The EMBO Journal vol.13 no.7 pp.1645–1653, 1994

Correction of xeroderma pigmentosum repair defect by basal transcription factor BTF2 (TFIIH)

A.J.van Vuuren[1], W.Vermeulen[1], L.Ma[2], G.Weeda, E.Appeldoorn, N.G.J.Jaspers, A.J.van der Eb[2], D.Bootsma, J.H.J.Hoeijmakers[3] and S.Humbert[4], L.Schaeffer[4] and J.-M.Egly[4]

Department of Cell Biology and Genetics, Medical Genetics Centre, Erasmus University, PO Box 1738, 3000 DR, Rotterdam, [2]Laboratory for Molecular Carcinogenesis, Medical Genetics Centre, Sylvius Laboratory, PO Box 9503, 2300 RA, Leiden, The Netherlands and [4]UPR 6520 (CNRS), Unité 184 (INSERM), Faculté de Médecine, 11 rue Humann, 67085 Strasbourg Cedex, France

[3]Corresponding author
[1]The first and second authors have contributed equally to this work

Communicated by D.Bootsma

***ERCC3* was initially identified as a gene correcting the nucleotide excision repair (NER) defect of xeroderma pigmentosum complementation group B (XP-B). The recent finding that its gene product is identical to the p89 subunit of basal transcription factor BTF2(TFIIH), opened the possibility that it is not directly involved in NER but that it regulates the transcription of one or more NER genes. Using an *in vivo* microinjection repair assay and an *in vitro* NER system based on cell-free extracts we demonstrate that ERCC3 in BTF2 is directly implicated in excision repair. Antibody depletion experiments support the idea that the p62 BTF2 subunit and perhaps the entire transcription factor function in NER. Microinjection experiments suggest that exogenous ERCC3 can exchange with ERCC3 subunits in the complex. Expression of a dominant negative K436 → R ERCC3 mutant, expected to have lost all helicase activity, completely abrogates NER and transcription and concomitantly induces a dramatic chromatin collapse. These findings establish the role of ERCC3 and probably the entire BTF2 complex in transcription *in vivo* which was hitherto only demonstrated *in vitro*. The results strongly suggest that transcription itself is a critical component for maintenance of chromatin structure. The remarkable dual role of ERCC3 in NER and transcription provides a clue in understanding the complex clinical features of some inherited repair syndromes**

Key words: BTF2/chromatin structure/ERCC3/nucleotide excision repair/repair syndromes

Introduction

An intricate network of DNA repair systems protects the genetic information from continuous deterioration due to the damaging effects of environmental genotoxic agents and inherent chemical instability of DNA. Thus these systems prevent mutagenesis leading to inborn defects, cell death and neoplasia, and may counteract the process of ageing.

Nucleotide excision repair (NER) is one of the major, cellular repair pathways. It removes a wide range of structurally unrelated lesions (such as UV-induced pyrimidine dimers and chemical adducts) in a complex multi-step reaction. The mechanistic details of this process in eukaryotes are—in contrast to *Escherichia coli*—poorly understood, but a general picture is emerging. After recognition of the DNA injury by a process not yet resolved, the damaged strand is incised on either side of the lesion, 27–29 nucleotides apart (Huang *et al.*, 1992). Excision of the patch, which appears to require one or more single strand binding proteins, such as HSSB (RP-A) (Coverley *et al.*, 1992) is followed by gap-filling, mediated by DNA polymerase δ and/or ϵ in a reaction dependent on PCNA (Nichols and Sancar, 1992; Shivji *et al.*, 1992). Finally, ligation is required to seal the newly synthesized repair patch to the pre-existing DNA (for recent reviews see Grossman and Thiagalingam, 1993; Hoeijmakers, 1993a,b; Sancar and Tang, 1993). In fact, in most, if not all, organisms at least two NER sub-pathways exist. Special factors allow for the rapid and efficient removal of lesions in the transcribed strand that interfere with ongoing transcription (transcription-coupled repair). The other sub-pathway deals with the slower repair of the rest of the genome (genome overall repair; Hanawalt and Mellon, 1993).

The dramatic consequences of impaired NER are illustrated by three distinct, inherited diseases characterized by sun (UV) hypersensitivity, elevated genetic instability and a striking clinical and genetic heterogeneity. These are the prototype repair disorder xeroderma pigmentosum (seven complementation groups: XP-A–XP-G), Cockayne's syndrome (two groups: CS-A and CS-B) and PIBIDS (at least two groups, one of which is equivalent to XP-D) (Stefanini *et al.*, 1986, 1993; for a review see Hoeijmakers, 1993b). XP is marked by severe cutaneous abnormalities, including a strong predisposition to skin cancer, and frequently progressive neurological degeneration (reviewed in Cleaver and Kraemer, 1989). CS exhibits poor general development and neurodysmyelination. No increased frequency of skin cancer is noted in this disorder (Lehmann, 1987; Nance and Berry, 1992). The repair defect in CS is limited to the sub-pathway of transcription-coupled repair (Venema *et al.*, 1990). Patients with PIBIDS manifest most of the CS symptoms and, curiously, the hallmark of another disease called trichothiodystrophy: ichthyosis and brittle hair and nails (the latter may be due to a reduced synthesis of a cysteine-rich matrix protein) (Peserico *et al.*, 1992; Bootsma and Hoeijmakers, 1993). In exceptional cases individuals display a combination of XP and CS. These have been assigned to XP complementation groups B, D and G (Vermeulen *et al.*, 1991, 1993, 1994).

Recently, the gene responsible for one of these, the *XP-B* gene: *ERCC3* (Weeda *et al.*, 1990), was unexpectedly found to be identical to the p89 subunit of basal transcription factor BTF2 (TFIIH) (Schaeffer *et al.*, 1993). Human TFIIH, its

rat counterpart factor δ and its yeast equivalent factor b are one of the seven or so components required for proper transcription initiation of RNA polymerase II *in vitro* from a number of model promoters (Conaway and Conaway, 1989; Feaver *et al.*, 1991; Gerard *et al.*, 1991; Flores *et al.*, 1992). The formation of an elongation-competent initiation complex involves a highly ordered cascade of reactions (reviewed in Sawadogo and Sentenac, 1990; Roeder, 1991; Gill and Tijan, 1992; Drapkin *et al.*, 1993), initiated by the binding of factor TFIID to the TATA box and completed by binding of TFIIJ. The multi-subunit BTF2 consists of a minimum of five proteins: p89, p62, p43, p41 and p35. The role of this factor is at least 2-fold. First, the human BTF2/TFIIH as well as the rat δ factor are associated with a protein kinase activity that specifically phosphorylates the C-terminal domain of the large subunit of RNA polymerase II (Lu *et al.*, 1992; Serizawa *et al.*, 1993b). Second, it exhibits a DNA helicase activity (Schaeffer *et al.*, 1993) that is functionally required for ATP-driven local denaturation of the transcriptional start site. The formation of an open configuration precedes the catalysis of the first phosphodiester bond of the transcript. Experimental evidence renders it likely that the ERCC3 gene product participates in the essential unwinding reaction (Schaeffer *et al.*, 1993). This is consistent with the identification of seven so-called 'helicase motifs' in the ERCC3 amino acid sequence (Weeda *et al.*, 1990).

