

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/RNA polymerase II/transcription-coupled repair

Introduction

Nucleotide excision repair (NER) is a universal and versatile 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), recog-

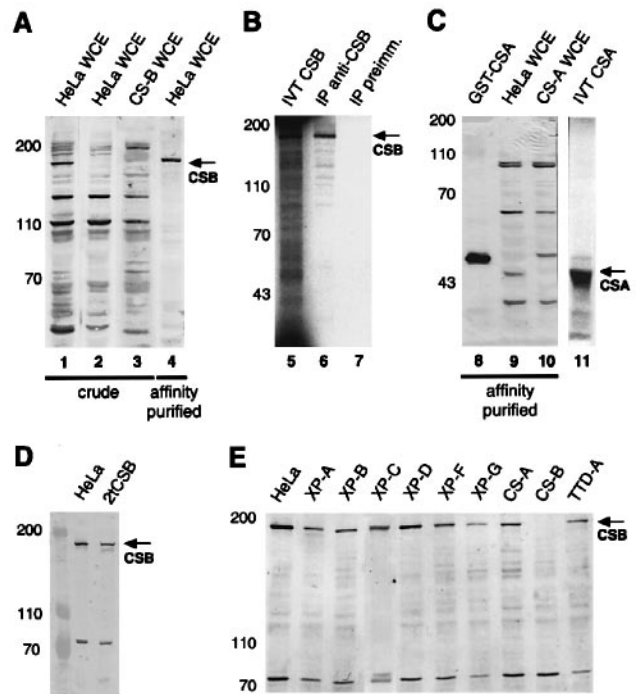


Fig. 1. Characterization of anti-CSB and anti-CSA antibodies.

(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 (2iCSB), 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.

nized 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.

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

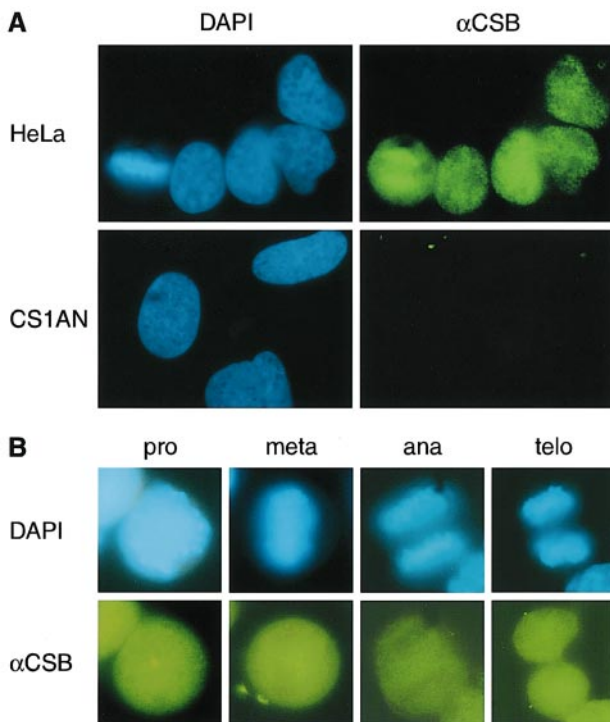


Fig. 2. The CSB protein is localized in the nucleus. (A) The affinity-purified anti-CSB antibody was used to stain the endogenous CSB protein in HeLa or CS-B (CS1AN-Sv) cells. The left panel displays the DAPI-stained chromosomal DNA, while the right panel depicts CSB staining, visualized by FITC-conjugated 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).

