Cockayne syndrome B protein: connection between repair, transcription and chromatin structure

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(Cockayne syndroom B eiwit: connectie tussen herstel, transcriptie en chromatinestructuur)

Proefschrift

ter verkrijging van de graad van doctor
aan de Erasmus Universiteit Rotterdam
op gezag van de
Rector Magnificus
Prof. dr. P.W.C. Akkermans M.A.
en volgens besluit van het College voor Promoties

De openbare verdediging zal plaatsvinden op
woensdag 24 mei 2000 om 13:45 uur

door

Elisabetta Citterio
geboren te Vimercate, Italië

Promotiecommisie

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Cover design: Jori de Ruiter

Dit proefschrift kwam tot stand binnen de vakgroep Celbiologie en Genetica van de faculteit der Geneeskunde en Gezondheidswetenschappen van de Erasmus Universiteit Rotterdam. De vakgroep maakt deel uit van het Medisch Genetisch Centrum Zuid-West Nederland. Het onderzoek is financieel ondersteund door Human Frontiers, de European Community (DNage project) en de Nederlandse Organisatie voor Wetenschappelijk Onderzoek (NWO).

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Chapter 1

General introduction and aim of the thesis

DNA repair mechanisms

DNA is the carrier of the genetic instructions in all living organisms. Its integrity is of vital importance for a faithful transmission of the genetic information and for the proper functioning of cellular processes. However, the DNA molecule is susceptible to alterations caused by both intrinsic chemical instability (e.g. deamination, depurination etc.) and by a wide variety of environmental and endogenous compounds. The most prominent DNA-damaging physical agents are ultraviolet (UV) light and ionizing radiation (X-rays and γ-rays). DNA damage can disturb cellular processes and can have severe consequences on human health. Its direct effect at the cellular level is inhibition of vital processes, most notably transcription, replication and cell cycle progression. Accumulation of lesions in DNA can either lead to cell death by apoptosis or to permanent mutations in the genetic code which can cause inborn diseases and contribute to premature aging. Importantly, mutations in proto-oncogenes and tumor suppressor genes are involved in the initial stages and subsequential progression of the multi-step process of carcinogenesis.

To safeguard the genetic information, a complex network of DNA repair mechanisms has evolved, most of which are strongly conserved from *E. coli* to yeast and mammals (46). Damage specificity is observed within the diverse repair systems. Briefly, *homology-dependent recombination* and *DNA-end joining* are the major repair pathways dealing with DNA double strand breaks, mainly induced by ionizing radiation (80, 223). Base mispairs and small insertions /deletions introduced during DNA replication are repaired by *mismatch repair*. *Base excision repair (BER)* removes several types of oxidatively damaged bases and small alkylating damage (152). These alterations in DNA bases can arise from challenge by reactive oxygen species, e.g. generated by ionizing radiation or, endogenously, by normal cellular metabolism or metabolism of several chemical carcinogens. Accumulating evidence has revealed the involvement of oxidative damage in the aging process of somatic cells (109). *Nucleotide excision repair (NER),* the repair system studied in this thesis, is responsible for the removal of numerous DNA-helix distorting lesions, among which UV-induced lesions are the most relevant (36).

The biological relevance of counteracting DNA damage is illustrated by the occurrence of inherited human syndromes caused by a NER defect: xeroderma

pigmentosum (XP), Cockayne syndrome (CS) and trichothiodystrophy (TTD). Extreme sensitivity to sunlight is the hallmark of all three clinical conditions. In addition, distinctive features characterize each disorder. XP patients show an elevated frequency (> 1000-fold) of sunlight-induced skin cancer and other mainly cutaneous abnormalities. In contrast, CS and TTD patients do not display an obvious cancer predisposition but suffer from severe neurodevelopmental abnormalities. Sulphur-deficient brittle hair and nails is the additional typical trait of TTD (11).

Transcription-coupled repair

Intimate links have been revealed between NER and other repair systems. In addition, to efficiently protect cells from DNA damage, NER activities are closely coordinated with other cellular processes, including cell cycle regulation and transcription by RNA polymerase II (RNAP II). The functional interplay with transcription was first disclosed by the discovery that actively transcribed genes, and in particular their transcribed strand, are repaired at a faster rate compared to silent sequences. This is accomplished by a NER mode known as transcription-coupled repair (TCR). A specific defect in TCR characterizes cells from Cockayne syndrome patients. Two genes have been shown to be specifically required for TCR in man, CSA and CSB, which are defective in CS complementation groups A and B respectively (62, 184).

A dual involvement in NER and transcription has been established for the basal transcription factor TFIIH, which has a pivotal role in both processes. Mutations in TFIIH subunits are associated with considerable clinical heterogeneity and can give rise to XP as well as to combined XP/CS symptoms and TTD. Except severe photosensitivity in all three diseases, the developmental and neurological abnormalities within CS and TTD are difficult to rationalize on the sole basis of a NER defect. Because of the dual function of TFIIH, it was proposed that some of the CS and TTD specific symptoms may arise from a defect in the transcription function of TFIIH (10, 203). In analogy with TFIIH, it has been suggested that the CS proteins may have an additional function, possibly in transcription (45, 193, 203); see chapter 4).

Chromatin structure

If stretched out, the DNA of a single human cell would extend approximately two meters in length. However, in order to fit within the nucleus, which is only a few micrometers in diameter, DNA is packaged by association with histones and non-histone proteins into a highly organized structure known as chromatin. This highly compacted organization of DNA adds more complexity to NER as well as to

all DNA transacting processes *in vivo*. At present, very little is known about how the NER enzymes deal with chromatin and how they gain access to DNA to perform the repair reaction. On the other hand, substantial experimental evidence has disclosed the existence of several mechanisms that modulate chromatin structure in order to facilitate RNAP II transcription (225). One of these mechanisms is based on the chromatin remodeling activity of a set of proteins known as SWI2/SNF2 DNA-dependent ATPases, which are usually part of large protein complexes (83). Interestingly, the SWI2/SNF2 protein family includes also proteins involved in various DNA repair pathways, such as NER and recombination repair, suggesting that these processes may require similar remodeling activities as transcription. Sequence analysis revealed that CSB, the topic of this thesis, belongs to the SWI2/SNF2 protein family (184).

Aim of the thesis

The work described in this thesis aims to gain insights into the function of the CSB protein in TCR. Specific attention was given to (i) investigation of the relationship of CSB with other repair and transcription factors within the context of cellular protein extracts and to (ii) isolation and biochemical characterization of CSB as a recombinant protein. (iii) Involvement of the isolated CSB in chromatin remodeling was addressed *in vitro*. (iv) Finally, aspects of CSB nuclear organization and its dynamic relationship with NER and transcription were studied in living cells.

In **Chapter 2**, an overview of the current literature on mammalian NER is presented. Particular attention is given to biochemical aspects of the NER mechanism. In addition, the recent application of confocal microscopy to the study of NER in living cells is described. **Chapter 3** focuses on the TCR pathway with emphasis on the biochemical characteristics of the proteins involved. In addition clinical aspects of CS and their relationship with mutations in TCR-specific genes will be discussed. Finally, aspects of the NER connection with transcription will be presented. A more detailed analysis of the current experimental data about the transcription-repair coupling reaction and the connection with transcription elongation is presented in **chapter 4**. **Chapter 5** deals with NER in the context of chromatin. In addition, aspects of chromatin remodeling related to transcription are summarized. The main observations described in the experimental part of the thesis are integrated and discussed in the above mentioned theoretical chapters (2, 3 and 4). The experimental work is outlined in the following chapters. In **Chapter 6** the physical interaction of CSB with other NER or transcription factors was investigated in the context of cellular protein extracts. **Chapter 7** describes the identification of a novel protein involved in TCR, XAB2, and its relationship with TCR-specific proteins, namely CSA, CSB and RNAP II. **Chapter 8** and **chapter 9** focus on the biochemical properties of the isolated CSB protein and on the analysis

of its involvement in chromatin remodeling *in vitro*, respectively. Finally, in **Chapter 10** aspects of CSB nuclear organization and dynamics are studied in living cells by using the green fluorescence protein (GFP) technology.

Chapter 2

Nucleotide Excision Repair (NER)

Introduction

Nucleotide excision repair (NER) is a versatile repair system whose major targets are DNA injuries induced by the short-wave UV component of sunlight: cyclobutane pyrimidine dimers (CPDs) and 6-4 photoproducts (6-4PP). In addition, NER repairs other lesions, including numerous bulky chemical adducts (such as the ones formed by polycyclic aromatic hydrocarbon carcinogens such as N-acetoxy-2-acetylaminofluorene, AAAF, N-2-acetylaminofluorene, AAF, and benzo[a]pyrene), and intrastrand crosslinks (caused by cis-platin and psoralens, among others). The common feature of NER lesions is significant distortion of the DNA helix. Both helical distortion and alteration of DNA chemistry are necessary for recognition by the NER machinery (63).

The above lesions are removed in a complex multistep reaction. Two NER subpathways can be distinguished: repair of lesions throughout the genome, referred to as global genome repair (GGR), and transcription-coupled repair (TCR), which removes damage in the transcribed strand of active genes that actually blocks ongoing transcription.

In humans, NER constitutes a major defense against the carcinogenic effects of sunlight. This is apparent from the high risk of developing skin tumors in individuals suffering from the NER-deficient disorder Xeroderma Pigmentosum (XP). At least two other distinct human syndromes are associated with defective NER, Cockayne syndrome (CS) and trichothiodystrophy (TTD). Extreme sensitivity to sunlight (UV) is the common hallmark for these rare, autosomal recessive conditions, which otherwise are highly heterogeneous in additional clinical features and in genetic make-up (see below; (11)). XP is due to mutations in one of 7 genes (designated *XPA* to *XPG*), CS to one of 2 genes (*CSA* and *CSB*). In addition, specific mutations in 3 XP genes (*XPB, XPD* and *XPG*) can cause a combination of XP and CS, two of which (*XPB* and *XPD*) can also give rise to the photosensitive form of TTD, in addition to *TTDA*. Most forms of XP and TTD are due to deficiencies in both subpathways of NER. Defective TC-NER is specifically associated with CS.

Human NER proteins and molecular mechanisms

The NER process involves the activity of 20-30 proteins. In the last two decades, all key NER genes have been cloned, the proteins isolated and the core NER reaction has been reconstituted *in vitro* on DNA templates containing positioned damage (recently reviewed in (36, 222)). In mammalian cells, six components are needed for the core GG-NER reaction *in vitro*. These are XPC-hHR23B, XPA, the heterotrimeric RPA protein (additionally involved in DNA replication and recombination), the multiprotein complex TFIIH (with dual involvement in transcription initiation and in NER), and the XPG and ERCC1-XPF endonucleases. The essential steps of the NER reaction are schematically depicted in Figure 1. Briefly, recognition of the DNA damage site is followed by the formation of an unwound intermediate DNA structure around the lesion termed "open complex". This is the substrate for structure-specific endonucleases that cleave the damaged strand on either side of the lesion (dual incision). After the excision of the damage-containing oligonucleotide, general replication factors perform gap-filling DNA synthesis and strand ligation.

Damage recognition

The NER reaction starts with recognition of the DNA injury. This step distinguishes the GGR from the TCR subpathway. The XPC-hHR23B complex is the damage sensor in GG-NER. It is probably the first protein that recognizes the DNA lesion and recruits the NER machinery to the damaged site (168). The XPC complex improved the efficiency of the NER reaction *in vitro* when it was allowed to bind to the damage before the addition of the other NER components (168). In addition, it has affinity for several NER lesions and is the only NER factor shown to make stable footprints around a lesion, namely 6-4 PP (168). XPC-hHR23B is thought to stably bind to a distorted lesion, thereby altering the DNA structure of the damaged region so that TFIIH and the other NER core proteins can bind (36, 222). The fact that XPC-hHR23B is not required when the DNA is sufficiently distorted or locally premelted around a lesion is consistent with this model (122).

Other NER proteins show preferential binding to damaged DNA and may assist XPC-hHR23B in the recognition of the damage. The UV-DDB protein complex, whose activity is lacking in some XPE patients, may facilitate the detection of lesions poorly recognized by XPC-hHR23B, such as CPDs, *in vivo* (71). XPA, the first human NER protein shown to have preferential affinity for several DNA lesions, was suggested to act as a lesion verifier in the subsequent steps of NER with the assistance of the single-strand DNA binding protein complex RPA (168).

XPC-hHR23B and probably UV-DDB (XPE) are the only NER proteins that are specific for GGR and whose function is not required for the TCR pathway. Within TCR, the elongating RNAP II complex that encounters a lesion is thought to detect the DNA damage and to activate the TC-NER pathway in a CSA/CSB-

dependent manner (57). The details of the TCR pathway will be discussed in the next chapter.

Open complex formation

Unwinding of the DNA around a lesion is an ATP-dependent process and requires the coordinated activities of XPC-hHR23B, TFIIH, XPA, RPA and XPG (42, 43, 123). XPC-hHR23B and TFIIH are required at the earliest steps of opening (43). The XPB and XPD subunits of TFIIH display ATPase and DNA unwinding activities and mediate the ATP-dependent melting of the DNA around the lesion (43). In addition to DNA unwinding, XPB and XPD may also function during subsequent steps, since premelted lesions still require TFIIH (122). The fact that mutations in XPB C-terminus selectively inhibits the 5' incision, while leaving the open complex formation and the 3' incision unaltered, suggests a structural role of TFIIH in facilitating the 5'cleavage reaction (43). XPA, RPA and XPG are needed for the formation of a full open complex of ~25 nucleotides across the lesion. XPG likely plays a structural function by stabilizing the preincision complex, since its nuclease activity is not required for opening (43, 123).

Dual incision

The XPG and ERCC1-XPF NER endonucleases perform the cleavage on the 3' and 5' side of the DNA lesion, respectively (120, 160). The 3' incision by XPG is made first and can be detected in the absence of ERCC1-XPF (120). On the other hand, ERCC1-XPF needs the physical presence, but not the catalytic activity, of XPG to perform the 5' incision, suggesting an additional structural role for XPG (123, 209). Both XPG and ERCC1-XPF are structure-specific endonucleases that cleave at the junctions between duplex and single-stranded DNA of an unwound DNA structure. During NER, RPA plays a crucial role in positioning both nucleases. RPA directs ERCC1-XPF cleavage activity to the damaged strand only, whereas its action is not sufficient to confer strand specificity to XPG (35). TFIIH has been proposed to participate in the positioning of the NER machinery, possibly through interaction with XPG (36).

Gap-filling and ligation

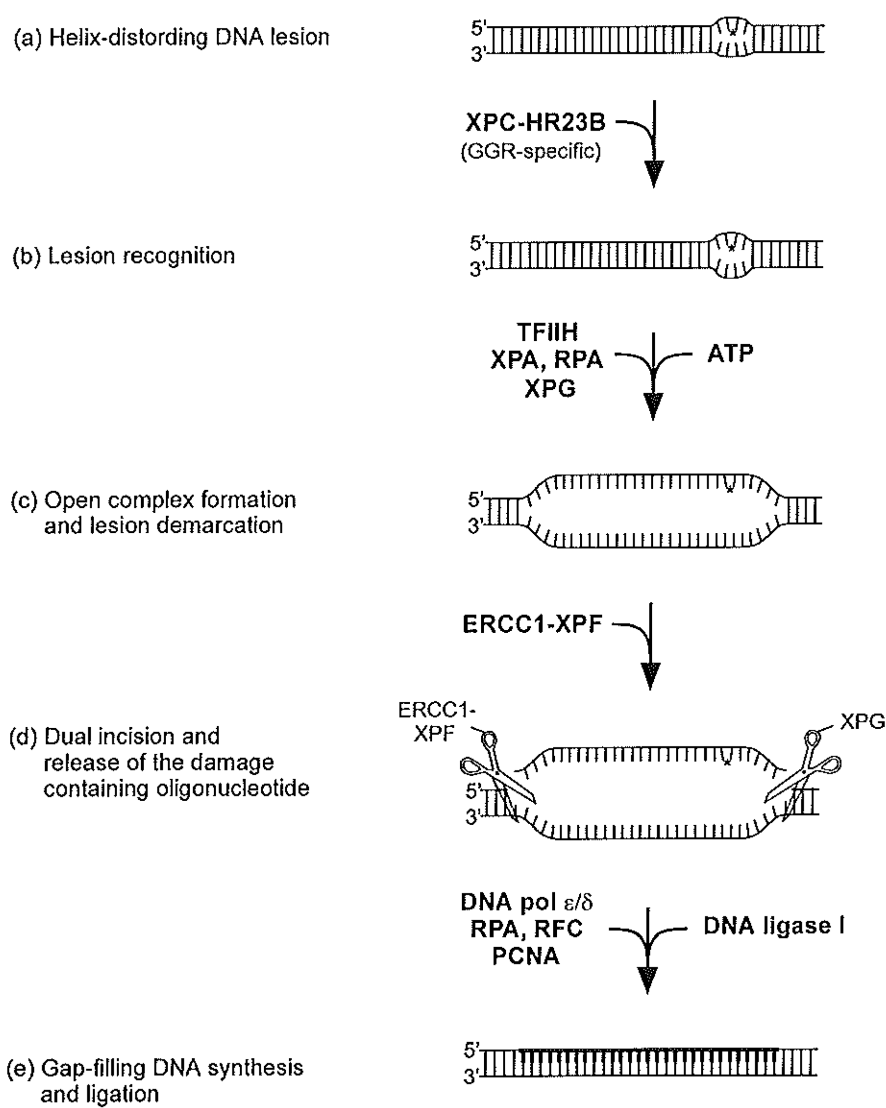
Dual incision is followed by the release of a 24-32 nucleotide fragment containing the damage. Gap-filling DNA synthesis is used for assaying NER *in vitro* and *in vivo* (unscheduled DNA synthesis, UDS) and is performed by the replication factors RPA, PCNA, RFC, and DNA polymerase / (159). Among the NER core factors, RPA is the only one required during the DNA repair synthesis. Ligation of the newly synthesized DNA is likely performed by DNA ligase I (3).

Connection between NER and other cellular processes

As presented above, the NER reaction has been analyzed in quite some detail *in vitro* using whole cell extracts or reconstitution by purified components on naked DNA templates (1, 121). However, the complexity of NER goes beyond the potential of these *in vitro* experimental systems and many questions are still open. *In vivo*, the NER enzymes have to deal with the highly compact organization of DNA in chromatin within the context of other processes and with the fact that lesions are rare and randomly distributed along the 6 x 10^9 base pairs of the typical (somatic) cellular DNA content.

The complexity of NER in vivo is further emphasized by its multiple connections with other cellular processes. NER is tightly coordinated to cell cycle progression, which is arrested by p53-dependent and independent pathways following the accumulation of high levels of, among others, UV-induced DNA damage (96). In addition, participation of several NER proteins in DNA transactions, other than NER, has been revealed. TFIIH is, as already mentioned, essential for basal RNAP II transcription. The TCR pathway, which will be discussed in the next chapter, also shows the strong functional link with transcription. The last step of the NER reaction shares components with the replication machinery. A functional interplay with other repair systems is illustrated by the probable involvement of the ERCC1/XPF complex in the repair of interstrand cross-links by mitotic recombination (single-strand annealing pathway) (26, 44) and by the requirement of CSA, CSB and XPG in the processing of oxidative damage, mainly repaired by base excision repair (BER), in a transcription-coupled manner (28, 92). The NER-BER link will be discussed in more detail in the next chapter. Finally, a connection between NER and the mismatch repair system has been proposed (91, 116).

FIG. 1. Model for the molecular mechanism of NER. Helix-distorting NER lesions (a) are recognized by XPC-hHR23B within global genome repair (GGR), leading to partial opening of the DNA helix (b). (c) The coordinated action of TFIIH, XPA, RPA and XPG mediates the formation of an unwound intermediate around the lesion. XPB and XPD helicase components of TFIIH catalyze ATP-dependent unwinding, whereas XPA and RPA stabilize the open complex and position the other factors. Most likely, XPA binds to the damage nucleotides and RPA to the undamaged strand. XPG presence is required for the stabilization of a fully opened complex. (d) Dual incision is performed by XPG and ERCC1-XPF structure specific endonucleases, which cleave the damaged strand 3' and 5' of the lesion, respectively. The lesion is released as part of a ~30mer oligonucleotide. (e) Gap-filling DNA synthesis by the replication machinery followed by ligation completes the NER reaction. * indicates a DNA lesion. III in bold indicates newly synthetized DNA. Model adapted from de Laat et al., 1999 and Wood, 1999 (36, 222).


(a) Helix-distording DNA lesion
5'
3'
XPC-HR23B
(GGR-specific)
(b) Lesion recognition
5'
3'
TFIIH
XPA, RPA
XPG
ATP
(c) Open complex formation
and lesion demarcation
5'
3'
ERCC1-XPF
(d) Dual incision and
release of the damage
containing oligonucleotide
ERCC1-
XPF
XPG
5'
3'
DNA pol ε/δ
RPA, RFC
PCNA
DNA ligase I
(e) Gap-filling DNA synthesis
and ligation
5'
3'

NER in living cells

To gain insight into the NER mechanism *in vivo*, confocal microscopy has been recently applied to study the dynamics of NER factors in living cells. This novel approach is based on the analysis of functional NER proteins fused to GFP (green fluorescence protein) that are stably expressed in human fibroblasts. The nuclear mobility of ERCC1-GFP/XPF was studied using fluorescence redistribution after photobleaching (FRAP) technology (68, 216). In the absence of DNA damage, ERCC1-GFP/XPF was found to diffuse rapidly in the nucleus, with a diffusion constant consistent with its molecular weight, arguing against the idea that ERCC1 is part of a NER holocomplex (171). UV-irradiation caused a fraction of the ERCC1-GFP/XPF molecules to become temporarily immobilized, likely for the duration of one repair event. On the basis of these data, the authors proposed a model in which individual NER proteins assemble at the site of DNA damage rather than being organized in a complete "repairosome". In addition, ERCC1-GFP/XPF seems to locate the damaged site not by processive scanning of the genome but in a distributive fashion by diffusion and collision with the other repair factors (68).

Chapter 3

Transcription-coupled DNA repair (TCR)

Molecular impact

An important consequence of inflicting DNA damage is rapid blockage of crucial DNA-transacting processes, including the vital process of transcription. A key step in understanding how the cell deals with this serious problem was the discovery, more than a decade ago, that processing of UV-induced injury in mammalian cells varies in accordance with the transcriptional status of the DNA. Quantitative analysis of CPD repair at the level of specific sequences reveals a faster removal of lesions from active genes (9) and more specifically from the transcribed strand (117) than from the genome overall. NER puts high priority to the elimination of transcription-blocking lesions from the genome to allow quick resumption of RNA synthesis. This process is called transcription-coupled repair (TCR), appears restricted to RNA polymerase II (RNAP II) transcribed sequences and requires RNAP II to be actively engaged in transcription elongation (recently reviewed in (91, 180, 193)). After damage 'recognition' by RNAP II, the lesion is removed by the core NER reaction (see Chapter 2, Figure1).

TCR is particularly relevant for lesions for which the global genome pathways are too slow or incomplete and that actually block transcription elongation, such as UV-induced CPDs (196). All available evidence points to the idea that release of blocked polymerases and subsequent recovery of transcription is the main 'raison d'être' of this system. The biological significance of the TCR pathway is emphasized both by the severe TCR-deficient human disease Cockayne syndrome and by its strong evolutionary conservation from man to mouse (191), *Saccharomyces cerevisiae* (93, 172, 194) and *Escherichia coli* (115).

Human genes, proteins and possible functions

The inability to perform TCR and the subsequent permanent arrest of transcription underlies the UV sensitivity of CS patients (195, 200). As indicated above, a defect in one of at least 5 genes can give rise to CS: CS-only (*CSA*, *CSB*) or in combination with XP (*XPB*, *XPD* and *XPG*). *CSA* and *CSB* are specifically required for TCR, whereas the three XP genes participate in both NER pathways. All these genes have been cloned, by human genomic DNA or cDNA library

transfections and subsequent functional complementation of the UV-sensitivity of NER-deficient cells (11).

CSA

The *CSA* gene has been mapped to the pericentromeric region of human chromosome 5 and encodes a 44 kDa protein mainly made up of five WD 40 repeats (Figure 1) (62). WD repeats containing proteins appear to have a regulatory rather than a catalytic function and are often components of multiprotein complexes involved in diverse aspects of cellular metabolism, including cell cycle regulation, RNA processing and gene regulation. The WD motif is thought to mediate protein-protein interactions (126). In line with other proteins of the family, size fractionation experiments of Hela whole-cell extracts (WCE) indicated that CSA resides in a multiprotein complex of about 450 kDa ((192) and chapter 6). Evidence for *in vitro* interaction of CSA with CSB and with the p44 subunit of TFIIH has been reported (62) (Table 1). CSA is also found to associate with XAB2 (XPA binding protein 2), a novel protein involved in TCR and in transcription (Chapter 7). XAB2 contains 18 repeats of the TPR (tetratricopeptide) motif, a degenerated 34 amino acid sequence identified in a wide variety of functionally different proteins, most of which are associated with multiprotein complexes. TPRs mediate protein-protein interactions and are often present in multiple copies organized in tandem arrays (8).

Table 1. Physical interactions between the CS proteins and other repair/transcription factors

	CSB	*XAB2*	*TFIIH*	*XPG*	*RNAPII*	*XPA*
CSA	IVT-IP Two-hybrid (62)	Pull-down WCE-IP (Chapter 6)	IVT-IP (p44) (62)			
CSB		WCE-IP (Chapter 6)	Pull-down (XPB) (155) Gel-shift (173)	IVT-IP (77)	WCE-IP (192; Chapter 6) Pull-down (154) Gel-shift (174; 173)	Pull-down (155)

(IVT-IP) Immunoprecipitation of *in vitro* translated proteins; (Pull-down) experiments: GST-, MBP-affinity chromatography; (WCE-IP) co-immunoprecipi-tation from whole cell extracts; (Gel-shift) experiments: binding to the transcribing ternary complex RNAP II/DNA/RNA or * to the CSB-bound ternary complex was visualized on native polyacrylamide gels.

Recently, the three-dimensional structure of the TPR motif has been determined and showed that each TPR consists of two antiparallel alpha-helices equivalent in length (33). Tandem arrays of TPR motifs are predicted to fold into a right handed helical structure with an amphipatic groove suited for interactions with target proteins. Specific combination of TRP repeats would allow TPR-containing proteins to simultaneously interact with multiple target proteins, thereby mediating the assembly of multiprotein complexes (33).

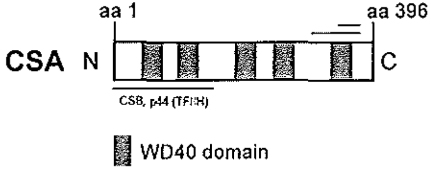
CSB

The CSB gene, also known as ERCC6, is located on chromosome 10.q11-21 and encodes a large protein of 168 kDa (184). Several domains have been identified in the predicted sequence, including an acidic region, two potential casein kinase II phosphorylation sites, and a nuclear localization signal. The middle third of the protein is comprised of a helicase-like domain highly conserved within the SWI2/SNF2 family of DNA-dependent ATP-ases (Figure 1) (184). Members of this family are associated with a wide range of nuclear processes, including transcription regulation, chromatin remodeling, and diverse DNA repair processes, such as NER, postreplication and recombination repair (40). SWI2/SNF2-related proteins have been proposed to function as molecular motors which use the energy of ATP hydrolysis to translocate on the DNA and to destabilize protein-protein or protein-DNA interactions (135). The ability of the SWI/SNF and ISWI subfamilies to destabilize reconstituted nucleosomes and the ability of the transcriptional regulator MOT1 to dissociate TBP from TBP-DNA complexes are consistent with such a mechanism (135). Involvement of SWI2/SNF2 proteins in chromatin remodeling will be discussed in chapter 5.

CSB is found in a large MW protein complex (larger than 700 kDa) in WCE and, like CSA, is able to associate with XAB2 ((192); Chapter 6 and 7). Using immunoprecipitations of *in vitro* translated proteins and GST pull-down, CSB was reported to interact with CSA (62), XPG (77), XPA, XPB and the p34 subunit of the transcription initiation factor TFIIE (155) (Table 1). However, no co-immunoprecipitation of CSB with any of the repair/transcription factors mentioned above could be detected in WCE ((192) and Chapter 6). In addition, CSA and CSB were found to reside in distinct protein complexes, of ~450 and > 700 kDa respectively, in WCE analyzed by size fractionation (Chapter 6 and 7). These results suggest that CSB has the potential to interact with these proteins *in vitro*. However, these associations may be transient in the cell nucleus, and consequently difficult to detect in WCE. On the other hand, the *in vitro* observations may not reflect the situation in the nucleus, where binding to other cellular proteins or to DNA may influence the native conformation and binding properties of the protein. During the TCR reaction, CSB is predicted to interact with the stalled RNAP II complex. In support of this idea, CSB has been found to associate with a fraction of RNAP II in WCE as well as *in vitro* in pull-down experiments ((154, 192); Chapter 6). Interestingly, in gel shift experiments CSB was shown to bind to transcribing

RNAPII molecules engaged in ternary complexes with DNA and nascent RNA (174). Subsequently, the resulting quaternary complex CSB/RNAP II/DNA/RNA was claimed to recruit TFIIH in analogous experiments (173).

Studies with purified recombinant CSB established that CSB is a DNA-dependent ATP-ase. CSB, like other SWI2/SNF2-related proteins, has no classical helicase activity, however it is able to alter the topology of plasmid DNA by introducing negative supercoils ((23, 155); chapter 8 and 9). The experiments described in chapter 9 also indicate that CSB functions as a chromatin remodeling factor on reconstituted nucleosomal templates. In addition, indications that CSB stimulates transcription elongation by RNAPolII *in vitro* have been reported (154); V. van den Boom and M. Timmers personal communication).



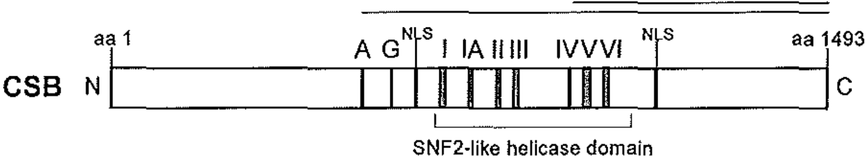


FIG. 1. Schematic representation of predicted functional domains in the CSA and CSB proteins. CSA. Five WD40 repeat motifs are depicted (126). Protein interaction domains are indicated below. Mutations found in CSA patients are indicated above. Data from Henning et al., 1995 (62). CSB. A, acidic region harboring ~60% glutamic or aspartic acid (amino acids 356-394); G, glycine rich region (residue 442-446); NLS, nuclear localization signal (amino acids 466-481 and 1038-1055) followed by potential casein kinase II phosphorylation sites; I-VI (amino acids 527-1007), helicase motifs conserved between RNA and DNA helicases (52), this region share high overall homology with proteins belonging to the SNF2 family of putative helicases (40). Data from Troelstra et al., 1992 (184). Most inactivating mutations described in CS patients results in truncated protein products (CSB truncations found in the CS1AN cells, see Chapter 6, 7 and 10 are shown above (184)). Single-amino-acid changes were also identified and were confined to the C-terminal two thirds of the protein (25, 108). For a complete overview of CSB mutations see Mallery et al., 1998 (108).

TFIIH: XPB and XPD subunits

TFIIH (transcription factor IIH), originally isolated as a basal transcription factor is a nine-subunit, multifunctional protein complex harboring helicase and protein kinase activities. TFIIH is essential for both transcription initiation by RNAPII and NER and is possibly involved in cell-cycle regulation (36, 47, 66, 220). The XPD and XPB proteins are DNA-dependent ATP-ases and exhibit opposite DNA helicase activity (3'-5' and 5'-3' respectively), involved in the local melting of the DNA helix both in transcription and in NER (43, 67). XPB and XPD appear to differently contribute to the two processes.

Recently, a functional TFIIH complex was reconstituted with recombinant proteins (178) and the individual role of XPB and XPD in *in vitro* transcription both in promoter opening and promoter escape (the transition from initiation to productive elongation) was investigated. XPB plays a crucial role in transcription initiation and its helicase activity is required for promoter opening and stimulatory for promoter escape (12, 178). In contrast, XPD catalytic activity is dispensable for *in vitro* transcription, as observed with both recombinant and native TFIIH complexes containing an ATPase-inactive XPD (178, 218). XPD was shown to play a structural role in promoter escape (12). On the other hand, NER requires both the XPB and the XPD helicase activities *in vitro* as well as *in vivo* (43, 218). Several indications for additional, possible structural roles of TFIIH in NER besides DNA unwinding have been reported (43, 122); see chapter 3, *open complex formation*). Multiple interactions of TFIIH with XPA, XPC, XPG (77, 121) and CSA (62) have been described.

XPG

The *XPG* gene encodes for a structure-specific endonuclease sharing sequence similarities with endonucleases of the FEN-1 family. It cleaves DNA 3'-junctions on bubble, splayed arms and stem loop substrates and makes the initial 3' incision during the NER reaction (160). *In vitro* and *in vivo* functional analysis of XPG mutants have defined its catalytic center (27) and have provided indications for an additional structural function in the NER reaction. As mentioned before, XPG's presence is required non-catalytically for subsequent ERCC1 incision during NER (123, 209) and direct interactions with TFIIH (77, 121), RPA, PCNA and CSB have been reported ((77); reviewed in (36)).

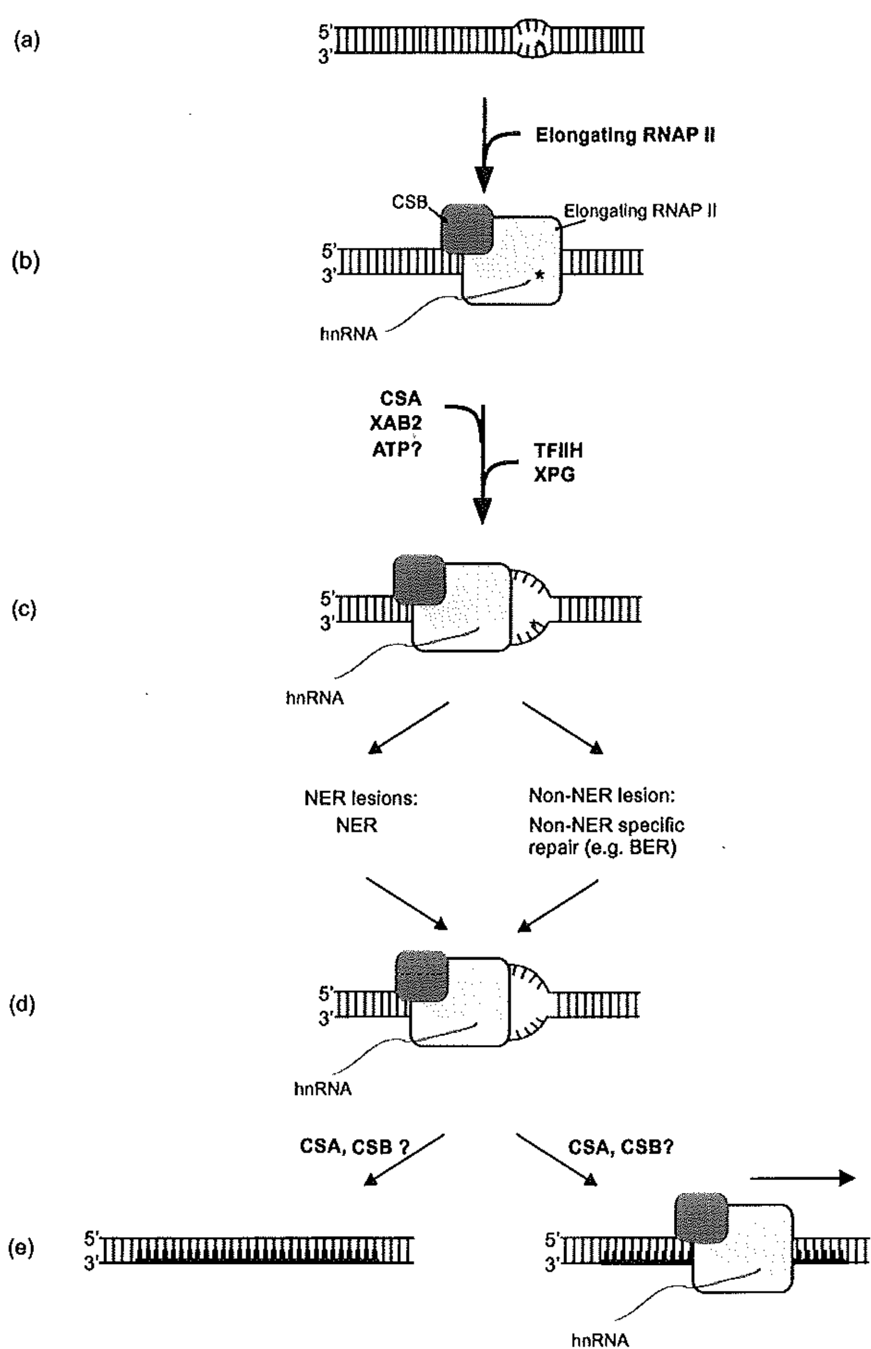
Cellular studies have revealed an additional function of XPG in the removal of oxidative damage by the BER pathway, that is distinct from its endonuclease activity in NER (28, 127). Recently, *in vitro* reconstitution of the BER reaction has shown that XPG can stimulate repair, acting as a cofactor for the activity of the human hNTh1 protein. HNTh1 releases the damaged base by its glycosylase activity and cleaves the abasic site by its AP lyase activity, during the first step of BER of thymine glycol containing DNA substrates. XPG protein promotes the binding of hNTh1 to the DNA substrate and strongly stimulates its catalytic activity (84). XPG is thought to play a structural rather than catalytic role in BER. This

hypothesis is supported by the fact that severe truncation of the protein, but not inactivation of its catalytic site by a point mutation, affects repair of oxidative damage both *in vivo* and *in vitro* (28, 84, 127).

Possible mechanisms of TCR

The mechanism by which the CSA and CSB proteins mediate the transcription-repair coupling reaction is still largely unknown, in contrast to the well-characterized TCR mechanism in *E. coli*. In prokaryotes, the transcription-repair coupling factor (TRCF) binds to and displaces the RNA polymerase stalled at a lesion, and subsequently promotes removal of the damage by recruiting the repair machinery (156). A similar sequence of events, has been proposed in a tentative model for mammalian TCR (57). The generally accepted view is that an elongating RNAP II complex that encounters a lesion detects the DNA damage and activates the TCR pathway in a CSA/CSB-dependent manner. An arrested RNAP II constitutes a steric hindrance for the repair machinery and needs to retract or to dissociate to allow repair of the damage (38, 57). The CSB protein, because of its limited sequence similarities with TRCF, was proposed to act catalytically as a coupling factor at the site of stalled transcription. Nevertheless, CSB, alone or in combination with CSA, cannot promote dissociation nor backtracking of RNAP II from a damaged site *in vitro* (154). On the contrary, the arrested elongation complex RNAP II/RNA/DNA, even when bound to CSB, is highly stable and competent to continue elongation (38, 154, 174). These findings suggest that mechanisms other than RNAP II dissociation may mediate TCR in human. Furthermore, additional proteins besides CSB and CSA may be required *in vivo*. One of these proteins is likely the recently identified XAB2 protein (chapter 6).

FIG. 2. Model for the molecular mechanism of TCR. Transcription-blocking lesions (a), e.g. CPDs (NER lesion) and thymine glycols (non-NER lesions) in the transcribed strand are detected by an elongating RNAPII complex, which initiates the TCR pathway (b). (c) CSA, CSB, TFIIH, XPG and probably other cofactors, XAB2, participate in the further processing of stalled RNAP II making the damage accessible to repair and subsequent resumption of transcription. This step could involve conformational changes or backward movement of RNAP II, release of RNAP II, or by-pass of the lesion (discussed in chapter 4). (d) Depending on the type of lesion, repair is completed by NER or by other repair pathways (e.g. BER). (e) The arrested RNAP II complexes might either have dissociated, with consequent release of the nascent transcript (left), or resume elongation (right). Dissociation may involve CSA- and CSB-dependent ubiquitination of RNAP II large subunits followed by proteosomal degradation (13, 143). CSB may facilitate re-start of RNA synthesis by its ability to enhance the rate of transcription elongation as determined in in vitro assays (154). For simplicity, only repair by NER, visualized as newly synthesized DNA patch, is illustrated. * indicates a DNA lesion. ꟾꟾꟾ in bold indicates newly synthesized DNA.



(a)
5'
3'
Elongating RNAP II
(b)
CSB
Elongating RNAP II
5'
3'
hnRNA
CSA
XAB2
ATP?
TFIIH
XPG
(c)
5'
3'
hnRNA
NER lesions:
NER
Non-NER lesion:
Non-NER specific
repair (e.g. BER)
(d)
5'
3'
hnRNA
CSA, CSB ?
CSA, CSB?
(e)
5'
3'
5'
3'
hnRNA

The sequence characteristic of both CSA and XAB2 are consistent with a structural function. Both the TPR repeats of XAB2 and the WD-40 motifs of CSA may be involved in mediating transient interactions between CSA/CSB/ RNAP II and the NER factors (Table 1). These interactions could be relevant in the recruitment of the repair machinery, thereby stimulating repair of the damage.

CSB' s properties are consistent with a catalytic role in the coupling reaction. CSB can alter DNA helix conformation upon binding, which may influence protein-DNA interactions. In addition, CSB can remodel nucleosomal templates in an ATP dependent manner, apparently without complete dissociation of the histone octamer from the DNA (Chapter 9). By an analogous mechanism, CSB may use the energy of ATP hydrolysis to induce conformational changes in the stalled RNAP II ternary complex or in the surrounding nucleosomal structure. Such alterations may allow the repair reaction without aborting transcription and\or may facilitate access or increase the affinity to other NER factors (chapter 4 and 9).

The role of TFIIH and XPG in TCR is largely unclear. Both could be recruited to the damage site by the stalled RNAP II at early stages of the repair reaction and are predicted to participate in the "displacement" of RNAP II from the damage. The altered DNA conformation at the site of arrested transcription, in analogy with the XPC-hHR23B-mediated conformational changes during GG-NER, may favor the binding of TFIIH and XPG (36). In addition, the above mentioned interactions observed between TFIIH and CSA, XPG and the stalled RNAPII/CSB complex may be relevant for the recruitment.

In recent years, substantial experimental evidence from cellular as well as *in vitro* studies revealed a broader role of TCR, not restricted to the NER repair pathway. CSA, CSB and XPG appear to be required for coupling transcription to at least two different systems, NER and BER (28, 92), as discussed below (see paragraph: 'Does the NER defect explain the CS phenotype?').

The emerging picture is that the elongating RNAP II may function as a general and efficient damage sensor that triggers different repair systems, including NER and BER, to the site of blocked transcription. In addition, blockage of transcription may constitute a sensitive signal for the induction of p53 activation followed by apoptosis ((100, 101, 111); see also next paragraph). TCR is predicted to act as a general mechanism with mainly two functions. First, it promotes fast repair of transcription blocking lesions, by the appropriate repair machinery. Second, and most important, it ensures the efficient progression of transcription elongation, possibly by releasing the blocked transcription machinery (193, 197). A tentative model for TCR is illustrated in Figure 2.