The discovery of the ERCC3−transcription connection provided an adequate explanation for several of the mysterious observations with respect to this gene, such as its unsolved essential function in yeast (Gulyas and Donahue, 1992; Park *et al.*, 1992) and *Drosophila* (Mounkes *et al.*, 1992) and a poorly defined involvement in expression of certain genes. In addition to a direct participation of ERCC3 in both transcription and repair, this finding opened a second possibility to explain the ERCC3−NER connection. When the expression of one or more essential NER genes critically depends on the functioning of *ERCC3*, mutations in this gene might indirectly result in a NER defect (Gulyas and Donahue, 1992; Schaeffer *et al.*, 1993). The aim of this study was to further define the role of *ERCC3* in transcription and repair *in vivo* and *in vitro*. The work presented here has implications for understanding the complex clinical picture of XP-B and other forms of NER syndromes and provides evidence for the involvement of an additional BTF2 component in NER.

Results

NER function of BTF2 measured by microinjection

The presence of XP-B specific NER correcting activity in protein fractions obtained during the purification of transcription factor BTF2 was assessed by microinjection into living XP-B fibroblasts in culture. The effect of the injected proteins on the repair capacity of the cells is measured by UV-induced unscheduled DNA synthesis (UDS), visualized by *in situ* autoradiography and quantified by counting silver grains above nuclei (De Jonge *et al.*, 1983). Figure 1A shows the purification scheme of BTF2 (Gerard *et al.*, 1991) together with the results of the microinjection in XP-B and as a control in XP-G. It is apparent that BTF2-containing fractions induce correction in XP-B. The multinuclear XP-B cell shown in Figure 1B has been injected with purified BTF2/TFIIH (hydroxyapatite fraction; Gerard *et al.*, 1991) and shows a level of UDS in the range of normal cells. In contrast, the non-injected neighbouring mononuclear cells exhibit the very low repair activity characteristic of XP-B. However, the correcting activity for XP-G fibroblasts, which is present in the whole cell extract (WCE), does not copurify with BTF2 (Figure 1A). To follow more closely the relationship between BTF2 and XP-B correction the fractionation profiles of the last two purification steps, the hydroxyapatite (HAP) chromatography and the glycerol gradient sedimentation were screened in a quantitative fashion. Figure 2 shows that there is a direct correlation between the repair complementing activity measured as UDS as well as *in vitro* and the transcription activation determined in an *in vitro* run-off assay using the adenovirus major late promoter with RNA polymerase II and all basal transcription factors except BTF2 (Fischer *et al.*, 1992). In

A Purification scheme of BTF2(TFIIH)

BTF2 purification step		Correction of NER-defect in XP-B	XP-G
HeLa WCE (Manley)		+	+
Heparin ultrogel	(0.22-0.4M KCl)	NT	NT
DEAE-spherodex	(0.07-0.17M KCl)	NT	NT
Sulphopropyl-5PW	(0.38M KCl, peak)	+	−
Phenyl-5PW	(0.2M $(NH_4)_2SO_4$, peak)	+	−
Hydroxyapatite	(0.37 M K-Pi, peak)	+	−
glycerol gradient		+	−

B

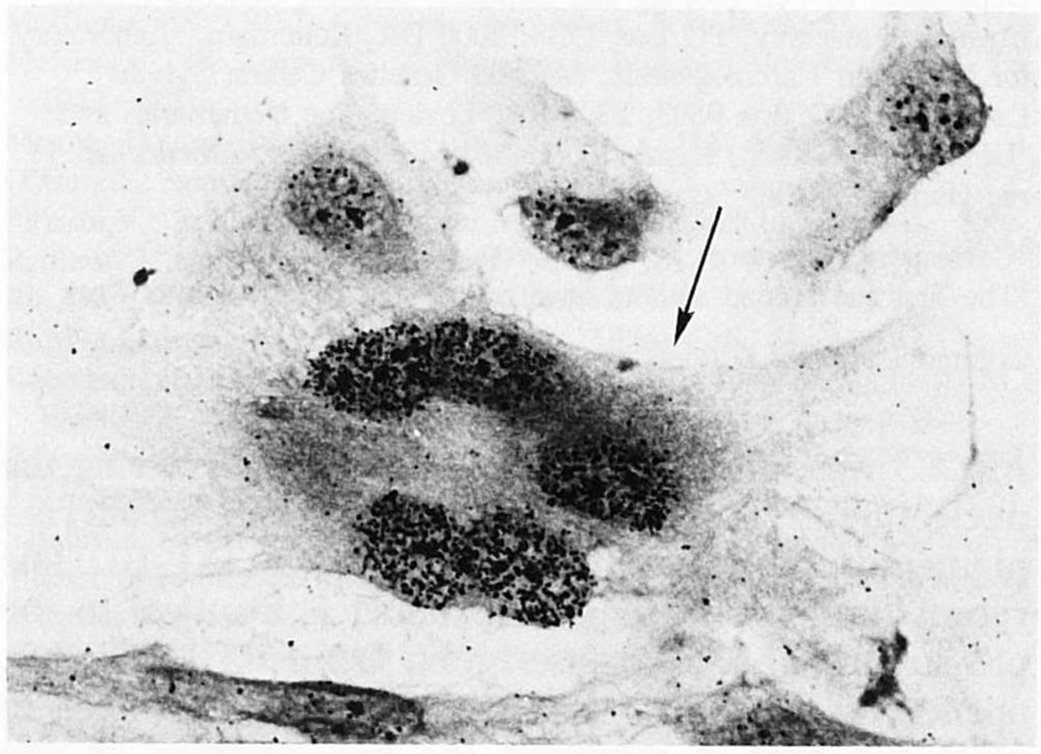

Fig. 1. Assessment of XP-B correcting activity by BTF2 using microneedle injection. (**A**) Copurification of XP-B repair correction and transcription stimulation of BTF2. Peak fractions of transcription stimulatory activity of each BTF2 purification step (for details on the BTF2 purification see Gerard *et al.*, 1991) were injected into the cytoplasm of XP-B or XP-G homopolykaryons, followed by UV irradiation, incubation in the presence of [^{3}H]TdR, fixation and processing for autoradiography (see Materials and methods for details). The number of silver grains above nuclei is a reflection of the repair capacity of the cell. Fractions were scored positive when the injected cells showed a level of UDS that was more than five times above the background of non-injected neighbouring fibroblasts. WCE: whole cell extract. The samples of the first two purification steps were very diluted compared with the WCE. (**B**) Micrograph of XP-B fibroblasts (XPCS1BA), microinjected with the highly purified fraction 12 of the HAP chromatography column (see Figure 2), showing induction of the UV dependent UDS in the injected multi-nucleated cell (arrow) compared with the typical XP-B residual UDS level as shown by the surrounding uninjected mononuclear cells.