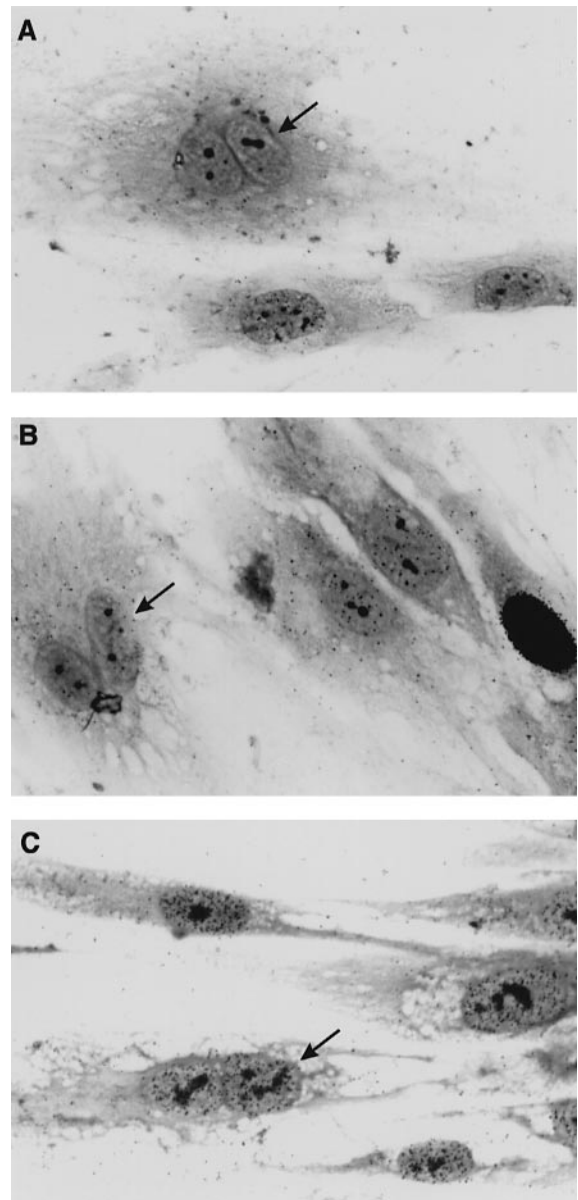


Fig. 3. Microinjection of anti-CSB and anti-CSA antisera inhibits transcription-coupled repair but not transcription *in vivo*. XP-C (XP21RO) fibroblasts were microinjected with anti-CSB (A) or anti-CSA (B) antisera to assay the effect on TCR (visualized by unscheduled DNA synthesis, UDS), or in wild-type (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 pre-immune 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.

^a100% corresponds to 19 grains, which represents 10% of the UDS observed in wild-type cells that were treated in parallel.

^bRecovery 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

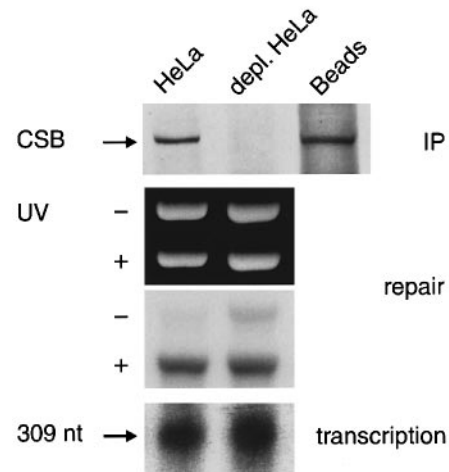


Fig. 4. Complete CSB immunodepletion has no significant effect on *in vitro* repair and basal transcription. A repair-proficient HeLa WCE was immunodepleted using crude anti-CSB serum under low stringency (buffer A) conditions. Complete CSB depletion from the extract, and binding to the beads was verified 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

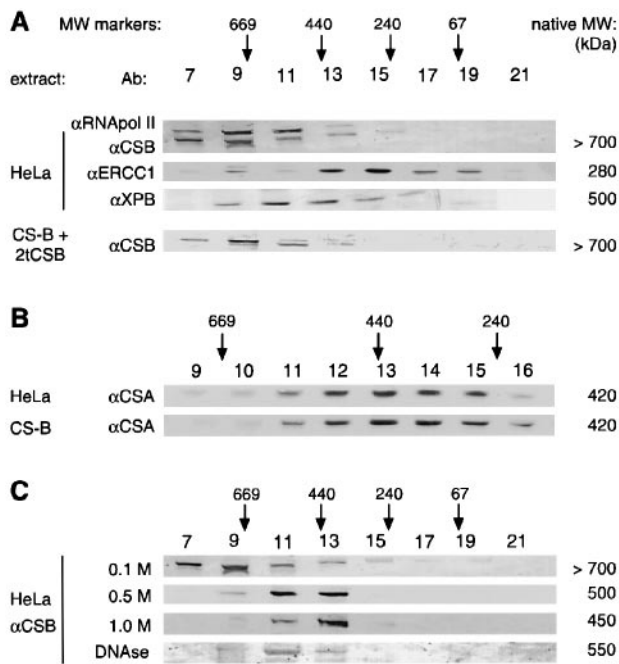


Fig. 5. CSB resides in a large molecular weight complex that does not include CSA. (A) Various WCEs were size-fractionated on a Superdex-200 column, loaded in the Manley-type buffer A containing 0.1 M KCl. Elution fractions were tested by immunoblotting using the indicated antisera. The native sizes 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, TFIIF 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

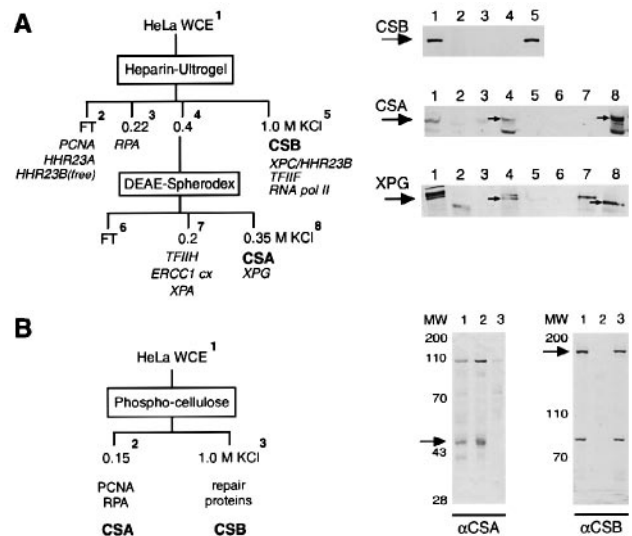


Fig. 6. CSB does not co-purify with the majority of the tested repair and transcription proteins. (A) Elution fractions of the indicated purification scheme were tested by immunoblot analysis for the presence of CSB, CSA and XPG. The presence of functional ERCC1, XPA, XPG and XPC was tested on immunoblots 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, TFIIF, 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,