Clinical aspects of transcription-coupled repair: Cockayne syndrome

Cockayne syndrome (CS) is a rare (about 200 cases have been described to date), autosomal recessive disorder with heterogeneous clinical manifestations. Characteristic features shared by CS patients include cutaneous sun-sensitivity, severe postnatal growth retardation and progressive neurologic dysfunction. Unlike patients suffering from the prototype NER-deficient disorder XP, CS individuals do not show clear predisposition to skin cancer. At least two considerations may account for this difference. CS cells still perform a proficient GGR that might be sufficient to reduce the low levels of UV-induced carcinogenic mutations to which CS patients are normally exposed (they are often hospitalized and have a very short life expectancy, the average life span being ~12 years). In addition, CS cells are induced to undergo apoptosis at significant lower UV-doses compared to TCR proficient cells (100). The TCR defect in CS cells enhances their p53-dependent apoptotic response, contributing to the elimination of cells that potentially carry oncogenic mutations (99, 101, 112). Nance and Berry presented in 1992 a comprehensive review of 140 patients, and proposed a subdivision of the disease into three clinically different classes: 1) a mild form, characterized by late onset and slow progression of symptoms; 2) a classical form (or CSI), which includes the majority of the patients; 3) a severe form (or CSII), characterized by early onset and severe progression of manifestations, with low birth weight and poor or absent development (125). The most common clinical symptoms associated with CS are summarized in Table 2. The clinical profile of the disease is further complicated by the existence of several atypical CS cases. Patients who exhibit the CS-like DNA repair characteristics (UV-sensitivity and defective recovery of RNA synthesis) without the hallmark clinical symptoms except photosensitivity are classified to belong to the distinct disorder "UV-sensitive" (Uv^s) syndrome (76). On the other hand, there are patients with clear CS hallmarks, but have neither photosensitivity nor RNA synthesis recovery defect (11, 25).

Does the NER defect explain the CS phenotype?

Apparently, CS clinical features are much more severe than the classical XP condition and go beyond photosensitivity (Table 3). Photosensitivity, together with some XP-specific features (such as pigmentation abnormalities and predisposition to skin cancer) can be explained on the basis of a NER defect, whereas the severe developmental and neurological manifestations typical for CS can not (10, 203). Consistently, these symptoms are not found in XP-A patients, which are totally deficient in NER (11). This and several other considerations (some of which are discussed below and in chapter 4) have led to the hypothesis that CS proteins may have additional functions beyond NER (193).

Table 2. Most common clinical features of Cockayne Syndrome patients

NER related
Skin abnormalities
Photosensitivity*
Thin, dry skin or hair
Non-NER related
Developmental
Growth failure
Microcephaly
Impaired sexual development
Chachetic dwarfism
Skeletal deformations
Neurologic dysfunctions
Accelerated neurological degeneration
Mental retardation
Delayed psychomotor development
Hearing loss
Wizened facial appearance
Calcification of basal ganglia of brain
Neurodysmyelination
Ocular abnormalities
Cataracts
Progressive pigmentary retinopathy
Optic atrophy
Dental abnormalities
Caries

Table adapted from (11). *Patients without photosensitivity, however suffering from severe CS features have been described (11, 25). On the other hand, CS patients showing only photosensitivity occur (11, 118).

In some cases, CS features are found in combination with XP, caused by specific mutations in the *XPB*, *XPD* or *XPG* genes. In addition, CS and TTD share most of the non-NER-related neurological and developmental clinical features except for the TTD-specific brittle hair and nails (see Table 3). On the basis of many of the non-NER related features, CS, together with TTD, is considered a progeroid syndrome. As discussed in more detail below (see also chapter 4), these observations have suggested additional involvement of the CS proteins in transcription itself (in analogy with XPB/XPD) and in coupling transcription to other repair processes (Table 4).

The dual involvement of TFIIH in NER and transcription is well established, and it has been proposed that mutations affecting its function in transcription initiation may be the cause of the typical CS and TTD features. In this scenario, CS and TTD are considered transcription/repair syndromes (10, 203). Similarly, it can be hypothesized that the CS proteins may have an additional function in

Table 3. Clinical features of NER syndroms

Feature	*XP*	*XP/CS*	*CS*	*TTD*
Skin abnormalities				
Photosensitivity	++	++	+ *	+*
Pigmentation abnormalities	++	+	-	-
Skin cancer	++	+	-	-
Brittle hair and nails	-	-	-	+
Ichtyosis	-	-	-	+
Developmental				
Growth defect	-	+	+	+
Hypogonadism	-	+	+	+
Neurologic				
Progressive mental degeneration	+/-	+	+	+
Wizened face	-	+	+	+
Primary defect:				
Neuronal loss	+	-	-	-
Neurodysmyelination	-	+	+	+

*Also CS and TTD patients exist without photosensitivity and NER defect.
Table adapted from Bootsma *et al.*, 1997 (11).

transcription. However, the fact that disruption of the CS genes is compatible with life in man (62, 184) as well as in mice (191) and in yeast (194) indicates that their role in transcription must be auxiliary. Mutations in the CS proteins may impair the release of blocked transcription (193). In addition they may cause subtle defects most likely during the elongation step of transcription (154, 174, 177, 188, 189, 192).

Cells from CS patients (CS-A and CS-B) and from XP-G patients with severe CS symptoms were shown to be sensitive to ionizing radiation in addition to UV-light (92). A specific defect in TCR of at least some type of ionizing radiation-induced oxidative damage, namely thymine glycols, was shown to underlie this sensitivity (28). These results have indicated an additional role of CSA, CSB and XPG in coupling arrested transcription to BER, besides NER, suggesting a general repair-transcription coupling deficiency to have a major impact on the severity of the CS phenotype. Since oxidative DNA damage can arise from intracellular metabolic processes, inefficient TCR of those subset of oxidative lesions which block transcription, such as thymine glycols, has been proposed to contribute to the developmental defects and the aging-related symptoms of CS (28, 109). Since specific mutations in XPB and XPD can give rise to the clinical symptoms associated with CS, TFIIH is predicted to function in TCR of oxidative damage as well.

Recently, several mouse models have been generated to study the consequences of NER and/or TCR deficiency *in vivo* (34). Mouse models for CS were generated by inactivating the CSB (CSB $^{-/-}$) or the CSA (CSA$^{-/-}$) gene and greatly contribute to the understanding of CS ((191); Van der Horst, unpublished results). CSB-deficient mice exhibit photosensitivity, impaired TCR, and specific ocular symptoms, whereas the other hallmarks of CS, such as growth failure and neurological dysfunction are present only in a mild form. Interestingly, in contrast to what is noted with the human condition, CS-B mice present predisposition to developing skin cancer after exposure to high UV doses. Several explanations can account for this seemingly inter-species discrepancy, including differences in apoptotic trigger, UV exposure (CS patients are often hospitalized), and repair parameters (GGR of CPDs is more potent in man), as discussed in details elsewhere (34, 191).

Importantly, a strong synergistic effect on neurological and developmental abnormalities was observed in double mutant mice CSB$^{-/-}$/XPC$^{-/-}$ and CSB$^{-/-}$/XPA$^{-/-}$, which suffer from severe neurological and developmental problems and die before weaning (G. van der Horst, unpublished observations). The fact that XPA$^{-/-}$ (totally defective in NER) and XPC$^{-/-}$ mice (specifically defective in GG-NER) do not show any obvious developmental abnormalities indicates that a NER defect itself, similarly to the human condition, does not cause the CS-specific symptoms (reviewed in (34)). However, the CSB defect in combination with a complete NER impairment, gives rise to the more severe CS phenotype, suggesting an additional function of CSB beyond NER. Similarly, inactivation of XPG, which has a CS-related additional function beside its role in NER (28), gives rise to a dramatic phenotype in mice, causing post-natal growth failure and premature death (60). In conclusion, the picture emerging from the analysis of mice with a CS defect combined with a NER deficiency (CSB$^{-/-}$XPA$^{-/-}$, CSB$^{-/-}$ /XPC$^{-/-}$ and XPG$^{-/-}$) support the hypothesis of an additional function of the CS proteins beyond NER.

Cockayne Syndrome: genotype-phenotype relationship

As discussed previously, the CSA and CSB genes are involved in the classical form of CS, the CS-B group comprising ~80% of the patients. However, no obvious gene-specific differences are apparent between CSA and CSB patients. Moreover, a wide clinical heterogeniety has been observed, including a 13-year-old boy with UV^{s}-like manifestations (118) and three patients with the severe form of CS but no sun-sensitivity (25) all due to mutations in the CSB gene. Molecular analysis of the inactivating mutations in CSB in 16 patients revealed a wide spectrum of mutations, most resulting in a severely truncated CSB protein (108). However, the severity of the disease does not seem to correlate with the nature of the mutation, suggesting that other factors are involved in determining the pathological phenotype (25, 108).

Mutations in the NER factors XPB, XPD and XPG can give rise to a complex phenotype with clinical features of both CS and XP (XP/CS) (11). XP-B patients

are extremely rare, probably reflecting the essential role of XPB in transcription initiation, and they all combine XP features either with CS or TTD (11, 12, 178). On the other hand, over 30 XP-D patients, including XP/CS and TTD, have been described. Consistent with a subordinate role of XPD in transcription, the consequences of a point mutation in XPD on transcription initiation are apparently

Table 4. Genetic heterogeneity of Cockayne syndrome

*Human Factor**	*Protein size (aa)*	*Requirement in NER*		*Properties of human factor*	*Additional involvement's beyond NER*	*Clinical implications*
		GGR	*TCR*			
CSA	396	-	+	Contains WD-40 repeats	RNAP II transcription? Base excision repair	CS
CSB	1493	-	+	Contains SWI/SNF-like helicase domain; DNA dependent ATPase	RNAP II transcription Base excision repair	CS
TFIIH:						
XPB	782	+	+	3'-5' helicase	RNAP II transcription Cell cycle regulation? Base excision repair?	XP-CS TTD
XPD	760	+	+	5'-3' helicase	RNAP II transcription Cell cycle regulation? Base excision repair?	XP XP-CS TTD
XPG	1186	+	+	Structure-specific endonuclease	Base excision repair	XP XP-CS

See text for references. *References for identification of these factors and their involvement in NER are listed elsewhere (11, 36).

less severe and are compatible with life. A complete deletion of the XPD gene has never been found. The genotype-phenotype relationship among XP-D patients is one of the most complex. Despite the complexity, distinct classes of mutation, specific for the disease, have been described and a correlation with the clinical phenotype has been proposed (10, 203, 219). A defect in XPD helicase activity is expected to affect NER, but not transcription, and consequently result in the classical XP phenotype (24, 219). Consistent with this view, XPD helicase activity

seems to be essential for NER, while being dispensable for transcription (178, 218). Mutations causing structural alterations of the XPD protein are thought to affect also its transcription function and give rise to the XP/CS phenotype (10, 24, 203). Finally, more severe mutations affecting XPD stability are thought to cause the additional symptoms of TTD patients (219).

Similarly to XPB and XPD, causative mutations in XPG are apparently linked to the clinical phenotype. The onset of the severe XP/CS symptoms correlates with the inability to produce full-length XPG protein and to perform TCR of oxidative damage (28, 127). On the other hand, missense mutations inactivating XPG endonuclease activity specifically affect NER and are found in patients with the classical form of XP (28, 84, 127). This suggested that certain domains of the XPG protein are involved in interactions important for its role in BER (28).

Interplay between NER and transcription: dual function of the CS proteins?

The discovery of the TCR pathway represented the first indication of a functional connection between DNA repair and basal transcription. The tight interplay between the two processes was further uncovered by the dual involvement of the transcription factor TFIIH in the NER reaction (reviewed in (66)). As mentioned before, a dual role has also been proposed for the CSA and CSB proteins (193). Several observations point to an additional, although not essential function in transcription regulation: 1) as discussed in this chapter, except sun sensitivity, the severe clinical symptoms of CS cannot be easily explained by a defect in DNA repair; 2) reduction of the RNA synthesis rate has been claimed in CS cells (4); 3) the CSB protein interacts with RNA polymerase II both *in vivo* and *in vitro* and with stalled ternary complexes *in vitro* (154, 174, 192): 4) CSB has been shown to enhance *in vitro* transcription (V. van den Boom and H.T. Timmers personal communication), and particularly the elongation step (154); 5) both CSA and CSB play a role in the UV-induced modification of RNA polII by ubiquitination (13, 143); 6) conventional and confocal microscopy analysis in living cells expressing a GFP-CSB tagged protein revealed a close correlation between the nuclear distribution of CSB with the transcriptional activity of the cell (chapter 10). In light of some of these findings, the link between CSB and transcription will be discussed in the next chapter.

Chapter 4

Effect of DNA damage on transcription elongation

General features of transcriptional elongation by eukaryotic RNA polymerase II

The synthesis of messenger RNA in eukaryotes is catalyzed by RNA polymerase II (RNAP II), a protein complex composed of nine subunits. Transcription requires the concerted action of a large number of proteins and protein complexes and proceeds in a multi-stage reaction. Five distinct steps have been recognized: preinitiation complex assembly on a promoter sequence, initiation, promoter clearance, elongation and termination. A set of proteins known as RNAP II basal transcription factors (TFII), including TFIID, TFIIB, TFIIE, TFIIF and TFIIH, are essential for recognition of the promoter sequences by RNAP II and subsequent transcription initiation (129, 145). In addition to optimal preinitiation complex formation and promoter-specific transcriptional activators, also the elongation step plays an important role in controlling gene expression (147, 157).

The carboxy-terminal domain (CTD) of RNAP II largest subunit is thought to play a decisive role in regulating the early stages of elongation. The CTD is composed of multiple heptad repeats (with the amino-acid consensus sequence YSPTSPS) which undergo reversible phosphorylation during the transcription cycle (32). The CTD is hypophosphorylated when RNAP II initiates RNA synthesis, and becomes hyperphosphorylated when the transcript is about 25 bases long (128). The hyperphosphorylated form of RNAP II is found engaged in ternary elongation complexes (15, 128), while the hypophosphorylated form is preferentially associated with preinitiation complexes (21). On the other hand, specific phosphatase activities have been purified from Hela cells and from *S.cerevisiae* which dephosphorylate the CTD, stimulating incorporation of RNAP II in preinitiation complexes and as a consequence transcription (22, 86).

Phosphorylation of the CTD facilitates the interaction of this domain with proteins that regulate the transition to productive elongation and with factors involved in pre-mRNA processing. At early elongation stages, the interplay between negative and positive elongation factors is thought to determine whether the RNAP II will enter processive elongation or will abort the transcript. At present, this regulatory network includes two negative elongation factors (DSIF and NELF)

which cooperate to repress RNAP II elongation, their action being counteracted by the CTD kinase P-TEFb (206, 207, 229, 230).

Recent experiments have revealed that RNAP II transcription and pre-mRNA processing are functionally coupled and may occur simultaneously. The current model suggests that mRNA synthesis and processing are coordinated by a "mRNA factory", in which the phosphorylated CTD may function as a platform for the assembly of pre-mRNA processing complexes that catalyze capping, splicing and cleavage/polyadenylation (7, 29). Interestingly, physical interactions have been discovered between the phosphorylated CTD and several RNA processing factors (7). Moreover, *in vitro* assays revealed that RNAP II participates directly in polyadenylation and splicing, suggesting that the CTD not only recruits processing factors, but also regulates their activity (64, 65).

Transcription elongation factors

An RNAP II engaged in processive elongation is target of an increasing number of elongation factors, whose function is to assure efficient transcription of active genes (147, 157). Most of the known elongation regulatory proteins seem to have a general function and not to be targeted to specific DNA sequences. Regulatory factors are needed to modulate elongation both on naked DNA templates as well as on reconstituted chromatin templates. Efficient transcription through nucleosomes is stimulated by FACT (for facilitates chromatin transcription) and by the human SWI/SNF chromatin remodeling complex ((14, 130, 131); see also chapter 5). RNAP II is very inefficient in *in vitro* transcription assays, proceeding at rates of about 100-300 nucleotides/minute on naked DNA, compared to the 1200-2000 nucleotides/ minute *in vivo* (157). Moreover, a number of obstacles can induce the RNAP II to pause or terminate. These includes natural pause sites where the DNA helix is bend, like in the c-Myc and H3.3 genes (82), DNA-binding drugs, DNA-binding proteins, nucleotide depletion and DNA lesions. *In vitro* transcription assays on naked DNA templates revealed the existence of at least two groups of elongation factors, most of which seem to target directly the RNAP II complex. One group includes factors that increase the catalytic rate of elongation and suppress pausing, like TFIIF, elongin, holo-ELL, ELL2, Tat-SF-1. Recently, also the CSB protein has been shown to enhance the rate of transcription (V. van den Boom and H.T. Timmers personal communication) and specifically stimulate elongation *in vitro* by suppressing pausing (154). Proteins that suppress transcriptional arrest, like SII and P-TEFb, belong to the second group (Figure 1). SII promotes the passage of RNAP II thorugh transcriptional arrest sites *in vitro* by activating a cryptic endoribonclease activity intrinsic of the RNAP II, which cleaves the nascent transcript upstream of its 3' end. This transcript shortening appears to be required for the realignment of RNAP II catalytic site with the new 3' hydroxyl terminus, followed by re-estention of the nascent transcript (146, 147). This mechanism provides repetitive opportunities for the arrested RNAP II to escape

transcriptional arrest. However, impediments like CPDs located on the transcribed strand (see below) cannot be overcome solely by SII activity (38, 181).

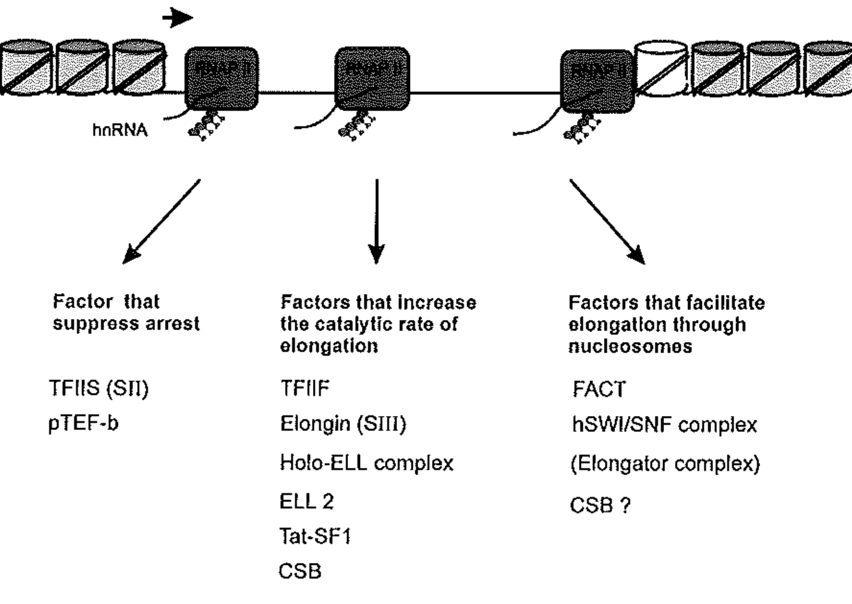


FIG. 1. RNA polymerase II (positive) transcription elongation factors. RNAP II that has already left the promotor and is engaged in processive elongation is schematically depicted. General elongation factors presumably act on the RNAPII ternary elongation complex and function together to sustain elongation at physiologic rates (14, 130-132, 146, 147, 154, 158, 221). Nucleosomes: a compact nucleosome structure is indicated in gray, whereas an altered conformation (unfolded and/or with putative acetylated histones) is represented in white.

Transcription elongation arrest by DNA lesions

A delicate situation arises when a transcribing RNAP II encounters a lesion on the DNA. It has been established *in vitro* that different types of lesion constitute an absolute physical block to RNAP II translocation. These includes bulky lesions as UV-induced CPDs (38) and adducts generated by chemical processes (N-2-acetylaminofluorene, psoralen crosslinks (37, 212)), as well as less distorting lesion such as oxidized bases (thymine glycol (61, 69)) (reviewed in (180)). Only DNA

injuries on the transcribed strand arrest RNA synthesis. The same lesions on the non-transcribed strand induce only temporary pausing of the RNAP II, which is eventually overcome, given time (37, 38).

What is the fate of RNAP II complexes stalled at a site of DNA damage?

One of the crucial aspects of transcription-coupled DNA repair is the processing of the RNAP II arrested at a lesion site. Different scenarios have been proposed. Theoretically, a stalled RNAP II constitutes a steric hindrance for the repair machinery and needs to be removed to allow repair of the damage (38, 57). Biochemical assays have been developed to gain insight into the molecular events that characterize this most likely, key step in TCR. They make use of DNA templates containing a specifically located lesion in the transcribed strand and purified RNAP II and general transcription initiation factors to perform RNA synthesis. Several experiments showed that RNAP II stops at CPDs as well as at AAF-induced adducts and at psoralen crosslinks (37, 38, 153, 212). RNAP II arrests very close to a CPD and covers ~ 35 nt around the lesion (153, 181). Interestingly, the conformation of RNAP II complexes arrested at a CPD and at an intrinsic pause site is similar, suggesting mechanistic similarities for the processing of the lesion (181). Indeed, elongation factor SII, when added in excess to the reaction induced backtracking of the RNAP II, as observed at natural arrest sites, sufficient for the repair enzyme photolyase to gain access to the lesion (181). However, no dissociation of RNAP II nor translesion bypass was observed. Most importantly, the ternary complex RNAP II/RNA/DNA was surprisingly stable and was competent in re-elongation (38, 153). This remarkable finding is supported by the results of Wang and colleagues who succeeded in isolating elongation complexes from HeLa extracts arrested at psoralen crosslinks (212). Extreme stability of the ternary complex is also observed at natural pause sites (205).

Based on the aminoacid sequence of CSB (SWI2/SNF2 family of ATPases), an enzymatic activity required for the release of arrested RNAP II was predicted. In addition, the structure of CSA suggests a regulatory role. However, CSB, alone or in combination with CSA, cannot promote the dissociation nor the backtracking of RNAP II from a damage DNA site *in vitro*, independent from the cause of transcriptional arrest (nucleotide starvation, the presence of a CPD or a natural pause site) (154, 155, 174). On the contrary, CSB seems to position and stabilize the RNAP II at a CPD site, by promoting the addition of 1 nt to the major transcript, enhancing transcription through a CPD-induced pause site, and by counteracting the transcript shortening catalyzed by TFIIS (154). Also, in a highly defined experimental system containing only purified RNAP II without transcription initiation factors, CSB can stably bind the ternary complex RNAP II/RNA/DNA engaged in transcription elongation (174). These findings strongly suggest that mechanisms other than RNAP II dissociation may mediate TCR in human.

However, at least two mechanisms exist that can potentially release a blocked RNAP II. First, a human transcription termination factor, known as factor 2 (HuF2),

has been isolated that releases short transcripts prematurely terminated at early elongation stages (98). Interestingly, HuF2 was also shown to rapidly disrupt ternary complexes arrested at a CPD (59). *In vivo*, this mechanism may account for the rapid processing of stalled complexes when repair is not accomplished. Second, RNAP II largest subunit was found to be ubiquitinated after UV-irradiation and to be subsequently targeted to proteosomal degradation (13, 143). Unreleased RNAP II ternary complexes may constitute an obstacle for further DNA-transacting processes. Since this modification of RNAP II is defective in CS cells, it has been proposed that TCR could involve degradation of arrested RNAP II and discard of the transcript (13, 143).

These findings indicate that the ternary complex stalled at a lesion is in a delicate situation and its fate *in vivo* is probably the result of a network of interactions between positive and negative regulatory factors. The state of a lesion-blocked ternary complex is presumably similar to elongation complexes arrested at natural pause sites, which are subjected to dynamic structural rearrangements (205). RNA polymerase (in prokaryotes as well as eukaryotes) is believed to function as an oscillating enzyme that can move backwards or forward depending on several parameters (182, 205). These parameters include the specific DNA sequence being transcribed, the relative stability of the DNA-DNA or RNA-DNA base pairing downstream of the transcription bubble, the interactions with regulatory proteins/transcription elongation factors and perhaps the chromosomal context (182, 205). During TCR, CSB is predicted to participate in the conformational changes of the ternary complex in order to allow repair and resumption of transcription. CSB's ability to induce topological changes in plasmid DNA (chapter 9) may be relevant in this respect, by influencing the stability of the DNA-DNA/RNA or protein-DNA interaction at the transcription bubble. The fact that ternary complexes are extremely stable, suggests that termination of transcription may be, in the majority of the circumstances, a less favourable solution. However, bypass of the lesion or fall off of the RNAPII may occur (99, 205). Besides being essential for TCR, CSB may have a more general function in the regulation of transcription elongation at natural occuring pause sites ((154, 174, 192); see also chapter 3).

The observed subtle transcription elongation activity of CSB might be more pronounced on a more natural transcription template, such as nucleosomal ones. The predicted CSB structure (as indicated in chapter 3) suggests a potential role of this protein in chromatin remodeling, analogous to the founder of this family, SWI2/SNF2 (31).

Chapter 5

Nucleotide excision repair in chromatin: affinities with transcription

Basic features of chromatin organization

The human genome contains about 3 x 10^9 nucleotide pairs containing more than 60.000 coding sequences (39). As mentioned before, if completely stretched, the DNA contained in a human cell would reach approximately 2 meters in length. Within the eukaryotic nucleus, association with histones and non-histones proteins allows the packaging of DNA into a highly compact and organised structure known as chromatin.

The nucleosome core particle is the fundamental unit of chromatin organization. It is composed of 146 bp DNA wrapped in 1.65 superhelical turns around an octameric core consisting of two copies of each histone protein, H2A, H2B, H3 and H4 (87). Nucleosome cores can be easily reconstituted *in vitro* by mixing core histones with DNA. This is because the histones are capable to self-assembly into octamers in solution and their binding to the DNA can be accomplished by simple salt gradient dilution (226). Recently, the high resolution (2.8 Å) crystal structure of an *in vitro* reconstituted nucleosome was determined and revealed the details of histone-histone and histone-DNA contacts within the core particle (106). Each histone consists of a three-helix domain (the histone fold) and of highly basic sequences of 25-40 aminoacids, referred as "tails". Except for histone H2A, the position of the "tails" is amino-terminal to the histone-fold domain. Histone "tails" do not participate in the structure of the core particle but rather protrude, forming a platform for interactions with protein complexes that regulate chromatin structure in a positive or negative manner (58, 107).

In addition to interacting with chromatin regulatory proteins, the core histone "tails" are involved in internucleosomal interactions required for chromatin condensation and formation of higher orders of chromatin structure, the highest order being the chromosome. Nucleosome core particles are connected by linker DNA, to which the linker histone H1 is bound, and repetitively occur every ~200 base pairs (bp) in the genome. A linear array of nucleosomes is folded in the nucleus to form a highly condensed chromatin fiber, known as the "30 nm" fiber, the three-dimensional organization of which is still poorly understood (233). Interestingly, recent scanning force microscopy studies of reconstituted chromatin fibers, have demonstrated the specific requirement of H3 and H1 for condensation of the nucleosome chain (95). Also, histone H3 N-termini have been shown to

occupy a strategic position in the nucleosome particle, being situated at the entry and exit points of the DNA, and have been proposed to interact with neighboring nucleosomes in the chromatin fiber. In the crystal structure, H4 tails contact the exposed face of an H2A-H2B dimer of an adjacent nucleosome (106).

The histone tails undergo a variety of post-translational modifications including acetylation, phosphorylation, methylation, ADP-ribosylation and ubiquitination (164, 165). These covalent modifications have an important role in altering chromatin higher order structure, thereby influencing nucleosomal accessibility and gene expression. In particular, numerous observations have revealed the link between histone acetylation and gene activation (53, 79, 225). Accordingly to the current view, the histone tails, by engagements in multiple interactions, play a crucial role in determining the chromatin organization and function of any given region of the genome.

Transcription and chromatin

Chromatin is a highly heterogeneous and dynamic structure. It undergoes reversible local rearrangements during all DNA-metabolic processes, including DNA replication, transcription, recombination and repair as well as chromosome condensation for mitosis or meiosis. It has become widely accepted that modulation of chromatin structure is needed to increase the accessibility of DNA processing enzymes to nucleosomal DNA and, as a consequence constitutes an important regulatory mechanism.

In transcriptionally inactive regions of the genome, chromatin is highly compacted and referred to as heterochromatin. However, when eukaryotic genes become transcriptionally active, the structure of the chromatin in their neighbourhood changes to allow binding of transcription factors and the passage of RNAP II. In vitro, both initiation and elongation are strongly inhibited by the presence of nucleosomes (78, 227). The modulation of chromatin structure requires both the actions of transcription factors and of protein complexes that support conformational changes within nucleosomes. Two major classes of chromatin modifying complexes have been characterized. The first includes complexes that covalently modify the core histones by acetylation or deacetylation, namely acetyltranferases (HATs) and deacetylases (HDACs) complexes respectively. Acetylation frequently correlates with activation of gene expression, while deacetylation correlates with transcriptional silencing (53, 85, 136). The second class includes chromatin remodeling complexes, which use the energy from ATP-hydrolysis to alter the nucleosomal conformation. These latter were originally discovered as positive regulators of transcription. However, their involvement in transcriptional repression has recently been shown as well (190). A complex network of functional interactions between HATs, HDACs, chromatin remodeling complexes and transcription factors regulates transcription in chromatin and is

subject of intensive studies (79, 83, 139, 225). An overview of the chromatin remodeling complexes isolated so far is discussed below.

ATP-utilizing chromatin remodeling complexes

Several different multisubunit chromatin remodeling complexes have been identified, most of which are strongly conserved through evolution (Table 1). They all share the ability to alter specific characteristics of chromatin structure using the energy of ATP hydrolysis. The common denominator is that each of these remodeling machines contains an ATPase subunit belonging to the SWI2/SNF2 family, which functions as enzymatic core (30, 138). The additional polypeptides possibly play a structural and/or regulatory role.

Homology within the central helicase-like domain defines the SWI2/SNF2 family members. Sequence similarities outside this domain characterize evolutionary distinct subfamilies (40). Proteins belonging to the SNF2, the ISWI (also known as SNF2L) and the CHD subfamilies are part of chromatin remodeling complexes (83). Other subfamilies comprise proteins involved in a variety of cellular processes, such as various aspects of DNA repair, including NER (RAD16 and CSB/ERCC6), recombinational pathways (RAD54) and post replication repair (RAD5) (Figure 1). The chromatin remodeling capacity of most of these latter proteins has not yet been established.

Different classes of remodeling complexes with specific activities can be identified (Table 1). The yeast SWI/SNF and the Drosophila NURF complexes are the founding members of the two best-studied classes of remodeling complexes, SWI/SNF and ISWI, respectively (17, 137, 187).

SWI/SNF complexes display similar activities in vitro. By altering nucleosome conformation they facilitate the binding of transcription factors to promoter sequences. This activity, known as nucleosome disruption, is visualized by widespread changes in the DNAse I cleavage pattern of reconstituted mononucleosomes and suggests an alteration of the DNA path around the histone octamer (18, 31, 88). Nucleosome disruption by SWI/SNF seems to proceed through the formation of a stable intermediate, a remodeled nucleosomal species that retains all the histones and can revert to the original stucture (104, 151). On reconstituted plasmid chromatin, SWI/SNF complexes reduce the negative supercoiling induced by nucleosomes (73, 140). In addition, some of these complexes disrupt the periodicity of regularly spaced nucleosome arrays (G.Schnitzler and R.E. Kingston personal communication; see also chapter 9).

In contrast to the SWI/SNF family members, the ISWI-containing remodeling complexes display markedly different properties in vitro (16, 198). One characteristic activity of all ISWI complexes except NURF is nucleosome spacing,

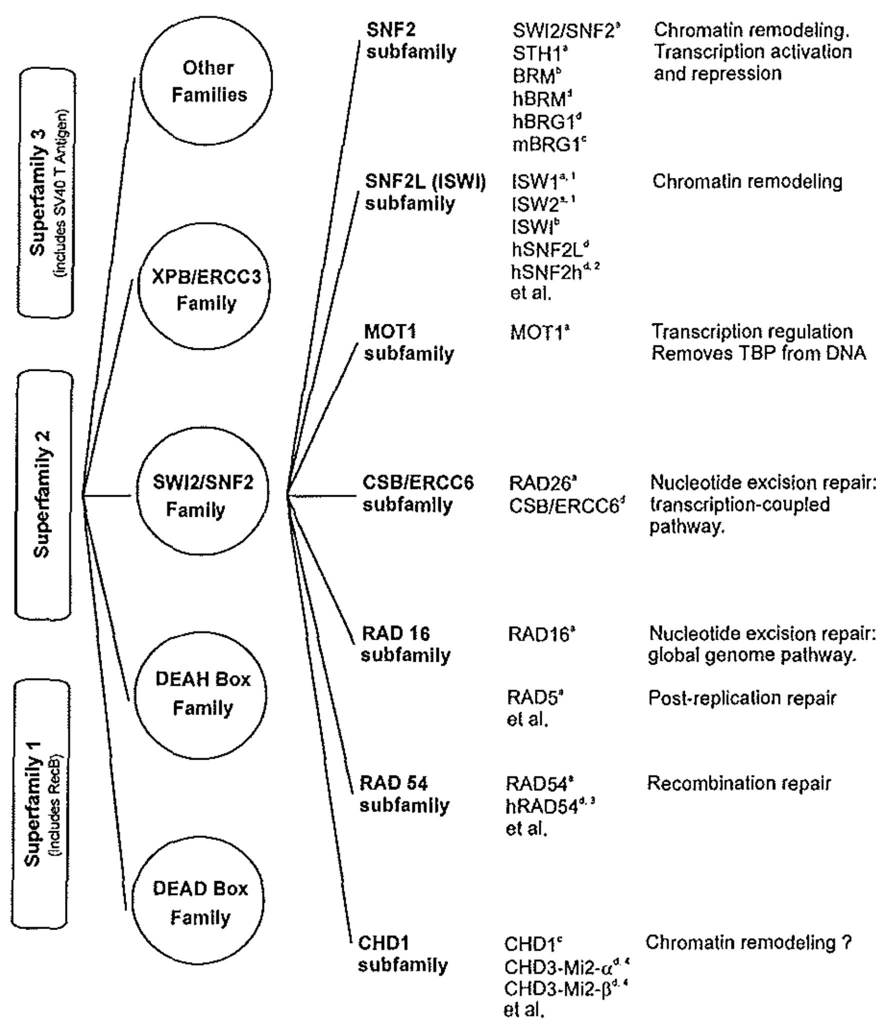


FIG. 1. Helicases and related protein families with conserved NTP binding motifs. Proteins belonging to the SWI2/SNF2 family contain conserved NTP-binding motifs present in several DNA and RNA helicases. However, none of the SWI2/SNF2-like proteins tested so far display true helicase activity. The figure is adapted from Pazin (1997) (135) and based on the classification of Gorbalenya (1993) and Eisen (1995) (40, 51). Selected examples of families and representative proteins are shown, as well as their involvement in cellular processes. A more complete list of proteins and their original references are available from Eisen (1995). References for the more recent identified proteins are: [1], (186); [2], (2); [3], (81); [4], (224) a: S.cerevisiae. b: D. melanogaster. c: Mus musculus. d: H. sapiens.

that is the ability to create an ordered array of nucleosomes with uniform spacing from a disordered nucleosomal template (74, 186, 199). On the other hand, only NURF, ACF and ISW1 can perturb chromatin structure and facilitate transcription factors binding to promoter sequences in different situations (74, 186, 187). In addition, each complex displays unique activities. NURF, like the SWI2/SNF2 complexes, can disrupt mononucleosomes in the absence of transcription factors (187). CHRAC does not possess this activity but facilitates access of restriction enzymes to plasmid chromatin (89, 199). ACF acts together with histone chaperones to mediate the assembly of regularly spaced nucleosome arrays, showing that chromatin remodeling and assembly are closely connected (74).

A third class of remodeling complexes is represented by the NuRD-Mi2 complex (also known as NURD or NRD) whose central ATPase belongs to the Mi2-CHD family (85). Interestingly, NuRD-Mi2 possesses both nucleosome remodeling and histone deacetylation activities and constitutes a physical and functional link between these two chromatin-modifying activities.

Basic models of chromatin remodeling

To explain the chromatin remodeling activity displayed by the SWI2/SNF2 related complexes, a general, unifying hypothesis has been proposed (83). According to this model, chromatin remodeling machines would facilitate the dynamic transitions between different nucleosomal states, which are already intrinsic to nucleosomes per se (113). These chromatin states could differ in either the position of nucleosomes or in the histone-DNA contacts. A remodeled or "activated" state, with weakened histone-DNA contacts has actually been detected during remodeling reactions by SWI/SNF complexes (104, 151). A similar intermediate has been proposed to be induced by the ISWI-based complexes (56).

Both the SWI/SNF complex, as well as the ISWI-based NURF and CHRAC can mobilize nucleosomes by a mechanism that involves sliding or tracking of the histone octamers along DNA (56, 89, 217). However, only SWI/SNF and RSC can actually displace histone octamers so that they can be transferred to acceptor DNA in trans (105, 133). Movement of the histone octamer involves the breaking of interactions between DNA and histones. How this is achieved and how exactly the histone-DNA contacts are altered is not known. Current models are based on the central idea that during remodeling, only a small stretch of histone-DNA contacts are broken at a time, in a mechanism similar to the passage of RNAP II through nucleosomes (166). Dissociation of DNA from the histone octamer is thought

Table 1. Overview of SWI2/SNF2-related chromatin remodeling complexes

Remodeling complex	*ATPase subunit*	*Organism*	*Size*
SWI/SNF family			
ySWI/SNF	SWI2/SNF2	*S.cerevisiae*	2 MDa
RSC	STH1	*S.cerevisiae*	~1 MDa
dSWI/SNF (Brahma)	BRM	*D. melanogaster*	2 MDa
hSWI/SNF	BRG1	*H. sapiens*	~2 MDa
	hBRM	*H. sapiens*	~2 MDa
ISWI family			
ISW1	ISW1	*S.cerevisiae*	N.D.
ISW2	ISW2	*S.cerevisiae*	N.D.
NURF	ISWI	*D. melanogaster*	500 kDa
CHRAC	ISWI	*D. melanogaster*	670 kDa
ACF	ISWI	*D. melanogaster*	220 kDa
RSF	hSNF2h	*H. sapiens*	~500 kDa
Mi-2/CHD family			
Mi2- complex	Mi-2/CHD	X. leavis	1-1.5 MDa
NURD	Mi-2/CHD	*H. sapiens*	~1.5 MDa

Table adapted from Tyler and Kadonaga, 1999 (190)

N. of subunits	*Comments*	*References*
11	Required for transcription activation and repression of specific genes	(31, 102, 133)
15	Essential for mitotic growth. Share subunits similarities with SWI/SNF complex; two forms of rRSC have been defined	(18, 19)
≥7	Essential for cell viability	(41)
~ 9 ~ 9	Human SWI/SNF complexes contain either BRG1 or hBRM, while sharing similarities in the additional subunits; tissue-specific subunit heterogeneity is observed. Alterations in the hSNF5/INI1 component are associated with malignant rhabdoid tumors. BRG1 is essential for viability.	(72, 88, 169, 204, 210, 211)
4	Nucleosome disruption and spacing activities.	(186)
2	Nucleosome spacing activity	(186)
4	Nucleosome disruption activity, thereby facilitating transcription factor binding to promotor sequences. Contains the WD protein p55	(50, 110, 185, 187)
5	Nucleosome spacing activity; contains topoisomerase II	(199)
2	Nucleosome spacing and assembly activities	(74, 75)
2	Facilitates transcription on nucleosomal templates *in vitro*	(94)
6 ≥7	NuRD, NRD, NURD and Mi2 complex are related or identical. Complex contains the ATPases Mi-2α/CHD3 and/or Mi2-β/CH4. Mi-2 is dermatomyosistis-specific autoantigen. Nucleosome disruption and histone deacetylase activity. HDAC1/HDAC2 subunits are histone deacetylase enzymes. Interacts with methylated DNA.	(208) (231, 179, 228, 232)

to start at one edge of the nucleosome, followed by the binding of an adjacent stretch of DNA to the exposed core histones. This would create a DNA loop that propagates through the chromatin template. Remodeling complexes could induce this movement directly by translocating along the DNA, or indirectly by changing the conformation of DNA (for example by twisting the DNA at the edges) or by influencing the histone octamer structure (135, 183, 190, 198).

A precise comparison of the activities of all known remodeling complexes is difficult, because of the variety of *in vitro* assays in which they have been tested so far. Some of the observed differences in activity may be in part related to the different assays applied. However, SWI/SNF and ISWI complexes substantially differ in the cofactors required for their ATPase activity (31, 90, 186, 187). In addition, they appear to target different sites on the nucleosome (50, 54, 103, 187). These differences, together with the distinct activities displayed *in vitro* suggest that SWI/SNF and ISWI complexes may use slightly different mechanisms to promote specific changes in chromatin structure (83).

Nucleotide excision repair and chromatin

The molecular mechanism of NER within chromatin is still poorly understood. The interplay between chromatin structure and DNA repair involves chromatin influences both on damage formation and on damage accessibility by the repair proteins. On the other hand, DNA lesions which induce distortions in the DNA helix may directly affect chromatin structure by influencing nucleosome stability or by altering the rotational setting of nucleosomal DNA (161). Thus, the specific chromatin configuration at a given damage site is the result of a complex network of different parameters.

Influence of nucleosome structure on NER

DNA repair enzymes need to gain access to damaged DNA in all chromatin domains immediately after DNA injury. Evidence that chromatin structure hinders the repair process comes from in vitro as well as in vivo studies.

In vitro repair of UV-induced lesions by NER on chromatin templates with a defined sequence has not yet been tested. However, two different studies using human whole cell extracts showed that repair efficiency is significantly reduced on SV40 minichromosomes and on plasmid DNA assembled into chromatin compared to naked DNA (167, 214). Nucleosome structure limits also damage accessibility by the photo-reactivating repair enzyme photolyase, since repair by photolyase is inefficient on *in vitro* reconstituted nucleosomes (150).

The *in vitro* data are supported by a number of *in vivo* experiments performed in a yeast strain containing a minichromosome that carries an active gene (URA1), a replication origin (ARS1) and has a well-characterized chromatin structure (162). A direct correlation between chromatin structure and DNA repair in the non-

transcribed strand of the URA1 gene was recently shown (215). Repair was much faster in linker DNA and in the 5', more accessible, region of the nucleosome compared to the internal protected regions (215). Also, photoreactivation is more efficient in non-nucleosomal regions and in linker DNA in a yeast minichromosome (170). Additionally, increasing chromatin accessibility by histone acetylation enhances overall repair synthesis, as shown in sodium-butyrate (which produce a global increase in the acetylation levels of chromatin) treated human fibroblasts (141).

Dynamic chromatin rearrangements at repair sites

Chromatin rearrangements during NER have been observed in cultured mammalian cells using biochemical or light microscopy analysis (114, 161). The transient changes in the nucleosomal organization during NER were analyzed by nuclease digestion, both by MNase and by DNAseI and led to the proposal of the "unfolding/refolding" model (119, 161). The initial "unfolding" step is characterized by increased nuclease sensitivity of newly repaired DNA and indicates the need for unfolding the nucleosomal structure to allow access to repair enzymes. Shortly after repair (approximately 20 minutes) the repaired DNA regions become nuclease resistant, indicative of nucleosomes deposition, and suggest a relative quick nucleosome "refolding". A later, slower phase is important for the correct repositioning of the nucleosomes to restore the original chromatin structure. This indicates the need for chromatin assembly machines/mechanisms for a proper completion of the repair event (48, 49).