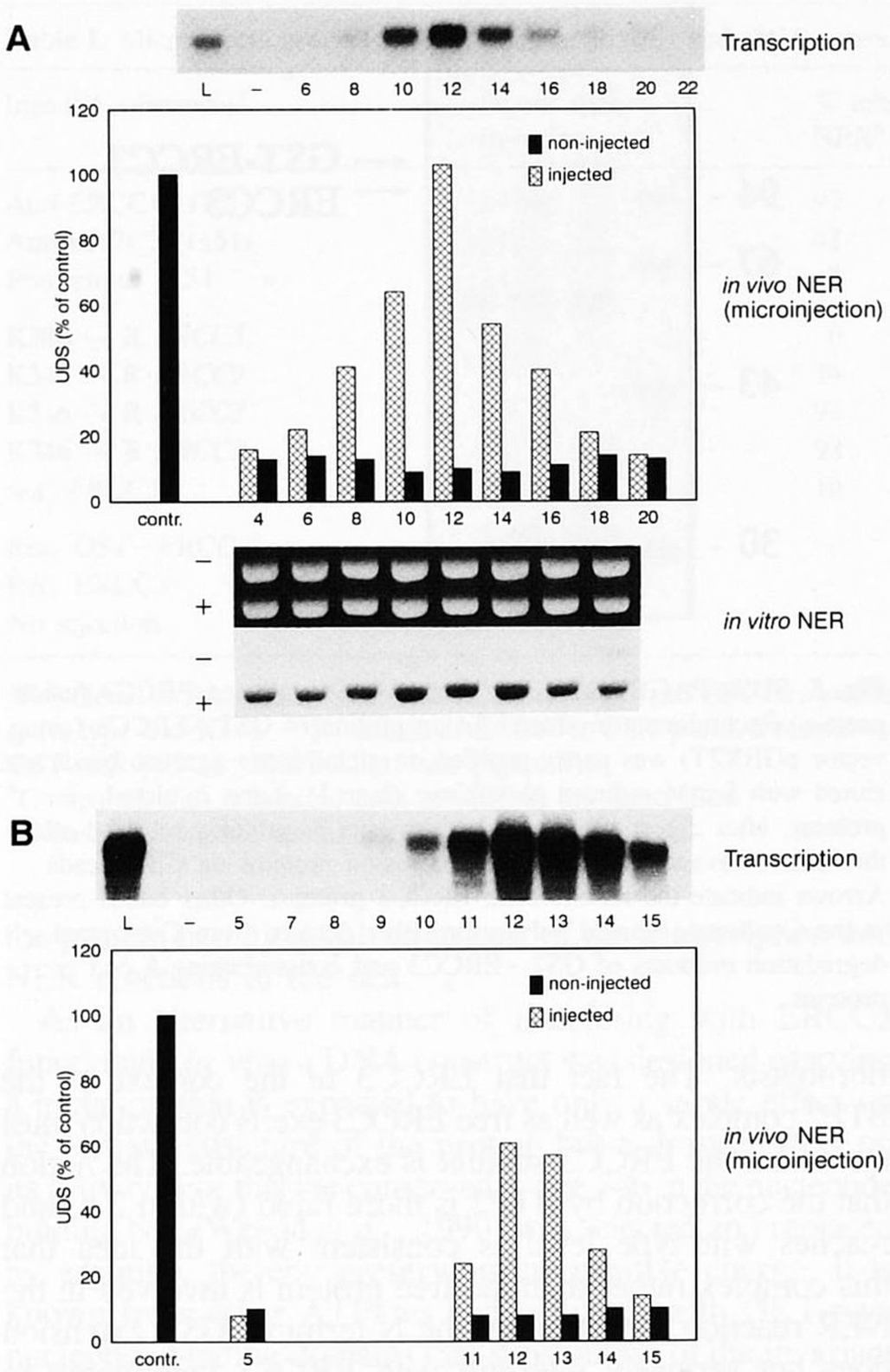

Fig. 2. Cochromatography and cosedimentation of BTF2 transcription stimulating and XP-B/ERCC3 repair-correcting activities. The transcriptionally active fractions that were eluted from the phenyl-5PW column were successively applied on a HAP column and a 15–35% glycerol gradient. All fractions were tested for transcription stimulation *in vitro* using the adenovirus major late promoter. Repair activity was determined *in vivo* by microneedle injection into living XP-B fibroblasts and *in vitro* using a cell-free NER assay. (**A**) HAP purification step. The upper part shows the autoradiogram of the transcription assay, the middle part presents the results of grain counting (average number of grains per nucleus, 50 nuclei counted) of XP-B cells microinjected with each fraction and assayed for UV-induced UDS. The lower two panels represent the *in vitro* NER activities of the same fractions in 27-1, a rodent NER deficient cell strain of complementation group 3. They show the stained gel to demonstrate equal loading and the autoradiogram to visualize repair synthesis. The AAF-damaged plasmid is indicated with '+', the undamaged internal control with '–'. Fractions 4 and 12 of the HAP profile had undergone an extra cycle of freezing and thawing prior to this test. (**B**) Glycerol gradient sedimentation. Transcription (upper part) and XP-B *in vivo* repair activity (lower part) of the glycerol fractions were assayed as above.

previous work it was shown that the transcription stimulation of BTF2 parallels its helicase activity (Schaeffer *et al.*, 1993). Thus, all BTF2 activities coincide with the XP-B specific NER correction over six purification steps. We conclude that microinjected BTF2 complex itself and not a minor contaminant causes restoration of the repair defect in living XP-B cells.

NER function of BTF2 in an in vitro repair assay

The results of the microinjection experiments do not exclude the possibility that ERCC3 is only indirectly involved in

