNA repair field that probably will be of major interest in the (near) future. In addition, possible functions of CSB are being discussed. Finally, future directions that emerge from the work described in this thesis are considered, which may shed more light on the function of the Cockayne syndrome B protein.

Samenvatting

Elk levend organisme heeft een stelsel van DNA herstel mechanismen ontwikkeld om te voorkomen dat mutaties optreden in het DNA, de drager van de erfelijke informatie. Deze veranderingen van de genetische code kunnen het gevolg zijn van intrinsieke instabiliteit van het DNA molecuul, van replicatie fouten, of van DNA schades, die geïntroduceerd zijn door DNA beschadigende agentia. DNA schades die niet verwijderd worden, kunnen interfereren met verschillende cellulaire processen, zoals de aanmaak van het RNA (transcriptie), of de verdubbeling van het DNA tijdens de celdeling. Dit kan ernstige gevolgen hebben, zoals voortijdige celdood, of juist tegengesteld, ongecontroleerde celgroei hetgeen kan ontaarden in de vorming van een tumor. Een van de meest belangrijke DNA herstel mechanismen is het nucleotide excisie herstel (NER), dat in staat is om velerlei verschillende DNA schades te verwijderen. Dit gebeurt door een klein stukje DNA met de schade te vervangen door een onbeschadigde copie. Het belang van een functioneel NER wordt geïllustreerd door de UV-gevoelige, erfelijke ziekten xeroderma pigmentosum (XP), Cockayne syndroom (CS) en thrichothiodystrophie (TTD). Patiënten die aan deze syndromen leiden hebben enkele symptomen gemeen, zoals huid abnormaliteiten en een verhoogde gevoeligheid voor zonlicht. Opvallend is dat alleen XP patienten een verhoogde kans op huidkanker vertonen. Daarentegen lijken bij CS en TTD patiënten ook andere processen aangedaan te zijn, aangezien deze patienten neurologische afwijkingen en ontwikkelingsstoornissen vertonen die niet eenvoudig verklaard kunnen worden door een NER defect.

Enige jaren geleden is gevonden dat het herstel van het DNA genoom niet uniform gebeurt. Genen die actief worden afgelezen (getranscribeerd) op het moment van beschadiging, worden sneller hersteld dan het inactieve deel van het DNA. Het bleek dat dit transcriptie gekoppeld DNA herstel (TCR) alleen optreedt voor de getranscribeerde streng van deze genen. Deze DNA streng wordt door een elongerende RNA polymerase afgelezen om het boodschapper RNA te maken, wat elders in de cel wordt vertaald in eiwit. Hoofdstuk 2 gaat dieper in op de huidige kennis omtrent TCR in verscheidene organismen en de factoren die hierbij een essentiële rol spelen. Genetische en biochemische studies hebben laten zien dat mutaties in de meeste XP genen leiden tot een algemeen DNA herstel defect, dus zowel het relatief snelle transcriptie gekoppelde alsmede het langzame globale genoom herstel zijn aangedaan. Daarentegen zijn CS cellen specifiek deficiënt in TCR. Dit betekent dat de produkten van de twee genen, die aangedaan zijn in Cockayne syndroom, het *CSA* en *CSB* gen, direkt betrokken zijn bij de koppeling tussen transcriptie en DNA herstel. Het onderzoek dat beschreven is in dit proefschrift had tot doel om opheldering te verkrijgen omtrent de rol van het Cockayne syndroom B (CSB) eiwit in transcriptie gekoppeld DNA herstel en andere processen in de cel.

De eerste stap was de bevinding dat mutaties in het eerder geïsoleerde *ERCC6* gen verantwoordelijk zijn voor het defect in Cockayne syndroom groep B (appendix I). Het *ERCC6* (*E*xcisie *R*eparatie *C*ross *C*omplementerend) gen was oorspronkelijk geïsoleerd doordat het de gevoeligheid voor UV licht van een hamster mutant kon corrigeren. Nadat het *ERCC6* gen vervolgens in cellen van alle bekende XP en CS mutant groepen werd ingebracht, werd specifieke correctie van CS groep B gevonden, waarna het gen hernoemd is als *CSB*. Vervolgens is de functie van CSB onderzocht in bakkersgist, muis en menselijke cellen. Gist is