Chromatin unfolding during NER: GGR versus TCR

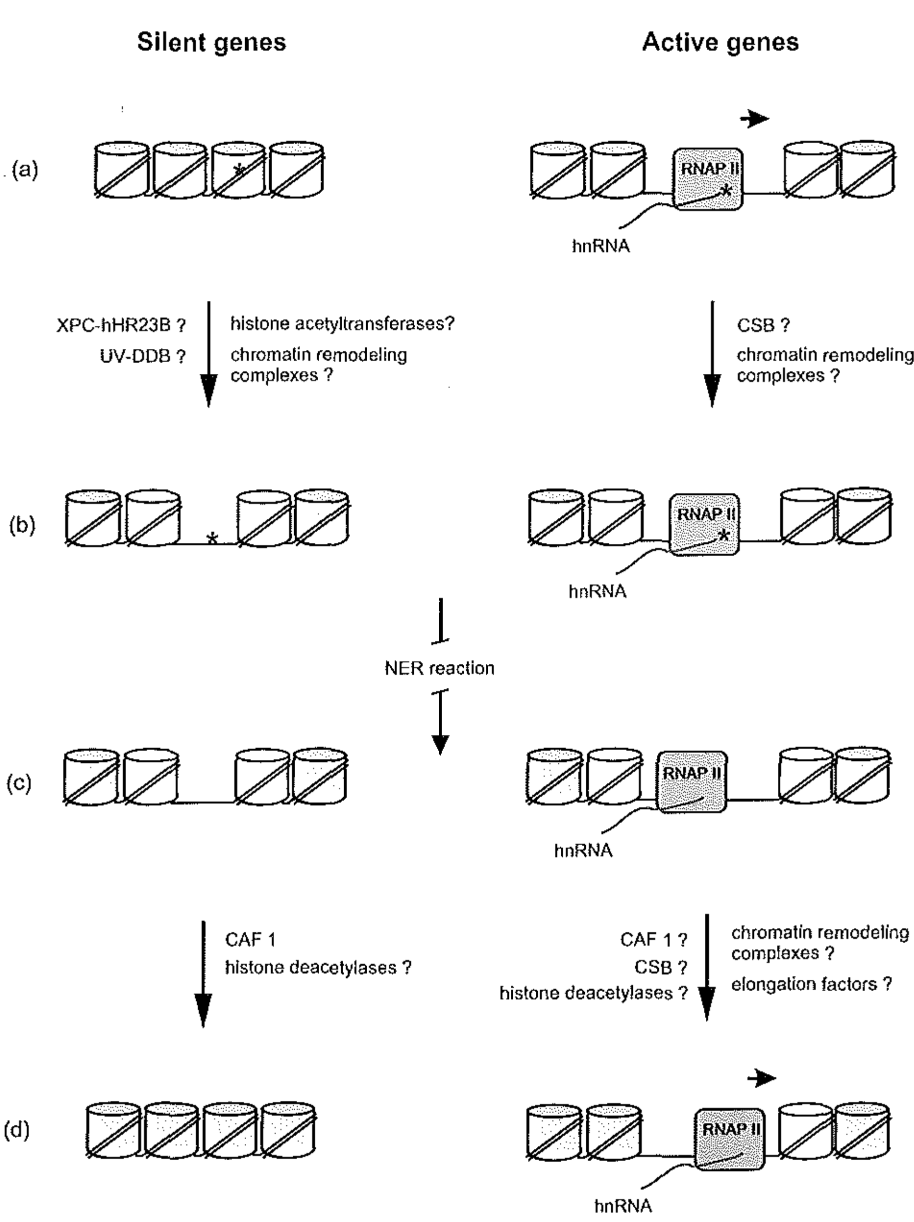
A repair event is associated with an unfolded and more accessible chromatin structure, both at the nucleosomal level as well as at higher levels of chromatin organization (161). However, very little is known about the proteins and the molecular mechanisms involved in these transitions. Nucleosome rearrangements are needed not only to allow recognition of the lesion but possibly also during further repair steps, since the human repair complex needs about 100 bp of DNA to excise a DNA adduct *in vitro* (70). In addition, not only nucleosome structure and positioning, but also the transcriptional activity of a given genomic region can influence the rate of repair. The more open chromatin structure associated with transcriptionally active sites may account for differences in accessibility to the repair enzymes both within GGR (between distinct genomic regions) as well as between GGR and TCR (see Figure 2). GGR and TCR may require different proteins/mechanisms to promote specific chromosomal rearrangements.

Concerning the GGR pathway, it has been suggested that chromatin unfolding may be partly supported by NER factors involved in early steps of the reaction. Such a role has been proposed for the human proteins UV-DDB (142) and XPC (5), as well as for the yeast Rad7-Rad16 protein complex (202). Human XPC cells and yeast *rad7* and *rad16* mutants have a similar phenotype, being defective in the

global genome repair of non-transcribed DNA (124, 201, 202). Mainly two properties of the Rad7-Rad16 complex have suggested a possible role in chromatin remodeling to facilitate access of the NER factors to DNA lesions in transcriptionally inactive DNA. First, Rad7 was found to interact with the SIR3 proteins, which are involved in the formation of transcriptionally silent DNA in yeast (134). Secondly, Rad16 belongs to the SWI2/SNF2 family of chromatin remodeling factors (40). In addition, Rad7-Rad16 is not required for *in vitro* NER on naked DNA templates (55). As mentioned before, also the TCR pathway requires the function of an SWI2/SNF2-like protein, namely CSB ((184); see also chapter 3). This raises the possibility that, in analogy with transcription, specialized chromatin remodeling factors may function in the regulation of NER in chromatin.

During TCR, the repair enzymes may take advantage of the more accessible chromatin conformation at actively transcribed genes, which would facilitate the recognition step. In support of this idea, studies using yeast minichromosomes have shown that the rate of repair is enhanced in transcribed regions (6, 162). However, within the chromatin context of an active gene, substantial differences are still observed between repair of the transcribed (TS) and the non-transcribed (NTS)

FIG. 2. Model for chromatin rearrangements associated with nucleotide excision repair. (a) a transcriptionally-inactive gene packaged in nucleosomes (left) and a transcriptionally active gene with elongating RNA polymerase and altered nucleosome structure. (b) The recognition and repair of DNA lesions is associated with unfolding of the chromatin structure to allow access to the repair machinery (161). During GGR within silent genomic regions (left) unfolding may involve the function of UV-DDB protein (142) and XPC-hHR23B complex (5), possibly with the help of nucleosome remodeling activities and histone modification by acetyltransferases (114, 141). Also a still unidentified human homologs of Rad7-Rad16 may be involved (202). Actively transcribed regions (right) are characterized by a more accessible nucleosome structure. Maintenance of this and/or the space required for the NER machinery might be provided by nucleosome remodeling factors, among which CSB (chapter 9). For simplicity only the transcribed strand including a lesion is illustrated (see text). (c) NER factors gain access to the damage and perform the repair reaction (see (36, 222)). (d) Regeneration of the original chromatin structure (left) probably occurs simultaneously with DNA repair synthesis and requires the function of chromatin assembly factors (CAF1) (48, 49). (Right) Within actively transcribed genes, chromosomal rearrangement may first require the action of nucleosome remodelling and/or transcription elongation factors (including CSB) to favor the re-start of transcription elongation. Alternatively, dissociation of RNAP II may have occurred after transcription blockage (b) (see chapter 3 and 4), followed by regeneration of an inactive chromatin structure (not illustrated). The figure is adapted fromThoma, 1999 and Meijer and Smerdon, 1999 (114, 175). Only factors potentially involved in chromatin rearrangements are illustrated. * indicates a NER lesion. Nucleosomes: a compact nucleosome structure is indicated in gray, whereas an altered conformation (unfolded and /or with putative acetylated histones) is represented in white.


Silent genes
Active genes
(a)
RNAP II
hnRNA
XPC-hHR23B ?
UV-DDB ?
histone acetyltransferases?
chromatin remodeling complexes ?
CSB ?
chromatin remodeling complexes ?
(b)
RNAP II
hnRNA
NER reaction
(c)
RNAP II
hnRNA
CAF 1
histone deacetylases ?
CAF 1 ?
CSB ?
histone deacetylases ?
chromatin remodeling complexes ?
elongation factors ?
(d)
RNAP II
hnRNA

strand. Studies both on minichromosomes (215) and on the chromosomal URA3 gene in yeast (176) have shown that repair of the TS is generally fast and independent from the chromatin environment, whereas removal of lesions from the NTS is less efficient and strongly influenced by positioned nucleosomes. This suggests that the transcription machinery involved in elongation plays a dominant role in TCR, likely determining the preferential repair rates in the TS. According to this model, the transcriptional activity prevails over the influence of chromatin environment in NER of the TS.

Transcription-dependent chromatin rearrangements are very dynamic. In particular, after transcription inactivation, re-deposition of nucleosomes occurs by a fast process, suggesting that DNA is rapidly reassembled into nucleosomes behind the transcribing RNAP II (20). As a consequence of transcription blockage at DNA lesions, chromatin rearrangements might take place around a stalled RNAP II. The chromatin remodeling activity of CSB, and possibly of other remodeling factors, may be needed to maintain and/or to generate an open chromatin conformation favorable to damage recognition and processing by the NER machinery (chapter 9) (see Figure 2). The *in vivo* studies based on minichromosomes do not allow this detailed analysis.

NER-dependent chromatin refolding: GGR versus TCR

Repair-associated chromatin assembly was addressed in vivo in Xenopus oocytes and in vitro using repair-competent human cell extracts (supplemented with Xenopus egg extracts to support chromatin assembly), or Drosophila embryo extracts (48, 49, 119). Interestingly, nucleosome deposition was initiated from a specific NER site on plasmid DNA and occurred simultaneously with repair-induced DNA synthesis. A single-strand nick in the DNA made by the NER apparatus might trigger nucleosome deposition. Importantly, these experiments suggest a mechanistic coupling between the two processes. Complementation studies revealed an essential role of the chromatin assembly factor 1 (CAF1) in this repair-dependent nucleosome assembly (48).

Concerning the NER-dependent chromatin assembly a fundamental difference distinguishes GGR from TCR. After repair of the DNA damage, silent genomic regions can be refolded into an inactive chromatin configuration. In contrast, transcription on active genes may resume and require factors that promote an open chromatin structure permissive to restart elongation (see Figure 2). CSB might participate in the chromatin remodeling/rearrangements at this stage to favor resumption of transcription by RNAPII (chapter 9).

Chapter 6

The Cockayne syndrome B protein, involved in transcription-coupled DNA repair, resides in an RNA polymerase II-containing complex.

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EMBO J. 16:5955-5965 (1997)

The Cockayne syndrome B protein, involved in transcription-coupled DNA repair, resides in an RNA polymerase II-containing complex

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Transcription-coupled repair (TCR), a subpathway of nucleotide excision repair (NER) defective in Cockayne syndrome A and B (CSA and CSB), is responsible for the preferential removal of DNA lesions from the transcribed strand of active genes, permitting rapid resumption of blocked transcription. Here we demonstrate by microinjection of antibodies against CSB and CSA gene products into living primary fibroblasts, that both proteins are required for TCR and for recovery of RNA synthesis after UV damage *in vivo* but not for basal transcription itself. Furthermore, immunodepletion showed that CSB is not required for *in vitro* NER or transcription. Its central role in TCR suggests that CSB interacts with other repair and transcription proteins. Gel filtration of repair- and transcription-competent whole cell extracts provided evidence that CSB and CSA are part of large complexes of different sizes. Unexpectedly, there was no detectable association of CSB with several candidate NER and transcription proteins. However, a minor but significant portion (10–15%) of RNA polymerase II was found to be tightly associated with CSB. We conclude that within cell-free extracts, CSB is not stably associated with the majority of core NER or transcription components, but is part of a distinct complex involving RNA polymerase II. These findings suggest that CSB is implicated in, but not essential for, transcription, and support the idea that Cockayne syndrome is due to a combined repair and transcription deficiency.
Keywords: Cockayne syndrome/CSB/nucleotide excision repair/RNApolymerase II/transcription-coupled repair

Introduction

Nucleotide excision repair (NER) is a universal and verstatile DNA repair pathway capable of removing a large variety of DNA lesions from the genome, including UV-induced cyclobutane pyrimidine dimers and bulky chemical adducts. NER entails a multistep cut and paste reaction in which damaged bases are excised from the DNA as a 24–32 base oligonucleotide, followed by gap-filling DNA synthesis and ligation (reviewed in Hoeijmakers, 1994; Wood, 1996; for repair in general, see Friedberg *et al.*, 1995). Although, in principle, NER acts on the entire genome, a profound heterogeneity exists in the efficiency with which at least some types of 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 (Smerdon and Thoma, 1990; Brouwer *et al.*, 1992), a clear link exists between transcription and repair efficiency (Bohr, 1991). Bohr *et al.* (1985) 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. Interestingly, it turned out that this transcription-coupled DNA repair (TCR) pathway enhances only the repair of the transcribed strand of active genes, while the non-transcribed strand is repaired at a slower rate, similar to that of the global genome (Mellon *et al.*, 1987).

Cells derived from Cockayne syndrome (CS) patients display a selective defect in the TCR pathway, while global genome repair is unaffected (Venema *et al.*, 1990a; Van Hoffen *et al.*, 1993). This strongly suggests that the two CS genes, *CSA* and *CSB*, are required for TCR. In support of this concept, we found that disruption of the *Saccharomyces cerevisiae* (Van Gool *et al.*, 1994) and mouse (Van der Horst *et al.*, 1997) homologues of *CSB* results in impairment of TCR. A suggested model for the TCR reaction (Mellon *et al.*, 1987) involves lesion detection by a transcribing RNA polymerase that is stalled because of the presence of DNA injury. Subsequently, the CSA and CSB proteins are thought to permit access to the damage by inducing either retraction (Hanawalt, 1992) or dissociation of the blocked RNA polymerase. Simultaneously, they may recruit the NER machinery, thus accomplishing the fast repair of the lesion and rapid resumption of the vital process of transcription (Troelstra *et al.*, 1992; Hanawalt *et al.*, 1994). The presence of the Swi2/Snf2-like ATPase domain in CSB is intriguing in this respect, since other members of the Swi2/Snf2 subfamily have been shown to be able to remodel or disrupt protein–DNA interactions (reviewed in Pazin and Kadonaga, 1997).

The clinical symptoms suggest that more processes than TCR alone are affected in CS, since a number of CS features cannot be attributed easily to a sole repair impairment. The consequences of a total NER deficiency are 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, frequently accelerated neurodegeneration is observed (Bootsma *et al.*, 1997). Despite the fact that the NER defect in CS patients is only partial, the syndrome displays many extra and more severe symptoms than the totally deficient XP-A individuals. CS shares increased photosensitivity with XP, but is in addition associated with seriously impaired physical and sexual development, and severe neurological abnormalities including mental retardation, spasticity, deafness and patchy demyelination of neurons (Nance and Berry, 1992; Bootsma *et al.*, 1997). Patients with CS features combined with XP have been found in XP groups B and D, which carry mutations in TFIIH, a multi-subunit factor involved in both NER and basal transcription (Vermeulen *et al.*, 1994). The origin of many of the CS features was postulated to be due to a subtle defect in transcription rather than in the repair function of the TFIIH complex, affecting the expression of a specific set of genes (Vermeulen *et al.*, 1994). Following this reasoning, CSA and CSB could also fulfil a (non-essential) role in the transcription process itself in addition to mediating transcription–repair coupling (discussed in Van Gool *et al.*, 1997). Here we present a characterization of the function of CSB in repair and transcription, including an analysis of proteins associated with CSB.

Results

Characterization of polyclonal anti-CSB and anti-CSA antibodies

The crude anti-CSB serum reacted with several proteins in immunoblot analysis of a HeLa whole cell extract (WCE), among which was a 168 kDa protein (Figure 1, lane 1). This represents the CSB protein, because: (i) it has the predicted molecular weight and co-migrates with *in vitro* translated CSB protein; (ii) the antiserum immunoprecipitates *in vitro* translated CSB (Figure 1B); (iii) the immunoreaction with the 168 kDa protein can be competed for specifically by pre-incubating the crude antiserum with a GST–CSB fusion protein (Figure 1, lane 2); (iv) the band is missing in a WCE derived from CS1AN-Sv cells that lack the C-terminal part (amino acids 337–1493) of CSB (Troelstra *et al.*, 1992) (Figure 1, lane 3) against which the antiserum was elicited, while the band reappears when the cells are transfected with the (double-tagged) *CSB* cDNA (Figure 1D, last lane); and (v) following affinity purification, the serum strongly stained the 168 kDa protein (Figure 1, lane 4), while occasionally an 80 kDa protein of unknown identity is recognized as well (Figure 1D).

The affinity-purified CSB antiserum was used to screen all known NER-deficient human complementation groups (XP-E not tested). Apart from the CS-B WCE, all other extracts contained comparable amounts of the CSB protein (Figure 1E), ruling out that mutations in other NER factors indirectly affect the cellular level of CSB, in contrast to what has been observed for, for example, the ERCC1/XPF (Van Vuuren *et al.*, 1995; Sijbers *et al.*, 1996a) and XRCC1/ligase III (Caldecott *et al.*, 1995) complexes.

The affinity-purified anti-CSA antiserum, strongly reacting with a very low amount (2 ng) of GST–CSA fusion protein on immunoblots (Figure 1, lane 8), recognized several proteins in a HeLa WCE, among which was one of 44 kDa (lane 9). This band is selectively absent in an extract of CSA-deficient CS3BE-Sv cells (lane 10), while the size matches *in vitro* translated CSA (lane 11), indicating that the 44 kDa protein is CSA.

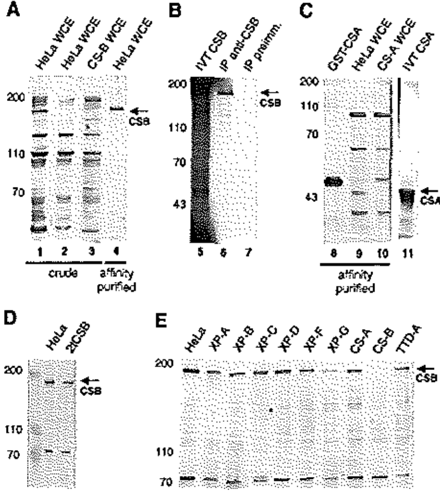

Fig. 1. Characterization of anti-CSB and anti-CSA antibodies.
(A) Specificity of the anti-CSB serum. Immunoblots of HeLa or CS-B (CS1AN-Sv) WCEs were incubated with: crude anti-CSB serum (lanes 1 and 3), crude anti-CSB serum pre-incubated with GST–CSB fusion protein (lane 2) and affinity-purified anti-CSB serum (lane 4). The molecular weight of pre-stained marker proteins is indicated. (B) The anti-CSB antiserum immunoprecipitates *in vitro* translated CSB protein. *In vitro* translated CSB (lane 5) was incubated with either crude anti-CSB serum (lane 6) or pre-immune serum (lane 7) and binding to protein A 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-Sv (CS-A) (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) Overexpression of *CSB* cDNA does not lead to elevated protein levels. A WCE of CS1AN-Sv cells transfected with the tagged *CSB* cDNA (2tCSB), under control of the SV40 promoter (see below), is analysed on immunoblot with a HeLa WCE. (E) CSB is specifically absent in CS-B WCE, but not in other repair-deficient extracts. Equal amounts (10 μg) of the indicated WCEs were analysed on immunoblots for the presence of CSB. The 80 kDa cross-reacting band provides a convenient internal control on differences in protein loading.

Intracellular localization of CSB

The presence of a consensus sequence for a nuclear localization signal in CSB and its central role in TCR predict that CSB is located in the nucleus. This was confirmed by immunofluorescence studies (Figure 2). Although the gene is very weakly expressed (Troelstra *et al.*, 1993), clear CSB staining was observed in the nuclei, but not the cytoplasm, of HeLa cells (Figure 2A). The CS1AN-Sv cells show no staining of CSB at all (Figure 2A), confirming the immunoblot results shown above. UV irradiation of HeLa cells, prior to fixation and

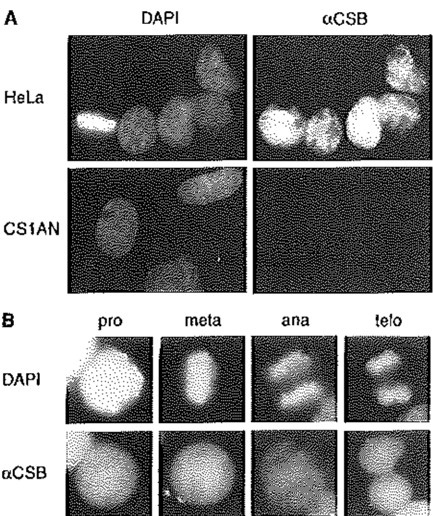

Fig. 2. The CSB protein is localized in the nucleus. (A) The affinity-purified anti-CSB antibody was used to stain the endogenous CSB protein in HeLa or CS-B (CS1AN-Sv) cells. The left panel displays the DAPI-stained chromosomal DNA, while the right panel depicts CSB staining, visualized by FITC-conjugated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

staining, did not alter the immunofluorescence pattern of CSB (data not shown). Previously, the DNA repair complex XPC/HHR23B was found to display an anaphase/telophase-specific association with chromatin (Van der Spek *et al.*, 1996). In contrast, in the majority of metaphase cells, CSB co-localizes with the microtubules of the mitotic spindle, which mediate the segregation of the chromosomes to the spindle poles (Figure 2A and B) (Hyman, 1995).

Function of the CSA and CSB proteins in vivo and in vitro

To gain more insight into the biological function of CSA and CSB *in vivo*, the specific antisera were microneedle injected into living primary human fibroblasts and the effects on TCR, transcription and RNA synthesis recovery were analysed (Figure 3, Table I). Repair activity is reflected by the level of UV-induced unscheduled DNA synthesis (UDS), determined by [^{3}H]thymidine incorporation after UV exposure, whereas transcription levels were quantitated by pulse labelling with [^{3}H]uridine (Van Vuuren *et al.*, 1994; Vermeulen *et al.*, 1994) (see Materials and methods). The contribution of TCR to total repair synthesis (measured as UDS 2 h after UV exposure) is small, because most repair synthesis is derived from the global genome repair subpathway, particularly from the efficient removal of UV-induced 6/4 photoproducts. This is apparent from the low residual UDS in XP-C cells, that are defective in global genome repair and only perform TCR (Venema *et al.*, 1990b; Carreau and Hunting, 1992).

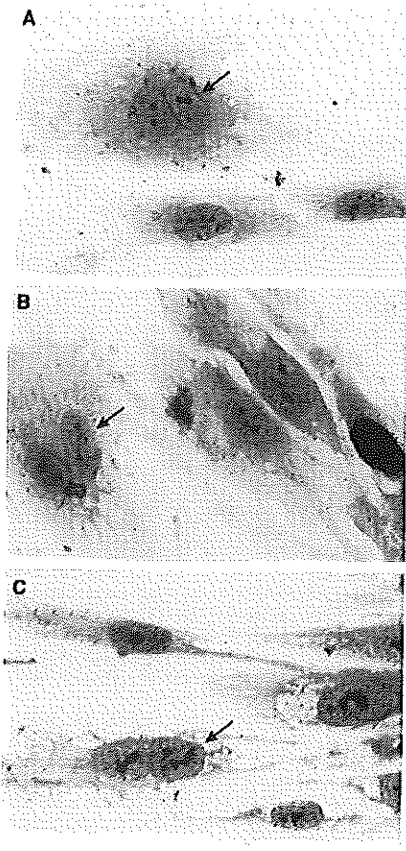

Fig. 3. Microinjection of anti-CSB and anti-CSA antisera inhibits transcription-coupled repair but not transcription *in vivo*. XP-C (XP21RO) fibroblasts were microinjected with anti-CSB (A) or anti-CSA (B) antisera to assay the effect on TCR (visualized by unscheduled DNA synthesis, UDS), or in wild-type (C5RO) fibroblasts to determine the effect on transcription levels (C). (A–C) show micrographs of injected cells (indicated by an arrow) and uninjected (surrounding mononuclear) cells, assayed for NER or transcription by [^{3}H]thymidine or [^{3}H]uridine pulse labelling respectively, as described in Materials and methods. The fibroblast with the dark nucleus in (B) was in S-phase during the [^{3}H]thymidine incubation. The quantification of the microinjections is shown in Table I.

Therefore, to analyse the effect on TCR, we used XP-C fibroblasts for microinjection. Two independent CSA antisera appeared to inhibit the residual UDS of XP-C fibroblasts (which is 10% of repair-competent cells analysed in parallel) by a factor of 2–2.5, while the preimmune serum had no effect (Figure 3B, Table I). 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 XP-C fibroblasts (Figure 3A, Table I).

Table I. Effect of CSA and CSB antibody injection on TCR, recovery of RNA synthesis and transcription

Injected antiserum	% Residual UDS XP-C[a]	% Recovery of RNA synthesis[b]	% Transcription
None	100	100	100
Pre-immune	100	95	–
Anti-CSA (#1)	47	–	109
Anti-CSA (#2)	40	65	104
Anti-CSB (#1)	22	–	105
Anti-CSB (#2)	15	61	99

Wild-type and XP-C fibroblasts were microinjected with anti-CSA and anti-CSB antisera as explained in Figure 3. Percentages are calculated by comparing injected versus uninjected cells on the same slide, with a typical SEM of 5%. '#1' and '#2' are independent antisera.
[a]100% corresponds to 19 grains, which represents 10% of the UDS observed in wild-type cells that were treated in parallel.
[b]Recovery of RNA synthesis is defined by the ratio of transcription levels in UV-irradiated wild-type cells relative to unirradiated cells. In wild-type cells, RNA synthesis at 24 h post-UV had recovered to 70% of that of untreated cells. This value is set at 100%. CS1AN-Sv (CS-B) cells that were included in the experiment displayed a recovery of 13%, relative to wild-type values.
–: not determined.

A hallmark of CS cells is the failure to recover RNA synthesis after UV irradiation, which is thought to be the consequence of the defect in TCR (Mayne and Lehmann, 1982; Troelstra *et al.*, 1992). Microinjection of the anti-CSA and anti-CSB (but not the pre-immune) antisera into repair-proficient fibroblasts significantly inhibited the recovery of RNA synthesis after UV irradiation (Table I). This indicates that both antisera are capable of inhibiting the function of CSA and CSB *in vivo*, and provides direct evidence for the involvement of these proteins in the TCR and RNA synthesis recovery pathways.

Similarly, both antisera were injected into wild-type fibroblasts to see whether inhibition of CS proteins has an effect on overall RNA synthesis. However, no significant difference was observed between injected and non-injected cells (Figure 3C, Table I), suggesting that neither CSA nor CSB make a major contribution to transcription of undamaged cells *in vivo*.

To test whether CSB (and/or CSB-associated proteins) are required for repair and transcription *in vitro*, we conducted immunodepletion experiments using repair- and transcription-competent HeLa whole cell extracts. As shown in Figure 4, depletion of CSB performed under low stringency conditions (upper panel) had no significant effect on *in vitro* repair (middle panels) or basal transcription activities (lower panel). The latter finding is in agreement with the microinjection experiments and confirms that CSB is not essential for RNA synthesis. The absence of a significant effect of CSB depletion on *in vitro* NER is consistent with the notion that the *in vitro* NER reaction mainly reflects transcription-independent NER. The above findings also imply that CSB in WCE is not associated with critical quantities of essential NER and basal transcription factors, as detectable in *in vitro* assays.

Superdex gel filtration of HeLa whole cell extract

We next investigated whether or not CSB is complexed with other proteins by performing fractionations of repair- and transcription-competent HeLa WCE (Figure 5) on hydrodynamic volume. Superdex S-200 gel filtration was

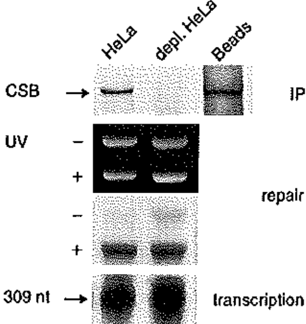

Fig. 4. Complete CSB immunodepletion has no significant effect on *in vitro* repair and basal transcription. A repair-proficient HeLa WCE was immunodepleted using crude anti-CSB serum under low stringency (buffer A) conditions. Complete CSB depletion from the extract, and binding to the beads was verified 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 vitro* transcription (bottom panel) assays. Immunodepletion using the pre-immune serum did not precipitate the CSB protein and, consequently, this had no effect on the *in vitro* repair activity (not shown).

performed under physiological conditions, identical to those in which *in vitro* activity assays are conducted. Although denatured CSB has a molecular weight of 168 kDa, the CSB protein present in HeLa WCE chromatographed at an estimated hydrodynamic size of >700 kDa (Figure 5A), suggesting that it may be part of a protein complex. Similar findings were made on Sephacryl S-300 and S-500 columns (data not shown). Furthermore, purified functional recombinant CSB analysed in parallel did behave very differently from the natural CSB in WCEs under various conditions (data not shown). The native elution profile of other protein complexes, such as ERCC1/XPF (majority running at a hydrodynamic size of 280 kDa) and XPB (TFIIH) (500 kDa) (Figure 5A), and also HHR23A (70 kDa) and HHR23B (140 kDa) (data not shown), were as found before and in the expected size range (Van Vuuren *et al.*, 1995; Van der Spek *et al.*, 1996), making aspecific aggregation of proteins in the extract unlikely. The migration profile of RNA polymerase II largely coincided with CSB (Figure 5A). These findings show that CSB in repair and transcription-competent WCE migrates in a manner distinct from many other NER factors.

Surprisingly, the 44 kDa CSA protein eluted at a hydrodynamic size of 420 kDa, distinct from CSB (Figure 5B). Moreover, in the CS1AN-Sv extract (lacking CSB), the same apparent size of CSA was found, strongly suggesting that CSA is also part of a complex and that the major part of CSA is not stably associated with CSB in these extracts.

To investigate the stability of the possible CSB complex, Superdex fractionation was performed under different salt conditions. When the salt concentration was increased from 0.1 to 0.5 and 1.0 M KCl, the apparent hydrodynamic size of CSB decreased from >700 kDa to ~500 and 450 kDa respectively, still considerably larger than the

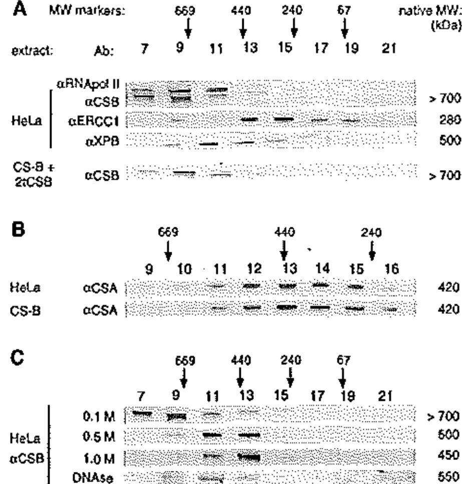

Fig. 5. CSB resides in a large molecular weight complex that does not include CSA. (A) Various WCEs were size-fractionated on a Superdex-200 column, loaded in the Manley-type buffer A containing 0.1 M KCl. Elution fractions were tested by immunoblotting using the indicated antisera. The native sizes 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 double-tagged CSB (see below). (B) The native size of CSA is unchanged by severe CSB 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 deletion of amino acid 337 to the end (amino acid 1493) of the CSB protein (Troelstra *et al.*, 1992). (C) The native size of CSB is dependent on salt concentration and DNase pre-treatment. 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 immunoblots using anti-CSB antiserum. Elution of the molecular weight marker proteins was hardly affected by the altered salt conditions.

denatured size of CSB and corresponding to the native size of CSB after pre-treatment of the HeLa WCE with DNase (Figure 5C).

Taken together, these results suggest that in repair- and transcription-competent cell-free extracts CSB resides in a protein complex, different from many NER proteins and complexes, that might be bound to DNA at low, but not at high salt.

Identification of CSB-co-purifying proteins

To determine the identity of the CSB-associated proteins, first co-purification of any known repair and transcription factors with CSB was investigated. For this, we assayed fractions of the purification scheme used to isolate basal transcription factors and complexes required for RNA polymerase II transcription (Gerard *et al.*, 1991). On the first column (heparin–Sepharose, Figure 6A), CSB eluted at 1.0 M KCl, excluding co-purification with TFIIA, TFIIB, TFIID, TFIIH and the ERCC1/XPF complex (Gerard *et al.*, 1991; Van Vuuren *et al.*, 1995). The XPA and XPG proteins eluted in the heparin 0.4 M and

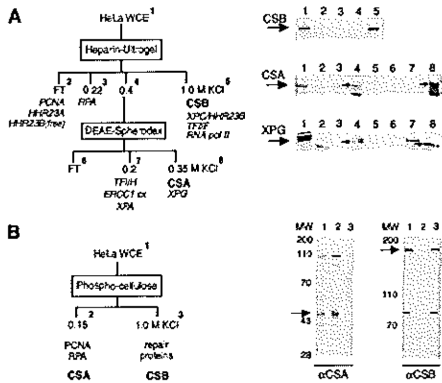

Fig. 6. CSB does not co-purify with the majority of the tested repair and transcription proteins. (A) Elution fractions of the indicated purification scheme were tested by immunoblot analysis for the presence of CSB, CSA and XPG. The presence of functional ERCC1, XPA, XPG and XPC was tested on immunoblots and by microinjection and *in vitro* complementation (Van Vuuren *et al.*, 1994, 1995; A.P.M.Eker and W.Vermeulen, personal communication). The purification of TFIIF, TFIIH, RNA polymerase II and other transcription proteins was described previously (Gerard *et al.*, 1991). (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 by immunoblot analysis for the presence of CSA and CSB, while the fractionation of the other proteins was described earlier (Shivji *et al.*, 1992).

subsequently in the DEAE 0.2 and 0.35 M KCl fraction respectively, as tested by immunoblot (Figure 6A) and *in vitro* and *in vivo* complementation (A.J.van Vuuren and W.Vermeulen, unpublished observations). Surprisingly, CSA eluted 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, which is clearly distinct from CSB. Apart from CSB, the heparin 1.0 M KCl fraction also contains the XPC/HHR23B complex (Van der Spek *et al.*, 1996) and, interestingly, also TFIIF and RNA polymerase II (Gerard *et al.*, 1991).

Next, we employed phosphocellulose column chromatography, frequently used to separate NER core factors (Shivji *et al.*, 1992; Aboussekhra *et al.*, 1995). The fraction that is not bound to the column at low salt (CF-I) contains replication protein A (RPA) and proliferating cell nuclear antigen (PCNA), while the bound fraction (CF-II) contains all other proteins required for *in vitro* NER (Shivji *et al.*, 1992). Remarkably, CSB is present exclusively in CF-II, while all detectable CSA is present in CF-I (Figure 6B). This again indicates that the majority of CSA and CSB are not stably associated. More specifically, CSB elutes between 0.4 and 0.6 M KCl (fraction FIII in Aboussekhra *et al.*, 1995), excluding co-purification with the vast majority of RPA, PCNA, XPG, XPA and ERCC1/XPF (data not shown).

In conclusion, in these fractionation schemes, CSB co-fractionates with the XPC/HHR23B, TFIIF and RNA polymerase II protein complexes, but not with the other tested repair and basal transcription proteins. However,

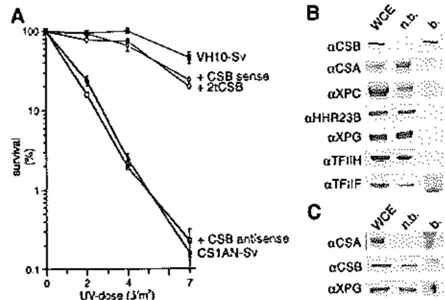

Fig. 7. Function and immunoprecipitation of tagged CSB. (A) Tagging of CSB does not interfere with its cellular function. CS-B fibroblasts were transfected with the indicated constructs, and the UV sensitivity of mass populations was determined by UV irradiation and pulse labelling with [^{3}H]TdR. □ CS1AN-Sv (CS-B), ● CS1AN-Sv + antisense CSB, O CS1AN-Sv + sense CSB, ◇ CS1AN-Sv + HA-CSB-His$_6$ (2tCSB), ■ VH10-Sv (wild-type). (B) None of the tested candidate proteins co-immunoprecipitates 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 antibodies. 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 of 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) No detectable quantities of CSB or XPG co-immunoprecipitate with CSA. The endogenous CSA protein from HeLa WCE was immunoprecipitated in buffer A using crude anti-CSA antiserum. The WCE, non-bound (n.b.) and bound (b.) proteins were tested on immunoblot using the indicated antisera.

the gel filtration studies described above suggest that the composition of the possible CSB complex changes upon increasing the salt concentration. Thus, the absence of co-purifying proteins does not exclude weak interactions that may be disrupted due to the high salt and other harsh conditions used for elution.

Construction and immunoprecipitation of HA-/His$_6$-double-tagged CSB

To investigate protein–protein interactions further and to allow isolation of CSB-associating proteins under physiological conditions, we generated CSB constructs containing an N-terminal hemagglutinin antigen (HA) epitope as well as a C-terminal histidine (His$_6$) tag (see Materials 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$_6$* cDNA showed a correction of UV sensitivity to wild-type level, identical to the non-tagged version (Figure 7A). Immunoblot analysis indicated that the transfected cells contain amounts of tagged CSB protein in the normal range, excluding significant over-expression (Figure 1D). Also, the native size of tagged CSB in a cell-free extract of these transfected cells is again very large (>700 kDa) (Figure 5A), indicating that the tagged protein behaves in a similar way to the non-tagged version in the cell.

Using the HA epitope, we could completely immunoprecipitate the tagged CSB protein from a WCE of the transfected CS1AN-Sv cells (Figure 7B, upper panel). Since this was done under the same (low salt) buffer conditions as the size fractionation, we expect that the CSB-associating proteins are also 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 (Figure 7B). Clearly, no significant quantities of CSA, XPC, HHR23B, XPG, TFIIH or TFIIF could be detected in the bound fraction that contained all CSB. Similar results were obtained when CSB was immunoprecipitated using the polyclonal 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 antibody disrupts the interaction of CSB with the proteins analysed. When CSA was immunoprecipitated from HeLa cell-free extracts using the anti-CSA antiserum, neither CSB nor XPG was co-immunoprecipitated (Figure 7C). This confirms the results above, and moreover indicates the absence of significant quantities of stable CSA–XPG interactions in these extracts.

Interestingly, when RNA polymerase II was tested using antiserum against the largest subunit, a minor but significant fraction (between 10 and 15%) appeared to be bound to the beads that could be eluted under physiological conditions using competition with the synthetic HA epitope (Figure 8A). Under the same conditions, no retention of TFIIH (XPB subunit) or TFIIF (RAP74 subunit) could be detected. The specificity of this binding is demonstrated further by the fact that when a similar co-immunoprecipitation was performed using a HeLa WCE containing a non-tagged version of CSB, no CSB or RNA polymerase II could be recovered from the eluate (Figure 8B) or from the beads (data not shown). The binding of RNA polymerase II to tagged CSB was found to be resistant to high salt concentrations (Figure 8C), suggesting a direct interaction of hydrophobic character. Moreover, when the immunoprecipitation reactions were supplemented with ethidium bromide, that is known to disrupt protein–DNA interactions without affecting protein–protein interactions (Lai and Herr, 1992), similar amounts of RNA polymerase II were found to be associated with CSB (Figure 8D).

In conclusion, the combined results from the size fractionation, co-purification and immunoprecipitation experiments suggest that CSB resides in a large molecular weight protein complex that is devoid of detectable amounts of CSA, XPA, XPB and XPD (TFIIH), XPC/HHR23B, ERCC1/XPF, XPG, TFIIA, TFIIB, TFIID, TFIIF, PCNA and RPA. In contrast, CSB seems to be associated in a stable, DNA-independent manner with a significant fraction of the RNA polymerase II molecules in these protein extracts.

Discussion

Little is known about the molecular mechanism that acts upon an elongating RNA polymerase II complex blocked by a lesion. In the prokaryote *Escherichia coli*, a single transcription–repair coupling factor (TRCF) was identified that is required and sufficient to mediate TCR *in vitro* (Selby and Sancar, 1994). TRCF was shown to bind and

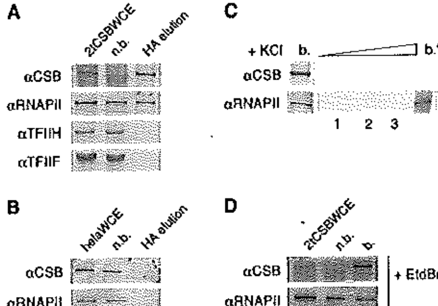

Fig. 8. A fraction of RNA polymerase II is stably associated with tagged CSB. (A) RNA polymerase II is immunoprecipitated with HA-tagged CSB. Proteins that were bound to the HA affinity beads after immunoprecipitation of HA-/His$_6$-double-tagged CSB from a WCE of UV-resistant CS1AN-Sv transformants (2tCSB WCE) were eluted using the synthetic peptide of the HA epitope. The WCE, non-bound (n.b.) and eluted proteins (HA elution) were tested on an immunoblot as indicated. (B) Binding of RNA polymerase II to HA affinity resin is specific for tagged CSB. A similar immunoprecipitation as in (A) was performed on a HeLa WCE that contains a non-tagged CSB protein. (C) The interaction between CSB and RNA polymerase II is resistant to high salt concentrations. Following immunoprecipitation of HA-tagged CSB from the 2tCSB WCE, beads with bound proteins were either boiled and analysed on immunoblot (b.), or incubated in buffer A with increasing amounts of KCl. Elution fractions containing 0.3, 0.6 or 1.0 M KCl (lanes 1, 2 and 3 respectively), as well as the boiled beads after salt incubation (b.*) were analysed on immunoblot as indicated. (D) CSB and RNA polymerase II do not interact via DNA. Immunoprecipitation of tagged CSB from the 2tCSB WCE was performed in the presence of ethidium bromide (EtdBr) at 50 mg/ml. Subsequently, the WCE, non-bound (n.b.) and bound (b.) proteins were tested on immunoblot.

displace a stalled RNA polymerase and, by recruiting the damage recognition protein UvrA, to stimulate repair (Selby and Sancar, 1995). In eukaryotes, the TCR reaction is probably much more complex and, hitherto, all efforts to reconstruct this system *in vitro* have failed. Even the composition of a paused transcription elongation complex is still largely unknown. Previously, cellular studies have suggested a specific role for the CSA and B proteins in TCR (Venema *et al.*, 1990a; Van Hoffen *et al.*, 1993). However, the clinical hallmarks of CS suggest that, in addition to TCR, the transcription process itself may also be affected (Vermeulen *et al.*, 1994; Hoeijmakers *et al.*, 1996; Van Gool *et al.*, 1997). Here we partially characterized the function of CSB by *in vivo* microinjection of antisera, *in vitro* immunodepletions and analysis of protein–protein interactions.

Identification of proteins interacting with CSB

Many methods used to identify protein–protein interactions employ overexpressed, *in vitro* synthesized or purified (parts of) proteins, often involving heterologous systems. One of the potential caveats in these approaches derives from the fact that the protein is studied outside of its natural context. Particularly, when the protein *in vivo* resides in a complex with multiple interaction domains, it may exhibit artificial association behaviour when examined in isolation. Moreover, overexpression may lead to incomplete synthesis or degradation of a fraction of the molecules, improper folding and lack of post-translational modification or natural partners. Therefore, it is important to verify interactions identified in such systems *in vivo* under physiological conditions or by valid genetic means.

To approach the *in vivo* situation closely, we utilized Manley-type WCEs to examine protein associations involving CSB. These extracts have been used for purification of protein complexes and are active in *in vitro* repair, transcription and splicing (Gerard *et al.*, 1991; Wood *et al.*, 1995), indicating that they contain functional multisubunit protein complexes. Indeed, we found evidence that CSB resides in a large molecular weight complex in such extracts. For analysis of complexes, it is important to utilize conditions that leave delicate protein–protein interactions as intact as possible. For this, we generated a cell line that stably expresses double-tagged CSB protein, and permits affinity purification using conditions under which *in vitro* repair and transcription are known to take place. We verified that the tags do not interfere with the CSB function (Figure 7A) and that the protein is not overexpressed (Figure 1D). Using similarly tagged TFIIH subunits, we recently have found that the HA affinity step yields a very high (>10 000-fold) purification (G.S. Winkler, G.Weeda and J.H.J.Hoeijmakers, in preparation). This implies that co-retention on the affinity column is highly specific. Our studies yielded several unexpected results.