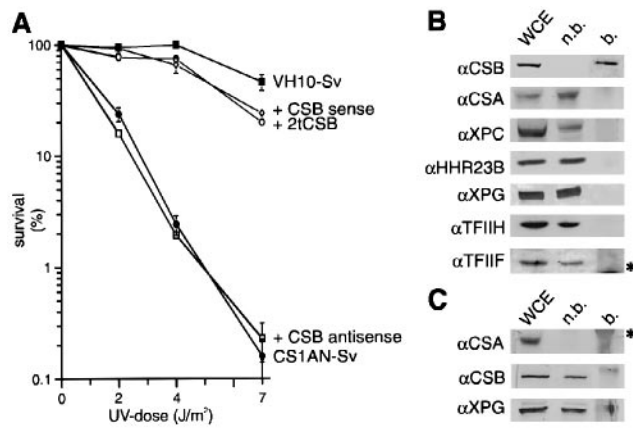


Fig. 7. Function and immunoprecipitation of tagged CSB. (A) Tagging of CSB does not interfere with its cellular function. CS-B fibroblasts were transfected with the indicated constructs, and the UV sensitivity of mass populations was determined by UV irradiation and pulse labelling with [³H]TdR. □ CS1AN-Sv (CS-B), ● CS1AN-Sv + antisense CSB, ○ CS1AN-Sv + sense CSB, ◇ CS1AN-Sv + HA-CSB-His₆ (2iCSB), ■ VH10-Sv (wild-type). (B) None of the tested candidate proteins co-immunoprecipitates with tagged CSB. HA-/His₆-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 TFIIF 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-/His₆-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 (His₆) 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-His₆ 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, TFIIF 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 TFIIF (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 (TFIIF), 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

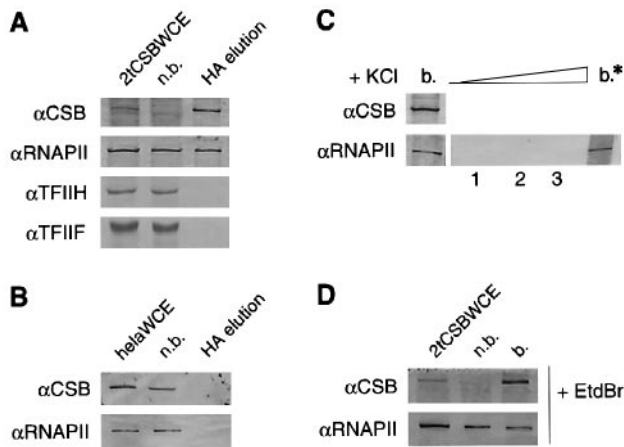


Fig. 8. A fraction of RNA polymerase II is stably associated with tagged CSB. (A) RNA polymerase II is immunoprecipitated with HA-tagged CSB. Proteins that were bound to the HA affinity beads after immunoprecipitation of HA-/His₆-double-tagged CSB from a WCE of UV-resistant CS1AN-Sv transformants (2tCSB 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 TFIIF 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 reconstituted *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 TFIIF 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 MgCl₂, 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/m²), pulse labelling for 2 h using [³H]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 [³H]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/m²) and, 24 h later, RNA synthesis was determined by [³H]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-Ultrolog 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 His₆-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' CATCGAGCTCATGTACCCATACGATGTTCCA-GATTACGCTAGCCCAAATGAGGGGAATCCCC 3' [encoding a *SacI* restriction site (underlined), start codon, HA epitope (double underlined) and CSB cDNA bp 4–21] and the antisense primer EC179-2, 5' CTCTGGCCTCATGTCTGACTCCA 3' (CSB cDNA bp 1062–1085). After DNA sequencing to check for the absence of PCR-generated mutations, a *SacI* fragment containing the HA-tagged N-terminus of CSB was exchanged with the corresponding *SacI* 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' CTGGGGCCCTTAGTGAT-GGTGATGGTGGTGACGACCTTCGATGCAAGTATCTGGCTTGAG 3' [encoding CSB cDNA bp 4460–4477, a factor Xa cleavage site, the His₆ stretch (double underlined), ochre stop codon and an *ApaI* restriction site (underlined)] were used. The His₆-tagged CSB C-terminus was isolated after *ApaI* 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/m² 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 × 10⁵ per 3 cm dish, 2–4 dishes per dose) and exposed to 0, 2, 4 or 7 J/m² UV 1 day after plating. Survival was determined after 4–6 days incubation at 37°C by [³H]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-His₆ 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 -³²P]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 -³²P]CTP-labelled run-off transcripts were visualized using autoradiography.

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