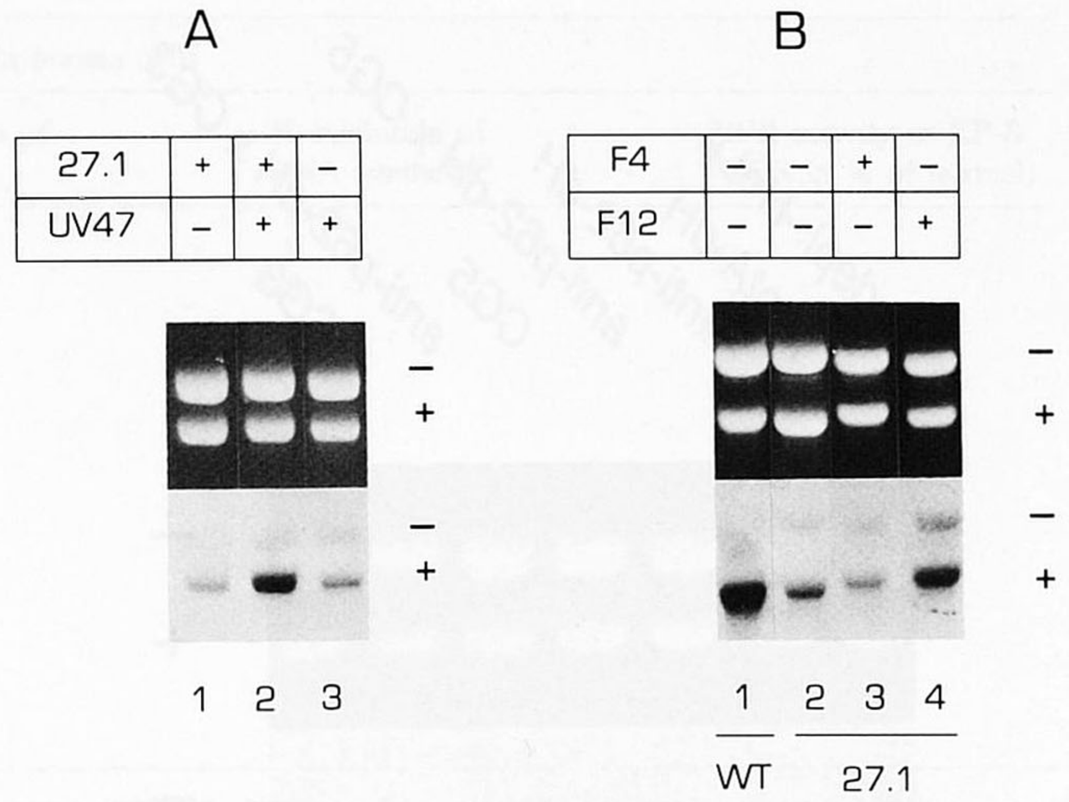

Fig. 3. BTF2 corrects NER defect of rodent complementation group 3 *in vitro*. (**A**) *In vitro* complementation of NER defect in CHO mutants of rodent complementation groups 3 (mutant 27-1) and 4 (mutant UV47). Reactions contained a damaged plasmid (pBKS, 3.0 kb) and a non-damaged internal control plasmid (pHM14, 3.7 kb), 250 ng each. Protein concentration in all reactions was 200 μg. In lane 2 100 μg of protein of each mutant extract were mixed. After incubation in the presence of [^{32}P]dATP to permit repair, DNA was isolated, linearized by restriction endonuclease treatment and size-fractionated by agarose gel electrophoresis. (**B**) *In vitro* correction of NER defect of rodent complementation group 3 (mutant 27-1) by purified BTF2 (fractions 4 and 12 of the HAP chromatography shown in Figure 2). Lanes 1 and 2 contained 200 μg protein, lanes 3 and 4 100 μg of extract was used. The amount of protein contributed by the BTF2 HAP fractions is negligible.

NER. It is feasible that introduced BTF2 corrects expression of one or more critical NER genes whose transcription is abolished by the *ERCC2* mutation in XP-B. Therefore, we also measured correction of the XP-B defect in an *in vitro* NER system (Wood *et al.*, 1988) where transcription and translation cannot occur. Figure 3B shows the outcome of administration of highly purified BTF2 (fraction 12 of the HAP chromatography) to cell-free extracts of CHO mutant 27-1. This mutant is a member of rodent complementation group 3, the equivalent of XP-B (Weeda *et al.*, 1990). In the presence of purified BTF2, repair synthesis in the damaged plasmid reaches a level similar to that achieved by mixing a complementing extract (Figure 3A). No such correction is observed with fraction 4 of the HAP eluate, which does not contain BTF2 activity. In further testing all fractions eluted from this column in the *in vitro* NER assay we find that the repair and transcription profiles are again superimposable, as they were using the microinjection assay (Figure 2A, lower panel). These experiments demonstrate that ERCC3 has a direct involvement in both transcription and NER.

Configuration of ERCC3 in the NER reaction

To determine whether the ERCC1 correcting activity in a whole cell extract resides in a BTF2(-like) configuration antibody depletion experiments were conducted. A repair proficient HeLa cell extract was incubated with a monoclonal antibody against the p62 component of BTF2 (Mab3C9), immobilized on protein A–sepharose beads. After removal of the beads by centrifugation the remainder of the extract was tested for repair capacity *in vitro*. Figure 4 shows that removal of p62 causes a clear antibody dependent reduction in repair activity (lane 3, compared with lane 1)

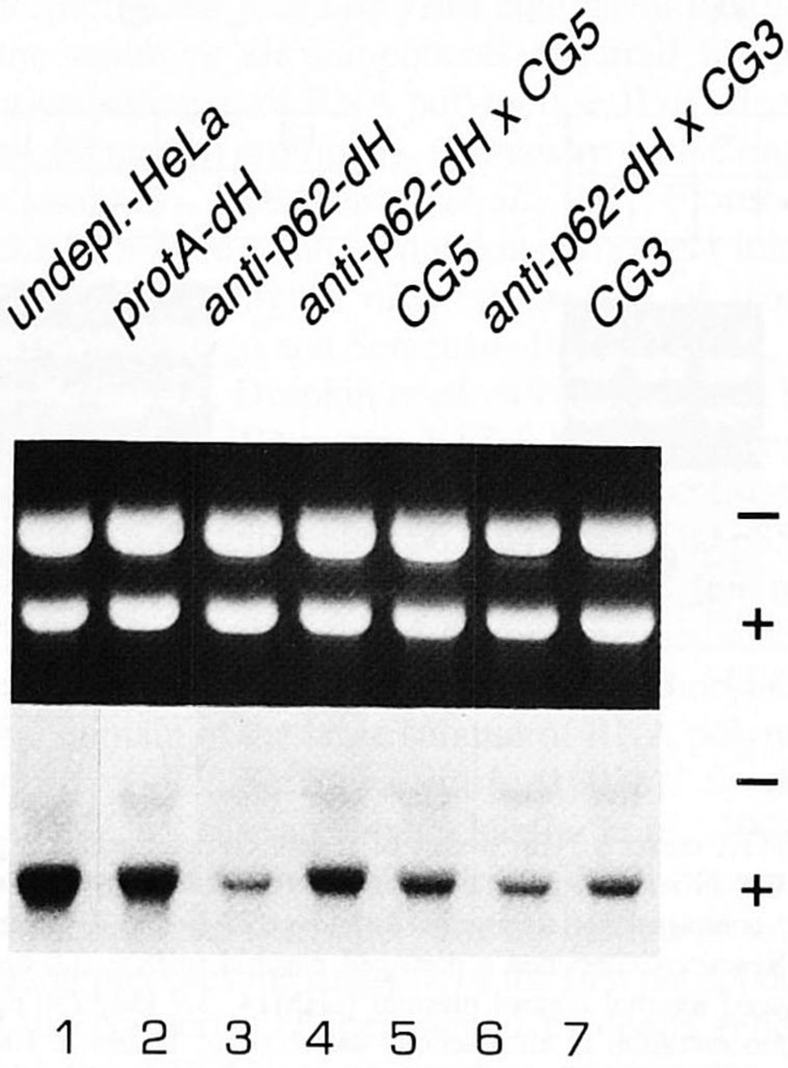

Fig. 4. Monoclonal antibodies against p62 deplete a repair-competent HeLa extract from repair and ERCC3 activity. HeLa cell-free extract (150 μg protein) was incubated with Mab3C9 against the p62 BTF2 subunit immobilized on protein A−sepharose CL-4B beads. After centrifugation to remove the bound proteins the supernatant was tested for its repair and complementing activities using the *in vitro* NER assay. Lane 1, HeLa extract (undepleted 100 μg); lane 2, HeLa extract treated with protein A beads alone (80 μg); lane 3, HeLa extract depleted with Mab3C9 (80 μg); lane 4, Mab3C9-treated HeLa extract (40 μg) mixed with extract of CHO mutant UV135 (complementation group 5, 100 μg); lane 5, UV135 extract alone (200 μg); lane 6, Mab3C9-treated HeLa extract (40 μg) mixed with extract of CHO mutant 27-1 (complementation group 3, 100 μg); lane 7, 27-1 extract alone (200 μg).