Since we showed that TCR and recovery of RNA synthesis after UV in living human cells requires both CS proteins (Table I), we anticipated these products to be stably associated with each other. However, unexpectedly, CSA and CSB were found to be part of different complexes. (i) Superdex gel filtration indicated that they migrate with a different hydrodynamic size. (ii) CSA and CSB fractionate differently on both heparin and phosphocellulose. The latter recently was also found in an independent study (Selby and Sancar, 1997). (iii) When immunoprecipitating CSA or CSB from different extracts using tags and various antibodies, no stable association was detected. However, binding of *in vitro* translated CSA and CSB proteins to each other was found recently while an interaction in the two-hybrid system was mentioned (Henning *et al.*, 1995), indicating that under certain conditions these proteins are able to interact.

The dual role of TFIIH in repair and transcription led to the suggestion that TFIIH also plays a central role in TCR (Schaeffer *et al.*, 1993; Drapkin *et al.*, 1994). 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 (Drapkin *et al.*, 1994; Vermeulen *et al.*, 1994; Van Oosterwijk *et al.*, 1996). However, in the analysis presented here, we failed to detect any stable 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 from Manley-type WCEs as well as from nuclear extracts using an HA-tagged XPB subunit, again no indication for an association with CSB was found (G.S.Winkler, G.Weeda and J.H.J.Hoeijmakers, in preparation). In addition, no interaction between the yeast

homologue of CSB, Rad26p, and yeast TFIIH could be observed (Guzder *et al.*, 1996). However, recently, a resin containing a GST–CSB (ATPase domain) fusion protein was shown to retain XPB from cell-free extracts (Selby and Sancar, 1997).

A number of XP-G patients display characteristic CS features (Vermeulen *et al.*, 1993), possibly reflecting a disturbed XPG–CSB interaction. We have tried to detect interactions between *in vitro* translated, full-length XPG and CSB proteins by co-immunoprecipitations under various conditions, but failed to detect any association of significant quantities of either protein (data not shown). Also, the analysis in cell-free extracts presented here does not indicate any (stable) association between XPG and CSB: (i) XPG fractionates differently on heparin (Figure 6A) and phosphocellulose (data not shown); (ii) XPG does not co-immunoprecipitate with CSB using the anti-HA monoclonal antibody (Figure 7B) or using the crude anti-CSB serum (not shown); and (iii) no co-immunoprecipitation of CSB with XPG is observed using a crude anti-XPG serum (not shown). In contrast to our results, binding of an *in vitro* translated XPG protein to unlabelled, *in vitro* translated CSB was reported recently (Iyer *et al.*, 1996).

The heparin column chromatography (Figure 6A) indicated co-fractionation of CSB with the XPC/HHR23B complex, involved in global genome repair (Venema *et al.*, 1990b; Masutani *et al.*, 1994). CSB and XPC/HHR23B are involved in complementary repair pathways and, as could be expected, were found not to be stably associated (Figure 7B).

Transcription initiation/elongation factor TFIIF and RNA polymerase II are present in the heparin 1.0 M fraction. Both factors play an important role in transcription elongation: RNA polymerase evidently is the core of the elongation machinery, while TFIIF is reported to increase elongation efficiency by suppressing the time an RNA polymerase molecule pauses at intrinsic pause sites (Aso *et al.*, 1995). TFIIF was not co-immunoprecipitated detectably with tagged CSB (Figures 7 and 8), but a significant portion of RNA polymerase II was (Figure 8). This interaction was shown to be specific for CSB, resistant to high salt, and not mediated via DNA. Recently, we found that the CSB-bound RNA polymerase II is fully functional in a reconstitued *in vitro* transcription reaction, implying that it is functionally intact.

In conclusion, in Manley-type WCEs, CSB appears to reside in a large complex that includes RNA polymerase II but none of the other core repair and transcription proteins investigated. Obviously, our studies do not preclude transient interactions that may occur in the course of the TCR reaction or very fragile complexes that are disrupted during the preparation of the extracts. This may explain the interactions found using other methods for detection of protein-protein binding in the studies cited above.

The contribution of CSB to transcriptional regulation

The stable association of CSB with RNA polymerase II described in this study supports the hypothesis that CSB is involved in transcription as well. However, at this stage, one can only speculate about the nature of this involvement. It will be important to establish which form of RNA polymerase is complexed with CSB. At present, several distinct RNA polymerase II-containing complexes have been identified in eukaryotes (Kim *et al.*, 1994; Koleske and Young, 1994; Ossipow *et al.*, 1995; Maldonado *et al.*, 1996; Shi *et al.*, 1997). Generally, they entail pre-assembled transcription initiation complexes that mediate activation of a subset of genes in response to transcriptional activators. Forms of RNA polymerase II engaged in transcription elongation or termination are poorly characterized. When the CSB complex was immunodepleted, no significant effect on *in vitro* basal transcription was found, despite the fact that a fraction of RNA polymerase II was found to be complexed to CSB. Several explanations can be considered. (i) The fraction of RNA polymerase II co-depleted, estimated to be between 10 and 15%, is too small to exert a detectable effect in the *in vitro* assay. Normally RNA polymerase is not the rate-limiting factor (J.-M.Egly, unpublished observation). (ii). This form of RNA polymerase II is not detectable in the *in vitro* transcription system. Our finding that none of the transcription initiation factors are co-immunoselected with tagged CSB argues that CSB does not interact with RNA polymerase molecules engaged in transcription initiation, and is consistent with the idea that the protein may be part of an elongating type of RNA polymerase complex. Furthermore, when antisera against CSB (and CSA) are microinjected in living cells, we clearly observed inhibition of TCR and recovery of RNA synthesis after UV, while no significant decrease in basal transcription levels was noted. In contrast, microinjections of antisera against TFIIH components resulted in a drastic decrease in transcription levels (Van Vuuren *et al.*, 1994; Marinoni *et al.*, 1997). These results imply that CSB does not have a major contribution to the normal basal transcription process. This does not rule out, however, the possibility that CSB modulates the efficiency of transcription in a more subtle manner, and as a second function mediates TCR. As suggested above, CSB might function as an elongation factor that is able to release a trapped transcription complex and thus stimulate transcription efficiency, while not being essential for this process (discussed further in Van Gool *et al.*, 1997). Release of the stalled elongating RNA polymerase may involve its ubiquitination, that recently was shown to occur after genotoxic treatment of cells, and to depend on the *CSA* and *CSB* gene products (Bregman *et al.*, 1996). It should be noted that our extracts are made from undamaged cells, which are therefore not expected to perform high rates of NER. The analysis of extracts from damaged cells performing maximal TCR that could reveal other protein interactions is in progress. A multiprotein complex that specifically associates with RNA polymerase in the elongating phase recently has been isolated from yeast (J.Svejstrup, personal communication). It will be of interest to know whether a similar complex exists in human cells and, if so, whether CSB is involved. Finally, the presence of CSB in an RNA polymerase II complex is consistent with the idea that CS is in part due to impaired transcription.

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 [trichothiodystrophy A (TTD-A)]. The fibroblasts were cultured in a 1:1 mixture of Ham's F10 and Dulbecco's modified Eagle's medium (DMEM), supplemented with antibiotics and 8–10% fetal calf serum. WCEs were prepared according to Manley *et al.* (1983) as modified by Wood *et al.* (1995), dialysed against buffer A containing 25 mM HEPES/KOH pH 7.8, 100 mM KCl, 12 mM $MgCl_2$, 1 mM EDTA, 2 mM dithiothreitol (DTT) and 17% (v/v) glycerol, and stored at –80°C. The protein concentration of these WCEs was 15–20 mg/ml.

Antibodies and immunoblot procedures

Rabbit polyclonal anti-CSB antibodies were raised against the C-terminal 158 amino acids of CSB, overproduced as a protein A fusion product in *Escherichia coli* using standard protocols (Harlow and Lane, 1988). Affinity purification was done using immunoblots of a purified GST fusion protein containing the same C-terminal region of CSB, and elution using a KSCN buffer [0.1 M KPi pH 7.0, 3 M KSCN, 1 mg/ml bovine serum albumin (BSA)]. The *CSA* cDNA (Henning *et al.*, 1995) was isolated via RT–PCR from human granulocyte RNA. Rabbit polyclonal anti-CSA antibodies were raised against the C-terminal half of the CSA protein (encoding amino acids 176–396), overproduced as a GST fusion product in *E.coli*. Affinity-purified anti-CSA antibodies were obtained by incubation of the crude serum with immunoblot strips containing purified GST–CSA fusion protein, followed by elution with acidic glycine buffer (0.1 M glycine pH 1.0, 0.5 M NaCl, 0.5 mg/ml BSA). The generation and characterization of the polyclonal anti-ERCC1 (Van Vuuren *et al.*, 1993), anti-XPG (O'Donovan and Wood, 1993) and monoclonal anti-p89/XPB (Schaeffer *et al.*, 1993) antibodies have been described before.

To visualize large molecular weight proteins such as CSB (168 kDa) on immunoblot, proteins were transferred to PVDF or nitrocellulose membrane by blotting for 2–3 h at 4°C in blot buffer without methanol (25 mM Tris–HCl pH 8.3, 0.2 M glycine) with the addition of SDS to 0.01%. After blocking, the blot was incubated overnight with crude (1:500) or affinity-purified (1:400) anti-CSB serum, followed by nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) detection. For other proteins, the standard procedure for immunoblotting was followed (Harlow and Lane, 1988).

Immunofluorescence

HeLa and CS1AN-Sv cells were grown on slides, washed with phosphate-buffered saline (PBS), fixed by incubation in 2% paraformaldehyde–PBS for 10 min and permeabilized 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 h 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 h. Slides were washed and embedded in vectashield mounting medium (Brunschwig) that contained 4'-6-diamino-2-phenylindole (DAPI). DAPI-stained DNA and FITC-labelled CSB was visualized using fluorescence microscopy.

Microneedle injection of antisera and analysis of repair and transcription levels

Microinjection of immune sera into cultured fibroblasts was performed as described previously (Van Vuuren *et al.*, 1994). 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 incubated further for 24 h 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 UDS) was determined by UV irradiation of the cells (254 nm; 15 J/m^2), pulse labelling for 2 h using [^{3}H]thymidine (60 µCi/ml, sp. act. 120 Ci/mmol), fixation and *in situ* autoradiography. Grains above the nuclei of injected (polykaryon) and non-injected (monokaryon) cells were counted and compared. Levels of RNA synthesis after microinjection in wild-type cells were analysed by pulse labelling with [^{3}H]uridine (10 µCi/ml; sp. act. 50 Ci/mmol) for 1 h 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 h at 37°C. Then, the cells were UV irradiated (254 nm; 10 J/m^2) and, 24 h later, RNA synthesis was determined by [^{3}H]uridine pulse labelling as described above.

Superdex gel filtration

To fractionate proteins and protein complexes on the basis of size and/or shape (hydrodynamic volume), HeLa or CS1AN-Sv WCEs (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, containing 0.1, 0.5 or 1.0 M KCl. Fractions were collected and tested on immunoblots as described above. DNase pre-treatment of the HeLa WCE was performed by incubating 1 mg of WCE with 10 µl (10 µg/µl) DNase for 10 min at 37°C. Complete digestion of all DNA was verified by agarose gel electrophoresis.

Column fractionations

Fractions of the heparin column were obtained as previously described (Gerard *et al.*, 1991). 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 fractionated further on a DEAE–Spherodex column by elution with 0.2 and 0.35 M KCl. Phosphocellulose column chromatography was performed as described (Shivji *et al.*, 1992) by loading the 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

HA and 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, the sense primer 5' CATCGAGCTCATGTACCCATACGATGTTCCAGATTACGCTAGCCCAAATGAGGGAATCCCC 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). After DNA sequencing to check for the absence of PCR-generated mutations, a *Sac*I fragment containing the HA-tagged N-terminus of CSB was exchanged with the corresponding *Sac*I fragment in the *CSB* cDNA. 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' GTGAAACAAGAGTGAGGCCAAGG 3' (*CSB* cDNA bp 3705–3729), and the antisense primer 5' CTGGGGCCCTTAGTGATGGTGATGGTGGTGACGACCTTCGATGCAGTATTCTGGCTTGAG 3' [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 after *Apa*I digestion and exchanged with the corresponding region in the *CSB* cDNA.

DNA transfections and UV survival

CS1AN-Sv fibroblasts were transfected with pSLME6(–) (antisense *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 (Graham and van der Eb, 1973). 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 characterized further by UV survival. For this, cells were plated (2×10^5 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 [^{3}H]thymidine pulse labelling as described elsewhere (Sijbers *et al.*, 1996b).

In vitro translation and immunoprecipitations

In vitro translated CSB and CSA protein were synthesized using the TNT T7 Coupled Reticulocyte Lysate System (Promega) as described by the manufacturer. The *in vitro* translated CSB protein was immunoprecipitated in a standard manner in NETT buffer (100 mM NaCl, 5 mM EDTA, 50 mM Tris–HCl pH 7.5, 0.5% Triton X-100) using crude anti-CSB serum. Immunoprecipitation of endogenous CSB or CSA from HeLa WCEs was achieved by first incubating crude antiserum with protein A–Sepharose beads (Pharmacia) in PBS–Tween (0.5%) for 1–2 h at 4°C, followed by extensive washing (twice with PBS–Tween and four times with buffer A) and a further incubation of the antiserum-coated protein A beads with 1 mg of HeLa WCE for 5 h at 4°C. The immunodepleted HeLa extract was recovered from the beads by spinning and analysed in activity assays or on immunoblot, together with the proteins bound to the beads. Immunoprecipitation of HA-CSB-His_6 was done by incubating the monoclonal anti-HA antibody 12CA5 overnight

at 4°C with a WCE of the CS1AN-Sv cells transfected with the double-tagged CSB construct, followed by addition of protein G beads (Pharmacia) and further incubation for 5 h at 4°C. Together with the depleted extract, bound proteins were analysed by SDS–PAGE and immunoblotting after boiling the beads. Alternatively, bound proteins were eluted by incubation with a synthetic peptide encoding the HA epitope (1 mg/ml) overnight at 4°C.

In vitro repair and transcription assays

Analysis of *in vitro* repair activity was performed as described in detail before (Wood *et al.*, 1995), by mixing 100 μg of (depleted) cell-free extract with a mixture of AAF-modified and non-damaged plasmids (Van Vuuren *et al.*, 1993). Repair activity, i.e. incorporation of [α-^{32}P]dATP into the damaged plasmid, was visualized by autoradiography.

In vitro transcription activity was assayed as described before (Gerard *et al.*, 1991), by incubating 100 μg of (depleted) cell-free extract with an Ad2MLP promoter-containing template, together with the required nucleotides. The 309 nucleotide [α-^{32}P]CTP-labelled run-off transcripts were visualized using autoradiography.

Acknowledgements

We are indebted to Leontine van Unen and André Hoogeveen for performing the Superdex-200 chromatography. We thank Karin Pouwels for the RT–PCR of the *CSA* cDNA, Peter van der Spek for help in the immunofluorescence experiments, Anja Raams for isolating WCEs, Esther Appeldoorn for help in the *in vitro* repair assay, Vincent Moncollin for *in vitro* transcription and Cécile Visser for technical assistance. Sandrine Humbert and Kaoru Sugasawa are acknowledged for providing respectively the heparin and phosphocellulose fractions, Bert van der Horst, Bas Winkler and André Eker for helpful suggestions, Jesper Svejstrup for sharing unpublished observations, and Mirko Kuit for photography. This research was supported by the Commission of European Community (contract number BJ6-141-NL and CHRX-CT94-0043) and by the Dutch Scientific Organisation through the Foundation of Medical Scientific Research (contract number 900-501-093), Human Frontiers, the Dutch Cancer Society (EUR 94-763) and the Swiss National Science Foundation (grant number 31-36481.92).

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Received on May 9, 1997; revised on July 9, 1997

Chapter 7

XAB2, a novel tetratricopeptide repeat protein, involved in transcription-coupled DNA repair and transcription.

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Submitted for publication in a modified version

Abstract

Nucleotide excision repair (NER) is a highly versatile DNA repair system responsible for elimination of a wide variety of lesions from the genome. It is comprised of two subpathways: transcription-coupled repair (TCR) which accomplishes efficient removal of damage blocking transcription and global genome repair. Recently, the basic mechanism of global genome repair has emerged from biochemical studies. However, little is known about TCR in eukaryotes. Here we report the identification of a novel protein designated XAB2 (XPA-binding protein 2) that was identified by virtue of its ability to interact with XPA, a factor central to both NER subpathways. The 855 amino acids XAB2 protein is mainly made up of 18 tetratricopeptide repeats. In addition to interacting with XPA, immunoprecipitation experiments demonstrated that a fraction of XAB2 is able to associate with the TCR-specific proteins CSA, CSB and RNA polymerase II. Furthermore, antibodies against XAB2 inhibited both TCR and transcription *in vivo* when microinjected into living fibroblasts. These results indicate that XAB2 is a novel component involved in TCR and transcriptionIntroduction

Introduction

Nucleotide excision repair (NER) is a highly versatile and strongly conserved DNA damage repair pathway. It maintains the genetic information by removing a wide variety of lesions from DNA including ultraviolet (UV)-induced cyclobutane pyrimidine dimers (CPD) and 6/4 photoproducts, as well as numerous chemical adducts (1). NER acts in a multi-step "cut-and-paste" manner involving lesion recognition, local opening of the DNA helix, dual incision of the damaged strand on each side of the lesion, excision of the damage-containing oligonucleotide followed by DNA synthesis filling the single stranded gap of 24-32 bases and ligation. Two subpathways can be discerned in NER: global genome repair (GGR) and transcription coupled repair (TCR) (2). Lesions that actually block transcription, such as CPDs (which are inefficiently removed by GGR), are rapidly repaired from the transcribed strand of active genes by TCR, to allow rapid recovery of RNA synthesis (3, 4).

The importance of NER is highlighted by the clinical features of rare human hereditary conditions caused by a deficiency in NER, such as xeroderma pigmentosum (XP) and Cockayne syndrome (CS). XP patients show striking hypersensitivity to sunlight and an extremely high incidence of skin cancer in sun-exposed areas, and frequently progressive neurological degeneration. XP is composed of seven genetic complementation groups, designated XP-A through XP-G. In addition, XP variant group (XP-V) are defective in post-replication repair (5). Cells from XP-C are deficient only in GGR but not in TCR (6, 7). In contrast, within CS the defect is restricted to TCR (9, 10). CS patients show photohypersensitivity, cachectic dwarfism and severe mental retardation but, unlike XP patients, no predisposition to skin cancer (8). Two genetic complementation groups exist: CS-A and CS-B.. To date, all genes responsible for XP and CS have been cloned (5).

Recently, the core reaction of NER in humans has been reconstituted *in vitro* with purified proteins (11-13), and the outlines of the mechanism of global genome repair have been elucidated (reviewed in 14). The XPC/HR23B complex is the main factor to initiate global genome repair by sensing and binding to various types of lesions. The UV-DDB protein affected in XP-E patients is required for recognition of a specific subset of damage, such as CPDs. The binding of XPC/HR23B complex to the lesion presumably induces a conformational change in the DNA around the injury. TFIIH, a general transcription initiation factor containing the XPB and XPD DNA repair helicases, is recruited to the recognized injury and locally unwinds the DNA duplex by its bi-directional DNA helicase activities to form an open reaction intermediate. XPA in a complex with replication protein A (RPA) is likely to be involved in verification of the damage, proper orientation of the NER machinery around the injury and stabilization of the opened intermediate. At the same time RPA positions the structure-

specific endonucleases at the appropriate sites for dual incision: XPG 2-8 bases at the 3' side and the ERCC1/XPF complex 15-24 nucleotides 5' of the lesion. After removal of the damage-containing 24-32 mer oligonucleotide, the resulting gap in the DNA is filled by general replication factors and the final nicked sealed by DNA ligase (see 14, 15 for recent reviews and specific references therein).

The molecular mechanism of TCR is only resolved for *Escherichia coli* (16-18). The Mfd gene product (containing helicase motifs) has been identified as a transcription-repair coupling factor, that displaces an elongating RNA polymerase blocked in front of a lesion and then recruits the UvrABC *E.coli* excinuclease which accomplishes removal of the lesion. In humans, genetic and cell biological evidence indicates that CSA and CSB play a key role in TCR (6, 7) but their functioning remains to be elucidated. CSA is a 44 kDa protein with WD-40 repeats which appears to have a potential for interaction with other proteins. It has been reported that CSA interacts with CSB and the p44 subunit of TFIIH *in vitro* (19). CSB is a 168 kDa protein with helicase motifs that belongs to the SWI/SNF family (20). We have previously shown that CSB is associated with RNA polymerase II *in vivo* (21), and we and others have shown that CSB has a DNA-dependent ATPase activity but no detectable classical helicase activity (22, 23). Since both CSB and Mfd contain helicase motifs, CSB may play a role equivalent to Mfd in mammalian cells. However, unlike Mfd, CSB has no detectable activity to dissociate RNA polymerase II stalled at the site of DNA lesion (23, 24). However, CSB has been shown in vitro to interact with RNA polymerase II in a complex containing DNA and nascent RNA (25). The resulting quaternary complex has an ability to recruit TFIIH, suggesting that CSB would recruit the NER proteins *in vivo* when RNA polymerase II encounters the lesion on the transcribed strand (26).

In the present study, we isolated a cDNA encoding a novel TPR protein, designated XAB2 (XPA-binding protein 2). We found that XAB2 is associated with TCR-specific factors CSA, CSB and with RNA polymerase II as well. Furthermore, microinjection of anti-XAB2 antibodies specifically inhibited transcription as well as TCR but not global genome repair, suggesting that XAB2 is a novel factor participating in TCR and transcription itself.

Results

XAB2 is an XPA-interacting protein

To identify protein interactions within NER and/or with other nuclear constituents, we performed a yeast-two-hybrid screen with XPA as bait (27)(see Methods). In addition to previously identified XPA-interacting NER proteins (such as: ERCC1 (28, 29), and the p34 subunit of RPA (27, 30)), we isolated a cDNA encoding a novel protein, designated XAB2 (XPA-binding protein 2). The specific interaction of XPA

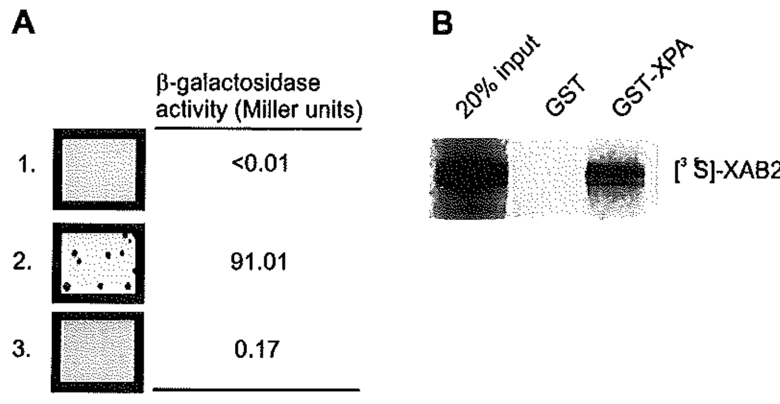


FIG. 1. A novel protein, XAB2, interacts with XPA. (A) Yeast two-hybrid assay showing a specific interaction of XPA and XAB2. A yeast strain expressing both XPA fused to the Gal4-DNA-binding-domain and XAB2 fused to the Gal4-activation-domain showed clear β-galactosidase activity (numerous blue colonies apparent in 2). No β-galactosidase enzyme activity was induced in yeast strains expressing 1: XPA fused to the Gal4-DNA-binding-domain and the Gal4-activation-domain (without XAB2) or 3: the Gal4-DNA-binding-domain (without XPA) and XAB2 fused to the Gal4-activation-domain. The enzyme activities measured by a quantitative liquid assay are shown in the table on the right. (B) *In vitro* pull-down assays using *in vitro* translated, [^{35}S]-Met labeled XAB2 with GST or GST-XPA.

and XAB2 in yeast (Fig. 1A) was confirmed by *in vitro* pull down using GST-XPA and *in vitro* translated XAB2 (Fig. 1B).

Sequencing of the complete cDNA (reconstructed after 5'-RACE, see methods) revealed a predicted acidic protein (pI 5.8) of 855 amino acids containing three stretches of acidic residues (Fig. 2A). Sequence homology searches using NCBI BLASTP to match the XAB2 sequence against sequences in the protein data bases (Non-redundant GenBank CDS translations, PDB, SwissProt, Spupdate, PIR) revealed two apparent homologs, an unidentified protein (GI:1707032; 47% identical) in *Caenorhabditis elegans* and SYF1(orf YDR416w; 26% identical) in *Saccharomyces cerevisiae* (31). These two proteins contain "tetratricopeptide repeat" (TPR; a degenerate

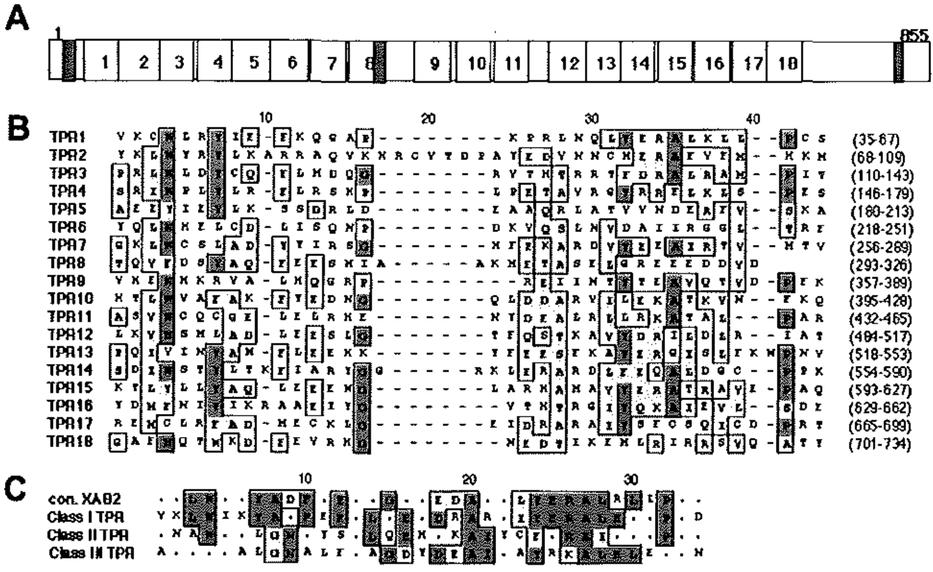


FIG. 2. XAB2 has 18 tetratricopeptide repeat (TPR) motifs. (A) Schematic representation of the XAB2 protein. Open boxes with numbers and hatched boxes indicate TPR motifs and acidic regions, respectively. (B) TPR sequence alignment. The 18 repeats in the predicted XAB2 protein were aligned, dark shading: amino acid residues found in >50 % of the repeats, boxes: conservative substitutions found in >40 % of the repeats. The substitutions were based on the following groupings: (F, Y, W); (S, T, A, G, P); (I, L, V, M); (D, E, N, Q); (R, H, K). Gaps in the sequence alignments are indicated by dashes. Numbering to the right corresponds to the amino acid positions of the TPRs in the XAB2 amino acid sequence. (C) Consensus sequence of TPRs in XAB2. The XAB2 consensus sequence is aligned with the consensus of the three classes of TRPs. The residues found most frequently at each position in the TPRs of XAB2 were used in the XAB2 consensus sequence. More variable positions are represented by dots.

repeat composed of 34 amino acids) motifs (32, 33). Sequence analysis of XAB2 revealed 18 TPR (class I) motifs covering most of the protein (Fig. 2A-C). TPRs are found in proteins of different organisms ranging from bacteria to human implicated in protein-complexes with diverged functions such as cell cycle control, transcriptional

regulation, RNA processing, and mitochondrial and peroxisomal protein transport (34, 35). Mutational and structural analyses suggest that TPR domains play a role in intra- and inter-molecular protein interactions (36-38). It is thus conceivable that XAB2, by virtue of its multiple TPRs, functions as an important factor for protein-complex formations in NER.

XAB2 interacts with CSA, CSB and RNA polymerase II

Since TPR proteins have been found in complexes with WD-40 repeat-containing polypeptides (39, 40), we focused on the CSA protein, the only known NER factor containing WD-40 repeats (19). As shown in Figure 3A, *in vitro* translated XAB2 was indeed able to bind to GST-CSA, and inversely *in vitro* translated CSA interacted with GST-XAB2. To verify the interaction *in vivo*, immunoprecipitations (IP) were performed using whole cell extracts (WCE) of CS-A cells stably expressing functional HA-tagged CSA (see Methods). Anti-HA monoclonal antibodies co-immunoprecipitated a small but significant fraction of XAB2 together with HA-tagged CSA (Fig. 3B), suggesting that at least part of XAB2 is associated with CSA *in vivo*.

The interaction with CSA prompted us to examine whether XAB2 interacts with CSB as well, since both CS proteins are specifically involved in the same TCR pathway. Using WCE of CS-B cells stably expressing physiological levels of functional HA-/His6-double-tagged CSB (2tCSB) (see 21, for documentation of these cells), IPs with anti-HA monoclonal antibodies revealed an association of significant quantities of XAB2 with CSB (Fig. 3C, upper part). This IP fraction also contained a significant proportion of RNA polymerase II as we have previously shown (21). The XAB2-CSB interaction is specific since neither endogenous-CSB nor XAB2 was precipitated with the anti HA-antibody when a WCE of HeLa without expression of 2tCSB was used (Fig. 3C, lower part).

Previously, we have shown that CSB together with RNA polymerase II is a part of a large protein complex (MW: >700 kDa) (21). Immunoblot analysis of HeLa WCE, fractionated under physiological salt conditions by Superdex-200, revealed that XAB2 is present in fractions with an estimated molecular weight of >700 kDa (Fig. 3D), whereas a monomer of XAB2 is approximately 100 kDa. The migration pattern of XAB2 largely coincides with that of RNA polymerase II and CSB and differed from other NER and transcription factors assayed in the same fractions, such as ERCC1 (which is complexed with XPF) and the XPB subunit of TFIIH, both migrating with a lower apparent MW. The association of XAB2 with RNA polymerase II is further supported by an identical co-migration of the two proteins in the presence of 1 M KCl (figure 3D, lower panel) suggesting that the interaction is highly salt-resistant. Furthermore, anti-RNA polymerase II (large subunit) antibodies were able to precipitate

XAB2 from HeLa WCE as shown in Figure 3E. These findings provide evidence that XAB2 interacts with the CSB/RNA polymerase II complex *in vivo*.

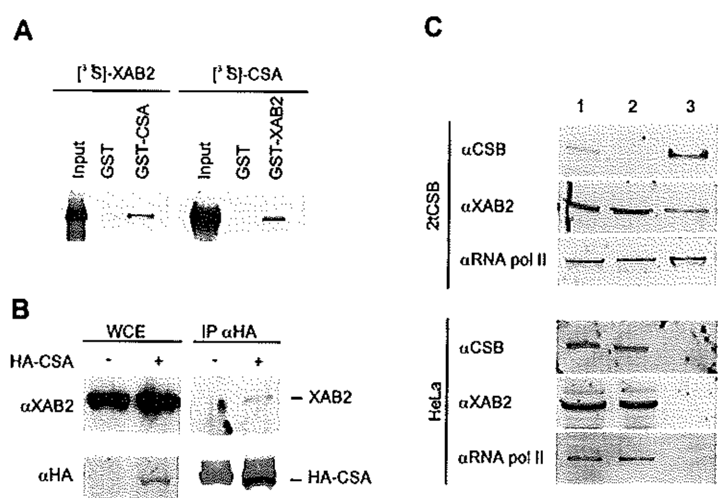
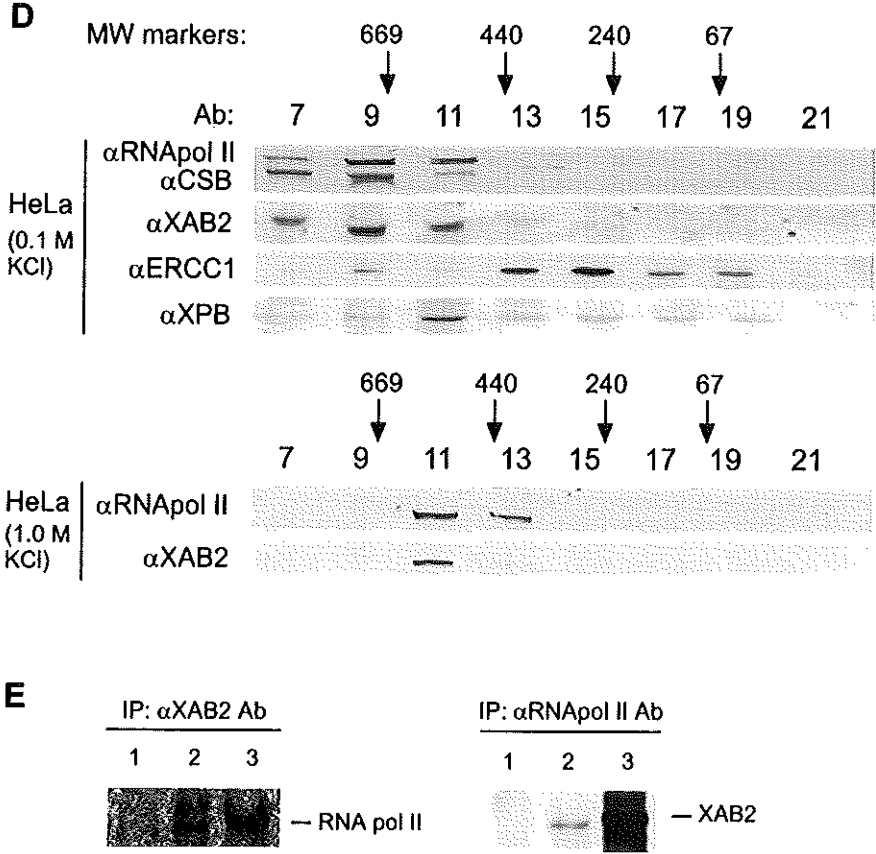


FIG. 3. Association of XAB2 with CSA, CSB and RNA polymerase II. (A) *In vitro* binding of XAB2 with CSA. *In vitro* pull-down assays were performed using *in vitro* translated, [^{35}S]-Met labeled XAB2 or [^{35}S]-Met labeled CSA with GST, GST-CSA or GST-XAB2. (B) Co-immunoprecipitation of XAB2 with CSA. WCEs and immunoprecipitated fractions of WCE from CS3BE-SV cells with (+) or without (-) the expression of HA-CSA were analyzed by immunoblotting using anti-XAB2FL antiserum (upper panels) or anti-HA rat monoclonal antibody (3F10; lower panels, the lighter signals both lanes are the heavy-chain of mouse monoclonal antibody 12CA5 used for the immunoprecipitation). (C) Co-immunoprecipitation of XAB2 with CSB. Immunoprecipitations were performed using WCE of CS1AN-SV (2tCSB) cells (upper) or HeLa cells (lower). WCE (lane 1), non-bound fraction (lane 2) and the fraction eluted with HA peptide (lane 3) were analyzed with the indicated antibody. (D) Immunoblot analyses of size-fractionated WCE. HeLa WCE was separated on a Superdex-200 column at 0.1 M KCl (upper panel) or 1.0 M KCl (lower panel). Immunoblot analysis of the collected fractions was performed with indicated antisera. The sizes and positions of molecular weight markers are shown at the top of the blots. (E) Co-immunoprecipitation of XAB2 with RNA polymerase II.



(E) Co-immunoprecipitation of RNA polymerase II and XAB2. Immunoprecipitated fractions from HeLa WCE with pre-immune serum (negative control; lane 1), anti-XAB2 antiserum (lane 2), and anti-RNA polymerase II monoclonal antibody 8WG16 (positive control; lane 3) were analyzed by 8WG16 (upper panel). Immunoprecipitated fractions from HeLa WCE with anti-HA mouse monoclonal antibody 12CA5 (negative control; lane 1), 8WG16 (lane 2), and anti-XAB2FL antiserum (positive control; lane 3) were analyzed by anti-XAB2FL antiserum (lower panel). Five percent of IP fractions were loaded in positive control lanes.

***In vivo* function of XAB2**

The interactions of XAB2 with XPA, CSA and CSB/RNA polymerase II complex suggest a possible role for XAB2 in the TCR subpathway of NER. To further analyze the XAB2 function in living cells, we examined the effect of microinjected anti-XAB2 antisera on various NER parameters. Two antisera were used, one raised against the full-length XAB2, and the other against the C-terminal part (residues 694-855): designated anti-XAB2FL and anti-XAB2C, respectively. Microinjection of both anti-XAB2FL and anti-XAB2C did not significantly inhibit UV-induced DNA repair synthesis (unscheduled DNA synthesis: UDS) of normal human fibroblasts, which is mainly derived from GGR (41) (Fig. 4A, table 1). In contrast, injection of anti-XAB2 antisera in fibroblasts of xeroderma pigmentosum group C patients, carrying a specific defect in GGR, induced a significant reduction of the residual UDS (Table 1). Since UDS in XP-C is derived only from TCR (6), these results suggest that anti-XAB2 antisera directly interfere with the TCR rather than the GGR pathway. Consistent with this observation is the finding that both anti-XAB2 antisera inhibited the recovery of RNA synthesis after UV-irradiation (RRS) in normal human cells (Fig 4B, Table 1). Anti-CSB antiserum induced a similar effect on the above NER parameters (Table 1), whereas injected anti-ERCC1 antiserum affected both subpathways of NER, consistent with its essential function both in TCR and GGR. Injection of pre-immune serum (Table 1), other non-immune sera and antibodies against various non-NER proteins (data not shown) did not induce any effect on DNA repair in wild-type fibroblasts. The inhibitory effect of anti-XAB2 antisera on the process of TCR indicates that this protein plays a role in the same pathway as the CS proteins. However, in contrast to anti-CSB antiserum, anti-XAB2FL also induced a significant inhibition of normal RNA synthesis (Fig. 4C, Table 1). This inhibitory effect was not observed using anti-XAB2C (Table 1), suggesting that the C-terminal region (694 - 855) of XAB2 may play an important role in TCR but not in transcription itself. As shown before, injection of non-immune sera as well as antibodies against other factors only involved in NER failed to exert inhibition of transcription, in contrast to antisera against various proteins implicated in both NER and transcription initiation (21). In conclusion, the results of the antiserum microinjection experiments suggest that XAB2 functions both in TCR and in normal transcription.

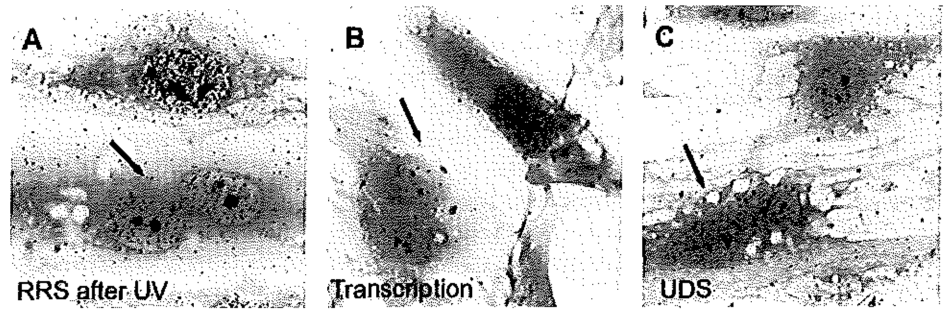


FIG. 4. Inhibition of recovery of RNA synthesis and transcription but not global genome repair by anti-XAB2 antiserum *in vivo*. Anti-XAB2FL antiserum was injected into the cytoplasm of bi-nuclear cells (indicated by an arrow) obtained after fusion of normal human fibroblasts. Subsequently the effect on (A) DNA repair synthesis after UV-irradiation (UDS) predominantly derived from global genome repair; (B) recovery of RNA synthesis after UV-irradiation (RRS); and (C) transcription (normal RNA synthesis without UV- irradiation) was assessed.

Discussion

We found a novel protein, XAB2, which interact with TCR-specific CSA, CSB proteins and RNA polymerase II as well as with the core NER factor XPA. Our microinjection experiments revealed that anti-XAB2 antisera caused specific inhibition of UV-induced UDS in XPC cells (which only have functional TCR) but had no significant effect on UV-induced UDS in normal human cells (predominantly derived from GGR). We also observed inhibitory effects of anti-XAB2 antisera on recovery of RNA synthesis after UV-irradiation in normal human cells. Together these results indicate that XAB2 is involved in TCR but not in GGR. In addition, antiserum against the entire XAB2 (anti-XAB2FL) inhibited transcription in non UV-irradiated normal cells, strongly suggesting that XAB2 could be a novel factor involved in the transcription process itself. Since transcription is essential for TCR, it is likely that the observed inhibition of TCR is (also) a consequence of the inhibitory effect of anti-XAB2FL on transcription. However, the anti-XAB2C (the antiserum against the carboxyl terminal portion of XAB2) inhibited the recovery of RNA synthesis after UV-irradiation without apparent inhibitory effects on transcription. These observations

suggest that besides being involved in transcription, XAB2 could work as a TCR-specific factor required for resumption of transcription after arrest by NER lesions, possibly mediated by the carboxyl terminal portion.

The molecular mechanism for the coupling of transcription and NER in eukaryotes is unknown. Presumably, a lesion on the transcribed strand is first noticed by a RNA polymerase II elongation complex (thus bypassing the need for the XPC/HR23B complex). Then core NER factors are recruited by TCR-specific proteins such as CSA and CSB (2). CSB was found *in vitro* and *in vivo* to reside in an RNA polymerase II complex, probably in an elongation mode (21, 25). A quaternary complex consisting of CSB, RNA polymerase II, template DNA and nascent RNA has been shown to be able to recruit TFIIH *in vitro* (26). The function of CSA is more obscure. *In vitro* associations of CSA with various NER factors have been reported (19) but no *in vivo* association to either the transcription machinery or to NER factors has been identified (21). In the present study, we found a dual interaction of part of XAB2 with a fraction of both CSA and CSB as well as the interaction with XPA. This raises the possibility that XAB2 links these TCR-specific proteins to assure recruitment and/or access of core NER factors to the lesion identified by the stalled RNA polymerase II in the elongation complex. The notion that these interactions are transient may explain our observation that only a small proportion of XAB2 is bound to CSA and RNA polymerase II (Fig. 3B, E). This is consistent with the fact that CSA and CSB appear to reside in different protein complexes (21).