een zeer geschikt modelorganisme om het effect van gen mutaties op cellulair niveau te bestuderen. Bovendien is het relatief eenvoudig om de effecten van beschadigingen (disrupties) van meerdere genen, betrokken in verschillende mechanismen, te onderzoeken. In appendix II staat beschreven hoe we de gist (*Saccharomyces cerevisiae*) homoloog van *CSB* hebben geïsoleerd, die *RAD26* genoemd is. Door middel van genetische manipulatie is een *rad26* disruptie mutant gemaakt, en uit analyse daarvan bleek dat ook de gist homoloog van CSB nodig is voor TCR. In tegenstelling tot de humane equivalent leidde disruptie van *RAD26* niet tot een verhoogde gevoeligheid voor DNA beschadigende agentia. Door dubbelmutanten te maken van *rad26* met *rad7* en *rad16*, twee factoren die nodig zijn voor het herstel van niet-getranscribeerd DNA, werd het mogelijk om de bijdrage van transcriptie gekoppeld en niet-transcriptie-geassocieerd DNA herstel aan de reparatie van één actief gen te bepalen (appendix III). Hieruit bleek dat deze twee mechanismen deels overlappen, wat waarschijnlijk de bovengenoemde ongevoeligheid van de *rad26* mutant verklaart. Bovendien onthulden deze experimenten een nog onbekende herstel activiteit, die specifiek is voor de getranscribeerde DNA streng. De mogelijke identiteit van deze residuele herstel activiteit wordt bediscussieerd in hoofdstuk 2.

Aangezien CSB een centrale rol inneemt in transcriptie gekoppeld DNA herstel en mogelijk ook in transcriptie zelf, is het waarschijnlijk dat het CSB eiwit interacties aangaat met andere transcriptie of DNA herstel factoren. CSB zou daarnaast ook geassocieerd kunnen zijn met CSA, aangezien mutaties in *CSA* en *CSB* tot eenzelfde DNA herstel defect leiden. In appendix IV worden biochemische experimenten besproken die tot doel hadden zulke CSB bindende factoren te identificeren. Antilichamen werden opgewekt tegen tegen het humane CSB eiwit en deze bleken TCR te kunnen blokkeren na micronaald injectie in gekweekte cellen. Deze microinjecties hadden opvallend genoeg geen effect op de transcriptie activiteit van de geïnjecteerde cellen, waaruit blijkt dat CSA en CSB geen essentiele bijdrage aan dit proces leveren. Interacties tussen CSB en andere eiwitten werden geanalyseerd onder pseudo-fysiologische condities, in eiwit extracten die erg actief zijn in *in vitro* DNA herstel en transcriptie reakties. Deze extracten werden gefractioneerd over scheidende kolommen of gebruikt om het CSB eiwit te isoleren met behulp van specifieke antilichamen. Er werden aanwijzingen verkregen dat CSB onderdeel is van een groot eiwitcomplex, waar CSA geen deel van uit maakt. Een stabiele interactie van CSB met een aantal andere kandidaat eiwitten werd evenmin gevonden in deze eiwit extracten. Dit sluit echter niet sluit dat een tijdelijke associatie onder de juiste omstandigheden kan plaatsvinden. Een verdere zuivering van het CSB-complex zal hopelijk leiden tot de identificering van de CSB-associërende eiwitten.

Tot slot, in hoofdstuk 3 worden verscheidene vraagstellingen in het DNA herstel onderzoek besproken, die hopelijk in de (naaste) toekomst beantwoord zullen worden. Tevens worden mogelijke functies van CSB bediscussieerd, alsmede vervolgexperimenten, die voortvloeien uit het werk dat in dit proefschrift beschreven staat, en die verdere aanwijzingen kunnen leveren omtrent de funktie van het Cockayne syndroom B eiwit.

Curriculum vitae

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aug. 1985 - aug. 1989
Higher Technician School at the Hogeschool Eindhoven
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Subject of masters thesis: 'Interaction of the *E.coli* UvrABC endonuclease with specific *cis*-Pt.GG and *cis*-Pt.GCG intrastrand cross-links *in vitro*',
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Ph.D. student, Department of Cell Biology and Genetics,
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Subject of thesis: 'The Cockayne syndrome B protein: Involvement in transcription-coupled DNA repair',
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Transgenesis, gene transplantation and gene therapy.
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Strategies for purification, sequencing and structural analysis of proteins.

Publications

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In preparation.

Werken in de DNA repair groep in Rotterdam heeft als voordeel dat er een enorme 'back-up' aan mensen, kennis en materiaal is, waardoor het een stuk eenvoudiger wordt om onderzoek te doen. Gelukkig is het AIO's, die in Rotterdam promoveren, toegestaan om die mensen met name te noemen, wat ik hieronder graag wil doen. Omdat iedereen toch dit nawoord als eerste gaat napluizen op zijn of haar naam, heb ik die maar in een titeltje gezet. Mocht ik iemand vergeten zijn, bedenk dan maar dat het echt geen opzet is geweest!

Promotoren Prof. Dick Bootsma en Prof. Jan H.J. Hoeijmakers, en commissieleden

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De rest van Genetica en Celbiologie

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