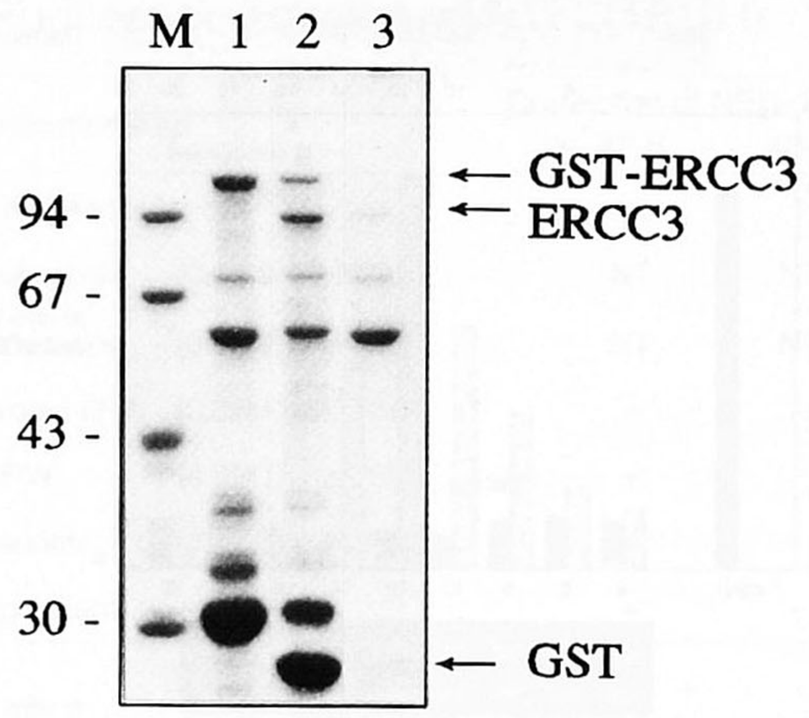

Fig. 5. SDS−PAGE analysis of recombinant-produced ERCC3 fusion protein. Recombinant-produced fusion protein of GST−ERCC3 (using vector pGEX2T) was partly purified on glutathione−agarose beads and eluted with 5 mM reduced glutathione (lane 1). Lane 2, eluted proteins, after digestion with thrombin; lane 3, proteins released after thrombin cleavage from immobilized fusion proteins on GSH beads. Arrows indicate the recombinant ERCC3 proteins. Other bands present in the Coomassie stained gel are probably derived from C-terminal degradation products of GST−ERCC3 and contaminating *E.coli* proteins.

suggesting that p62 is also involved in NER. To see whether ERCC3 activity is still present the treated extract was mixed with an extract of CHO group 3. It is apparent from Figure 4 that the $(p62)^{depleted}$-HeLa extract had also lost the ability to complement the group 3 (lane 6) but not the group 5 defect (lane 4), which is equivalent to XP-G (O'Donovan and Wood, 1993; Scherly *et al.*, 1993). These findings indicate that ERCC3 correcting activity in a repair-competent HeLa whole cell extract is associated with p62 and that the ERCC5/XP-G factor is not tightly bound to the p62−ERCC3 complex.

Effect of free ERCC3 protein

The purified BTF2 complex is quite stable, at least *in vitro*, as the proteins involved remain associated even in 1 M KCl (Schaeffer *et al.*, 1993). To find out whether the ERCC3 protein can only function in NER when offered in the form of the BTF2 complex we tested the ability of ERCC3 protein overproduced in *E.coli* to complement the XP-B defect. Figure 5 shows a glutathione−agarose bound fraction containing the GST−ERCC3 fusion protein after SDS−PAGE (lane 1) and the partially purified ERCC3 gene product released from the GST fusion protein after cleavage by thrombin (lane 3). The results summarized in Table I (lower part) demonstrate that the recombinant GST−ERCC3 fusion protein as well as free ERCC3 induce significant correction soon (within 4 h) after microinjection into XP-B fibroblasts. The fact that ERCC3 in the context of the BTF2 complex as well as free ERCC3 exerts correction must mean that the ERCC3 subunit is exchangeable. The notion that the correction by BTF2 is more rapid (within 2 h) and reaches wild-type level is consistent with the idea that this complex rather than the free protein is involved in the NER reaction. Apparently, the N-terminal GST extension does not seriously interfere with ERCC3 functioning in the BTF2 complex.

Consequence of interference with ERCC3 functioning for NER and transcription

The involvement of ERCC3 in transcription is derived from results using an *in vitro* transcription assay and a limited set of promoters (Gerard *et al.*, 1991). It is important to verify whether the protein performs such a function *in vivo* as well. Therefore, we assessed the effect of antibody injection on RNA synthesis (determined by incorporation of [^{3}H]uridine) and on NER (as determined by the level of UV-induced [^{3}H]TdR incorporation) into normal fibroblasts. As a control, cells on the same slide were injected with preimmune serum of the same rabbit and with antibodies against the ERCC1 protein, a NER component not residing in the BTF2 complex. Previously, we presented the specificity of the antibodies (Schaeffer *et al.*, 1993; van Vuuren *et al.*, 1993). The results are summarized in Table I (upper part). The ERCC3 preimmune serum has no significant effect on the number of grains derived from transcription or NER. The ERCC1 control serum induces a total inhibition of excision repair, demonstrating that this protein is indispensable for NER. However, no reduction of general transcription is observed, demonstrating that transcription does not drop as a non-specific consequence of the inhibition of repair. The ERCC3 serum causes a significant but incomplete repression of transcription as well as repair synthesis. The partial effect can be explained in several ways. The antibodies cannot reach or block the activity of all ERCC3 molecules, e.g. because the antigenic sites are not accessible. Alternatively,

Table I. Microinjection of ERCC3 protein, antibodies and DNA constructs in human cells

Injected substances[a]	Hours after injection	% inhibition of NER[b]	% inhibition of RNA synthesis[b]	NER activity in XP-B (UDS in % of normal)
Anti-ERCC1 (476)	24	97	0	–
Anti-ERCC3 (1151)	24	43	48	–
Preimmune 1151	24	2	5	–
K346 → R *ERCC3*	6	0	0	–
K346 → R *ERCC3*	22	74	70	–
K346 → R *ERCC3*	30	93	78	–
K346 → R *ERCC3*	48	98	95	–
w.t. *ERCC3*	30	10	5	–
Rec. GST–ERCC3[c]	4–6	–	–	39
Rec. ERCC3[c]	4–6	–	–	43
No injection	–	–	–	11

[a]Polyclonal rabbit serum (476, anti-ERCC1; 1151, anti-ERCC3; preimmune 1151), *ERCC3* cDNA in the mammalian expression vector pSVL (wild-type and K346 → R mutant of *ERCC3*); GSH-purified recombinant fusion protein of GST–ERCC3 and thrombin-cut fusion protein of GST–ERCC3 on GSH column (see Figure 5).
[b]Compared with uninjected cells present on the same slide.
[c]Estimated protein concentration 0.05–0.1 μg/ml.

the protein is only involved in part of the transcription and NER reactions in the cell.