Sequence homology searches using NCBI BLASTP revealed that XAB2 showed homology with an unidentified protein (GI: 1707032; 47 % identical) in *Caenorhabditis elegans* and Syf1p (26% identical) in *Saccharomyces cerevisiae*. Dix et al. reported that Isy1p, interactor of Syf1p, was required for optimal pre-mRNA splicing in yeast (42). They also mentioned that the SYF1 gene had been identified as a synthetic lethal mutant with the CDC40/PRP17 gene which is involved in S phase progression of the cell cycle and pre-mRNA splicing in yeast (YPD protein report for SYF1) (43). In addition, McDonald et al. reported that cwf3p, the *Schizosaccharomyces pombe* ortholog of Syf1p, is associated with cdc5p which is required for G2/M progression of the cell cycle and essential for pre-mRNA splicing (44). Moreover, it has been reported that Syf1p and cwf3p are essential for viability in *S. cerevisiae* and *S. pombe*, respectively (43, 44). The requirement of XAB2 in transcription may account for the essential role of Syfp for viability in yeast because Syfp is seemingly a yeast homolog of XAB2. It is also possible that XAB2 is involved in processes associated with cell cycle control and pre-mRNA splicing in mammalian cells. Since the coupling of transcription elongation and pre-mRNA splicing has been observed (45, 46), a potential involvement of XAB2 in pre-mRNA splicing raises the possibility that the inhibition of RNA synthesis observed after microinjection of anti-XAB2FL is a consequence of an impeded transcription

affected by impaired splicing processes. However, it has been reported that transcription occurs at a normal rate in the absence of efficient splicing of nascent pre-mRNA during transcription elongation in human cells (46, 47). Thus, it is also possible that the inhibition of RNA synthesis by anti-XAB2FL resulted from impaired transcription rather than disturbed pre-mRNA splicing. The above findings in yeast fit nicely with our observation that a significant proportion of XAB2 is in a complex with the fraction of RNA polymerase II that is complexed with CSB and is thought to be in an elongation mode (21).

Based on our experimental data and an apparent similarity with yeast Syfp, it is likely that XAB2 is a multifunctional protein involved in cellular processes such as cell cycle control and pre-mRNA splicing as well as TCR and transcription in mammalian cells. Das et al. reported that tandemly arranged TPR motifs are organized into a regular right-handed super-helix with a helical repeat of approximately seven TPR motifs (38). It is proposed that proteins with these structures could simultaneously interact with multiple target proteins, utilizing specific combinations of TPR motifs within the super-helix (38). Since XAB2 harbors 18 tandem arrays of TPR, a possible scaffolding function for XAB2 within cellular processes including NER and transcription is in line with its deduced amino acid sequence. XAB2 may function as a (transient) bridging protein, by simultaneously interacting with several other proteins or protein complexes. Besides being involved in transcription and TCR, XAB2 may participate in other cellular responses provoked by DNA damage in the transcribed strand; such as transcription-coupled base excision repair (48), ubiquitination of RNA polymerase II (49) and activation of p53 (50). In addition, it would be of interest to find out whether defects in XAB2 also give rise to a human condition, since both CSA and CSB are associated with the severe neuro-developmental, UV-sensitive TCR disorder Cockayne syndrome.

Materials and methods

Yeast two-hybrid system

Screening of a HeLa cDNA library was performed using the yeast two-hybrid system as described (27). Positive transformants were classified into several groups based on cross-hybridization. Out of 281 positive clones, 54 belonged to the group of XAB2. To obtain full-length cDNA of XAB2, we screened a HeLa cDNA library in lambda ZAP (provided by Dr. H. Nojima, Osaka University) using the 939 bp SmaI fragment of XAB2 cDNA as a probe. In addition, 5'-RACE was performed with 5'-AmpliFINDER RACE KIT (Clontech) using the P1 primer (5'-TTCATAGGCAGGGTCGGTCACACAG-3') and P2 primer (5'-TGTGCCCGACGCGCCTTCAGGTATC-3') according to the attached protocol. The full-length cDNA of XAB2 was reconstructed in pBluescript SK(-) by insertion of the EcoRI-KpnI fragment

from the RACE product into the EcoRI and KpnI sites of the cDNA from the two hybrid or HeLa lambda Zap library screenings.

***In vitro* pull-down assay**

GST-XAB2 fusion was obtained by in-frame-cloning the full-length XAB2 cDNA into pGEX-5X-2. Referring to the published data (19), the CSA cDNA was isolated from WI38 VA13 cells with RT-PCR using an upper primer (5'-CGAATTCTCGAGGATATGCTGGG-GTTTTTGTC-3') with an EcoRI site and a lower primer (5'-TTGGTCGACTCTGTTTT-AGGATTTTATGCAAA-3') with a SalI site. The amplified product was digested with EcoRI and SalI, and inserted into pBluescript SK(-) and pGEX-5X-2 for *in vitro* translation and GST fusion protein, respectively. *In vitro* translation of proteins and pull-down assays using GST, GST-XAB2 or GST-CSA fusion protein were performed as described (27).

Immunoprecipitation

To examine the interactions of XAB2 with CSA, we used the SV-40 transformed CS-A fibroblast line CS3BE-SV and CS3BE-SV(dtCSA) cells. CS3BE-SV expressed no endogenous CSA, while CS3BE-SV(dtCSA) stably expressing haemagglutinin (HA)-, His6-double-tagged CSA (HA-CSA) at physiological levels showed a normal UV sensitivity (E. C., unpublished). Whole cell extracts (WCE) of these cells were prepared as previously described (27). To examine the interactions of XAB2 with CSB, the SV40 transformed CS1AN-SV (2tCSB) cell line (stably expressing functional and physiological levels of HA-/His6-double-tagged CSB) (21) and HeLa cells were used for preparing Manley's WCE. The WCE (4 mg) was incubated with 5 μg of anti-HA mouse monoclonal antibody (12CA5) at 4°C for 6 hr. For co-immunoprecipitation of XAB2 with RNA polymerase II, HeLa WCE (4 mg), prepared as described (27), was incubated with anti-RNA polymerase II mouse monoclonal antibody (8WG16; a kind gift from Dr. J-M. Egly, CNRS/INSERM/Université Louis Pasteur) or anti-XAB2FL at 4°C for 12 hr. The immuno-complexes were subsequently precipitated with 40 μl (bed volume) of Protein G-Sepharose beads and further incubated at 4°C for 1 hr. After extensive washing, bound proteins were eluted by SDS sample buffer or by HA-peptide, separated on SDS-PAGE and subjected to immunoblot analyses. The WCEs (20 μg) were also subjected to immunoblot analyses with IP fractions.

Microinjection

Microinjections were performed into homopolykaryons of DNA repair-proficient control primary fibroblasts (C5RO) and XP21RO (XP-C) cells obtained after cell fusion as described (21). NER activity (unscheduled DNA synthesis: UDS) was measured 24 hr after injection. Injected samples were UV-irradiated at 15 J / m^2, then subjected to a 2 hr incubation in culture medium containing 20 μCi / ml of [^{3}H]-Thymidine, washed with PBS, fixed and processed for autoradiography. Recovery of RNA synthesis (RRS) after UV-irradiation was determined as follows; 24 hr after injection, cells were exposed to 15 J / m^2 of UV-light, incubated for an additional 24 hr in normal culture medium, washed with PBS and subsequently incubated for another 1 hr in culture medium containing [^{3}H]-Uridine (10 μCi / ml), fixed and processed for autoradiography. Overall normal RNA synthesis (transcription) was determined, 48 hr after injection as described above for RRS but without prior UV-irradiation. UDS-, RRS-, and

transcription-levels were quantified by counting the autoradiographically induced silver grains above the nuclei (at least 100 nuclei). The relative levels of repair and transcription in the injected cells were obtained by dividing the mean grain count number by the number of grains above the nuclei of surrounding non-injected cells.

Acknowledgements

We thank Dr. Jean-Marc Egly for kindly providing anti-RNA polymerase II monoclonal antibody (8WG16), and Dr. Hiroshi Nojima for the HeLa cell cDNA library. This work has been supported by grants from the Ministry of Education, Science, Sports and Culture of Japan, and Health Science Research Grants for Research on Human Genome and Gene Therapy from Ministry of Health and Welfare of Japan (Grant-in-Aid for Scientific Research (A) (11307056), Grant-in-Aid for Scientific Research on Priority Areas (08280101) and H10-Genome-021 to K.T., Grant-in-Aid for Scientific Research(C) (11680679) to Y. N.). This research was also supported by the Dutch Cancer Society (project EUR94-763, and 1800), the Dutch Science Foundation, EC contracts, a SPINOZA premium and the Louis Jeantet Foundation to J. H. J. H.

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Chapter 8

Biochemical and biological characterization of wild-type and ATPase-deficient Cockayne syndrome B repair protein

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J. Biol. Chem. 273:11844-11851 (1998).

Biochemical and Biological Characterization of Wild-type and ATPase-deficient Cockayne Syndrome B Repair Protein*

(Received for publication, November 5, 1997)

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Cockayne syndrome (CS) is a nucleotide excision repair disorder characterized by sun (UV) sensitivity and severe developmental problems. Two genes have been shown to be involved: *CSA* and *CSB*. Both proteins play an essential role in preferential repair of transcription-blocking lesions from active genes. In this study we report the purification and characterization of baculovirus-produced HA-His$_6$-tagged CSB protein (dtCSB), using a highly efficient three-step purification protocol. Microinjection of dtCSB protein in CS-B fibroblasts shows that it is biologically functional *in vivo*. dtCSB exhibits DNA-dependent ATPase activity, stimulated by naked as well as nucleosomal DNA. Using structurally defined DNA oligonucleotides, we show that double-stranded DNA and double-stranded DNA with partial single-stranded character but not true single-stranded DNA act as efficient cofactors for CSB ATPase activity. Using a variety of substrates, no overt DNA unwinding by dtCSB could be detected, as found with other SNF2/SWI2 family proteins. By site-directed mutagenesis the invariant lysine residue in the NTP-binding motif of CSB was substituted with a physicochemically related arginine. As expected, this mutation abolished ATPase activity. Surprisingly, the mutant protein was nevertheless able to partially rescue the defect in recovery of RNA synthesis after UV upon microinjection in CS-B fibroblasts. These results indicate that integrity of the conserved nucleotide-binding domain is important for the *in vivo* function of CSB but that also other properties independent from ATP hydrolysis may contribute to CSB biological functions.

Nucleotide excision repair (NER)[1] is an evolutionary strongly conserved pathway responsible for the removal of a wide variety of lesions from the DNA, including the major types of UV-induced DNA injuries: cyclobutane pyrimidine dimers and (6–4) photo products. Removal of these lesions proceeds via a multi-step reaction; lesion recognition is followed by local opening of the DNA helix, dual incision in the damaged strand on each side of the lesion, release of the damage-containing fragment, gap-filling DNA synthesis, and ligation. A large number of gene products is involved in this repair reaction (reviewed in Refs. 1–3). The dramatic consequences of impaired NER are illustrated by several hereditary human diseases including xeroderma pigmentosum (XP) and Cockayne syndrome (CS). Patients with XP manifest a high sun (UV) skin sensitivity associated with a strong predisposition to skin cancer and frequently progressive neurodegeneration. Besides these common features, a significant clinical and genetic heterogeneity is observed among XP patients. Seven excision-deficient complementation groups (XP-A to XP-G) have been identified so far on the basis of cell fusion experiments (4). Patients with CS show, like XP, increased sensitivity to UV light. In addition, they suffer from severe developmental problems, which include growth retardation, skeletal and retinal abnormalities, and severe neurological deficiencies due to dysmyelination of neurons (4–6). Unlike XP patients and unlike a recently generated mouse model for CSB (7), no significant skin cancer predisposition has been noted for CS so far.

Several observations have revealed functional connections between DNA repair and basal transcription. Firstly, at least three subunits of the basal transcription factor TFIIH (XPB, XPD, and TTDA) are also required for NER (8–10). Secondly, lesions in the transcribed strand of active genes hamper or block the vital process of transcription. For some lesions such as cyclobutane pyrimidine dimers the normal (global genome) NER pathway is very slow. Therefore, a special subpathway of NER has evolved that accomplishes rapid and efficient removal of these types of damage from the transcribed strand of active genes (11, 12). This process, called transcription-coupled repair is highly conserved in evolution, because it has been described in *Escherichia coli*, yeast and mammalian cells (7, 13–16). In humans, two genes have been shown to be specifically required for transcription-coupled repair: *CSA* and *CSB*, defective in CS complementation groups A and B, respectively (17, 18). CS cells exhibit an elevated UV sensitivity correlated with a specific defect in transcription-coupled repair. Consequently, unlike normal cells, they are unable to recover RNA synthesis after UV irradiation (19, 20).

The CSB gene encodes a protein of 1493 amino acids containing a 500-amino acid region highly homologous to members of the SWI2/SNF2 subfamily of DNA-dependent ATPases (18, 21). Interestingly, SNF2-related proteins are involved in a wide variety of cellular functions, notably transcription regulation, chromatin remodelling, and all known multi-step DNA repair pathways, such as nucleotide excision repair of both transcribed and nontranscribed regions of the genome, post replication repair, and recombination repair (22, 23). Importantly,

* This work was supported by European Community Training and Mobility Research Network Project Number CHRX-CT94-0443, Dutch Cancer Society Grant EUR 94-763, Human Frontiers Science Program Grant RG 373, and funds from the Louis Jeantet Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviation used are: NER, nucleotide excision repair; CS, Cockayne syndrome; XP, xeroderma pigmentosum; HA, hemagglutinin; ssDNA, single-stranded DNA; dsDNA, double-stranded DNA; wt, wild type; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis.

we found recently that the CSB protein resides in a large molecular weight complex that includes RNA polymerase II but no transcription initiation factors (24).

The mechanism by which CSB protein mediates transcription-coupled repair is still largely unknown. Availability of a recombinant CSB protein is essential for studying CSB activity in *in vitro* assays. Here we present the generation, purification, and functional characterization of a double-tagged wt and ATPase-deficient mutant CSB protein. Surprisingly, the DNA-dependent ATPase activity was found to be partially dispensable for the *in vivo* CSB function in RNA synthesis recovery.

EXPERIMENTAL PROCEDURES

Cell Culture—The immortalized human cell lines used in this study were: HeLa, C5RO (wt), GM2965 (CS-A), CS1AN (CS-B), and XPCS1BA(XP-B). The fibroblasts were cultured in Ham's F-10 medium, supplemented with antibiotics and 12% fetal calf serum. Cell-free extract from HeLa cells was prepared as described previously (25, 26).

An insect cell line, *Spodoptera frugiperda* Sf21, was cultured at 27 °C in Hanks' medium supplemented with antibiotics and 10% fetal calf serum. Hanks' medium was prepared from Grace's insect cell culture medium (Life Technologies, Inc.) supplemented with 0.33% tissue culture yeastolate (Difco), 0.33% tissue culture lactalbumin hydrolysate (Difco), and 0.55% bovine serum albumin.

Plasmid Constructs—Previously we have generated a CSB cDNA construct (2tpSLME6) encoding a N-terminal hemagglutinin antigen (HA) epitope as well as a C-terminal histidine (His_6) tag (24). For overexpression of the HA-CSB-His_6 in the BAC to BAC Baculovirus Overexpression System (Life Technologies, Inc.), the N-terminal *SacI* fragment and the C-terminal *SacI-XbaI* fragment were cloned together in the pFASTBAC vector (Life Technologies, Inc.), giving rise to construct 2tpFBACE6. Construct 2tpFBACE6-K538R, containing a mutation changing the Lys-538 codon to Arg, was generated as described below.

Site-directed Mutagenesis—The K538R mutation in the CSB cDNA was introduced using the PCR-mediated site-directed mutagenesis method (27). For this purpose, two fragments of the CSB cDNA containing the helicase domain (1330-base pair *KpnI-SmaI* fragment and 1914-base pair *KpnI-KpnI* fragment) were independently subcloned into pBluescript KS and pTZ18 vector, respectively. The primers used in the PCR reactions are: the mutagenic primer (5' GGATTGGGCAGGACCATCCAG 3') and the universal primers SK and M13 universal. The final PCR product was cloned into the pBluescript vector, and the internal *AsuII* fragment was used to replace the wild-type fragment in 2tpFASTBACE6. Sequencing of the entire PCR fragment ruled out PCR-derived errors and confirmed the presence of the desired mutation.

Baculovirus Infection and Purification of Recombinant Proteins—Suspension cultures of Sf21 cells at 1.5 × 10^6 cells/ml were infected with the recombinant baculoviruses at 27 °C for 1 h at a multiplicity of infection of 5–10. At 3 days post-infection, cells were collected and washed twice with ice-cold phosphate-buffered saline. For fractionation, the cell pellet was resuspended into 8 (packed cell) volumes of ice-cold NP lysis buffer (25 mM Tris-HCl, pH 9.0, 1 mM EDTA, 10% glycerol, 1% Nonidet P-40, 0.3 M KCl, 1 mM dithiothreitol, 0,1 mM phenylmethylsulfonyl fluoride, 0,2 μM chemostatin, leupeptin, antipain, and pepstatin A). After incubation on ice for 30 min with occasional agitation, the suspension was centrifuged at 12,000 × *g* for 15 min to obtain the supernatant fraction and the precipitate fraction. To examine the protein remaining in the precipitate fraction, the pellets were homogenized in NP buffer by sonication. For purification, the supernatant fraction was diluted with NP buffer at pH 6,8 in a 1:1 ratio and loaded on a heparin-Sepharose (CL-6B; Amersham Pharmacia Biotech) column equilibrated with buffer A (25 mM HEPES-KOH, pH 7, 9, 0,01% Nonidet P-40, 10% glycerol, 1 mM β-mercaptoethanol, 0, 1 mM phenylmethylsulfonyl fluoride) containing 0.3 M KCl. The column was washed with the same buffer, and the adsorbed proteins were eluted with buffer A containing 0.8 M KCl. The eluate was directly loaded on a Ni^{2+}-nitrilotriacetic acid-agarose column equilibrated with buffer A containing 0.8 M KCl and 5 mM imidazole. After washing the column with 20 mM Imidazole, bound proteins were eluted with buffer A containing 0.3 M KCl and 60 mM/250 mM imidazole.

In the final step of purification, the Ni^{2+}-nitrilotriacetic acid elution fractions were incubated with a 5-fold excess of monoclonal anti-HA (12CA5) antibody O/N at 4 °C with rotation. The protein-antibody complexes were subsequently bound to protein G-Sepharose beads. After extensive washing of the beads with buffer A containing 0,1% Nonidet P-40, CSB protein was specifically eluted using the synthetic HA peptide (YPYDVPDYA) at 1 mg/ml in buffer A containing 0.2 M KCl. Proteins were stored at −80 °C. The behavior of the recombinant protein during the purification was monitored by SDS-PAGE and immunoblot blot analysis.

ATPase Assay—Standard reactions (10 μl) were carried out in buffer B (20 mM Tris-HCl, pH 7.5, 4 mM $MgCl_2$, 40 μg/ml bovine serum albumin, 1 mM dithiothreitol), 1 μCi of [γ-^{32}P]ATP (5000 Ci/mmol; Amersham Pharmacia Biotech) and the specified amounts of CSB protein. When indicated, reactions were carried out in the same buffer B supplemented with 50 μM cold ATP and 0.25 μCi of [γ-^{32}P]ATP. 150 ng of DNA cofactor/reaction was added. After 1 h at 30 °C the reactions were stopped with 5 μl of 0.5 M EDTA, and a 1-μl sample was analyzed on polyethylenimine-cellulose thin layer chromatography plates developed in 0.75 M KH_2PO_4. The extent of ATP hydrolysis was determined by image analysis and quantification on a PhosphorImager (Molecular Dynamics).

Helicase Assay—The partial duplex DNA substrates were constructed by hybridizing a 5' ^{32}P-labeled complementary fragment to the viral (+)-strand of M13 DNA: (i) 18-base oligonucleotide; (ii) 36-base oligonucleotide; (iii) 50-base oligonucleotide with a 5' 15-base noncomplementary overhang; and (iv) 50-base oligonucleotide with a 3' 15-base noncomplementary overhang. Helicase substrates with a 5'-3' and a 3'-5' polarity were made by hybridizing a 5'-^{32}P-labeled complementary fragment to a 36-base oligonucleotide (F36), respectively: (i) 19-base oligonucleotide (F19) and (ii) 17-base oligonucleotide (F17) (see Table I). Also, a double-stranded 75-base DNA fragment was used. Free oligonucleotides were removed by gel filtration on a Sephadex G50 (Amersham Pharmacia Biotech) column. The substrates, 5 ng each, were incubated with the indicated amount of CSB protein in buffer B containing 1 mM ATP (total volume 25 μl). After incubation at 37 °C for 45 min, reactions were stopped and subjected to electrophoresis on 12% polyacrylamide gel and autoradiography as described (8).

Microneedle Injection of Recombinant Proteins and Analysis of Repair and Transcription Levels—Microneedle injection into cultured fibroblasts was performed as described previously (10, 28). Comparable amounts of purified recombinant CSB, wild type, and K538R mutant dissolved at 20 ng/μl were injected into the cytoplasm of CS1AN (CS-B) homopolykaryons (obtained after cell fusion of CS1AN (CS-B) fibroblasts with each other), multinucleated cells of GM2965 (CS-A), or XPCS1BA (XP-B) homopolykaryons. The recovery of RNA synthesis post-UV was measured. Cells were UV-irradiated with 10 or 15 J/m² (UV-C 254 nm) and allowed to recover for 16–20 h. RNA synthesis was measured after pulse-labeling with [^{3}H]uridine (10 μCi/ml; specific activity, 50 Ci/mmol), fixation, and autoradiography. Grains above the nuclei of injected cells (polykaryons) and noninjected neighboring cells (monokaryons) were counted and represent a quantitative measure for RNA synthesis.

Antibodies—Anti-CSB antibodies used in this study were raised against the C-terminal 158 amino acids of CSB. Characterization of affinity-purified anti-CSB and immunoblotting procedures were performed as described (24).

DNA Substrates—DNA cofactors used in the ATPase assay are described in Table I and kindly provided by Wouter de Laat (29). HeLa polynucleosomes were a generous gift of Robert Kingston and Gavin Schnitzler.

RESULTS

Overexpression and Purification of Recombinant wt and Mutant CSB Proteins—To facilitate purification of CSB and to allow isolation of a full-length protein, we generated a double-tagged CSB (dtCSB) construct (24) in a baculovirus expression system. A 9-amino acid HA epitope was introduced at the N terminus of the cDNA sequence, and a hexameric histidine stretch (His_6) was introduced at the C terminus. It was important to verify whether the addition of tags *per se* did not interfere with the biological function of CSB. Therefore, we established by cDNA transfection a CS-B cell line stably expressing dtCSB in a mammalian expression vector. The level of expression of dtCSB protein was similar to the endogenous CSB level in normal cells. We found that the tagged protein conferred wild-type UV resistance to the CS-B cells and was incorporated into a large complex including RNA polymerase II (see Ref. 24). Thus, these findings confirm that dtCSB was

TABLE I
DNA substrates used as cofactors in ATPase assays and helicase templates
Helicase partial duplex templates were made by hybridyzing 5′ ^{32}P-labelled F19 or F17 oligonucleotides to the F36 fragment.

DNA substrate	Sequence	Structure
stem19-loop8	5′-TCGGGTCGCCAGCGCTCGG(T)$_8$CCGAGCGCTGGCGACCCGa-3′	19, 8
stem20-loop15	5′-TGGCACATCCTGGGTCGCCAGCGCTCGG(T)$_{15}$CCGAGCGCTGGCGACCCAGGATG-3′	20, 15
splayed-arm	5′-(T)$_{12}$GCCATCGCGAGTCCGGACTCGCGATGGC(T)$_{12}$-3′	14, 12, 3′, 12, 5′
ss-oligo	5′-GATCCATCGATTCAGCTAGCGTAATCTGGAACATCGTATGGGTAAGCGCTGCCTCCGTGATGGTGATGGTGGTG-3′	74
RNA-oligo	5′-CUAGGUAGCUAAGUCGAUCGCAUUAGACCUUGUAGCAUACCCAUUCGCGACGGAGGCACUACCACUACCA-3′	70
F19	5′-GCGATGCGGATCCAAGTCT-3′	F19 3′, F36
F36	5′-CCTAGACTTAAGAGGCCAGACTTGGATCCGCATCGC-3′	
F17	5′-GGCCTCTTAAGTCTAGG-3′	5′ F17, F36

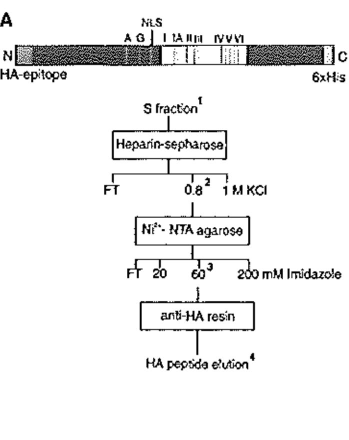
biologically active *in vivo* and functionally indistinguishable from the untagged wt protein.

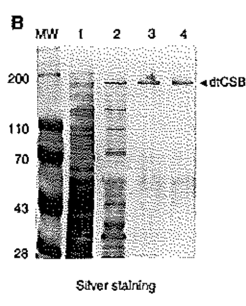
To investigate the importance of the ATPase activity for the various CSB functions we introduced by site-directed mutagenesis a strategic amino acid substitution in the Walker-type A motif (30) by replacing the invariant lysine residue (Lys-538) in the GXGKT sequence by the physicochemically related arginine, yielding dtCSB (K538R). It has been shown for other ATPases that such an alteration does prevent ATP hydrolysis but still permits ATP binding (31–33). Importantly, this conservative amino acid change is not expected to drastically modify the overall protein conformation.

Both the wild-type and the mutant CSB protein were overproduced using the baculovirus/Sf21 insect cell system. Infected Sf21 cells were extracted with hypertonic buffer containing 0.3 M KCl, and the dtCSB protein was isolated from the soluble fraction. The purification was monitored by immunoblot analysis using polyclonal anti-CSB antibodies and by silver staining of SDS-PAGE. After two chromatographic steps, a heparin-Sepharose and a Ni^{2+}-nitrilotriacetic acid-agarose column that selects for the C-terminal His_6 stretch, we obtained a protein fraction consisting of a major band of about 170 kDa, corresponding to the predicted molecular weight of dtCSB (Fig. 1, *A* and *B*, *lanes 1–3*). Some degradation products were visible after immunoblot analysis using antibodies raised against the C terminus of CSB (data not shown; similar findings made by Selby and Sancar (34)). The presence of an HA epitope at the N terminus of dtCSB allowed us to select only for full-length protein during the last highly specific monoclonal affinity purification step. When the final fraction was analyzed by SDS-PAGE and silver staining, no protein species other than dtCSB were detected, indicating that the protein was purified to near homogeneity (Fig. 1*B*, *lane 4*). The same purification procedure was used for both wild-type dtCSB and dtCSB (K538R) mutant, obtaining both proteins at comparable high levels of purity, intactness, concentration, and yield (Fig. 1*C*). The three-step purification scheme described above yielded about 22 μg of protein from 10^8 infected cells. Immunoblot analysis of purified wt and mutant dtCSB and HeLa WCE using antibodies against CSB confirms the identity of the purified proteins (Fig. 1*D*, note the slight increase in size of the dtCSB as compared with the nontagged wt CSB in HeLa whole cell extract; for characterization of the antiserum, see Ref. 24).

Functionality of the Purified Recombinant dtCSB Protein in Vivo—Prior to the biochemical characterization of the recombinant dtCSB protein, it was important to verify whether overproduction in the heterologous Baculo system and/or the purification procedure had not inactivated the biological function of the protein, *e.g.* by improper folding or lack of post-translational modification.

One of the characteristic features of CS cells is their inability to recover RNA synthesis after UV exposure (18, 35). To assess the *in vivo* function of the recombinant wt CSB, the purified protein was microinjected into the cytoplasm of living CS-B (CS1AN) fibroblasts, and its ability to transiently correct the DNA repair defect of CS-B cells was analyzed. The experimental protocol is outlined in Fig. 2*A*. CS1AN (CS-B) fibroblasts were fused to generate homopolykaryons. After injection of at least 50 multi-nucleated fibroblasts, the cells were irradiated with 15J/m^2 UV light and incubated for 16–20 h to allow recovery from UV-induced inhibition of RNA synthesis. Transcription was analyzed by a 1-h pulse labeling with [^{3}H]uridine followed by autoradiography. Transcription levels were quantified by counting silver grains above the nuclei (10, 28). As shown in Table II and Fig. 2*B*, in injected CS-B polykaryons the recovery of UV-induced inhibition of transcription is resumed to levels observed in wild-type cells assayed in parallel. In contrast, the neighboring noninjected cells (monokaryons in





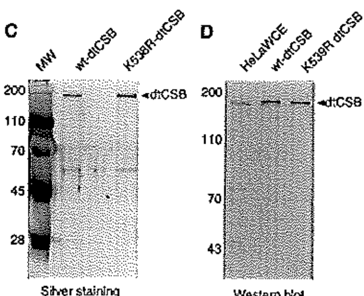


FIG. 1. **Purification of recombinant dtCSB.** *A*, dtCSB purification scheme (see "Experimental Procedures") and schematic representation of the predicted functional domains of the CSB protein: *A*, acidic amino acid stretch; *G*, glycine-rich region; *NLS*, nuclear location signal. The *lightly shaded area* represents the central CSB domain with overall high homology to the region shared between all members of the SNF2 family of DNA-dependent ATPases. Roman numbers *I–VI* within this region refer to conserved "helicase" motifs that have been identified in numerous known and putative DNA and RNA helicases. *B*, purification of recombinant wt dtCSB. Purification was monitored by SDS-PAGE. Aliquots of samples at each purification step (for numbering of the corresponding steps see *panel A*) were analyzed on a 8% SDS-PAGE gel, and protein bands were visualized by silver staining. (The faint staining at around 60 kDa is an artifact; see *panel C*). *C*, purified recombinant wild-type dtCSB and dtCSB (K538R) mutant proteins. Aliquots of the last purification step (HA elutions) of both proteins (200 ng each) were resolved by SDS-PAGE followed by silver staining. (The lane between the dtCSB samples is empty showing a faint staining artifact at the position of ~60 kDa). *D*, immunoblot analysis of HeLa whole cell extract (*WCE*, 10 μg) and purified wt dtCSB and dtCSB (K538R). Affinity-purified anti-CSB polyclonal antibodies were used (24). The positions of the molecular mass markers (*MW*) in kDa are shown.

Fig. 2*B*), exhibit a very low level of transcription, which is typical for UV-exposed CS-B fibroblasts.

When CSB was microinjected in CS-A and XP-B fibroblasts, no significant complementation of their RNA-synthesis recovery defect was observed, proving that the correcting activity is specific for CS-B cells (Table II).

These experiments demonstrate that the purified recombinant dtCSB protein is able to exert its biological function(s) *in vivo*, including transportation to the nucleus and participation in recovery of RNA synthesis, thus presumably also in transcription-coupled repair and complex formation with RNA polymerase II (24). The microinjection results confirm our earlier findings with the transfected CS-B cells stably expressing the dtCSB protein (24) and provide a solid basis for analysis of the biochemical properties of the purified protein and its mutant version.

In Vitro Biochemical Characterization of the Purified Recombinant wt and Mutant dtCSB Proteins—The presence of a SNF2 domain in the CSB protein starting with a Walker A-type nucleotide hydrolysis motif strongly suggested that CSB harbors an ATPase activity (21, 30). The mutant dtCSB (K538R) protein should serve as an optimal negative control for this assay. As shown in Fig. 3*A*, in the absence of nucleic acids, both proteins exhibited a comparable background level of activity. However, ATPase activity of wild-type dtCSB was highly stimulated by the presence of ds λ DNA in the reaction, in contrast to the mutant protein (Fig. 3*A*). The extent of ATP hydrolysis was proportional to the amount of wt dtCSB added to reactions in which ATP concentration was kept constant and the DNA cofactor was in excess (Fig. 3*B* and 5*A*). Quantification of ATP hydrolysis showed that the conservative K538R substitution resulted in a complete loss of the catalytic ATPase function of CSB (Fig. 3*B*). Because wt and mutant proteins (purified in parallel) are at the same level of purity and equally concentrated (Fig. 1*C*), this result indicates that DNA-dependent ATPase activity is an intrinsic property of CSB protein and that the K538R substitution abolishes ATP hydrolysis.

To get insight into the optimal structure of the DNA cofactor

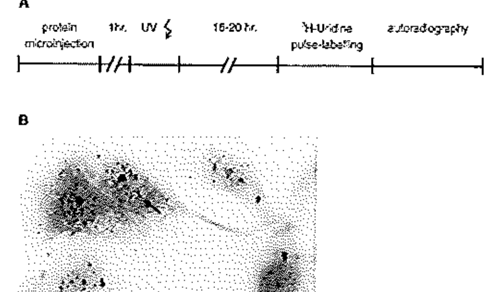


Fig. 2. Assessment of recombinant wt dtCSB *in vivo* function using microneedle injection. *A*, schematic representation of the microinjection RNA-synthesis recovery assay. wt dtCSB protein (0.02–0.05 μg/μl) purified from insect cells was injected into the cytoplasm of CS-B fibroblasts (homopolykaryons were used for the injection). RNA synthesis was assayed 16–20 h after UV irradiation by a 1-h pulse labeling with [^{3}H]uridine, fixation, and autoradiography (see "Experimental Procedures" for details). *B*, micrograph of a CS-B binuclear fibroblast (homopolykaryon) injected with wt dtCSB protein and assayed for RNA synthesis recovery by autoradiography. The injected binuclear CS-B cell (indicated by the *arrow*) exhibits a high level of transcription, as indicated by the high number of silver grains above both of its nuclei, whereas the noninjected surrounding CS-B fibroblasts (monokaryons) show the low level of RNA synthesis recovery typical for UV-exposed CS cells.

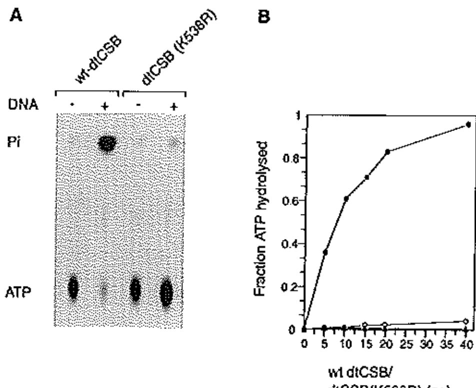


Fig. 3. ATPase activity of recombinant wt and mutant dtCSB protein. *A*, ATPase activity assay. Recombinant wt dtCSB and dtCSB (K538R) proteins (15 ng) purified from Sf21 insect cells were assayed for ATPase activity in the presence (+) or in the absence (−) of double-stranded λ DNA (150 ng) (see "Experimental Procedures"). Incubation was for 30 min. *B*, effect of increasing dtCSB protein concentration on ATP hydrolysis. Reactions were performed as in *A* in the presence or absence of ds λ DNA (150 ng) and the indicated protein concentration for 30 min. Release of P_i was quantified using a PhosphorImager (Molecular Dynamics). ●, wt dtCSB + λ DNA; ○, dtCSB (K538R) + λDNA; ▲, wt dtCSB and dtCSB (K538R) without DNA (data points are superimposed).

Table II
Effect of microinjection of recombinant dtCSB protein on RNA synthesis recovery

Cell strain	Complementation group	Injected protein[a]	UV irradiation	Grains per nucleus	Recovery of RNA synthesis
			J/m²	*mean ± S.E.*	*% of normal*[b]
C5RO	wt		15	69 ± 3	100
C5RO	wt	wt-CSB	15	63 ± 3	91
CS1AN	CS-B		15	15 ± 1	22
CS1AN	CS-B	wt-CSB	15	62 ± 5	90
CS1AN	CS-B	K538R-CSB	15	26 ± 2	38
GM2965	CS-A		15	20 ± 1	29
GM2965	CS-A	wt-CSB	15	22 ± 2	32
C5RO	wt		10	78 ± 4	100
XPCS1BA	XP-B		10	13 ± 1	17
XPCS1BA	XP-B	wt-CSB	10	15 ± 1	19

[a] Recombinant dtCSB protein purified from insect cells (see Fig. 1). Estimated protein concentration, 0.02–0.05 μg/μl.
[b] As a control, basal transcription was also measured in unirradiated cells. wt and CS1AN fibroblasts showed the same RNA synthesis level under these experimental conditions: 123 ± 4 grains/nucleus in wt C5RO; 123 ± 4 grains/nucleus in CS1AN.

required for CSB ATPase activity, we tested several DNAs with different structural properties for their ability to stimulate the CSB ATPase. Various double-stranded DNAs, including ds λ DNA and supercoiled circular pBluescript DNA, elicited high activity (Fig. 4*C*). Also M13 single-stranded DNA appeared a potent activator (Fig. 4*A*, *lane 1*). Because dsDNA and ssDNA are structurally very different, it is surprising that both stimulate the CSB ATPase activity to a closely comparable extent. Therefore we decided to premelt the M13 ssDNA to reduce secondary structure. Fig. 4*A* shows that the stimulatory activity of M13 ssDNA was strongly decreased after boiling the ssDNA followed by quenching on ice. These findings indicate that unmelted M13 ssDNA is structurally ill defined and probably contains considerable double-stranded character. We conclude that the DNA structure is important for CSB ATPase activity and that the protein is primarily stimulated by dsDNA. To further investigate the substrate requirement of CSB, structurally more defined oligonucleotides were tested for stimulation of ATP hydrolysis by dtCSB. Using synthetic oligonucleotides we observed that the presence of secondary structure such as stem-loop and fork-like structures or DNA hairpins enhanced CSB ATPase activity (Fig. 4*B* and Table I), whereas single-stranded molecules of the same length and sequence, as well as RNA oligonucleotides and RNA/DNA hybrid molecules, are only very weak activators (Fig. 5*B* and Table I). In this respect, it is interesting to note the difference between the activity elicited by a single-stranded poly-d(T)$_{>80}$ molecule (Fig. 5*B*, *open circles*), which has little secondary structure, and a single-stranded oligonucleotide containing palindromic sequences, which can give rise to stem-loop structures (Fig. 5*B*, *open triangles*, for sequence see Table I). In addition, we observed that CSB DNA-dependent ATPase activity was stimulated to the same extent by both naked DNA and nucleosomal DNA (Fig. 4*C*). Presence of $MgCl_2$ was essential for catalytic activity, which was optimal at a pH value of 7.5 and at 100 mM salt (not shown).

Under the experimental conditions used, a turnover rate ranging from 27 to 33 ATP min^{-1} was calculated in the presence of dsDNA (Fig. 4*B*). The turnover number was calculated in three independent experiments, including an ATPase kinetics analysis with constant protein concentration and ds λ DNA in which ATP concentration ranged from 0.025 to 0.2 mM. When ssDNA (poly(dT)$_{>80}$) was used as cofactor, the turnover number was in the order of 3.7–6 ATP min^{-1} (Fig. 4*B*).

In conclusion, our *in vitro* data indicate that CSB is a DNA-dependent ATPase, which is specifically stimulated by double-stranded DNA and double-stranded DNA structures with a partial single-stranded character as well as nucleosomal DNA but not by true ssDNA. Moreover, the conservative amino acid substitution in the NTP binding motif impairs CSB ability of

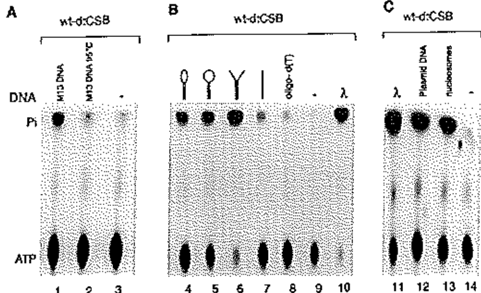
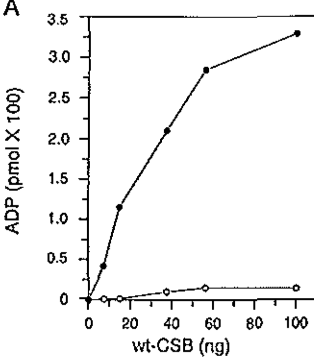


FIG. 4. **Requirement of DNA cofactors for dtCSB ATPase activity.** *A*, secondary structure in M13 ssDNA is required for dtCSB ATPase activity. wt dtCSB (10 ng) was assayed for ATP hydrolysis in standard ATPase reactions (as in Fig. 3) containing the following DNA molecules (150 ng): *lane 1*, M13 ssDNA; *lane 2*, M13 ssDNA premelted at 95 °C and subsequently quenched on ice; *lane 3*, without DNA. *B*, effect of different synthetic DNA molecules on dtCSB ATPase activity. ATP hydrolysis by wt dtCSB (15 ng) was analyzed in the presence of synthetic oligonucleotides with different secondary structures (150 ng): *lane 4*, stem 19-loop 8; *lane 5*, stem 20-loop 15; *lane 6*, splayed arm; *lane 7*, single-stranded oligonucleotide (74 nucleotides); *lane 8*, oligo(dT)$_{15}$; 9, no DNA; 10 λ DNA. See Table I for oligonucleotides sequences. *C*, nucleosomal DNA acts as cofactor for CSB ATPase activity. wt dtCSB ATPase activity was analyzed in the presence of naked and nucleosomal DNA (150 ng): *lane 11*, λ DNA; *lane 12*, double-stranded pBluescript DNA; *lane 13*, bulk HeLa polynucleosomes; *lane 14*, without DNA.



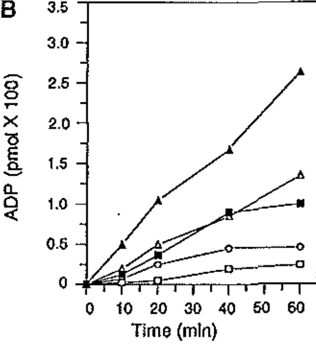


FIG. 5. **Kinetics of ATP hydrolysis.** *A*, ATP hydrolysis as a function of wt dtCSB protein concentration. Reactions contained 50 μM cold ATP supplemented with 0.25 μCi of [γ-^{32}P]ATP (see "Experimental Procedures") and were incubated for 1 h at 30 °C. ○, no DNA was added; ●, λ DNA (150 ng) was added. *B*, ATP hydrolysis as a function of incubation time. wt dtCSB protein (35 ng) was assayed for ATP hydrolysis in the presence of different nucleic acid cofactors (150 ng). Aliquots were taken after incubation for 10, 20, 40, and 60 min and analyzed for release of P$_i$. Reaction mixture contained synthetic oligonucleotides (74 nucleotides long): △, ssDNA; ▲, dsDNA; □, RNA; ■, RNA-DNA hybrid; ○, poly(dT)$_{>80}$. See Table I for oligonucleotide sequences. The single-stranded oligodeoxynucleotide described in Table I was annealed to its complementary sequence and used as dsDNA cofactor. The synthetic RNA oligonucleotide described in Table I was used either alone or annealed to its complementary ssDNA oligonucleotide (RNA-DNA hybrid).

hydrolysing ATP, showing that ATPase activity is not due to a contaminating polypeptide.

The highly conserved common domain of the family of SNF2/SWI2 proteins shares significant homology with seven sequence motifs identified in numerous DNA and RNA helicases that require NTP hydrolysis for disrupting the hydrogen bonds that hold the two DNA strands together (for review see Refs. 36 and 37). To test whether functional dtCSB displayed any DNA helicase activity, the protein was incubated with M13 ssDNA to which a ^{32}P-labeled 18- or 36-base primer was annealed. Release of the bound oligonucleotide from M13 DNA was analyzed by polyacrylamide gel electrophoresis (see "Experimental Procedures"). Under the assay conditions used, which were able to detect bona fide DNA helicase activity of purified TFIIH used as positive control, no displacement of the helicase templates was observed (not shown). Also, CSB failed to displace a 50-base primer with a 15-nucleotide 3′ or 5′ noncomplementary overhang (data not shown). The same negative results were obtained with a 75-base double-stranded oligonucleotide and a 36-base oligonucleotide, to which a 5′ ^{32}P-labeled 17-base or 19-base fragment (F17-F36; F19-F36; Table I) was annealed (data not shown). Absence of detectable helicase activity in the presence of DNA-dependent ATP-ase is in agreement with recent findings reported by Selby and Sancar (34) using a non-tagged, partially full-length CSB preparation.