As an alternative manner of interfering with ERCC3 functioning *in vivo* a DNA construct was designed carrying a mutation that is expected to have only a subtle effect on the tertiary structure of the protein but a drastic effect on its activity. For this the conserved lysine 346 in the nucleotide binding box (Weeda *et al.*, 1990) was selected and replaced by arginine, thereby preserving the positive charge. It is known from other ATPases and helicases with GKT-type nucleotide binding domains that substitution of the invariant lysine reduces or completely abolishes ATP hydrolysis but does not necessarily affect ATP binding as such (Azzaria *et al.*, 1989; Reinstein *et al.*, 1990; Tijan *et al.*, 1990). In the yeast RAD3 repair helicase it has been demonstrated that a similar K → R replacement does not interfere with ATP binding but blocked the hydrolysis step and helicase activity (Sung *et al.*, 1988). Transfection of the mutant *ERCC3* cDNA into the UV-sensitive, repair-deficient rodent group 3 cell line 27-1 demonstrated that the K346 → R protein was unable to restore the repair defect (L.Ma, A.Westbroek, A.G.Jochemsen, G.Weeda, D.Bootsma, J.H.J.Hoeijmakers and A.J. van der Eb, manuscript submitted). An analogous mutation in the yeast homologue of ERCC3, RAD25, showed that the essential function of the protein was also inactivated (Park *et al.*, 1992). To see whether this mutation exerts a dominant effect the K346 → R *ERCC3* cDNA was microinjected into repair-competent fibroblasts. As a control the wild-type cDNA in the same vector was injected into cells on the same slide. Although at different time points some heterogeneity was seen in the magnitude of the effects, presumably due to differences in the level of expression, a sharp drop in UDS was registered within 22 h which was total by 48 h (see Figure 6D–F and for quantitative results Table I, middle part). Concomitantly, transcription fell down to undetectable levels (Figure 6A–C, Table I). This was closely followed by a dramatic change in nuclear morphology. At first (22 h) the nucleoli increased in size and became less densely stained. At later times the entire Giemsa-stainable chromatin material clumped in a small area, leaving the remainder of the nucleus empty. In contrast, the cytoplasm stayed remarkably normal in morphology (see Figure 6). None of these effects were observed in the cells injected with the wild-type cDNA construct which even exerts correction of the repair defect when injected into XP-B fibroblasts (Weeda *et al.*, 1990). We conclude that the K346 → R substitution confers a dominant-negative effect on both transcription and NER and induces a dramatic chromatin collapse.

Discussion

The findings reported here demonstrate that the ERCC3 gene product functions directly in two quite different aspects of nucleic acid metabolism: basal transcription and nucleotide excision repair. This confirms and extends earlier observations by Egly and coworkers who identified the ERCC3 protein as one of the components of the BTF2/TFIIH complex required for proper transcription initiation of RNA polymerase II *in vitro* (Schaeffer *et al.*, 1993). Thus these results rule out the theoretical possibility raised by these and other remarkable observations with respect to the *ERCC3* gene in the yeast and *Drosophila* systems, namely that the ERCC3–NER connection could be indirect, i.e. ERCC3 might control the expression of one or more (real) NER genes without being involved itself in this process (Gulyas and Donahue, 1992; Mounkes *et al.*, 1992; Schaeffer *et al.*, 1993). In addition, this work establishes that the ERCC3 protein participates in the transcription machinery *in vivo*; the evidence for this was hitherto only based on an *in vitro* transcription assay. Finally, this paper reveals the dramatic consequences of interfering with proper ERCC3 functioning *in vivo* via overexpression of dominant-negative ERCC3 mutant protein or by antibody injections. Our findings have direct implications for the functioning of ERCC3 at the molecular level and for the interpretation of some of the clinical manifestations of the associated human syndrome.

Involvement of BTF2 in NER

In what configuration does ERCC3 function in NER? The fact that purified BTF2 is able to correct the XP-B/rodent

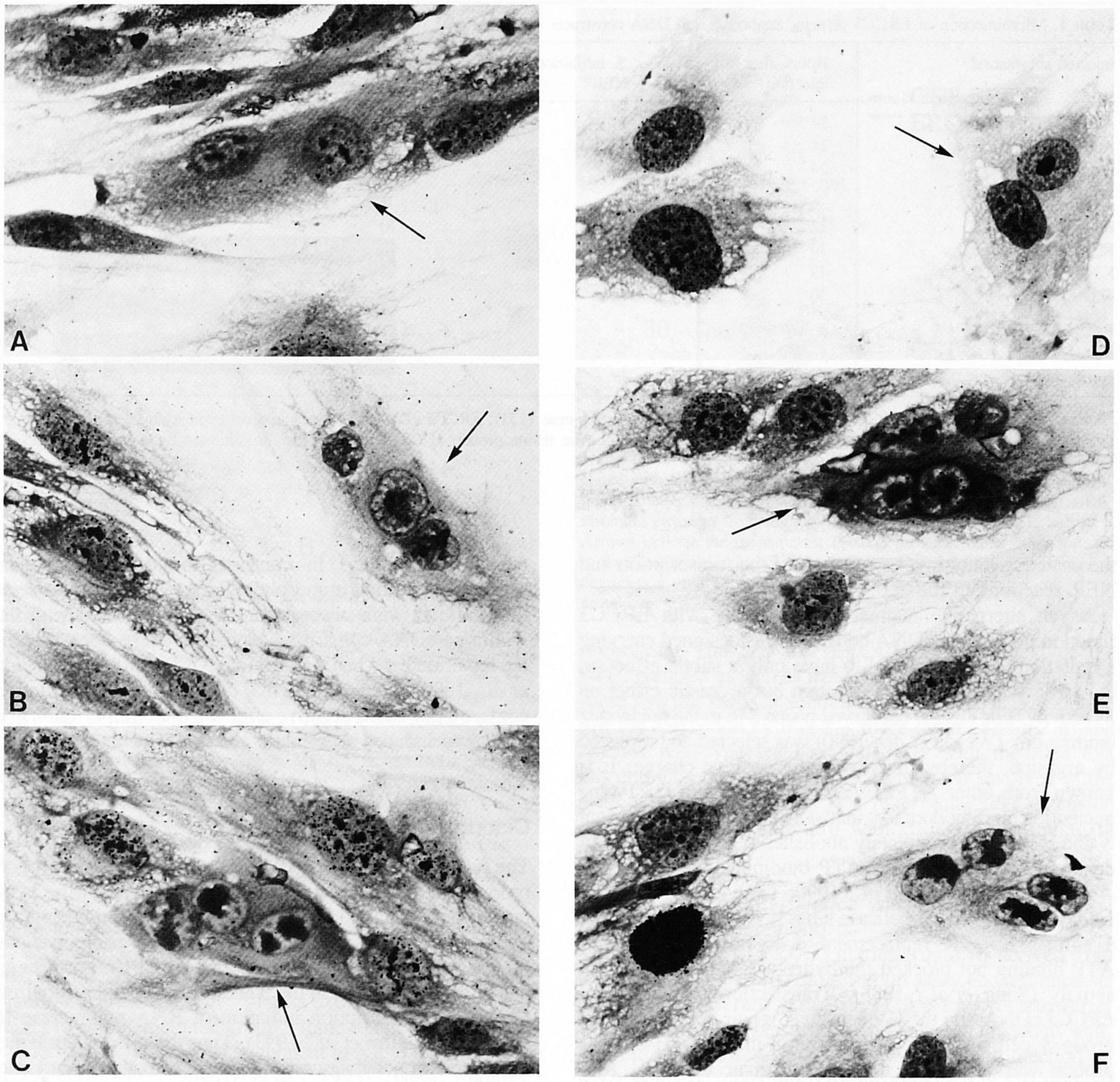

Fig. 6. Effect of K346 → R ERCC3 mutant on transcription and NER. Micrographs **A**, **B** and **C** show the time dependent effect on RNA synthesis, assayed by pulse labelling with [^{3}H]uridine, in control (wild-type) fibroblasts injected with the *ERCC3* cDNA encoding the K346 → R mutated protein. Micrographs **D**, **E** and **F** demonstrate the effect on NER, as revealed by UV-induced UDS. A and D were assayed 22 h, B and E 30 h and C and F 48 h after microinjection. Injected polykaryons (arrows) demonstrate a complete inhibition of RNA synthesis (no incorporation of [^{3}H]uridine) as well as NER (absence of UDS in injected polykaryons) and a dramatic chromatin collapse.

group 3 repair defect *in vivo* and *in vitro* suggests, but does not prove, that the complex as a whole participates in the NER process. It is, however, not excluded that the ERCC3 subunit dissociates from the complex and either functions alone or in a different complex in the NER reaction mechanism. An argument in favour of the involvement of BTF2 in NER is our finding that monoclonal antibodies against p62 are able to deprive a HeLa WCE of its NER capability and all detectable ERCC3 activity. This suggests that at least the p62 subunit of BTF2/TFIIH is tightly associated with the majority of the ERCC3 molecules that are required for *in vitro* NER. A second inference from this observation is that the p62 protein may be yet another NER factor. Whether the same holds for the entire BTF2 complex has to be established. The dual involvement of ERCC3 extends the emerging notion that the eukaryotic NER system recruits many factors from other systems that operate in the nucleus. From parallels with yeast it is likely that the recently discovered ERCC1, ERCC4, ERCC11 and XPFC complex functions simultaneously in NER and in a mitotic recombination pathway (Schiestl and Prakash, 1990; Bailly *et al.*, 1992; Bardwell *et al.*, 1992; Biggerstaff *et al.*, 1993; van Vuuren *et al.*, 1993). Using the *in vitro* NER assay Wood and coworkers have shown that the replication factors PCNA and HSSB (RPA) also participate in the post-incision stages of the NER reaction (Coverley *et al.*, 1992; Shivji *et al.*, 1992) in addition to DNA polymerase δ or ϵ and ligase I. The dual usage of these proteins for different processes

may be for economical reasons. Alternatively, it may be a reflection of the tight links of excision repair with other processes in the nucleus.

Possible role of ERCC3 in NER

What can be the role of ERCC3 and BTF2 in transcription initiation and NER? From the foregoing it is most logical to search for a common functional step for ERCC3 and perhaps the entire BTF2/TFIIH complex in both processes. It has been demonstrated that the human BTF2/TFIIH complex as well as the rat counterpart exhibit two activities: a protein kinase that is able to phosphorylate the C-terminal domain of the large subunit of RNA polymerase II (Lu *et al.*, 1992; Serizawa *et al.*, 1993a,b) and a helicase activity (Schaeffer *et al.*, 1993). The latter is likely to be associated with the p89 ERCC3 subunit. In this light at least two possibilities (or a combination) can be envisaged for a common catalytic function of ERCC3 in transcription and NER. (i) Induction of a locally melted DNA conformation for RNA polymerase to be loaded onto the template. In the context of NER a similar function can be imagined for putting a scanning complex onto the DNA or the incision complex at the site of a lesion. (ii) Unwinding of the helix as part of the translocation of the transcription or NER scanning complex along the chromatin, as suggested for the $UvrA_2B$ complex in *E.coli* NER (Grossman and Thiagalingam, 1993). In both cases the ERCC3 protein or the BTF2 factor has to interact with components of the transcription as well as the NER machinery. Mutations in the ERCC3 protein found in XP-B patients (such as alteration of the C-terminus: Weeda *et al.*, 1990) primarily inactivate NER. It is tempting to speculate that these regions are specifically involved in the interaction with other NER components.

Interference with ERCC3 functioning

Microinjection of the K346 → R *ERCC3* mutant construct induces a strong reduction or even a complete blockage of NER and general transcription. A similar effect—although quantitatively not as pronounced—was seen with anti-ERCC3 antibodies. Selective inhibition of NER is not the reason for the dramatic decline of transcription, because antibodies against the ERCC1 protein also cause a total blockage of NER but have no measurable impact on transcription. The inhibition of NER and transcription by K346 → R ERCC3 occurs with similar kinetics, indicating that both processes are disturbed in an analogous fashion. Furthermore, it is observed relatively shortly after microinjection when taking into account that the injected gene has to be transcribed, the mRNA translated and the protein incorporated in a sufficient number of BTF2 complexes to exert the observed effect. For many genes expression is first registered 16−24 h after injection. Also the correction of the XP-B repair defect by injection of the purified ERCC3 protein is a quite rapid phenomenon: significant increase in UDS is seen within 4 h after injection. This suggests that exogenous ERCC3 is able to exchange with endogenous ERCC3 in BTF2 complexes in a relatively fast manner. One possibility is that the BTF2 complex disassembles and reassembles at regular times as part of its reaction cycle and in this way incorporates new exogenous ERCC3 protein. For the dominant effect of mutant ERCC3 the following scenario seems most plausible. The conservative K346 → R mutation is not expected to perturb dramatically the tertiary conformation and fitting of the protein into the BTF2 complex. After incorporation into BTF2 and assembly of the preinitiation complex the reaction proceeds up to the stage in which the ATPase activity of ERCC3 is required. At this point the system is paralysed, frustrating the normal progression of both transcription and NER. When a critical threshold of poisoned transcription units is reached this process culminates in a complete and irreversible inhibition of transcription. Ironically, this catastrophic event will also shut down the expression of the injected *ERCC3* mutant construct itself.