Inactivation of the ATPase Activity Does Not Completely Impair the in Vivo Function of CSB—As shown above, the specific K538R substitution in the CSB nucleotide-binding domain abolishes its catalytic ATPase activity *in vitro*. To investigate the biological consequences of this mutation, purified dtCSB (K538R) protein was microinjected into living CS-B fibroblasts in parallel to injected wild-type dtCSB. The same type of mutation (Lys to Arg) in the ATP hydrolysis domain of the dual functional XPB DNA repair helicase was shown to possess a dominant-negative *in vivo* effect; this mutant subunit of the TFIIH repair/transcription complex completely paralyzes both repair and basal transcription in a dominant fashion in normal cells (28). Apparently, the mutant XPB protein is incorporated in the TFIIH complex but is unable to exert its function, thereby actively blocking both processes. The CSB protein too is incorporated in a RNA polymerase II complex, and thus substitution by an enzymatically dead molecule could actively disrupt the processes in which it is engaged. However, in striking contrast to the effect of mutant XPB, injection of the recombinant dtCSB (K538R) protein even partially rescued the defect in RNA synthesis recovery after UV irradiation in CS-B fibroblasts (Table II). Although the activity is much lower when compared with wt dtCSB, still a significant (approximately 2-fold) stimulation of resumption of transcription was observed in repeated experiments (Table II). This partial *in vivo* activity is in sharp contrast with the completely inactive ATP hydrolysis of (K538R) mutant protein.

DISCUSSION

Purification of the dtCSB Protein Using Tags—Here we describe the successful isolation of double-tagged CSB protein from baculovirus-infected SF21 cells. We have utilized a novel purification procedure that has a number of advantages above classical purification protocols: (i) The use of the HA and His_6 tags allows a highly selective, rapid, standardized three-step procedure. This avoids lengthy purification protocols involving a large number of column chromatography steps. In independent experiments we have found that the HA-monoclonal affinity chromatography by itself already can provide an >1000-fold purification when starting from mammalian whole cell extracts (38). The Ni^{2+}-nitrilotriacetic acid agarose column chromatography specific for the hexameric histidine tag has the benefit that it is very versatile because it can be carried out under a wide range of conditions. (ii) The strategy used in this work has also the advantage that it selects only for full-length protein avoiding inclusion of partial degradation products in the final purified protein preparation. (iii) The purification procedure is very efficient: the dtCSB approach used yielded 22 μg of intact CSB protein from 10^8 infected cells, appearing at least 15-fold more efficient compared with classical protocols in which CSB was successfully purified involving seven chromatographic steps (34). A prerequisite for the use of tags is that one should test whether the addition of tags interferes with the function of the protein. In the case of dtCSB, we demonstrate *in vivo* and *in vitro* that the tagged protein was fully functional. Microinjection and DNA transfection experiments showed that the dtCSB protein completely corrected the defect in recovery of UV-induced inhibition of RNA synthesis and UV sensitivity of CS-B cells.

Biochemical Properties of the Purified dtCSB Protein—Purified wt dtCSB possesses an *in vitro* DNA-dependent ATPase activity, as also reported recently by Selby and Sancar (34). We investigated the DNA cofactor requirement for CSB ATPase activity using structurally defined DNA substrates. The highest stimulation of CSB ATPase activity was observed when dsDNA or structured oligonucleotides with a partial double- and single-stranded character were used as DNA substrates (Figs. 4*B* and 5*B*). Unexpectedly, M13 ssDNA also exerts a strong stimulation. The notion that true dsDNA and ssDNA are physicochemically very different rendered it unlikely that both should be equally efficient in enhancing CSB-ATPase. However, the ATPase stimulation by M13 ssDNA was drastically reduced after removing secondary structure by melting. This indicates that the double-stranded character in M13 ssDNA was responsible for the stimulation and that true ssDNA is a poor activator for CSB ATPase. This was confirmed by the use of $poly(dT)_{>80}$, which is known to have little secondary structure and "single-stranded" oligonucleodides with multiple palindromic sequences. In addition to differences in protein preparations, the above observation may explain the findings of Selby and Sancar, who reported a strong stimulation of CSB-ATPase by ssDNA (34) and the yeast equivalent RAD26, which was reported by Guzder *et al.* to be stimulated preferentially by ssDNA (39). In fact, for any ssDNA-dependent ATPases for which nonpremelted ssDNA substrates were used, it should be considered a possibility that the stimulation was not derived from ssDNA but from double-stranded structures within it. The fact that the CSB ATPase activity is strongly enhanced by ds/ss DNA substrates such as Y-structures containing hairpins opens the possibility that a double-stranded to single-stranded transition is a biological relevant DNA conformation for CSB. In addition, we find that ATP hydrolysis by CSB is stimulated equally by naked dsDNA and nucleosomal DNA (Fig. 4*C*). A similar observation is made for the ySWI-SNF complex (40, 41). Specific activity of dtCSB in the presence of dsDNA was approximately 160 pmol ADP formed/μg CSB/min (K_{cat} = ~27 min^{-1}), which is within the range of activities measured for other DNA-dependent ATPases of the same family. In particular, CSB appears to be a stronger ATPase than the recombinant ySNF2 (20 pmol ADP/μg/min (42)) as well as the *Drosophila* NURF complex (K_{cat} = ~8.5 min^{-1} in the presence of nucleosomal DNA) (43).

As observed with other members of the SNF2-like family of ATPases, no overt helicase activity could be attributed to CSB, using classical helicase DNA substrates as well as DNA molecules with different types of noncomplementary overhangs (39, 42). The high homology of CSB with members of the SNF2 family suggests that these proteins may function via a similar mechanism. SNF2-like proteins have been reported to be involved in chromatin remodelling and to be able to disrupt protein-DNA interactions (44–47). On this basis, it has been proposed that they function as DNA-translocating factors that use the energy derived from ATP hydrolysis to move along the DNA, destabilizing proteins bound to the DNA (48). Similarly, we can speculate that CSB uses energy from ATP hydrolysis to induce some type of structural alteration in the DNA, involving local strand separation that diminishes the affinity of bound protein for dsDNA. It has been shown that NTP-binding proteins undergo conformational changes upon NTP binding and hydrolysis. Recently, a structural homology was found between a DNA helicase of *Bacillus stearothermophilus* and the recombination protein RecA. This suggested a common basis for the coupling of ATP binding/hydrolysis to conformational changes of the protein, affecting the affinity of the enzyme for different DNA substrates, thereby driving the catalytic reaction (49). In agreement with this idea we recently found CSB to reside in a large MW complex *in vivo*. This complex contains RNA polymerase II but is devoid of transcription initiation factors (24), and its further characterization indicates that it most likely represents an elongation mode of RNA polymerase II.[2] Direct *in vitro* association of CSB with RNA polymerase II was reported recently by Tantin *et al.* (50) and by Selby and Sancar (51). The latter study also provided evidence that CSB stimulates transcription elongation *in vitro*. The DNA structure requirements of CSB presented here are consistent with the transitions from ssDNA to dsDNA at the site of an elongating or stalled RNA polymerase. At this location the CSB protein may help destabilize nucleosome-DNA interactions, thus facilitating transcription elongation at pause sites. In addition, CSB may enable resumption of stalled transcription elongation after DNA damage removal by dissociating NER (such as TFIIH) and other repair factors and/or nucleosomes bound to the DNA.

Biological Consequences of ATPase Deficiency on the Functioning of CSB—By site-directed mutagenesis the invariant lysine 538 residue in CSB ATP binding motif was replaced with the physicochemically related arginine. The conservative K538R mutation leads to a (virtual) complete loss of dtCSB ATPase activity *in vitro* (Fig. 3, *A* and *B*), as also has been shown for a variety of other ATPases (31, 42, 52). Surprisingly, we found that the ATPase-dead CSB protein still exerts a partial function *in vivo*, as shown by microinjection experiments in CS-B fibroblasts (Table II). Interestingly, the *in vivo* results suggest that this specific mutation can partially uncouple ATP hydrolysis from other biological activities of CSB, implying that other properties of CSB are also important for its function. In this respect is relevant to note that CS-B cell line (CS1AN) used in this study carries two alleles, each leading to a severely truncated CSB protein product (18). In fact, system-

[2] E. Citterio and J. H. J. Hoeijmakers, unpublished observations.

atic mutation analysis of a large number of CSB patients has revealed that most patients produce severe CSB truncations. Our microinjection experiments suggest that having an enzymatically dead but physically intact CSB molecule is still better than having no protein at all. We suggest that the partial activity observed may be due to the fact that the enzymatically inactive CSB mutant protein may help stabilize a CSB-containing complex or otherwise permits this complex to function better when CSB is physically present. In fact, this is very similar to findings made with a comparable Lys to Arg substitution in the XPD helicase of TFIIH.[3] For the transcription function of the TFIIH complex, this protein must be physically present to permit complex formation or stability, but it does not have to be enzymatically functioning. For the NER function of the TFIIH complex, the XPD helicase activity is at least in part required. Also for other protein complexes it has been shown that absence of one subunits leads to instability of the complex (*e.g.* Ref. 53). Consistent with the above interpretations for the partial activity of mutant CSB is the notion that the conservative Lys to Arg amino acid substitution is not expected to dramatically alter the overall structure of the CSB protein. Because we observe a biological effect, the dtCSB (K538R) mutant protein probably still fits into the RNA polymerase II complex. Our finding that the same type of amino acid substitution in the XPB and XPD helicase subunits of TFIIH exerts at least some biological effects strongly suggests that also these mutant proteins are still capable of complex formation. The intriguing finding that an enzymatically inactive CSB protein still exert some function *in vivo* supports the idea that interaction of CSB with other transcription/repair proteins *per se* contributes to its function(s).

Acknowledgments—We are grateful to Robert Kingston and Gavin Schnitzler for providing the purified HeLa polynucleosomes. Wouter de Laat is acknowledged for providing the oligonucleotides substrates and for useful discussion. We thank Roland Kanaar, Bas Winkler, and André Eker for helpful suggestions. Sigrid Swagemakers and Cécile Visser are acknowledged for technical assistance, and Mirko Kuit is acknowledged for photography.

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[3] G. S. Winkler, manuscript in preparation.

Chapter 9

ATP-dependent chromatin remodeling by the Cockayne syndrome B DNA repair-transcription coupling factor

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Submitted for publication

Abstract

The Cockayne syndrome B protein (CSB) is required for coupling nucleotide excision DNA repair (NER) to transcription in a process known as transcription-coupled repair (TCR). Cockayne syndrome patients show severe UV-sensitivity and neurodevelopmental abnormalities. CSB is a DNA-dependent ATPase of the SWI2/SNF2 family. SWI2/SNF2-like proteins are implicated in chromatin remodeling during transcription. Here, we used purified recombinant CSB protein to investigate whether it can remodel chromatin *in vitro*. We show that binding of CSB to DNA results in an alteration of DNA double-helix conformation. In addition, we find that CSB is able to remodel chromatin structure at the expense of ATP hydrolysis. Specifically, CSB can alter DNase I accessibility to reconstituted mononucleosome cores and disarrange an array of nucleosomes regularly spaced on plasmid DNA. In this respect, CSB resembles the chromatin remodeling properties of other SWI2/SNF2-related proteins. Nucleosome remodeling by CSB does not involve complete dissociation of the histone octamer. This study extends the knowledge of chromatin remodeling factors within the SWI2/SNF2 family to DNA repair proteins. CSB is the first NER protein found to play a direct role in modulating nucleosome structure. The relevance of this finding at the interplay between transcription and repair is discussed.

Introduction

In eukaryotes, nuclear DNA is compacted into an organized chromatin structure, whose fundamental unit is the nucleosome (24). This packaging of DNA negatively influences most processes on DNA, including transcription, recombination and DNA repair (13, 22). To relieve nucleosomal repression different mechanisms have evolved, of which chromatin remodeling by large multiprotein complexes is one of the best studied. The role of remodeling complexes in transcription regulation is well documented (58). Several *in vitro* studies have shown the ability of these remodeling "machines" to change the nucleosome structure in an ATP-dependent manner (23). As a common feature the remodeling machines contain a DNA- and/or nucleosome-dependent ATPase of the SWI2/SNF2 family (12). This family is divided into a number of different classes, including SWI2/SNF2 and ISWI. Proteins belonging to these latter classes are highly homologous to yeast SWI2/SNF2 and *Drosophila* ISWI, respectively (34, 51). Recently, three ATPases, the human Brg1 and hBrm together with the *Drosophila* ISWI, have been reported to exhibit chromatin remodeling activities on their own (8, 35). Brg1 and hBrm are the ATPase components of two distinct human SWI/SNF complexes (25, 55), while ISWI is part of three *Drosophila* remodeling complexes, ACF, CHRAC, and NURF (5). These experiments have led to the view that the ATPase subunits are the catalytic core of the remodeling "machines", while the other associated proteins may have regulatory or auxiliary functions. Interestingly, the SWI2/SNF2 family includes also proteins involved in various DNA repair pathways, such as nucleotide excision repair (NER), post-replication repair, and recombination repair (12), suggesting that these processes may require similar remodeling activities as transcription.

NER is a versatile DNA repair pathway that removes a remarkably wide range of DNA lesions, including the main ultraviolet (UV) light-induced lesions, i.e. cyclobutane pyrimidine dimers and 6/4 photoproducts. Approximately 30 proteins are involved in mammalian NER, that proceeds via a well characterized, step-wise 'cut and paste' reaction (for recent review see (11)). One of the immediate consequences of DNA damage is blockage of transcription. To allow a rapid resumption of this vital process, a subpathway of NER has evolved, that preferentially recognizes and repairs transcription-blocking lesions (2, 30). This process is known as transcription-coupled repair (TCR). Some aspects of TCR are highly conserved from *Escherichia coli*, to yeast and mammals (50). In addition, evidence has been obtained that oxidative damage, mainly processed by the base excision repair pathway, is removed in a transcription-coupled manner (7). Thus, the importance of TCR is not limited to NER.

Inherited defects in TCR form the molecular basis of Cockayne syndrome (CS). CS patients are UV-sensitive and show severe developmental and neurological dysfunctions (3, 32). Two human genes are specifically required for TCR, *CSA* and *CSB*. These genes are defective in CS complementation groups A

and B (17, 47). Several recent observations suggest that the CS proteins may have a subtle, additional role in transcription regulation (1, 38, 46, 49).

CSA codes for a five WD 40 repeat-containing protein (17). The *CSB* gene encodes a nuclear protein of 168 kDa and its central part is highly homologous to the helicase domain of SWI2/SNF2 (12, 47). The recombinant CSB protein is a double-stranded DNA-dependent ATPase (activated by both naked and nucleosomal DNA), but, like other members of the SWI2/SNF2 family, is not a classical helicase (6, 9, 33, 39). Changing the invariant lysine to an arginine residue within the Walker A motif, responsible for NTP-binding and hydrolysis, abolished CSB ATPase activity, but only partially affected its biological function in living cells (6). Both *in vivo* and *in vitro* it was found that CSB interacts with RNA polymerase II (39, 46, 49).

Chromatin remodeling is thought to play a role in DNA repair. Partial decondensation of chromatin is suggested to provide access to DNA damage processing factors (29). However, little is known about potential candidate activities. In this study, we address the possibility that CSB, because of its similarities to SWI2/SNF2, may play a role in such a process. We examined the involvement of CSB, as an isolated recombinant protein, in chromatin remodeling by *in vitro* accessibility assays on nucleosomal templates. Our results show that CSB can alter DNA conformation and is able to induce changes in chromatin structure in an ATP-dependent fashion. Mechanistic similarities between CSB and the hSWI/SNF complex are discussed.

Results

In this study, we utilized highly purified, recombinant CSB protein. Wild type and ATPase-deficient (CSB^{K538R}) mutant CSB proteins, both epitope–tagged (HA-CSB-his_6) were isolated from baculovirus infected Sf21 insect cells as described previously. The tags do not interfere with the biological function of CSB *in vivo* (6). Both proteins were purified to near homogeneity, as determined by silver staining (Fig. 1A). As previously described, CSB ATPase activity was stimulated by double stranded but not by single stranded DNA. Both naked as well as nucleosomal DNA gave a similar stimulation (data not shown and (6)). When HeLa core histones were tested as cofactors, no increase of ATP hydrolysis by CSB was detected (data not shown; see Materials and Methods). This emphasizes that CSB ATPase activity is strictly dependent on DNA. The hSWI/SNF complex was isolated from HeLa cells and functionally characterized as described (37).

CSB influences DNA topology by inducing negative supercoiling

CSB contains seven conserved motifs characteristic of the SNF2/SWI2 family of putative helicases (12, 47). Nevertheless, CSB, as well as most of the SWI2/SNF2-related proteins tested to date, fail to show classical helicase activity as

assayed by oligonucleotide strand displacement (6, 9, 39, 44). However, the lack of overt helicase activity does not exclude the possibility that these proteins may work by altering the DNA conformation thereby inducing a local separation of the DNA strands. To address this possibility, we tested CSB activity in a topological assay (45). Singly-nicked DNA plasmid was incubated with increasing amounts of purified CSB or CSB^{K538R} (Fig. 1B). Subsequently, DNA ligase was added to covalently close the nick in order to preserve any protein-induced change in linking number (ΔLk) in the duplex DNA. To determine the extent and the direction of induced ΔLk, the topoisomer population was analyzed by agarose gel electrophoresis in the absence or presence of the intercalating agent chloroquine (Fig. 1B and data not shown). As visible in Figure 1B lane 5, addition of 40 ng of CSB shifted the topoisomers distribution. The amount of slowly migrating DNA topoisomers in the gel increased with increased CSB concentration (Fig. 1B, lane 6, and data not shown). We calculate that the presence of CSB at an approximate molar ratio of 10:1 per plasmid DNA molecule, was sufficient to induce the topological effect seen in Figure 1B, lane 6.

The hydrolysis of ATP was not required to induce the topological change, since the same ΔLk was observed in the absence of ATP (not shown), and in the presence of the ATPase-deficient CSB^{K538R} mutant (Fig. 1B, lanes 8-10). However, ATP hydrolysis by CSB was detected in the reactions (data not shown).

Furthermore, in control experiments we did not detect any contaminating topoisomerase activity in the CSB preparations (data not shown, see Materials and Methods). The chloroquine included in the gels shown in Figure 1B intercalates in the DNA double helix and introduces positive supercoiling in the covalently closed relaxed plasmid. CSB counteracted chloroquine action, causing an electrophoretic retardation of the DNA topoisomers (Fig. 1B, lane 3). We conclude that binding of CSB causes a change in DNA conformation detected as negative supercoiling in our topological assay. The direction of CSB-induced ΔLk was confirmed by analysis of the topoisomers on two-dimensional gels (data not shown) (45).

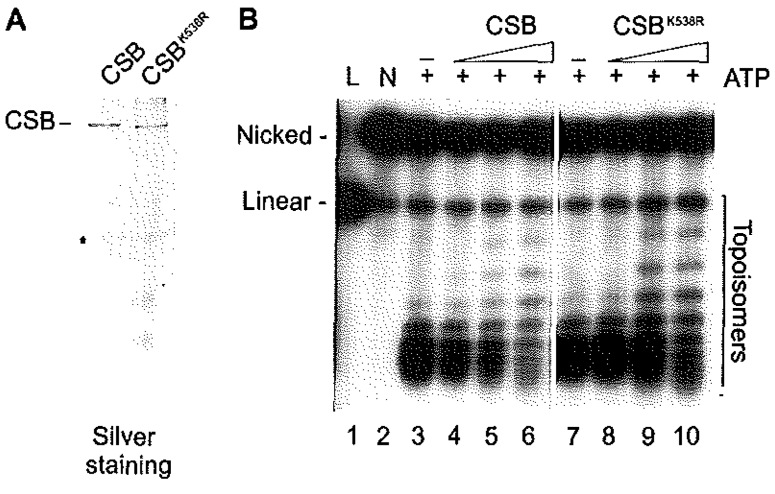


FIG. 1. CSB introduces negative supercoils in plasmid DNA upon binding. A. Purified recombinant CSB and CSB^{K538R} proteins. Aliquots of the Mono Q fractions of both proteins were separated by SDS-PAGE (8%) and visualized by silver staining. In addition to the major CSB band, two degradation products (as determined by Western blotting) were visible. *represents keratins which were also present in empty lanes. B. Shift in the topoisomers distribution upon CSB binding. Singly-nicked plasmid DNA (100 ng) (lane 2) was incubated with increasing amounts of CSB or CSB^{K538R} (20, 40, 80 ng, lanes 4-6 and 8-10, respectively) in the presence of ATP. The DNA molecules were closed by the addition of *E. coli* DNA ligase and the topoisomers were resolved by electrophoresis on a 1% agarose gel containing 0.5 μg/ml chloroquine.

CSB remodels a nucleosome core in an ATP-dependent manner

The ability of CSB to influence DNA conformation, together with the high homology shared with the SWI2/SNF2-related proteins, suggested that CSB might play a role in chromatin remodeling. We first investigated this possibility at the basic level of chromatin organization, the nucleosome. For this purpose, we reconstituted rotationally phased mononucleosomes with purified histones on a radioactively labeled 155-bp DNA template using a salt dilution procedure. Alterations of the nucleosomal structure were assessed by DNase I footprinting (19), (see Materials and Methods). In the absence of remodeling activities, DNase I digestion of nucleosomes gave rise to the characteristic pattern of DNA fragments with a periodicity of 10 bp, which is clearly distinct from the pattern generated on naked DNA (Fig. 2A, compare lanes 1 and 11). Mononucleosomes were incubated with increasing amounts of purified CSB protein. In the presence of ATP, CSB visibly altered the DNase I accessibility to nucleosomal DNA. Remodeling is

particularly evident from the dramatic change in intensity of some specific DNA bands (Fig. 2A, lanes 2-4, note bands marked by arrows). The changes in the digestion pattern were proportional to the amount of CSB added (Fig. 2A, lanes 2-4). We estimate that an approximately equimolar ratio of CSB versus core particles promote alterations of the nucleosomal structure (Figure 2A, lane 3). No alterations were observed in the absence of ATP (Fig. 2A, lanes 5-7), or with the addition of the non-hydrolyzable ATP analogue, ATPγS (Fig. 2B, lane 3), nor was the ATPase-deficient CSB^{K538R} able to promote the remodeling (Fig. 2A, lanes 8-10). The ATP-dependent changes in the DNAse pattern cannot simply be due to CSB binding because CSB binding to nucleosomes was independent from ATP, and was observed also with the CSB ^{K538R} mutant protein in gel shift experiments (data not shown). These results strongly indicate that nucleosome remodeling is catalyzed by CSB at the expense of ATP hydrolysis.

When apyrase, which hydrolyses ATP, was added to a reaction containing nucleosomes before addition of CSB, ATP-dependent nucleosome remodeling was inhibited (Fig. 2B, compare lanes 2 and 4). To analyze the stability of the altered nucleosome, we first incubated identical samples containing ATP, nucleosomes and CSB for 45 min, exposed them to apyrase, and subsequently digested with DNase I at time points ranging from 2 min to 40 min (Fig. 2B, lanes 5-7). The specific DNase I digestion pattern was conserved until 40 min past the addition of apyrase (Fig. 2B, lanes 5-7). These data suggests that the CSB-induced structural changes in the nucleosome are stable and that maintenance of the remodeled state does not require continuous ATP hydrolysis.

In order to qualitatively compare CSB activity with other chromatin remodeling factors, we assayed CSB and the isolated hSWI/SNF multiprotein complex (37) in identical disruption reactions. The results presented in Figure 2C reveal that the DNase I digestion patterns mediated by CSB and by hSWI/SNF are very similar. However, subtle differences in DNase accessibility were visible in the central portion of the DNA fragment (Fig. 2C, compare lane 3 and 4). Specific DNase cleavage at these sites was repeatedly observed in different reactions (data not shown). Quantitative comparison of the two remodeling activities is complicated by the fact that CSB is tested as an isolated recombinant protein, while hSWI/SNF as a native multiprotein complex. In addition to this, it was recently shown that the remodeling activity of the entire hSWI/SNF complex is much stronger than that of the separate ATPases subunits (35).

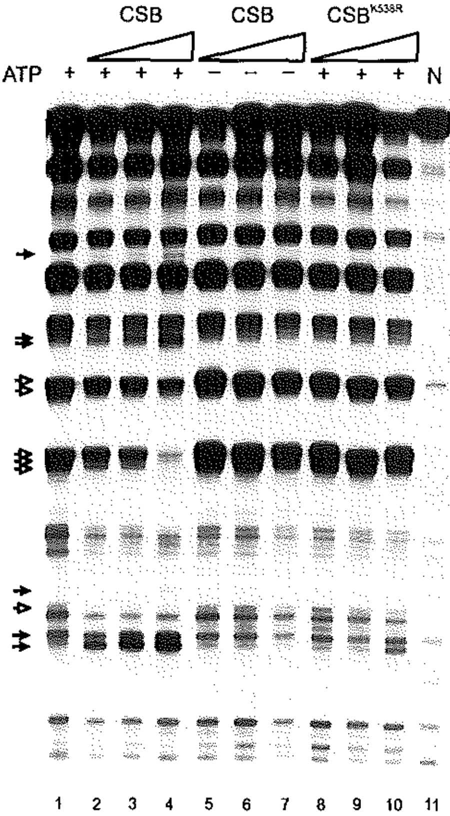
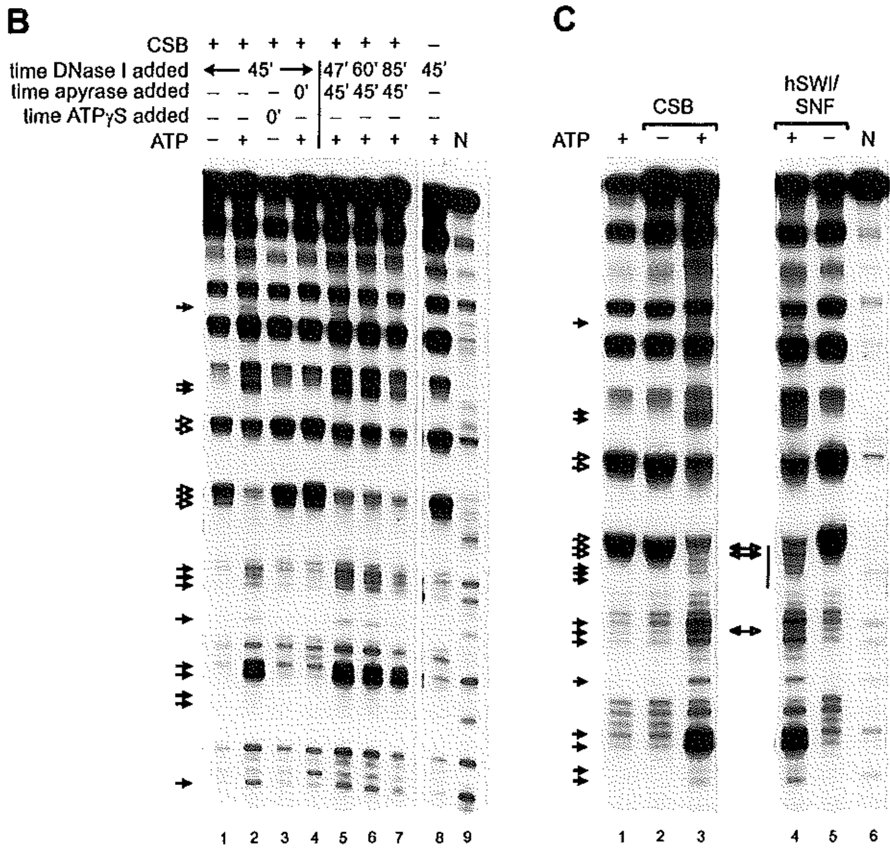


FIG. 2. Mononucleosome remodeling by CSB. A. Mononucleosome remodeling by CSB is ATP-dependent. End-labeled nucleosome particles (approximately 3 ng of total nucleosomes) were incubated with increasing CSB amounts (10, 20 and 40 ng), in the presence or absence of ATP (lanes 2-7). Similar reactions were performed with CSB^{K538R} in the presence of ATP (lanes 8-10). Remodeling was assessed by DNase I digestion. Filled arrows represents sites of enhanced cutting due to the presence of CSB, while open arrows indicate sites of reduced cleavage. N represents naked control DNA.



B. Nucleosome remodeling by CSB is stable upon removal of ATP by apyrase. Reactions contained 40 ng CSB and were performed as in A, except that, where indicated, ATP S (2 mM), or apyrase (1 U) were added. ATP S did not support remodeling (lane 3). Similarly, addition of apyrase (1 U) prior to CSB inhibited nucleosome remodeling (lane 4). However, addition of apyrase (1 U) after CSB had been present for 45 min did not inhibit nor reverse nucleosome disruption, as evidenced by DNAse I digestion after 2 min (lane 5), 15 min (lane 6), and 40 min (lane 7). N represents naked DNA. C. CSB nucleosome remodeling pattern is very similar but not identical to the one generated by the hSWI/SNF complex. Reactions were performed as in A, and contained CSB (60 ng, lanes 2 and 3) or the isolated hSWI/SNF complex (300 ng, lanes 6 and 7), in the presence or absence of ATP as indicated. Sites of enhanced (filled arrows) and of decreased (open arrows) DNase I digestion are indicated. Double-headed arrows between lane 3 and 4 represent cleavage sites that distinguish the two nucleosome disruption activities. Bar: approximate position of the nucleosomal dyad axis. N is naked control DNA.

Plasmid chromatin remodeling by CSB

To examine whether CSB could reorganize an array of nucleosomes, which more closely resembles the *in vivo* situation, we used *Drosophila* embryo extracts to reconstitute chromatin on a DNA plasmid *in vitro* (52). The chromatin assembly and spacing complexes (20, 53) active in these extracts catalyze deposition of nucleosomes with uniform spacing, as visualized by Micrococcal nuclease (MNase) digestion (see Fig. 3A and Materials and Methods). Before the chromatin templates were incubated with CSB, the endogenous *Drosophila* remodeling activities were disrupted by sarkosyl treatment (53). Sarkosyl and ATP were subsequently removed by gel filtration (Fig. 3A, Materials and Methods). The regularity of the nucleosomal array, which was maintained after the sarkosyl treatment, was significantly perturbed by the addition of CSB in the presence of ATP (Fig. 3A). The loss of periodic spacing between the nucleosomes was visible when CSB was present in a ~20 fold molar excess compared to the template. Since this plasmid contains an average of 16 nucleosomes, we estimate that CSB is active at an approximate equimolar ratio with nucleosome particles. A similar irregular MNase pattern was induced by the hSWI/SNF complex in an ATP-dependent reaction (Fig. 3A and data not shown). In contrast, in the presence of the CSB^{K538R} ATPase-deficient mutant, the nucleosomes were found in the original regular array (Fig. 3A). Similar results were obtained in control reactions in which ATP was omitted (not shown). These data indicate that CSB uses the energy from ATP-hydrolysis to remodel nucleosomes on large DNA molecules. This change could result from repositioning of nucleosome octamers and/or generation of an altered nucleosome that is more accessible to nucleases.

One hallmark of the SWI2/SNF2 complexes is the ability to change the topology of nucleosomal plasmid DNA in an ATP-dependent manner (19, 25, 57), activity which is also displayed by the isolated human ATPases Brg1 and hBrm (35). To assay CSB for this activity, closed circular plasmid chromatin was reconstituted with *Xenopus* oocyte heat-treated extracts (19) (see Materials and Methods). Nucleosomes introduce negative supercoils in plasmid DNA, which can be visualized after deproteinization as a rapid migration (relative to relaxed DNA) in agarose gel electrophoresis (Fig. 3B, lane 1). As previously characterized, addition of hSWI/SNF, in the presence of ATP and Topoisomerase I, reduces the supercoiling of the reconstituted plasmid. This is detected as the appearance of

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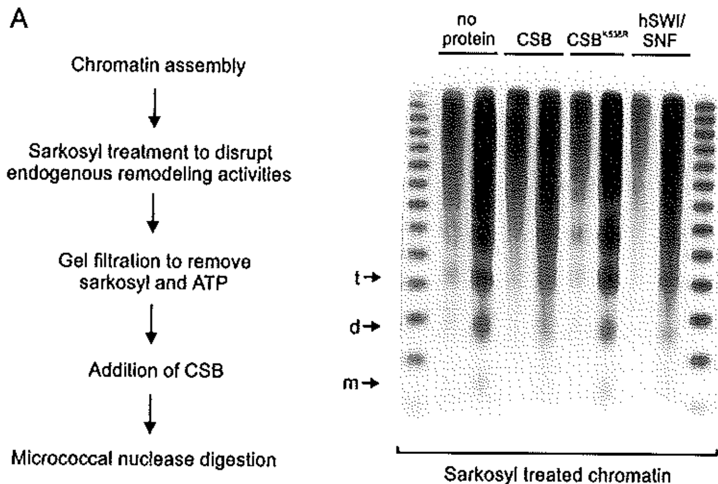
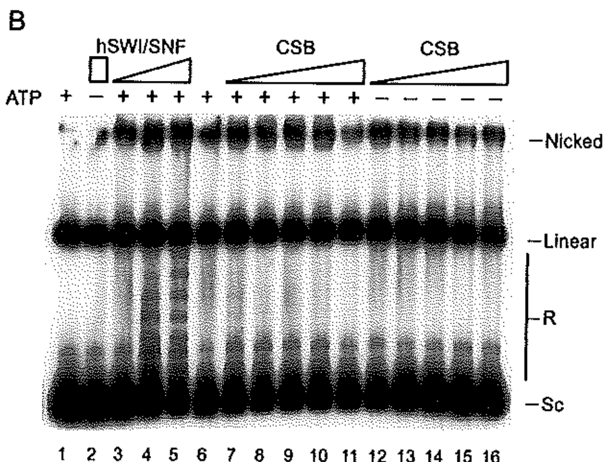
FIG. 3. Remodeling of plasmid chromatin by CSB. A. Remodeling of nucleosome arrays detected by Micrococcal nuclease (MNase) digestion. CSB induces loss of the regular nucleosome repeat characteristic of *Drosophila* reconstituted chromatin in an ATP dependent manner. Left panel: schematic outline of the assay. Right panel: Sarkosyl-stripped chromatin (40 ng) was incubated either in the absence of proteins or with CSB, CSB^{K538R}, and hSWI/SNF in the presence of ATP (as described in Materials and Methods). Nucleosome organization was studied by MNase digestion (8 units), performed for 60 and 120 seconds, respectively. The position of mononucleosomes (m), dinucleosomes (d) and trinucleosomes (t) are indicated by





arrows. The DNA size marker represents a ladder of 123 bp repeats. B. Remodeling of nucleosome arrays visualized as ATP-dependent changes in supercoiling. CSB, unlike hSWI/SNF, does not induce visible changes in the topology of nucleosomal plasmid DNA. Nucleosomal template was incubated with topisomerase I and either hSWI/SNF (200 ng in lane 2; 20, 60 and 200 ng, respectively in lanes 3, 4 and 5) or increasing amounts of CSB (3-fold increment starting from 2.9 ng in lanes 7 and 12), in the presence or absence of ATP. The molar ratio of CSB (in lane 11) to the ATPases subunits of hSWI/SNF (in lane 5) was approximately 20:1, as determined by silver staining (not shown). N, nicked DNA; L, linear; Sc, supercoiled; R, relaxed or partially supercoiled.

topoisomers that have a reduced mobility (Fig. 3B, lanes 3-5; (19)). In contrast, CSB showed no detectable activity in this assay (Figure 3B, lanes 7-11) even when it was present in an ~20-fold molar excess compared to the hSWI/SNF ATPase subunits, as determined by silver staining (Fig. 3B, compare lane 11 with lane 5; data not shown). Similar results were obtained on chromatin templates assembled with *Drosophila* embryo extracts (52) (data not shown; see Materials and Methods). These data might indicate mechanistic differences in chromatin remodeling by CSB and hSWI/SNF.

Nucleosome remodeling by CSB does not result in a complete disruption of the DNA-histones contacts, nor in octamer transfer to free DNA in trans

The above reported results establish the ability of CSB to influence chromatin structure. To gain insight into the mechanism of remodeling by CSB, we used gel mobility shift experiments to determine whether CSB dissociates histones from DNA. As it has been seen with other remodeling complexes, mixing CSB with labeled nucleosomes (or bare DNA) creates a mixture that does not enter the gel (Fig. 4, lanes 2-6 and 12-16 respectively) (see Materials and Methods). To determine whether histones have dissociated from DNA, we performed the remodeling reactions, incubated with an excess of unlabeled competitor DNA, and then analyzed the reaction products on native gels. Importantly, addition of bare DNA (see Materials and Methods) to the samples after remodeling but prior to electrophoresis, reverts the nucleosomes-CSB complexes and regenerates the two DNA species in the original ratio (Fig. 4, lanes 7-10). A comparison of lanes 7 and 8 with lane 1 indicates that the remodeling reaction did not release appreciable amounts of labeled non-nucleosomal DNA. This suggests that, under the experimental conditions used, nucleosome remodeling by CSB does not involve a complete dissociation of the core histones from the DNA.

Yeast SWI/SNF, yeast RSC and human SWI/SNF ((28, 56); G. Schnitzler *et al.*, unpublished observations) can transfer histone octamers from excess donor nucleosomes to labeled bare DNA to form nucleosomes. We could not detect octamer transfer activity by CSB under experimental conditions in which transfer was readily detected by SWI/SNF (data not shown; see Meterials and Methods).

Discussion

In this study, we further characterize the biochemical activities of CSB by analyzing its interaction with DNA and chromatin.

CSB alters DNA double helix conformation upon binding

Our results show that CSB binds to double-stranded DNA and that this binding causes a significant change in linking number as evidenced by the appearance of negatively supercoiled DNA in a topological assay (Fig. 1B). This activity is

reminiscent of the hRad54 recombination-repair protein, another member of the SWI2/SNF2 family (45). It also resembles the effect of binding of HMG-box-containing polypeptides, such as HMG-1, mtTF1 and LEF-1 to DNA (14, 15, 43). In contrast, DNA binding of the yeast SWI/SNF complex induces positive supercoils. However, CSB and ySWI/SNF share the property that ATP hydrolysis is not required to alter DNA topology (36). Relevant to this point is our observation that ATPase-deficient CSB (CSB^{K538R}) still retains partial activity when microinjected into living CS-B cells (6). In theory, CSB binding can induce negative supercoiling in two ways. One possibility is that CSB wraps the DNA around its surface in a left-handed manner, which will cause a change in writhe. Alternatively, CSB DNA binding could induce a change in twist, which may result in local unwinding of the DNA double helix. At present, we can not discriminate between these two possibilities.

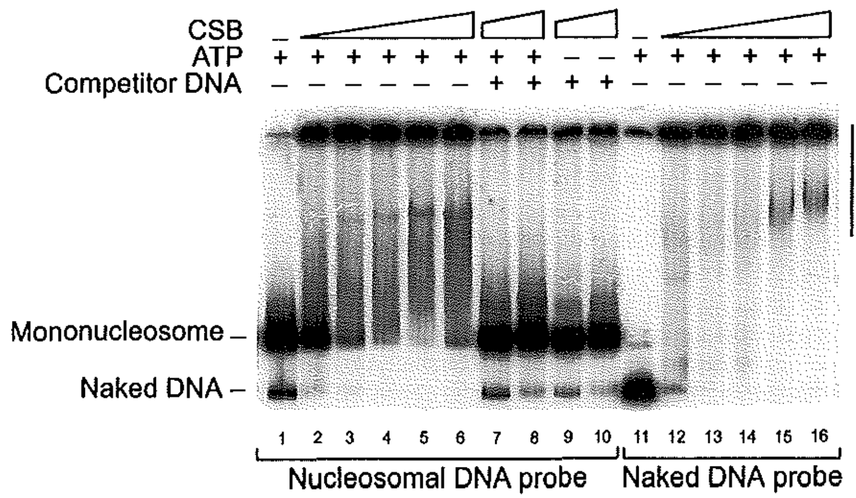


FIG. 4. Gel-shift analysis of CSB mononucleosome remodeling reactions. CSB does not catalyze the complete dissociation of histone octamers from DNA upon nucleosome binding and remodeling. Mononucleosomes (lanes 1-10) or free DNA (lanes 11-16) were incubated with increasing amounts of CSB (10, 20, 30, 60, 100 ng for lanes 2-6 and 12-16; 60, 100 ng, respectively in lanes 7-8 and 9-10) and reactions were performed as those shown in Figure 2, with (+) or without (-) ATP. Reactions were analyzed directly on native polyacrylamide gels or, where indicated, were treated with excess of cold competitor plasmid DNA before loading (lanes 7-10) (see Materials and Methods). Multiple DNA-protein complexes are visible (bar).

CSB shares chromatin remodeling properties with both the SWI2/SNF2 and the ISWI containing complexes

Purified, recombinant CSB has intrinsic chromatin remodeling activities *in vitro*. It catalyzes the remodeling of both reconstituted mononucleosomes, as well as nucleosomes uniformly spaced on plasmid DNA. Both activities require the energy of ATP hydrolysis and are detected at an approximate equimolar ratio of CSB and nucleosome particles. As demonstrated for hBRM and BRG1, additional proteins may be needed to increase the rate of remodeling by CSB (35). Our findings are consistent with the current view that SWI2/SNF2-related ATPases form the functional core of chromatin remodeling "machines" (8, 35).

Although ATP-dependent chromatin remodeling complexes perform similar activities, mechanistic differences distinguish the SWI2/SNF2 family from the ISWI-based complexes (23). Additionally, the three ISWI-family remodeling complexes display different activities *in vitro* (5). An interesting question is whether the CSB remodeling activity resembles more that of the SWI2/SNF2 type proteins or that of ISWI.

CSB activity on mononucleosomes is similar to that of the SWI2/SNF2 containing complexes. In fact, CSB induces significant changes in the DNase I cleavage pattern on reconstituted mononucleosomes (Fig. 1 and (9, 25)). In contrast, the ISWI-based CHRAC complex does not support nucleosome remodeling in similar experiments (26). The DNase I digestion patterns induced by CSB and by the hSWI/SNF complex are very similar (Fig. 2 C). Only subtle differences are observed near the nucleosomal dyad axis, the suggested location of SWI/SNF binding (36, 37), possibly because CSB and hSWI/SNF bind slightly different sites on the nucleosome. During the mononucleosome remodeling reaction, both CSB and hSWI/SNF generate an altered nucleosome structure that is stable after removal of ATP (Fig. 2B and (19)). This activity has not been tested for ISWI-based complexes. CSB activity on reconstituted plasmid chromatin presents two aspects. On one hand, CSB shows the same behavior of hSWI/SNF and the Drosophila NURF as they all disorganize the regular repeat of nucleosome arrays (Fig. 3; G. Schnitzler, unpublished observations; (48)). This activity distinguishes CSB from ISWI (8) and from the ISWI-based remodeling complexes CHRAC and ACF (20, 53). On the other hand, CSB does not induce detectable loss of superhelical density of nucleosomal plasmid, a characteristic activity displayed by SWI2/SNF2 complexes (Fig. 3B; (19)). This might reflect differences in the details of the remodeling reactions. Generally, it is argued that chromatin remodeling complexes work without removal of histone octamers from the DNA. CSB results are consistent with this idea (Fig. 4). However, SWI2/SNF2 containing complexes (RSC, hSWI/SNF, ySWI/SNF) can transfer histone octamers to an acceptor DNA molecule *in trans* (28, 56)(Schnitzler *et al.*, unpublished results). We were not able to detect this type of activity for CSB (data not shown). In this respect, CSB is more like the ISWI-based complexes NURF and CHRAC (16, 26). In conclusion, the data presented here indicate that CSB remodeling activity has some properties in

common with both the SWI2/SNF2-based and the ISWI-based family. This may be due to the fact that CSB was tested as isolated polypeptide and not as a complex such as the others. An additional interesting possibility is that CSB may be distinct in its capabilities from both classes of remodeling complexes.

Implications for transcription-coupled repair

A dual functionality has been proposed for CSB. First, CSB is specifically required for the transcription-repair coupling reaction. Second, several observations support an additional, non-essential role of CSB in transcription itself (1, 50). The capacity of CSB to remodel nucleosome structure may be relevant for both functions, as discussed below.

In relation to the role of CSB in repair, CSB may be involved in chromatin rearrangements at repair sites. These rearrangements may include both opening of chromatin, to favor accessibility of NER enzymes to the damage, and the rapid refolding of nucleosomes following repair synthesis (29, 31). Since transcriptionally active genes already present a more accessible chromatin structure, a more likely hypothesis is that CSB may be implicated in chromatin reorganization after repair allowing the stalled RNA polymerase II (RNAP II) to efficiently resume transcription elongation. In addition, the capability of CSB to modulate DNA double helix conformation may directly facilitate TCR. In a mechanism similar to nucleosome disruption, CSB could use ATP hydrolysis to weaken the RNAP II-DNA contacts at the site of a DNA lesion, thereby inducing a conformational change in the ternary complex favorable to repair. In this scenario, CSB would counteract the steric hindrance of the stalled RNAP II complex without displacing it from the template, and without aborting transcription. In accordance with this idea, no removal of RNAP II from a damaged DNA template is detected *in vitro* (39). Specific targeting to sites of blocked transcription might be mediated by interaction with RNAP II, which is observed both *in vivo* and *in vitro* (38, 46, 49).

In respect to the role of CSB in transcription, it is well documented that nucleosomes constitute a strong barrier to transcription elongation (4, 21). A mild stimulation of transcription elongation by CSB on naked DNA templates has recently been reported *in vitro* ((38); our unpublished observations). The data presented here open the possibility that CSB may play a role in facilitating transcription by RNAP II through pause sites on natural chromatin templates *in vivo*.

The experiments presented extend CSB function to chromatin remodeling, and, together with the previous knowledge, place the protein at the crossroad between DNA repair, transcription and chromatin structure. Recently, inactivation of a component of the hSWI/SNF complex (54) and alterations in proteins that regulate histone acetylation (27, 41) have been reported to correlate with certain human cancers. It is possible that defective chromatin rearrangements during DNA repair or transcription may contribute to the severe clinical symptoms of Cockayne syndrome patients, that cannot be explained solely by a DNA repair defect.

Materials and methods

Proteins

Recombinant, epitope tagged (N-terminal hemagglutinin antigen epitope, HA, and C-terminal histidine stretch, His_6) CSB and CSB^{K538R} mutant proteins were overexpressed using the Baculovirus system and purified as described (6), except that the final purification step was substituted by a Mono Q column. The eluate from the Ni^{2+}-nitrilotriacetic acid-agarose column was loaded on a Mono Q column equilibrated with buffer A (25mM HEPES-KOH, pH7.9, 0.05% Nonidet P-40, 10% glycerol, 1mM EDTA, 1mM DTT, 0.1mM PMSF) in 0.1 M KCl and the adsorbed proteins were eluted by a 0.1M-1M KCl gradient. The elution profile and purity of the CSB fractions was monitored by SDS-PAGE followed by silver staining. Protein concentration was ~20 ng/µl.

The hSWI/SNF complex was purified from HeLa cells by affinity chromatography to a FLAG epitope tag on the Ini1 subunit and its functional characterization was performed as described (37).

HeLa core histone octamers were purified as described by (60). H1- depleted HeLa polynucleosomes were isolated and quantified as described previously (37).

ATPase assay

Standard reactions (10 µl) were performed as described (6). Plasmid DNA (300 ng), HeLa polynucleosomes (300 ng), and HeLa core histones (40 ng - 400 ng) were tested as cofactors. No ATPase activity intrinsic to HeLa polynucleosomes or core histones was detected. Incubation was for 30 min at 30°C, followed by separation on thin layer chromatography. ATP hydrolysis was determined by image analysis on a Phosphoimager (Molecular Dynamics).

Topological assay on plasmid DNA

pBlueScript II KS was singly nicked by treatment with bovine pancreatic DNAse I (Boehringer Mannheim) (10) and purified by phenol/chloroform extraction and ethanol precipitation. Reactions (60 µl) containing 100 ng of nicked plasmid and the indicated amounts of CSB or CSB^{K538R} were performed as described (45) except for the KCl concentration, which was adjusted to 60 mM. After 10 min, one unit *E.coli* DNA ligase was added and the incubation was continued for 50 min. Topoisomers were resolved by electrophoresis on 1 % agarose gels containing 0.5 µg/ml chloroquine. Gels were run in 1X TBE for 20 hrs at 70 V, followed by Southern blotting, hybridization with a pBluescript probe and autoradiography. Two-dimensional gel electrophoresis was performed as described (42, 45). In a control experiment, purified CSB was incubated with closed, relaxed plasmid DNA under the above experimental conditions. Reactions were analyzed on a 0.8% agarose gel. No formation of supercoiled DNA was observed.

Mononucleosome assembly and DNAse I accessibility assay

Mononucleosome cores were assembled by step-wise salt dilution (19) on MluI-EcoRI DNA fragments obtained by digestion of the TPT plasmid (37). The DNA sequence includes two GT-phasing sequences (40) and was 155 bp after labeling with Klenow and ^{32}P-dCTP. DNAse I footprinting reactions (25 µl) were performed as described (19, 37) except that 0.5 mg/ml BSA was added. Where indicated, the reactions contained the non-hydrolysable ATP analog ATPγS (Sigma) or 1U of apyrase (Sigma) (dissolved in 20 mM HEPES, pH 7.9, 1mM

MgCl2, 1mM DTT, 1mM EDTA, 1 mg/ml BSA at a concentration of 1U/μl). KCl concentration was adjusted at 60 mM in all reactions. After incubation at 30°C for 45 minutes, followed by 5 min at room temperature, the reactions were subjected to digestion with DNase I. Processing and denaturing PAGE were performed as described (19).

Gel-shift and histone octamer transfer reactions

For gel shift analysis, remodeling reactions (containing either 0.3 ng labeled nucleosome particles or labeled naked DNA, and the indicated amount of CSB) were directly loaded onto 4% polyacrylamide gels (80:1 acrylamide/bisacrylamide ratio) containing 12.5 mM Tris, 100 mM glycine, 0.5 mM EDTA. Gels were run at ~150 V for ~2.5 hours at 4 °C. Where indicated, reactions were stopped by the addition of KCl (160 mM final), and competitor DNA (2 μg plasmid DNA, 0.5 μg polynucleosomes) prior to loading on the gel. Octamer transfer reactions contained unlabeled HeLa polynucleosomes in excess (10 ng), naked labeled TPT MluI-EcoRI fragment (1 ng) as possible acceptor of core particles, CSB (40 ng) or hSWI/SNF (200 ng) and were performed under the same conditions as for the DNase I footprinting. After 60 min at 30 °C, KCl and 2 μg of plasmid DNA (as described above) were added to stop the reactions. Samples were further incubated at 30 °C for 10 min and analyzed by EMSA using 5% polyacrylamide gels (18). Product analysis was performed using a PhosphoImager. Both in the presence or absence of ATP, no *de novo* formation of core particles on the naked DNA could be detected in the CSB reactions, whereas octamer transfer was catalyzed by hSWI/SNF in an ATP dependent manner (data not shown).

Plasmid chromatin remodeling assays

Chromatin was assembled on the 3.35-Kb pG_5HC_2AT plasmid (59) in *Drosophila* embryo extracts and subsequently treated with sarkosyl to disrupt endogenous remodeling activities according to published protocols (52, 53). Sarkosyl and ATP were removed by gel filtration on Micro Bio-spin columns (Bio-Gel polyacrylamide P-6, Biorad). Sarkosyl-treated chromatin (~40 ng DNA) was incubated with CSB or CSB^{K538R} (estimated amount 160 ng) or with hSWI/SNF complex (300 ng) for 90 min at 30 °C in 70 μl of EX buffer (52) containing 60 mM KCl. Micrococcal nuclease (MNase) digestion and agarose-gel electrophoresis were performed as described (52). Southern blotting, hybridization with ^{32}P-labelled total plasmid DNA and autoradiography were used to visualize the DNA fragments.

The supercoiling assay on reconstituted plasmid chromatin was performed as described (19). The pG_5HC_2AT plasmid was internally labeled and assembled into nucleosomes using purified HeLa core histones and a heat-treated Xenopus egg extract (19). Glycerol gradient purified template (1 ng DNA) was incubated with the given amounts of CSB and hSWI/SNF and remodeling reactions were carried out in 12.5 or 25 μl for 90 min at 30 °C as described (19). Reactions contained 60 mM KCl. Similar results were obtained when the pG_5HC_2AT plasmid was assembled with *Drosophila* embryo extracts (52), sarkosyl treated and reaction were carried out under the same experimental conditions described above for the MNase analysis.

Acknowledgments

We are grateful to the entire Kingston lab for indispensable help, especially to J. Guyon and L. Corey for sharing reagents and protocols, and M. Phelan and G. Narlikar for fruitful suggestions. We thank P. Becker for *Drosophila* facilities. We thank the members of our lab for constant support and helpful discussion and Prof. D. Bootsma for his continued interest. S. van Baal is acknowledged for his assistance in computer work and M. Kuit for photography. This work was supported by EC grant n.7010-212; the Dutch Science Foundation (NWO 901-501-151); R.K. is a fellow of the Royal Netherlands Academy of Arts and Sciences; J.H.J.H. is recipient of a SPINOZA award from NWO.

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Chapter 10

Transcription- and UV-dependent changes in mobility of the Cockayne syndrome B DNA repair-transcription coupling factor in living cells

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Abstract

The Cockayne syndrome B protein (CSB) plays an essential role in coupling nucleotide excision repair (NER) to transcription by RNA polymerase II. This process is known as transcription-coupled repair (TCR). Cells from patients with Cockayne syndrome (CS) show a specific defect in TCR and are unable to resume transcription after UV exposure. CS patients suffer from cutaneous photosensitivity and severe neurodevelopmental abnormalities. Previous studies have shown that CSB possesses transcriptional and chromatin-remodeling activity. Here we investigate the intranuclear organization and dynamic properties of CSB in living human fibroblasts by tagging CSB with the green fluorescent protein (GFP). With confocal and conventional microscopy we show that CSB is organized into multiple distinct foci in the nucleus. UV-irradiation as well as drug-induced inhibition of transcription causes CSB to relocalize into a completely homogeneous distribution throughout the nucleoplasm. Quantitative fluorescence redistribution after photobleaching (FRAP) indicated that CSB is mobile within the nucleus. The observed diffusion rate of CSB is slower than predicted by the size of the single polypeptide, suggesting that CSB is associated with other proteins. Preliminary mobility measurements reveal a partial UV-dependent immobilization of CSB molecules, indicative of CSB engagement in TCR. Alterations in CSB mobility were also detected in transcriptionally inactive cells. Our observations provide evidence that CSB nuclear organization is dynamic and changes after UV-induced DNA damage. In addition, they suggest that the transcriptional activity of the cell directly influences CSB nuclear localization and mobility in the absence of DNA damage.

Introduction

Nucleotide excision repair (NER) is a versatile DNA repair mechanism that removes a wide variety of structurally unrelated lesions from DNA. The most relevant among these are cyclobutane pyrimidine dimers (CPD) and 6/4 photoproducts (6-4PP), both induced by the shortwave ultraviolet (UV) component of sun light (8, 12). NER requires the concerted and coordinated action of approximately 30 proteins in mammals and operates via a multistep reaction well characterized in an *in vitro* system (8, 61).

One of the immediate consequences of UV-induced DNA damage is the blockage of different DNA transactions, including the essential process of transcription (29). NER gives high priority to the elimination of transcription-blocking lesions to allow the rapid resumption of transcription. This NER mode is known as transcription-coupled repair (TCR) and accounts for the preferential repair of lesions from the transcribed strand of active genes (2, 30). In addition to TCR, also a global genome repair (GGR) pathway exists, which removes lesions from the entire genome. An elongating RNAP II stalled at a site of damage is thought to initiate the TCR pathway (15). TCR is particularly relevant for those lesions which are slowly repaired by the GGR pathway and that actually block transcription, such as CPDs (48, 52, 54). In addition, evidence has been obtained that some types of oxidative damage, mainly processed by the base excision repair pathway can be removed in a transcription-coupled manner as well (7). Thus, the importance of TCR is not limited to NER.

Inherited defects in the TCR pathway form the molecular basis of the human genetic disease Cockayne syndrome (CS) (53, 55). Besides cutaneous hypersensitivity to UV-light, CS is characterized by very severe developmental and neurological abnormalities (3, 33). The *CSA* and *CSB* genes are specifically required for TCR in man and are defective in CS complementation groups A and B (16, 49). *CSA* codes for a five WD 40 repeat-containing protein (16). The *CSB* gene encodes a nuclear protein of 168 kiloDalton (kDa) belonging to the SWI2/SNF2 family of DNA-dependent ATPases (10, 49). Similarly to the yeast SWI2 and to several SWI2-like proteins, CSB has the ability to remodel chromatin templates at the expense of ATP hydrolysis *in vitro* (6, 22).

The discovery of the TCR pathway and the dual involvement of transcription factor TFIIH in the NER reaction indicate a tight functional interplay between DNA repair and basal RNAP II transcription (17, 35). Except from photosensitivity, the severe neurodevelopmental symptoms associated to CS are difficult to explain on the basis of a sole NER defect. These non-NER-related symptoms are proposed to be caused by a subtle transcriptional defect (57), suggesting an additional, non-essential role of the CS proteins in transcription (52). The recent observations that CSB can interact both *in vivo* and *in vitro* with RNA polymerase II (39, 47, 51) and that it enhances transcription elongation *in vitro* (38) are consistent with this hypothesis. However, it is still unknown whether the CSB protein is an intrinsic

component of RNAP II complexes engaged in elongation *in vivo*. In contrast to the detailed knowledge of the core NER process (8, 61), little is known about the TCR mechanism. Moreover, the dynamic interplay between transcription (required for TCR), and the nuclear architectural (chromatin) organization is largely unknown.

Light and electron microscopy studies have shown that many proteins involved in diverse nuclear processes, such as replication, transcription and RNA processing are differently organised in the nucleus of mammalian cells and are often found concentrated in specific domains (23, 42, 50). Immunofluorescence experiments localizing sites of RNA polymerase II (RNAP II)-derived nascent transcripts relative to both RNAP II and to factors involved in pre-mRNA processing revealed how transcription, pre-mRNA splicing and 3' processing are spatially related with each other within the nucleus (19, 32, 36).

In recent years, fusion of the green fluorescent protein (GFP) to specific proteins has allowed their direct observation within living cells. GFP-technology is being applied to study *in vivo* organization and dynamics of numerous nuclear proteins, among which transcription and pre-mRNA processing factors and proteins involved in DNA repair (1, 18, 31, 41).

Recently, photobleaching studies on human living cells expressing functional ERCC1-GFP/XPF (a core NER factor) provided evidence that NER is a highly dynamic process (18). The results support a model in which individual NER factors freely diffuse in the nucleus in the absence of DNA damage and, following UV-challenge, assemble successively at sites of lesions, where they become temporarily engaged in the repair reaction (18).

In order to study the nuclear organization of the CSB protein, its dynamics during TCR and its possible engagement in transcription in living cells, we tagged CSB with EGFP (enhanced GFP variant) (64). Using confocal microscopy and photobleaching experiments (60) we investigated CSB nuclear mobility in relation to both DNA repair and transcriptional activity of the cell.

Results

Fibroblast expressing EGFP-CSB are fully repair competent

To study the nuclear organization and dynamic properties of the CSB protein in living cells, the cDNA encoding enhanced green fluorescent protein (EGFP) was fused in-frame to the amino-terminus of CSB. The fusion cDNA (EGFP-CSB) was stably transfected into CS-B deficient human fibroblasts (CS1AN-Sv) (see Materials and Methods) (49). Immunoblot analysis with anti-CSB and anti-GFP antibodies showed that the vast majority of detectable CSB was in the EGFP-tagged form and was expressed at levels comparable to the endogenous CSB in HeLa cells (Figure 1A and data not shown). The EGFP-CSB cDNA was able to fully correct the extreme UV-sensitivity of CS-B cells to a wild-type, TCR-proficient level

(Figure 1B), indicating that addition of the EGFP tag did not detectable interfere with CSB function *in vivo*.

EGFP-CSB is preferentially localized in discrete foci within the nucleus

EGFP-CSB intracellular localization in living cells was studied by conventional (epifluorescence) and confocal laser scanning microscopy (see Materials and Methods). The tagged proteins predominantly localized within the nucleus and appeared homogeneously distributed (Figure 1C), consistent with previous observations by immuofluorescence labelling with anti-CSB antibodies (51). Interestingly, higher image resolution revealed that EGFP-CSB fluorescence was not uniformly diffuse in the nucleoplasm, but instead sites of more intense fluorescence were visible overlaying the diffuse pattern (Figure 1C and D). Mainly, two categories of EGFP-CSB preferential localization sites (referred as EGFP-CSB foci in this study) were recognized, including large more fluorescent domains of variable size and small very brightly fluorescent spots, suggesting that they represent high local concentration of EGFP-CSB (a representative cell is shown in Figure 1D). The observed EGFP-CSB pattern was heterogeneous in a population of exponentially growing cells, with a large fraction of cells (> 70 %) presenting foci (either large domains or small dots or both). A precise quantification of both the number of foci per cell as well as the frequency of these spots containing cells in the entire population was difficult because foci are not always very apparent due to fast bleaching during imaging. Importantly, EGFP-CSB foci were not due to overexpression of the protein for three reasons: 1) they appeared also in cells with a relatively low expression levels (Figure 1C); 2) human fibroblasts expressing physiological levels of functional CSB protein tagged with both an N-terminal hemagglutinin antigen (HA) epitope as well as a C-terminal histidine (His_6) stretch (HA-CSB-His_6) (51) showed a CSB distribution pattern reminiscent of EGFP-CSB in living cells when stained with anti-HA antibodies (data not shown); 3) CSB foci were also visualized by immunofluorescence (IF) studies using anti-CSB antibodies (51) within wild-type human fibroblasts and HeLa cells (data not shown). In conclusion, the IF studies are consistent with the observations made on EGFP-CSB in living cells. The data indicate that the apparent CSB distribution observed with both methods is not due to tagging artifacts, and most likely reflects the endogenous nuclear organization of the CSB protein.

CSB nuclear localization pattern is distinct from "speckles"

EGFP-CSB small foci were present with an estimated average of 20-50 per nucleus, and were dispersed throughout the entire nucleoplasm. The number of preferred EGFP-CSB sites is reminiscent of the number of "speckles", irregularly shaped domains, visualized with antibodies to many pre-mRNA splicing factors (42). A splicing factor which has been shown to prominently localize in speckles is the SR protein SC-35 (13, 43). IF using anti-SC-35 specific antibodies showed a pattern with weak overall staining and very prominent "speckles" in fixed EGFP-

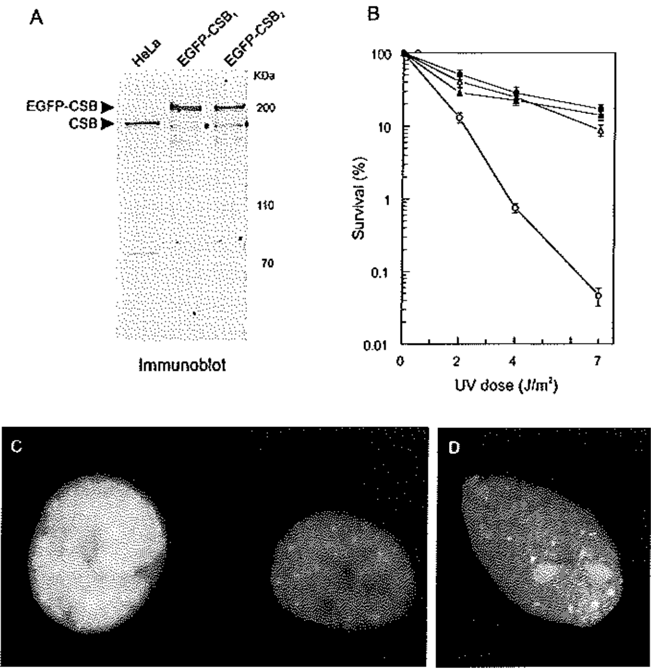


FIG. 1. Characterization of EGFP-CSB fusion protein. A. Immunoblot analysis of EGFP-CSB expression. Equal amounts (10 μg protein per lane) of whole cell extracts (WCE) from HeLa and from EGFP-CSB transfected CS1AN-Sv fibroblasts (two independent clones, 1 and 2 respectively) were probed with polyclonal anti-CSB antibodies. Molecular weight (MW) of protein markers is indicated in kilo-Dalton (kDa). * indicates a degradation product of EGFP-CSB protein. B. UV-survival of EGFP-CSB expressing cells. The percentage of surviving cells is plotted against the applied UV dose. Survival of two isolated EGFP-CSB expressing clones ($EGFP_1$and $EGFP_2$) and control cell lines after UV-treatment was determined by pulse labeling with ^{3}H-thymidine (see Materials and Methods): CS1AN-Sv, CS-B (o); VH10-Sv, wt (Δ), CS1AN-Sv + $EGFP\text{-}CSB_1$ (▲), CS1AN-Sv + $EGFP\text{-}CSB_2$ (•). C and D. Nuclear localization of EGFP-CSB. Epi-fluorescence image (C) and a stack of confocal planes (D) of EGFP-CSB expressing CS1AN-Sv fibroblasts. Multiple discrete sites of preferential fluorescence, visible as small bright foci and larger domain, can be distinguished on top of a diffuse nuclear signal.

CSB expressing cells, whereas EGFP-CSB showed an entire nuclear fluorescence and additional bright foci (Figure 2, A and B). Most of the EGFP-CSB foci appeared smaller in size and morphologically different from the SC-35 speckles. No significant co-localization between the sites of preferential staining of the two

proteins was observed, although a number of foci were found in close proximity and only very few EGFP-CSB foci overlapped with speckles (overlay in Figure 2C). These results suggest that the majority of EGFP-CSB appeared not localized in the SC-35 "speckles".

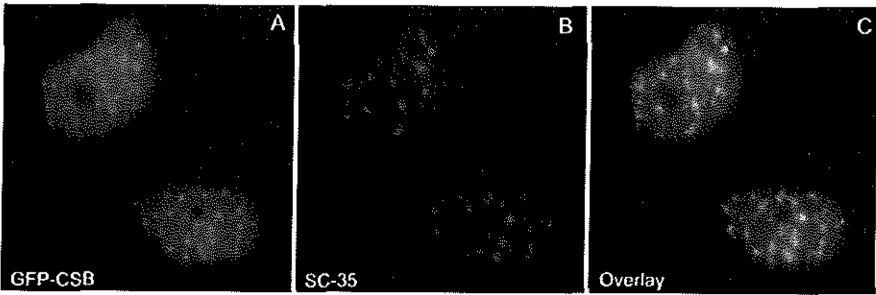


FIG. 2. Localization of EGFP-CSB relative to the splicing factor SC 35. After fixation, CS1AN-Sv + EGFP-CSB cells were immunolabeled with anti-SC 35 antibodies. EGFP-CSB fluorescence (A) was compared with that of anti-SC 35 immunostaining (B, red); colocalization would appear in yellow in the overlay image (C). Although EGFP-CSB foci and SC-35 speckles are frequently found in each other proximity, EGFP-CSB foci differ in dimension and shape compared to the SC-35 speckles and only very limited colocalization is observed.

UV-irradiation and transcription inhibition cause redistribution of EGFP-CSB in the nucleus

The localization of EGFP-CSB in preferential sites, suggested that EGFP-CSB is partially compartmentalized in the nucleus in the absence of DNA damage. To investigate the functional significance of the EGFP-CSB foci and their potential relationship with CSB activity during TCR, we challenged the EGFP-CSB cells with UV-light. After UV exposure ($15J/m^2$), we observed a redistribution of EGFP-CSB in the nucleus (Figure 3B). Within 2 hours after irradiation, EGFP-CSB appeared uniformly distributed throughout the nucleoplasm in most of the cells and sites of preferential staining became undetectable (representative cells are shown in Figure 3B). Monitoring of fluorescence 24 hrs post-UV revealed that the cells that survived the UV treatment had regained the typical EGFP-CSB pattern (data not shown). At this time point repair of DNA damage, in particularly lesions subjected to TCR, is completed and the RNAP II- mediated transcription activity recovered (51, 53). Similar results were obtained by IF analysis using anti-HA antibodies on UV-irradiated HA-CSB-his_6 fibroblasts (data not shown).

To determine whether other types of DNA damage influenced CSB distribution, GFP-CSB or HA-CSB-his$_6$ cells were exposed to ionizing radiation (IR) (6Gγ and 12Gγ, see Materials and Methods). IR treatment has been shown to induce nuclear foci formation by proteins which are specifically required for repair of IR-induced DNA double-strand breaks (DSB), such as the mammalian hMre11, hRad50, mRad51 and mRad54 ((28, 46) and references within). In contrast, similar doses of IR did not significantly altered the CSB nuclear pattern (data not shown; see Materials and Methods). This is consistent with the fact that CSB is not required for DSB repair ((21), see also discussion).

One of the direct consequences of UV-induced DNA damage is the temporary blockage of transcription, whose recovery specifically requires the function of the CSB and CSA proteins (29, 51, 53). In contrast, IR has little effect on transcription. We therefore investigated whether the cellular transcriptional activity could directly influence GFP-CSB spatial organization, independently from the presence of DNA damage. For this purpose, transcription in EGFP-CSB cells was arrested by incubation with drugs able to selectively inhibit RNA synthesis by RNAP II, 5,6-dichloro-β-D-ribofluoranosyl-benzimidazole (DRB) and alpha-amanitin (α-amanitin) (Materials and Methods). DRB, a nucleoside analogue, is reported to reversibly inhibit transcription at the level of elongation (5) as a result of the inhibition of one or more protein kinases able to phosphorylate the C-terminal domain of the RNAP II large subunit (27, 34, 65). Positive and negative elongation factors required for DRB-sensitive transcription have been recently identified (62, 63). Permanent general transcription inhibition by α-amanitin involves direct binding of the drug to the large subunit of RNAP II (25). Blockage of transcription by incubation of EGFP-CSB cells with either α–amanitin or DRB (see Materials and Methods), caused the concomitant disappearance of EGFP-CSB foci (Figure 3C and E respectively) similar to UV-irradiation (Figure 3B). Actual significant inhibition of transcription was verified by ^{3}H-uridine pulse labeling (data not shown; D.Hoogstraten unpublished observations).

In contrast to α-amanitin, DRB easily penetrates through the cellular membranes and can be rapidly washed out in order to reverse the transcription inhibition (45). Interestingly, DRB wash out experiments revealed a reversible and dynamic transcription-dependent distribution of EGFP-CSB, as shown in figure 3D, E and F (see Materials and Methods). A similar transcription-related relocation of CSB was visualized by anti-HA antibodies staining in HA-CSB-his$_6$ expressing cells (data not shown).

Nuclear mobility of EGFP-CSB in living cells

The above findings indicate that at least a fraction of CSB is mobile in the nucleus and able to respond to external stimuli. To further investigate the EGFP-CSB nuclear mobility we applied fluorescence redistribution after photobleaching (FRAP) procedure to EGFP-CSB expressing cells (11, 18, 60). Briefly, EGFP-CSB fluorescent molecules were bleached by an intense laser pulse within a defined strip

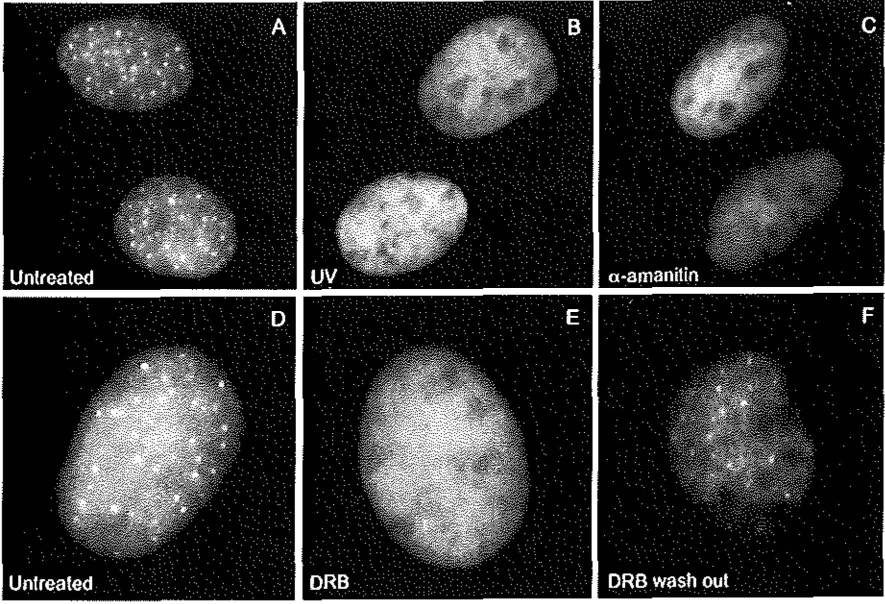


FIG. 3. Redistribution of EGFP-CSB in living CS1AN-Sv cells upon UV-irradiation and transcriptional arrest. Epifluorescence images of CS1AN-Sv + EGFP-CSB cells were taken before (A and D) and after irradiation with UV-light (2 hrs, 15 J-m^2, B), or incubation with RNA polymerase II -specific transcription inhibitors -amanitin (6 hrs, C) or DRB (2 hrs, E). DRB treated cells were subsequentely washed and cultured with DRB-free medium for an additional 3 hrs (F). UV-irradiation and inhibition of transcription cause the disappearence of EGFP-CSB foci. The original fluorescence pattern is regained after restoration of the transcriptional activity by DRB wash out or after completion of transcription-coupled repair.

through the nucleus. The redistribution of fluorescent and bleached molecules into the strip was subsequently monitored (see Materials and Methods). Plotting the recovery of fluorescence intensity in the bleached strip as a function of time allowed to evaluate EGFP-CSB mobility and to compare it with XPB-EGFP or XPA-EGFP mobility (Figure 4A) (D. Hoogstraten and S.Rademakers respectively unpublished results and (18, 60)). EGFP-CSB fluorescence recovery in the strip was significantly slower compared to both to XPB-EGFP and to XPA-EGFP, as observed within the first two seconds post-bleach. In addition, after recovery, EGFP-CSB fluorescence intensity decreased at a slower rate than XPB-EGFP and XPA-EGFP fluorescence during the period of analysis (Figure 4A). This also

indicates that EGFP-CSB molecules move slower than XPB-EGFP and XPA-EGFP molecules, since molecules with a high relative mobility will pass through the strip more frequently in a given period of time than relatively slow molecules. This causes a faster decrease of fluorescence intensity of fast compared to slow molecules, because of bleaching due to monitoring. In conclusion, EGFP-CSB nuclear diffusion rate appeared to be much slower than XPA-EGFP and slightly slower compared to XPB-EGFP (Figure 4A; D. Hoogstraten and S.Rademakers unpublished observations). The estimated diffusion coefficients (*D*) were respectively 4.8 ± 0.5 $\mu m^2/s$ for EGFP-CSB, 6.6 ± 1.2 $\mu m^2/s$ for XPB-EGFP, and 14 ± 3.1 $\mu m^2/s$ for XPA-EGFP. This is consistent with a difference in molecular size between the NER proteins analyzed (37). In addition the estimated EGFP-CSB *D* suggests that the approximate size of the EGFP-CSB protein (complex) in the nucleus is larger than the XPB-containing complex TFIIH (>400kDa), and larger than ~800 kDa (18). Such a protein size exceeds the predicted MW of 190 kDa of a single CSB polypeptide chain and correlates well with the high MW complex in which CSB was observed to reside in our previous studies using size fractionation (51).

Alteration of EGFP-CSB mobility after DNA damage and transcription inhibition

To investigate the effect of DNA damage on EGFP-CSB mobility, cells were exposed to 16 J/m^2 UV light. Within 5-30 min, FRAP measurements were performed (Figure 4B). The initial fluorescence recovery within UV-damaged cells as measured within 2 seconds after the bleach pulse was slightly faster than in non-challenged cells (Figure 4B), indicating that induction of DNA damage slightly increases the diffusion rate of EGFP-CSB molecules. Inhibition of transcription by DRB incubation altered the EGFP-CSB diffusion rate to the same extent as after UV-irradiation (Figure 4B). Since transcription is arrested by both treatments (UV-irradiation and DRB), the results suggest that the diffusion rate of EGFP-CSB is related to the transcription activity.

To investigate whether UV damage and/or transcription inhibition induced transient immobilization of EGFP-CSB molecules, analogous to damage-dependent ERCC1-GFP/XPF immobilization (18), the same set of data (as shown in figure 4B) was computed differently. In this case the mean fluorescence intensity immediately before bleaching was set to one (see Materials and Methods and Figure 4C). The absence of full recovery of fluorescence into the bleached strip using this normalization is a measure for an immobile fraction (in addition to the fraction of permanently bleached molecules) (11, 60). The immobile fraction was deduced from the resulting diffusion plots. The apparent reduced level of recovery within UV-irradiated cells when compared to non-treated cells (Figure 4C) showed that a significant fraction (~15-20%) became transiently immobilized upon damage induction (60). The fraction of immobile molecules was proportional to the applied UV-dose (data not shown). Interestingly, despite the comparable influence of both

UV-irradiation and DRB treatment on the diffusion rate EGFP-CSB, no significant immobilization of EGFP-CSB molecules was detected in DRB treated cells (figure 4C).

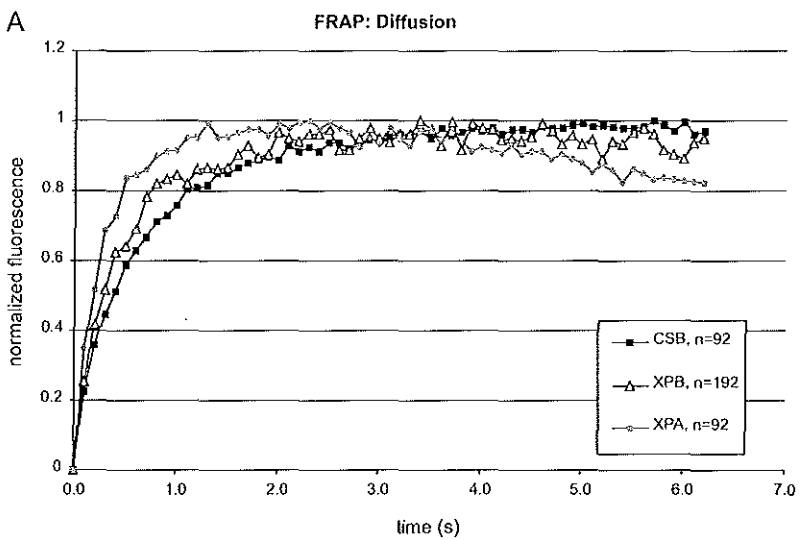
Discussion

Here we report the transcription-dependent dynamic organization of a functional EGFP-CSB fusion protein. Fusion of CSB to the green fluorescent protein allowed us to visualize CSB and to study some aspects of its intranuclear organization and dynamic behavior in living cells, where the tagged protein remains fully functional and is expressed at physiological levels.

The intranuclear organization of EGFP-CSB is dynamic and is related to the cellular transcriptional activity

With conventional and confocal microscopy, a homogeneous nuclear distribution of EGFP-CSB is apparent, in addition to numerous distinct domains of preferential localization visible as sites of more intense fluorescence (Figure 1, C and D panels). The data in living cells are consistent with immunofluorescence microscopy on HA-CSB-his_6-expressing human fibroblasts (data not shown) (51). No striking colocalization of the EGFP-CSB foci with "speckles" (visualized by the splicing factor SC-35) (42) was found, although a fraction of EGFP-CSB foci appeared localized in close vicinity of the SC-35 foci (see Figure 2 overlay). The significance of this close but not precise colocalization needs to be further investigated. In addition, the presence of numerous foci in the nuclear organization of EGFP-CSB appears to be different from the reported, predominantly homogeneous distribution of other NER factors, among which, ERCC1-GFP/XPF (18) and XPA-EGFP (S. Rademakers, manuscript in preparation). This is again different from the pattern found with TFIIH, which presents a smaller number of larger nuclear foci besides a homogeneous distribution all over the nucleus as detected by EGFP-XPB (D. Hoogstraten, manuscript in preparation) and by antibody staining (14).

In the majority of cells, the most abundant type of EGFP-CSB foci were small in size, appeared uniform in shape and were dispersed throughout the entire nucleoplasm (Figure 1C). In addition, a few EGFP-CSB large nuclear domains were detected in many cells (Figure 1D). Induction of DNA damage by UV-irradiation caused relocation of EGFP-CSB since the bright foci disappeared within 2 hrs after UV exposure (Figure 3A and B and data not shown). The uniform EGFP-CSB fluorescence pattern was reversible upon accomplishment of repair of the damage and recovery of the RNA synthesis. Importantly, other DNA damaging agents, such as IR (data not shown) did not induce EGFP-CSB re-localization. The same experimental conditions for IR were previously reported to induce nuclear foci formation by mRad51 and mRad54 recombination-repair mammalian proteins (46).



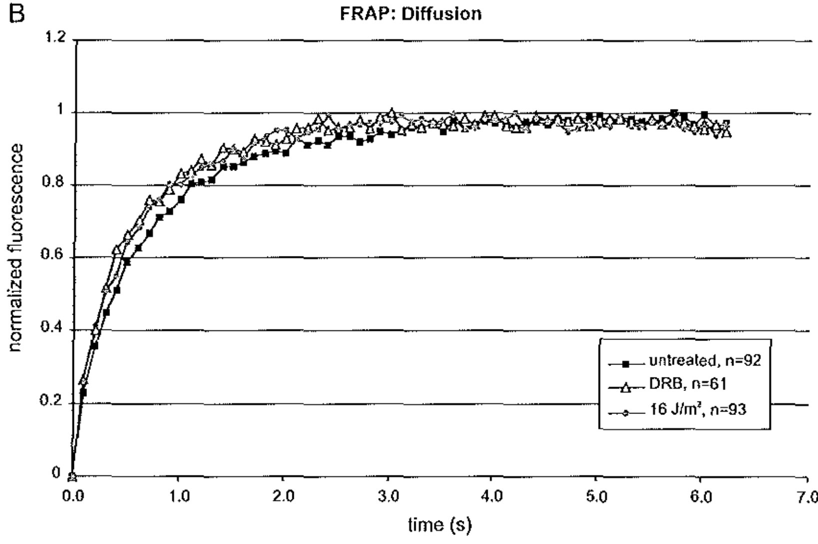
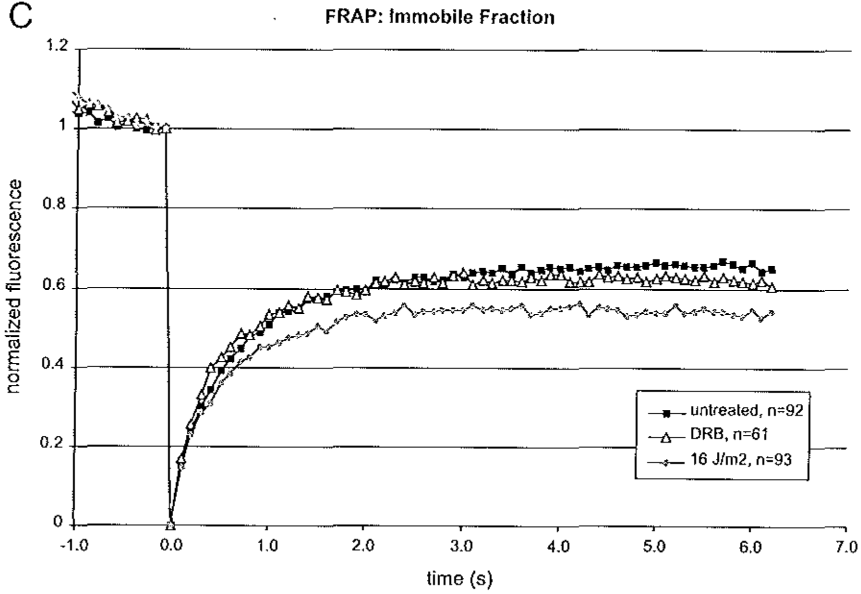


FIG. 4. Dynamic measurements of EGFP-CSB by fluorescence redistribution after photobleaching (FRAP). The recovery of the relative fluorescence intensity within the bleached strip through the nucleus is plotted as a function of time (seconds). A. Application of FRAP (see Materials and Methods) to living human cells expressing EGFP-CSB, XPB-EGFP or XPA-EGFP. For evaluation of diffusion rates, the mean



fluorescence intensity immediately after high laser intensity bleaching (postbleach) was set to zero and the maximum intensity reached after recovery was set to 1. EGFP-CSB fluorescence (solid squares) shows a slower recovery compared to XPA-EGFP (gray circles) and XPB-EGFP (open triangles) (as observed within approximately two seconds after bleaching) and once reached its maximum value, maintains it for the complete recording time. In contrast, a decrease in both XPB-EGFP and XPA-EGFP fluorescence is observed. This suggests that EGFP-CSB molecules diffuse less rapidly compared both to XPB-EGFP and to XPA-EGFP (see text). XPA-EGFP molecules show the highest relative mobility. B. Effect of UV-irradiation and DRB treatment on EGFP-CSB diffusion rate. FRAP was applied to EGFP-CSB expressing cells without treatment (solid squares) or after treatment with UV light (16 J/m^2) (gray circles) or DRB (open triangles) (see Materials and Methods). Normalization of the mean fluorescence intensity was as applied in A. A slight increase in recovery of EGFP-CSB fluorescence is detected after both treatments. This indicates that a significant fraction of EGFP-CSB molecules diffuses faster in UV-or DRB- treated cells compared to untreated cells. C. Effect of UV-irradiation and DRB treatment on EGFP-CSB mobility. FRAP was applied to EGFP-CSB expressing cells without treatment (solid squares) or after treatment with UV light (16 J/m^2) (gray circles) or DRB (open triangles) (see Materials and Methods). To determine a potential immobile fraction the mean intensity immediately before bleaching was set to 1 and the intensity immediately after bleaching was set to zero. A decrease in recovery of EGFP-CSB fluorescence intensity is observed in cells challenged with UV compared to untreated cells. This decrease is indicative of immobilization of a small fraction of EGFP-CSB molecules due to induction of DNA damage. In contrast, incubation with DRB did not induce significant changes in the EGFP-CSB mobile fraction.

These results indicate that EGFP-CSB redistribution is predominantly linked to DNA damage that requires CSB for its repair. CSB was shown to be involved in TCR of some IR-induced oxidative damage, such as thymine glycols (7, 24). However, this type of lesions constitutes only a fraction among the complex spectrum of DNA damages induced by IR (which include single- and double-strand DNA breaks, and a large variety of bases and sugar lesions) (58) and CSB redistribution might remain under the detection threshold of this *in vivo* system.

When RNAP II-mediated transcription was arrested by drug treatment (α-amanitin or DRB), EGFP-CSB became uniformly dispersed in the nucleus, in a pattern indistinguishable from that induced by UV-irradiation (Figure 3). Furthermore, EGFP-CSB reorganized into the typical foci after reversion of DRB inhibitory effect (Figure 3, C-E). A similar relocation of HA-CSB-His_6 was detected by IF studies (data not shown). These results reveal a dynamic functional relationship between CSB spatial distribution and the transcriptional activity of the cell, notably in the absence of DNA damage.

Several investigators have demonstrated that the nuclear distribution of RNAP II, as well as of multiple factors involved in pre-mRNA splicing and 3' cleavage varies in relation to transcription (4, 9, 31, 36, 43, 66). We find that the transcription-related dynamic behavior of EGFP-CSB is opposite to the reported redistribution of RNAP II and of several splicing factors. In response to transcriptional arrest, EGFP-CSB abandons the foci and homogeneously diffuses throughout the nucleus (Figure 3). In contrast, splicing factors relocate from a dispersed punctuated pattern which strikingly coincides with nascent mRNA sites in actively transcribing cells, into enlarged speckles (66). Speckles, which are more prominent within poorly transcribing cells, are believed to represent storage/recycling compartments for pre-mRNA splicing factors (19, 50, 66).

At present, the function of EGFP-CSB foci is unknown. Since, in addition to its specific role in TCR, CSB is thought to be involved in transcription elongation (38, 51, 52), an attractive possibility is that EGFP-CSB bright small foci might, at least in part, overlap with sites of active transcription. However, detection of nascent RNA by BrUTP incorporation, has revealed numerous focal sites scattered all over the nucleus, typically much more in number (~300-500) and smaller in size compared to the EGFP-CSB foci (20, 59). These EGFP-CSB foci may represent genomic loci of specific transcriptional units that preferentially require CSB in contrast to the more randomly distributed general transcriptional factors. Alternatively, areas of high EGFP-CSB concentration may represent storage compartments from which CSB is recruited to sites of blocked transcription.

EGFP-CSB is mobile within the nucleus of living cells

The above observations demonstrate that at least part of the CSB molecules can redistribute over the nucleus. To directly investigate the mobility of EGFP-CSB we utilized the ability to photobleach the EGFP-CSB molecules in a defined part of the nucleus and follow the redistribution of bleached and non-bleached proteins in

time. Fluorescence recovery after photobleaching (FRAP) measurements show that the majority of EGFP-CSB molecules are mobile in the nuclei of living undamaged cells (Figure 4). EGFP-CSB diffusion rate is clearly slower than that of XPB-EGFP and much slower than XPA-EGFP mobility, which were measured in parallel experiments (Figure 4A; D. Hoogstraten manuscript in preparation; S. Rademakers manuscript in preparation; (18)). This difference in mobility points to different molecular size of the NER factors (subcomplexes) analyzed (37), indicating that these proteins are (at least for the major part) not in the same complex. This argues against the existence of a stable NER holocomplex involving a significant fraction of NER factors (44). The calculated diffusion coefficients of XPB-EGFP, XPA-EGFP and ERCC1-GFP/XPF, were found to be proportional to the known molecular weight (MW) of the correspondent NER factor/complex, with the largest complex, namely XPB-EGFP (>400 kDa, in TFIIH) showing the slowest mobility (18). EGFP-CSB estimated diffusion rate is consistent with a protein size larger than ~800 kDa. Since the CSB protein has a predicted MW of 168 (kDa) (49), the observed EGFP-CSB slow mobility suggests that CSB molecules do not behave as a monomer, but instead associate with other protein/s in the nucleus. These data are in agreement with our previous observations, indicating that CSB in WCE has a hydrodynamic size of > 700 kDa when chromatographed on size fractionation columns (51). The observed mobility may reflect the dynamic behavior of a mixture of molecules of different sizes, in which part of the CSB molecules are incorporated into different complexes. Analysis of these complex diffusion patterns will be subject of future studies. Finally, the finding that most of the CSB molecules appear to be mobile under normal (non-damaged) conditions strongly suggests that the majority of EGFP-CSB is not part of a stable RNAP II transcription elongation complex. Either the fraction of EGFP-CSB involved in transcription elongation is too small to be detected using this method or the interaction with elongating RNAP II complex is transient or highly dynamic.

Induction of DNA damage by UV-irradiation causes a small but significant increase of the diffusion rates of the EGFP-CSB molecules that are mobile. This suggests that at least a fraction of EGFP-CSB molecules may undergo changes in protein complex composition (Figure 4B). In addition, FRAP and FRAP-FIM measurements (18) on UV-treated EGFP-CSB, provide evidence that a small (15-20%), nonetheless significant fraction of EGFP-CSB molecules becomes immobilized as a consequence of DNA damage (Figure 4C and data not shown). Our findings that this immobilization seems to be UV-dose dependent (data not shown) is consistent with the idea that the immobilization reflects actual engagement of EGFP-CSB in TCR at sites of DNA damage. The relatively limited small fraction of EGFP-CSB molecules that becomes immobile when compared to the core NER factor ERCC1 (18) could be explained by the fact that CSB is only involved in TCR whereas ERCC1 is implicated in GGR as well. TCR only accounts for the removal of a small percentage of the total amount of DNA lesions, which are mainly processed by the GGR pathway (56).

Interestingly, DRB-induced transcription inhibition caused a slight increase of EGFP-CSB nuclear diffusion rate, similarly to UV-treatment (Figure 4B). The fact that transcription inhibition (either induced by UV-irradiation or drug-treatment) causes alterations in EGFP-CSB diffusion rate indicates that EGFP-CSB mobility is directly related to the cellular transcriptional activity. The slower diffusion of EGFP-CSB molecules observed in cells with normal levels of transcription compared to transcriptionally inactive cells may be the result of a combination of two parameters. Firstly, we hypothesize that interaction of a small fraction of EGFP-CSB molecules with RNAP II complexes temporarily engaged in transcription elongation may contribute to the reduced mobility observed within the long term period of fluorescence recovery (Figure 4). The fact that CSB has been shown to interact with RNAP II by different investigators supports this hypothesis (39, 47, 51). In addition, involvement of CSB in transcription elongation in an *in vitro* system has been reported (38). Secondly, in actively transcribing cells, CSB may associate with specific proteins possibly involved in regulating and/or targeting CSB function in transcription elongation. Blockage of transcription either by UV or by DRB would cause the release of transcriptionally engaged molecules and/or potential changes in protein partners, resulting in an increase of the measured diffusion rate (Figure 4B). As discussed above, immediately after DNA damage induction, a fraction of CSB molecules was found to be immobilized possibly at sites of DNA lesions (Figure 4C and data not shown), analogous to the transient immobilization observed for the core NER factor ERCC1-GFP/XPF (18). The fact that, in contrast to UV-irradiation, DRB-induced transcriptional arrest did not cause significant immobilization of EGFP-CSB molecules suggests that (part of) elongation complexes containing CSB dissociate and become free to diffuse throughout the nucleoplasm (Figure 4C). The observations also suggest that there might be subtle differences in composition of CSB complexes involved in TCR compared to transcription.

In conclusion, we provide evidence that EGFP-CSB nuclear organization is highly dynamic and is related to both TCR of UV-induced DNA damage and to transcription, further establishing a direct role of CSB in transcription.

Materials and methods

Cell culture and specific treatments

The cell lines used in this study were HeLa, and VH10-Sv (wild-type) and CS1AN-Sv (CS-B) SV40 immortalized human fibroblasts. Fibroblasts were grown at 37° C in 5% CO_2 atmosphere in a 1:1 mixture of Ham's F10 and DMEM (Gibco) supplemented with antibiotics and 10% fetal calf serum. CS1AN-Sv cell line stably expressing a HA-CSB-his_6 fusion cDNA was generated and characterized by van Gool et al., 1997 (51).

Transcription inhibitors were added to the culture medium according to the following conditions: 5,6-dichloro-1β-D-ribofuranosyl benzimidazole (DRB) (100 μM, during 2 hrs),

α-amanitin (50 μg/ml, for 6 hrs). For recovery after DRB treatment, cells were washed twice and incubated in fresh preheated culture medium for 3 hrs.

Treatment with ultraviolet (UV) light was at 254 nm using a germicidal lamp at the indicated doses. For ionizing radiation (IR) treatment, cells were exposed to γ-rays from a ^{137}Cs source. Single doses of 6 and 12 Gy were used. After exposure, cells were incubated at 37 °C for 2 or 6 additional hours before light microscopy analysis. Whole-cell extracts (WCE) were prepared according to Manley et al., 1983 (26) with modifications as described by van Gool et al., 1997 (51).

Generation of EGFP-CSB fusion cDNA construct

A construct encoding N-terminal-EGFP tagged CSB was generated to study the cellular distribution and the dynamic behaviour of the CSB protein in living cells. The N-terminal HA-tagged CSB cDNA sequence (51) was cloned in-frame downstream of the EGFP cDNA sequence in the SacI-SalI sites of the pEGFP-C3 eukaryotic expression vector (Clontech). A two step approach was followed, involving first cloning of the C-terminal SacI-SalI CSB fragment, followed by insertion of the SacI-SacI fragment containing the HA-tagged N-terminus of CSB. pEGFP-HA-CSB plasmid was obtained; for simplicity, the tagged cDNA construct is referred to as EGFP-CSB.

DNA transfection and UV-survival

Plasmid pEGFP-HA-CSB was transfected into CS-B-deficient (UV-sensitive) fibroblast (CS1AN-Sv) using SuperFect transfection reagent (Qiagen) according to the manufactures' protocol. After selection with G418 (300 μg/ml), stable transfectants were isolated and further selected for UV-resistance by exposing cells three times to a UV dose of 4 J/m^2 UV-C (254 nm) with daily intervals. UV-selected pEGFP-HA-CSB expressing CS1AN-Sv clones were further characterized for protein expression and by UV-survival together with VH10-Sv (wt) and CS1AN-Sv untransfected fibroblasts. UV-survival was performed as described (40, 51); the expression level of the tagged protein was determined by immunoblot analysis (51).

Immunofluorescence labelling and antibodies

Cells were grown on glass coverslips at 60-80% confluency. After washing twice with phosphate-buffered saline (PBS), cells were fixed with 2% paraformaldehyde in PBS for 10 minutes at room temperature (RT) and permeabilized with 0.5 % Triton X-100 in PBS for 10 minutes at RT. Cells were subsequently washed three times (5 minutes each) with PBS$^+$ (PBS, 0.15% glycine, 0.5% BSA). Incubations with the primary antibody were performed for 1.5 hour in PBS$^+$ in a moist chamber at RT. After washing five times in PBS$^+$, cells were incubated with the secondary antibodies for 1.5 hour in PBS$^+$ at RT. Next, coverslips were washed five times with PBS$^+$ and once in PBS, and mounted in Vectashield Mounting Medium (Vector Laboratories) containing 4'-6-diamino-2-phenylindole (DAPI).

Primary antibodies used for immunolabelling were: high affinity rat anti-HA antibodies, clone 3F10 (Boehringer); affinity purified-rabbit polyclonal anti-CSB antibodies (51); mouse anti-SC35 antibodies (generously supplied by Dr. T. Maniatis).

Secondary antibodies were: goat anti-rat Alexa 594-conjugated and goat anti-rabbit Alexa 594-conjugated antibodies (Molecular Probes); goat anti-mouse Cy3-conjugated antibodies (Jackson ImmunoResearch Laboratories).

Antibodies used for immunoblot analysis were: rabbit anti-GFP antibodies (Clontech); affinity-purified rabbit polyclonal anti-CSB antibody (51).

Light microscopy and image analysis

Fluorescent microscopy images were obtained with either a Leitz Aristoplan microscope equipped with epi-fluorescence optics and a PLANAPO 63x/1.40 oil immersion lens (Figure 2, immunofluorescence experiments) or an Olympus 1X70 microscope equipped with epi-fluorescence optics and Olympus Plan Apo 60x/1.40 oil immersion lens (Figure 1C and Figure 3, EGFP-CSB live cells). Confocal laser scanning microscopy images (Figure 1D, EGFP-live cells) were recorded with a Zeiss LSM 410 microscope (see also below, FRAP).

GFP images were obtained after excitation with 455-490 and long pass emission filter (>510 nm). Cy3 images were obtained after excitation with 515-560 and long pass emission filter (580 nm).

Fluorescence recovery after photobleaching (FRAP)

A Zeiss LSM410 microscope was used for the FRAP experiments. Recovery curves for evaluation of protein mobility were obtained as described before (11). Briefly, a strip 2 μm wide, spanning the entire nucleus was bleached for 200 ms at highest intensity of the 488 nm line of a 15 mW Ar-laser focused by a 40X 1.3 n.a. oil immersion lens. Subsequently the recovery of fluorescence in the strip was monitored at intervals of 100 ms with the same laser at 10% of the intensity applied for bleaching, using a dichroic beamsplitter (488/543nm) and an additional 515-540 nm band pass filter for emmision detection. The intensity profiles of a varying number of cells (as indicated in Figure 4) were averaged and normalized in two different ways. For calculation of immobile fraction the intensity immediately before bleaching (prebleach) was set to 1 and the intensity immediately after bleaching (postbleach) was set to 0 (see Figure 4C). For evaluation/calculation of diffusion rates, the mean postbleach fluorescence intensity was set to zero and the maximum intensity reached after bleaching was set to 1 (see Figure 4A and B).

Aknowledgments

We are grateful to D. Hoogstraten and S. Rademakers for communicating unpublished results. We thank the members of Hoeijmakers lab for fruitful discussion and Prof. D. Bootsma for his continuous support. We also thank P. Verschure and R. van Driel (E.C. Slater Institute, University of Amsterdam) for valuable suggestion. S. van Baal is acknowledged for his assistance in computer work. This work was supported by EC grant n.7010-212; the Dutch Science Foundation (NWO 901-501-151); J.H.J.H. is recipient of a SPINOZA award from NWO.

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Chapter 11

Concluding remarks and future directions

Substantial experimental evidence has established the important role of TCR in ensuring the efficient progression of transcription by RNAP II after DNA damage. The generality of this mechanism, that seems to target different transcription-blocking DNA lesions and to involve multiple distinct repair pathways, such as NER and BER, has been recently revealed (91). Furthermore, a complex interplay between transcription, repair and chromatin organization is likely to determine the efficiency of TCR *in vivo*. However, many fundamental questions, including the molecular mechanism of TCR, are still unanswered.

Although the requirement of both CSA and CSB genes in TCR has been established at the genetic and cellular level, their precise molecular function in the repair-transcription coupling reaction remains unclear. Further characterization of CSB biochemical properties and isolation of the CSA protein are prerequisites to the elucidation of their role in TCR as well as in transcription. Size fractionation experiments of whole cell extracts (WCE) provide evidence that CSA and CSB are part of protein complexes of different sizes, CSB being partially associated with RNAPII (chapter 6). Also, the newly identified TCR protein XAB2 appears to reside in a large protein complex that co-fractionates both with CSB and with RNAP II (chapter 7). Interestingly, XAB2 was identified by its ability to associate with the core NER factor XPA. The observation that XAB2 is also able to interact with CSA, CSB and RNAPII (chapter 7) suggests that XAB2 may act as a dynamic bridging factor between the core NER machinery and the TCR proteins. The isolation of native CSA, CSB as well as XAB2 from human cells and the identification of their protein partners constitute an essential step in the understanding of their role *in vivo*.

The experiments described in chapter 9 provide evidence that CSB functions at the crossroads between repair, transcription and chromatin. To further investigate the precise role of CSB in chromatin transitions (including a possible involvement in chromatin assembly in addition to the observed remodeling activity) in relation to transcription and repair, it is important to determine the structural requirements for nucleosome binding and remodeling by CSB. The latter will involve a more detailed analysis of the physical interactions between CSB and the histones and the potential influence of covalent modifications (i.e. acetylation, phosphorylation, ubiquitination, ADP ribosylation) of the histone "tails" on such interactions. Also, the use of DNA templates with a higher level of chromatin compaction, such as

reconstituted plasmid chromatin containing linker histone H1, will provide insight into CSB function in chromatin. CSB might be able to influence both the individual nucleosome structures as well as the global organization of chromatin fibers.

Several studies (including the experimental work described in chapter 6 and 10 and as summarized in chapter 3 and 4) support the hypothesis that CSB is involved in transcription. In addition, as mentioned above and described in chapter 9, CSB displays nucleosome-remodeling activity. The fact that many members of the SWI2/SNF2 family, to which CSB belongs, are chromatin disrupting transcription factors, suggests that CSB may also play a role in facilitating transcription in a chromatin environment. It will be of great interest to test this hypothesis on reconstituted nucleosomal templates *in vitro*. The fact that large deletions or inactivating point mutations of the CSB gene are compatible with life, indicates that CSB is not essential for transcription (108, 184, 191). Differences in chromatin structure and/or in pausing-inducing sequences/factors within genomic regions may dictate differential requirements of genes for CSB, as well as for other chromatin- remodeling and/or elongation factors. Mutations in CSB may lead to a reduced transcription of a subset of genes that need elevated transcription levels in specific tissues or at certain stages of development. These may include, for instance, expression of some of the genes responsible for the formation of the myelin sheath. Reduced transcription of these genes and subsequent diminished proteins levels are perhaps the basis of neurodysmyelination and consequently the neurological abnormalities observed in CS patients (144, 148). The discovery that mutations in genes encoding chromatin-modifying proteins are associated with specific human diseases (97, 163, 204) leads to the attractive hypothesis that misregulated chromatin rearrangements may underlie the complex clinical symptoms of CS patients.

Despite the availability of *in vitro* assays for both transcription and NER, which are based on whole cell extracts competent for both processes, to date no *in vitro* system reliably mimicking TCR has been successfully developed (149, 213). The failure in observing a transcription-dependent increase in repair rates *in vitro* may reflect both the need for high transcription levels, not supported by the *in vitro* system, and the need for more natural DNA templates, such as nucleosomal templates, versus naked DNA. In addition, these extracts could lack specific activities required for TCR *in vitro*.

Understanding the NER process in the context of chromatin is one of the next challenges within the repair field. A prerequisite is the development of appropriate DNA templates, containing a positioned lesion, as well as precisely positioned nucleosomes. Interestingly, a reconstituted DNA sequence with a well-characterized nucleosome structure and a long stretch of pyrimidines has been recently used to address damage accessibility by *E. coli* photolyase *in vitro* (150). The potential of such a defined nucleosomal template is that it allows the monitoring of CPDs formation and repair. Similar templates could be used to

investigate damage recognition in chromatin by the complex NER machinery of mammals.

The recent implementations of the green fluorescent protein (GFP) technology in addition to microscopic and spectroscopic developments add an extra dimension to the current methods of investigation, providing a powerful tool to study NER dynamics in living cells (68). Application of this technology to the CSB protein (chapter 10), as well as to CSA, is in progress. The experiments presented in chapter 10 (based on fluorescence redistribution after photobleaching) provide evidence that CSB is mobile within the nucleus of living cells and likely associates with other proteins, in agreement with the biochemical data previously discussed (chapter 6). In addition, these experiments indicate a direct link between CSB both nuclear mobility and organization with the cellular transcriptional activity, further supporting a direct involvement of CSB in transcription (chapter 10). Additional studies on both CSB and CSA dynamics will provide valuable insight into their function in TCR and in transcription *in vivo*. Another interesting extension is the analysis of CSB behavior in a CSA deficient cellular background. This may disclose aspects of the potential functional relationship between the two proteins in living cells. In addition, analysis of the effect of mutations, including the synthetic aminoacid substitution in the ATP-binding site (chapter 8) as well as inactivating missense mutations found in CS patients (108), on both the CSB biochemical activity and behavior in living cells will contribute to elucidation of the role of CSB.

A combined effort of biochemical analysis, which allows the dissection of protein function in a well-defined system, with dynamic studies within the complex metabolic environment of living cells will be necessary to reach understanding of the TCR mechanism. Finally, several mouse models have been generated to study the consequences of NER and/or TCR deficiency (34, 191). *In vivo* analysis of these mice will greatly contribute to understanding of the relationship between the molecular defects and the clinical phenotype of the human excision repair syndromes.

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Abbreviations

aa	amino acid
ATP	adenosine triphosphate
BER	base excision repair
CS	Cockayne syndrome
CSA/CSB	Cockayne syndrome A/B protein
CPD	cyclobutane pyrimidine dimer
CTD	C-terminal domain of RNA polymerase II large subunit (hepta-peptide repeat)
DNA	deoxyribonucleic acid
DRB	5,6-dichloro-1β-D-ribofuranosyl benzimidazole
EGFP	enhanced green fluorescent protein variant
ERCC	human excision repair cross complementing gene
FRAP	fluorescence redistribution after photobleaching
GFP	green fluorescent protein
EGFP	enhanced green fluorescent protein
GGR	global genome repair
HA	hemagglutinin
IP	immunoprecipitation
ISWI	imitation switch
kDa	kiloDalton
NER	nucleotide excision repair
NURF	nucleosome remodeling factor
nt	nucleotide
6-4 PP	(6-4) pyrimidine-pyrimidone photoproduct
RAD	radiation sensitive
(hn)RNA	(heterogeneous nuclear) ribonucleic acid
RNAP II	RNA polymerase II
SNF	refers to *S. cerevisiae* sucrose non-fermenting mutants
SWI	refers to *S. cerevisiae* mutants defective in mating type switching
TCR	transcription-coupled DNA repair
TFIIH	transcription factor IIH
TPR	tetratricopeptide repeat
TTD	trichothiodystrophy
UV	ultraviolet light
(UV)DDB	(UV-light) DNA damage binding protein
WCE	whole cell extract
XAB2	XPA binding protein 2
XP	xeroderma pigmentosum
XPA to -G	xeroderma pigmentosum group A to -G protein

Summary

DNA, the carrier of genetic information, is continuously susceptible to changes, as a consequence of intrinsic chemical instability, replication errors or the action of genotoxic agents. Lesions in the DNA can arise both from endogenous metabolic processes as well as from environmental factors, including chemical compounds and physical agents such as the short-wave UV component of sunlight and ionizing radiation. DNA damage directly disturb vital cellular processes such as transcription, replication and cell cycle progression, is implicated in cancer and contributes to premature aging.

A complex network of DNA repair processes exists to safeguard the genome from the deleterious effect of damage. Nucleotide excision repair (NER) is one of the major DNA repair pathways. NER is highly conserved from yeast to man and is responsible for the removal of a wide diversion of DNA lesions, including UV-induced photoproducts and bulky DNA adducts. The NER process requires the coordinated activity of ~30 proteins and proceeds by a multistep reaction in which a short segment of the damage-containing DNA strand is excised and replaced by copying the intact complemetary strand. Briefly, recognition of the damage is followed by local unwinding of the DNA double helix and cleavage of the damaged strand on both side of the lesion. After excision of the damage-containing oligonucleotide, the undamaged strand serves as template for gap-filling DNA synthesis by the replication machinery.

At least three human syndromes are associated with defects in NER, illustrating the biological importance of this repair pathway: xeroderma pigmentosum (XP), Cockayne syndrome (CS) and thricothiodystrophy (TTD). Extreme sensitivity to sunlight is the hallmark of these human disorders, which however are very heterogeneous in other clinical manifestations. Only XP patients present high predisposition to skin cancer, whereas CS and TTD patients suffer from severe developmental and neurological abnormalities, symptoms that are difficult to explain on the basis of a defect in NER. The dual involvement of the basal transcription factor TFIIH both in NER and in transcription suggested that a transcriptional defect may underlie some of the clinical symptoms of CS and TTD patients.

It has become apparent that NER, as well as other repair mechanisms, are closely coordinated with other cellular processes. In particular, a close link between NER and transcription has been revealed. Lesions located on the transcribed strand of active genes which actually block transcription by RNA polymerase II (RNAP II) are preferentially repaired by a fast NER mode known as transcription-coupled DNA repair (TCR), which allows the rapid resumption of transcription. A specific defect in TCR is responsible for Cockayne syndrome. At least two genes are required for TCR in man, CSA and CSB. CSA harbors five WD repeats. The WD motif is present in proteins involved in various aspects of cellular metabolism, most

of which have regulatory rather than enzymatic functions. CSB belongs to the SWI2/SNF2 family of DNA dependent ATPases, several of which are implicated in remodeling of chromatin structure during transcription. In analogy with TFIIH, the CS proteins are thought to play an additional role in transcription.

The molecular mechanism of NER is reviewed in **chapter 2**. In addition, new developments in the study of NER within living cells are briefly outlined. **Chapter 3** and **4** provide a review of the TCR pathway, the proteins involved in TCR and the clinical consequences of a TCR defect in CS patients. Also, aspects of the NER connection with transcription are discussed. The organization of DNA into a highly compacted chromatin structure influences all DNA transacting processes *in vivo*, including DNA repair and transcription. The molecular mechanism of NER within chromatin is still poorly understood. **Chapter 5** focuses on the interplay between chromatin structure and NER. In addition, the activity of chromatin remodeling protein complexes in relation to transcription is summarized. The main observations described in the experimental part of the thesis are integrated and discussed in the above mentioned theoretical chapters.

The experimental part of this thesis aims to gain insights into the function of CSB in TCR. CSB activity was investigated both *in vitro* as well as in living cells. The central role of CSB in TCR suggests possible interactions with other NER and/or transcription factors. The relationship of CSB with such proteins was investigated in the context of cellular protein extracts, as described in **Chapter 6.** Gel-filtration experiments provided evidence that CSA and CSB are part of protein complexes of different size, ~450 kDa and > 700 kDa respectively. The generation of a human cell line stably expressing a tagged version of the CSB protein (HA-CSB-His_6) allowed specific immunoprecipitation (IP) of CSB from cellular extracts. CSB was found to interact with RNAP II but, surprisingly, not with other NER/transcription factors. The results from both the gel-filtration and immunoprecipitation experiments indicate that CSB is part of a protein complex involving RNAP II. **Chapter 7** describes the identification of a novel tetratricopeptide repeat containing protein involved in TCR, XAB2. This protein was identified by its ability to associate with the core NER factor XPA. XAB2 resides in a large protein complex that co-fractionates both with CSB and with RNAP II. Evidence is presented that support physical interaction of XAB2 with TCR specific proteins, CSA, CSB and RNAPII, suggesting that XAB2 may act as a dynamic bridging factor between the core NER machinery and the TCR proteins. Microinjection of anti-XAB2 antibodies in human fibroblast revealed the involvement of XAB2 not only in TCR but likely also in transcription *in vivo*.

The biochemical properties of recombinant CSB protein were investigated, as presented in **chapter 8** and **9. Chapter 8** describes the purification of baculovirus-produced Ha- his_6-tagged CSB (HA-CSB-His_6) using an efficient three-step procedure. Recombinant CSB displays double-stranded DNA-dependent ATPase activity, activated by both naked as well as nucleosomal DNA. No classical helicase activity was found, in agreement with results obtained with other SNF2-like

proteins. By site-directed mutagenesis the invariant lysine within the Walker A motif, responsible for ATP binding and hydrolysis was substituted with an arginine. This mutation abolished CSB ATPase activity, as expected, but only partially affected its biological function in living cells. Further characterization of CSB enzymatic activities is presented in **chapter 9**. In a topological assay, CSB is able to alter the DNA double helix conformation, introducing negative supercoils in a relaxed plasmid upon binding. The possibility that CSB plays a role in chromatin remodeling, based on its similarities with SWI2/SNF2 proteins, was examined by *in vitro* accessibility assays on reconstituted nucleosomal templates. CSB appears to function as an ATP-dependent chromatin remodeling factor, being able to alter DNAse I accessibility to mononucleosome cores and to disorder an array of nucleosomes regularly spaced on plasmid DNA. CSB remodeling activity has some properties in common with both SWI2/SNF2 and ISWI complexes, two of the best-characterized families of remodeling complexes.

As described in **chapter 10**, stable expression of a functional CSB-green fluorescent protein (GFP) fusion protein in human fibroblasts allowed the study of some aspects of CSB nuclear organization and dynamic properties in living cells. Confocal and conventional microscopy showed that CSB is concentrated in multiple distinct foci in the nucleus, which redistribute into a homogeneous pattern upon both UV-irradiation and transcription inhibition by drug treatment. The nuclear mobility of CSB molecules was investigated by quantitative fluorescence redistribution after photobleaching (FRAP). CSB diffuses in the nucleus with a diffusion rate slower than predicted by the size of a single polypeptide. This suggests that CSB is associated with other proteins in the nucleus, in agreement with the biochemical observations described in chapter 6. Alterations in CSB nuclear mobility were detected in both UV-irradiated and transcriptionally inactive cells. The results suggest that CSB nuclear organization and mobility are dynamic and are directly related to both UV-induced DNA damage and to transcription, further supporting a direct involvement of CSB in transcription. The results described in this thesis, together with the knowledge present in the literature, place CSB at the crossroad between DNA repair, transcription and chromatin structure.

Samenvatting

DNA, de drager van genetische informatie, is continu onderhevig aan wijzigingen door intrinsieke chemische instabiliteit, replicatiefouten of de inwerking van gen-toxische stoffen. Zowel endogene metabole processen als omgevingsfactoren, waaronder chemische verbindingen, UV stralen uit het zonlicht en ioniserende straling, kunnen beschadigingen in het DNA veroorzaken. Een rechtstreeks gevolg van DNA schades is de verstoring van enkele vitale processen zoals transcriptie (de aanmaak van het RNA), replicatie (verdubbeling van het DNA tijdens de celdeling) en progressie van de celcyclus. Ook speelt DNA beschadiging een rol bij het ontstaan van kanker en premature veroudering.

Er bestaat een uitgebreid netwerk van DNA herstel mechanismen dat het genoom moet beschermen tegen de nadelige effecten van DNA schade. Één van deze DNA herstel mechanismen is nucleotide excisie reparatie (NER). NER is aanwezig van bacterie en bakkergist tot aan de mens en is verantwoordelijk voor de herkenning en de verwijdering van een grote verscheidenheid aan DNA beschadigingen, die veroorzaakt worden door onder meer zonlicht (UV), sigarettenrook en uitlaatgassen. Het NER-proces vereist de samenwerking van ongeveer 30 eiwitten. In opeenvolgende stappen wordt de schade herkend, vervolgens wordt de dubbele DNA helix plaatselijk geopend, daarna wordt het korte fragment met de schade uit de DNA streng geknipt en vervangen, waarbij de tegenoverliggende intacte streng als voorbeeld wordt gebruikt voor het opvullen van het "gat" door de replicatie-mechanismen.

Het belang van een goed functionerend NER mechanisme is duidelijk zichtbaar bij drie erfelijke ziektes die veroorzaakt worden door defecten in dit mechanisme: xeroderma pigmentosum (XP), Cockayne syndroom (CS) en trichothiodystrophy (TTD). Deze drie ziektes hebben gemeen dat ze een extreme overgevoeligheid voor zonlicht veroorzaken. Er zijn ook duidelijke verschillen in de symptomen van patienten. Alleen XP patienten vertonen een verhoogde kans op kanker. CS en TTD patienten op hun beurt lijden aan neurologische afwijkingen en ontwikkelingsstoornissen die niet eenvoudig door een NER defect kunnen worden verklaard. De tweezijdige betrokkenheid van de basale transcriptiefactor TFIIH in zowel NER als transcriptie deed vermoeden dat een defect in het transcriptieproces aan de basis ligt van een aantal symptomen van CS en TTD patienten.

Het is duidelijk geworden dat NER, alsook andere herstelmechanismen, goed samenwerkt met andere processen in de cel. Het meest opvallende voorbeeld hiervan is een directe relatie tussen NER en transcriptie. DNA schades aanwezig in de over te schrijven streng van actieve genen, blokkeren de door RNA plymerase II (RNAP II) geïnduceerde transcriptie. Deze schades worden door een efficiente variant van NER versneld verwijderd. Dit proces wordt transcriptie gekoppeld DNA herstel (transcription coupled DNA repair (TCR)) genoemd. Door TCR kan de geblokkeerde transcriptie snel worden hervat. Een specifiek defect in TCR is

verantwoordelijk voor het Cockayne syndroom. Voor TCR zijn bij de mens tenminste twee genen belangrijk, CSA en CSB. CSA bevat een geconseveerd domein van ~35 aminozuren (WD motief) dat vijf keer gerepeteerd aanwezig is. Het WD motief bevindt zich in eiwitten die betrokken zijn bij verschillende aspecten van het celmetabolisme. CSB behoort tot de SWI2/SNF2 familie van DNA afhankelijke ATPases, waarvan sommigen betrokken zijn bij het herschikken van de chromatine structuur tijdens transcriptie. Naast TFIIH spelen de CS eiwitten waarschijnlijk ook een rol in transcriptie.

Het NER mechanisme wordt in **hoofdstuk 2** besproken. Tevens worden nieuwe ontwikkelingen in het onderzoek van NER in levende cellen kort uiteengezet. **Hoofdstuk 3** en **4** gaan in op het TCR mechanisme, de daarbij betrokken eiwitten en de klinische consequenties van een TCR defect in CS patienten. Bovendien worden er nog een aantal andere aspecten van de betrokkenheid van transcriptie met NER besproken. De organisatie van het DNA in de zeer compacte chromatine structuur beïnvloedt *in vivo* alle processen langs het DNA, inclusief DNA herstel en transcriptie. Momenteel is er nog weinig bekend over de wijze waarop de chromatine structuur het NER proces beïnvloed. In **hoofdstuk 5** wordt hier nader op ingegaan. Tevens wordt er een samenvatting gegeven van de verschillende chromatine reorganiserende eiwit-complexen die betrokken zij bij transcriptie. De belangrijkste waarnemingen die worden beschreven in het experimentele gedeelte van dit proefschrift zijn opgenomen en worden besproken in bovengenoemde theoretische hoofdstukken.

Het experimentele deel van dit proefschrift beoogt het begrip van de functie van CSB in TCR te vergroten. De CSB activiteit werd zowel *in vitro* als in levende cellen onderzocht. De centrale rol van CSB in TCR suggereert een mogelijke interactie met andere NER en/of transcriptie factoren. De relatie van CSB met zulke eiwitten werd onderzocht aan de hand van cellulaire eiwit-extracten, zoals beschreven in **hoofdstuk 6**. Gel-filtratie experimenten toonden aan dat CSA en CSB deel uitmaken van verschillende eiwit-complexen van verschillende grootte, respectievelijk ~450 kDa en >700 kDa. Ook werd gebruik gemaakt van een cellijn die een gemarkeerde versie van het CSB eiwit (HA-CSB-His_6) stabiel tot expressie brengt, waarbij het gemarkeerde eiwit m.b.v. specifieke immunoprecipitatie (IP) uit celextracten kon worden geïsoleerd. Hieruit bleek een interactie tussen CSB en RNAP II, echter niet tussen CSB en andere NER/transcriptie factoren. De resultaten van zowel de gel-filtratie als de immunoprecipitatie-experimenten geven aan dat CSB deel uitmaakt van een eiwitcomplex waarbij RNAP II betrokken is.

Hoofdstuk 7 beschrijft de identificatie van een nieuw tetratricopeptide repeat bevattend eiwit dat ook betrokken is bij TCR, namelijk XAB2. Dit eiwit wordt gekenmerkt door zijn vermogen zich te binden aan XPA, de centrale NER factor. XAB2 bevindt zich in een groot eiwitcomplex samen met CSB en RNAP II. Er wordt verder bewijs geleverd dat er een fysieke interactie tussen XAB2 en TCR-specifieke eiwitten CSA, CSB en RNAP II bestaat. Dit suggereert dat XAB2 een dynamische overbruggingsfunctie vervult tussen de centrale NER machinerie en de

TCR eiwitten

De biochemische eigenschappen van recombinant CSB eiwit werden onderzocht, zoals beschreven in **hoofdstuk 8** en **9**. **Hoofdstuk 8** beschrijft de isolatie van door baculovirus geproduceerde HA-His_6-gemarkeerde CSB door middel van een efficiente drie-staps zuiveringsmethode. Recombinant CSB bleek een DNA-afhankelijk ATPase te zijn, dat zowel door kaal DNA als nucleosomaal DNA wordt gestimuleerd. Daarnaast werd aangetoond dat CSB geen klassieke helicase is, wat overeenkomt met de resultaten van andere SNF2-achtige eiwitten. Uit de karakterisering van een mutant CSB eiwit, dat niet in staat is om ATP te hydrolyseren, bleek dat de ATPase activiteit slechts ten dele nodig is voor de biologische functie van CSB in levende cellen. Nadere karakterisering van de enzym-activiteit van CSB wordt beschreven in **hoofdstuk 9**. In een topologisch assay bleek CSB in staat te zijn de vorm van de dubbele DNA helix te wijzigen door binding. Gebaseerd op de overeenkomsten met SWI2/SNF2 eiwitten, zou CSB ook een rol kunnen spelen in chromatine reorganisatie. Deze mogelijkheid werd onderzocht door *in vitro* DNAse toegankelijkheids-assays uit te voeren op gereconstitueerde necleosomen. CSB lijkt te werken als een ATP-afhankelijke chromatine reorganiserende factor, in staat om DNAse I toegankelijkheid tot mononucleosomale kernen te wijzigen en om een serie van gelijkmatig verdeelde nucleosomen op plasmide DNA te verstoren. De reorganiserende activiteit van CSB heeft een aantal eigenschappen die overeenkomen met die van SWI2/SNF2 en ISWI complexen, twee van de best-gekarakteriseerde families van reorganiserende complexen.

In **hoofdstuk 10** wordt omschreven hoe, met behulp van stabiele expressie van een functioneel gemarkeerd CSB met het groen fluorescent eiwit (GFP) in humane fibroblasten, een aantal aspecten van de organisatie van CSB in de celkern en zijn dynamische eigenschappen in levende cellen kon worden onderzocht. Confocale en conventionele microscopie toonde aan dat CSB is geconcentreerd in meerdere discrete foci in de kern, die zich herverdelen in een homogeen patroon na zowel UV-bestraling als transcriptie-onderdrukking door chemische stoffen. De mobiliteit van CSB moleculen in de kern werd onderzocht door kwantitatieve "fluorescentie redistributie na foto-bleking" (fluoresence redistribution after photobleaching, FRAP). CSB diffundeert langzamer in de kern dan op grond van de grootte van het enkele polypeptide verwacht wordt. Dit suggereert dat CSB is geassocieerd met ander eiwitten in de kern, overeenkomstig met de biochemische observaties zoals omschreven in hoofdstuk 6. Veranderingen in de mobiliteit van CSB in de kern werden gedetecteerd in zowel UV-bestraalde als transcriptie-onderdrukte cellen. De resultaten suggereren dat de organisatie en mobiliteit van CSB in de celkern direct gerelateerd zijn aan DNA schade door UV bestraling en aan transcriptie, hetgeen de hypothese van een directe betrokkenheid van CSB bij transcriptie verder ondersteunt. Samen met de kennis die in de literatuur aanwezig is, plaatsen de resultaten die in dit proefschrift worden beschreven CSB op het kruispunt tussen DNA herstel, transcriptie en chromatine structuur.

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Curriculum Vitae

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1991-1993	Dipartimento di Genetica e di Biologia dei Microrganismi, Universita' degli Studi di Milano, Milan, Italy. Subject of master thesis: *"Molecular characterization of the human Plasminogen-Apolipoprotein(a) gene family by YAC cloning."* Supervisors: Prof. Sergio Ottolenghi and Prof. Roberto Taramelli
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Acknowledgements

It is a pleasure to take the opportunity to thank all the people who contribute to the realization of this work and who were at my side during these four years of intense both scientific and social experience.

First, I would like to express my gratitude to my promotors, Prof. Jan Hoeijmakers and Prof. Dick Bootsma. It is a privilege for me to work in your lab. Thank you for the innumerable scientific opportunities you offered me and the trust during these years. Jan, science under your supervision is a continuously stimulating experience. Your enthusiasm for research is contagious and it will accompany me in the future years. Thank you also for always being available for me, no matter your more than busy schedule. It is a great pleasure to have you Wim (Vermeulen) as co-promotor. I'm glad you have listened to my SOS a couple of years ago. Thank you for your constant and stimulating scientific support along these years and for your attentive eye also on not-scientific aspects of life in and outside the lab. I'm also indebted with you for your tireless patience during the reading and revision of the manuscript. I also appreciated the constructive scientific contribution of Roland to my research project. Roland, thank you for your valuable advice. Only recently I had the opportunity to be introduced to the world of "nuclear dynamics" by Adriaan Houtsmuller and to benefit of his knowledge and creativeness. Adriaan, thanks for your help during the writing of the last GFP results.

Special thanks also for the people of the CS team. Alain and Vincent, you both greatly contributed to this thesis. Alain, thanks for introducing me to the CSB project and for your enthusiasm. No GFP-CSB mobility without Vincent's efforts during the last months. Thank you for this and for your flexibility in switching from GFP to chromatin and back when needed. I wish you good luck with the continuation of the CSB project and with your thesis. Suzanne, thanks for the several microinjections, de "gezelligheid" and your precious help in the daily lab work. Martijn, thanks for your effort on the CSA project. I wish you success with your thesis.

In the lab I could always count on the support of my colleagues and the friendly atmosphere. Deborah (thanks for the "cultural" evenings), Claire, Ana, Niels, Josef (computer languages are much easier with your help), thanks for the support and for the fun (like during our excursions to Dutch natural beauties, artistically revised by Josef; see next page). As you noticed, I couldn't miss you one day even during the writing of the thesis. Of course I'd like to extend my thanks also to all the members of the previous labs, Bas (thanks for the opportunity to practice part of this last year), Thierry, Cecile, Sigrid, Jeroen, Peer, Kaoru, Orlando, Mies, Andre, Michael, to the repair group and to all the staff of the 7th floor.

Edgar, thanks for your essential help with "chromatin". Rita, thank you for always helping me with a cheerful smile. Mirko, thanks for your photographic advice.

Lisa and Manja, I'm very happy to have you as paranimfen. I could not desire better ones.

I'd like to thank Prof. F. Grosveld, Prof. P. Verrijzer and Dr. M Timmers for critical reading of the manuscript and useful suggestions.

The collaboration with foreign labs was an essential contribution to my research. I'm indebted with Prof. K. Tanaka for the opportunity to work in his lab and his great hospitality during my stay in Japan. My thanks are extended to the all the members of the lab, in particular Dr. Y. Nakatsu. I also appreciate very much the collaboration with Prof. J.M. Egly. Jean-Marc, thank you for your friendly hospitality, the precious protein samples and your advice. Frederic (Coin), thanks for the transcription assays. I'm grateful to Prof. R. Kingston and Dr. G. Schnitzler for introducing me to the chromatin field. Bob and Gavin, thank you for the constant support during the revision of the CSB "chromatin remodeling" manuscript.

A special thank for Ardi's family. Lisbeth and Wim, thank you for making me feel at home in Holland. I'd like to finish with thanking all my friends and especially my family: ' carissimi mamma, papa', Umberto, Fulvia, Vittoria, Elisa e Marco, grazie per la vostra continua comprensione e amore, viva anche attraverso il filo telefonico.'
Ardi, a te il merito di avermi fatto atterrarre qui, e quindi a te va anche anche il primo e l'ultimo posto in questa tesi. Grazie che posso sempre contare su di te.

Grazie a Tutti

Betty

Appendix

Table belonging to chapter 7

Table 1. Effect of XAB2 anti-serum microinjection on DNA repair and transcription

Antiserum	% UDS[1]		% RRS[3]	% transcription[4]
	wt	XPC[2]		
Preimmune	100	100	100	100
αXAB2FL[5]	94-98	30-55	25-33	41-64
αXAB2C[5]	96-98	74	40-55	95-106
αCSB	100	20	37	100
αERCC1	3-10	ND[6]	21	100

[1]Unscheduled DNA synthesis (DNA-repair synthesis) levels expressed as a percentage of UDS compared to non-injected neighboring cells.
[2]Percentage of the residual UDS in XP-C cells was 25±5% of normal level.
[3]Percentage of RNA synthesis recovery (RRS) after UV-exposure in injected cells compared to non-injected neighboring cells.
[4]Percentage of overall RNA synthesis in non-irradiated injected cells.
[5]All experiments were repeated at least three times, except for αXAB2FL and αXAB2C injection into XP-C cells. These latter were performed twice and once, respectively. Given percentages, expressed as the observed (maximum) variation between different experiments, are derived from autoradiographic counting of at least 100 nuclei.
[6]ND: not done.