BTF2 has been demonstrated to be involved in transcription by RNA polymerase II (Gerard *et al.*, 1991). It is not known whether it is implicated in RNA polymerase I and III transcription as well. The effects of the dominant-negative *ERCC3* mutant indicates that total RNA synthesis becomes impaired. This does not necessarily mean that ERCC3 participates directly in all three modes of transcription. It is possible that the inhibition of e.g. RNA polymerase I is an indirect result of the blockage of RNA polymerase II.

The time-resolved consequences of selective inhibition of transcription by RNA polymerase II have hitherto escaped detection because severe mutants in this process are obviously not viable. In our transient microinjection system the most dramatic and earliest morphological effects concern the nucleus where the nucleolus seems to swell first and eventually all chromatin appears to become clumped in one or a few regions, leaving the remainder of the nucleus empty. These alterations initiate already at a stage when inhibition of transcription is still not complete suggesting that the chromatin structure critically depends on ongoing transcription and functioning ERCC3. Probably all cellular processes which require proteins with a high turnover and a short mRNA half-life will be affected first. Morphologically, the affected cells show features resembling an early stage of apoptosis. However, further studies are warranted to establish a relationship with apoptosis.

Consequences for the clinical features of XP-B

The very rare XP-B complementation group exhibits a number of clinical characteristics that are atypical for a NER defect and difficult to rationalize on the basis of a DNA repair problem. This includes many of the features these patients share with Cockayne's syndrome, such as neurodysmyelination, immature sexual development, absence of subcutaneous fat and a general growth deficiency (Cleaver and Kraemer, 1989; Scott *et al.*, 1993; Vermeulen *et al.*, 1994). In view of the additional function of ERCC3 it is tempting to assign at least some of these features to a subtle impairment of transcription. In mouse models evidence has been collected that the production of the myelin sheet is strongly determined by the amount of mRNA for myelin basic protein (Popko *et al.*, 1987). Thus the neurodysmyelination may be related to a reduced expression of this protein. Viable mutations in the *Drosophila* homologue of *ERCC3* designated '*haywire*' display sterility which is likely to be caused by reduced expression of β-tubulin, required for spindle formation in meiosis (Mounkes *et al.*, 1992). Perhaps the immature sexual development in XP-B could be due to the same problem. Recent findings in the field of basic transcription support the notion that the requirement of transcription factors may vary from promoter to promoter, depending on the sequence around the initiation site, the topological state of the DNA and perhaps other factors such

as the local chromatin structure (see Stanway, 1993 and references therein). This may explain the above features and the poor general development characteristic of this form of XP. In fact two other XP complementation groups, XP-D and XP-G, also display the remarkable clinical features of XP-B (Vermeulen *et al.*, 1993). As demonstrated here the XP-G factor is not present in the BTF2 complex. If it is involved in transcription it is either present in another complex or it is only loosely associated with components of BTF2 and not essential for transcription. The latter idea is in agreement with the notion that the yeast homologue of XP-G, RAD2 (Scherly *et al.*, 1993) is also not an essential gene (Madura and Prakash, 1986). As noted before, XP-D and the corresponding gene *ERCC2* have many parallels with XP-B and *ERCC3*. This has prompted the idea that the ERCC3 and ERCC2 proteins may interact and have a similar function (Weeda *et al.*, 1990). Clinical heterogeneity in XP-D is even more pronounced than in XP-B and includes the peculiar brittle hair symptoms of trichothiodystrophy. We have recently obtained evidence that the functional overlap between repair and transcription also includes ERCC2. This is consistent with the idea that some of the clinical features of these rare pleiotropic disorders are due to basal transcription problems.

Materials and methods

General procedures

Purification of nucleic acids, restriction enzyme digestion, gel electrophoresis of nucleic acids and proteins, transformation of *E.coli*, etc. were performed according to standard procedures (Sambrook *et al.*, 1989).

Purification of BTF2/TFIIH

The purification of BTF2 and all other general transcription factors required for the transcription assay using the adenovirus 2 major late promoter as template was performed starting from HeLa cells as described earlier (Gerard *et al.*, 1991).

Microneedle injection and assay for RNA synthesis and UV-induced unscheduled DNA synthesis

Microneedle injection of XP-B fibroblasts as well as control cells (C5RO) was performed as described (Vermeulen *et al.*, 1994). Briefly, at least 3 days prior to microinjection cells were fused with the aid of inactivated Sendai virus, seeded onto coverslips and cultured in Ham's F-10 medium, supplemented with 12% fetal calf serum and antibiotics. After injection of at least 50 homopolykaryons cells were incubated for the desired time in normal culture medium before being assayed. NER activity was determined after UV-C light irradiation with 15 J/m^2, incubation for 2 h in [^{3}H]thymidine (10 μCi/ml; s.a.: 50 Ci/mmol)-containing culture medium, fixation and exposure to autoradiography. Grains above the nuclei (>100) were counted and represent a quantitative measure for NER activity. RNA synthesis was determined also by counting autoradiographic grains above the nuclei of injected cells, after labelling with [^{3}H]uridine (10 μCi/ml; s.a.: 50 Ci/mmol) during a pulse labelling period of 1 h in normal culture medium. Protein preparations (including antisera) were injected into the cytoplasm, cDNAs were microinjected into one of the nuclei of polykaryons.

In vitro DNA repair assay

Plasmids pBKS (3.0 kb) and pHM14 (3.7 kb) were isolated from *E.coli* and extensively purified as closed circular DNA (Biggerstaff *et al.*, 1991). pBKS was treated with 0.1 mM *N*-acetoxy-2-acetylaminofluorene (AAF) (a kind gift of R.Baan, TNO, Rijswijk), inducing mainly *N*-(guanine-8-yl)−AAF adducts. AAF-modified plasmids were collected by repeated di-ethyl-ether extractions and ethanol precipitation (Landegent *et al.*, 1984) and repurified on a neutral sucrose gradient. pHM14 was mock-treated in parallel. There are 15−20 AAF−guanine adducts per damaged plasmid.

Repair-proficient cell lines: HeLa, Chinese hamster ovary (CHO) cell strain CHO9 and CHO NER mutants: 27-1 (complementation group 3), UV47 (CG 4) and UV135 (CG 5) were cultured in a 1:1 mixture of Ham's F-10 and DMEM medium (Gibco) supplemented with 10% fetal calf serum and antibiotics. Cells were harvested and extracts were prepared from 2−5 ml of packed cell pellet by the method of Manley as modified by Wood (Manley *et al.*, 1983; Wood *et al.*, 1988). Extracts were dialysed in 25 mM HEPES−KOH, pH 7.8, 0.1 M KCl, 12 mM $MgCl_2$, 1 mM EDTA, 2 mM DTT and 17% (v/v) glycerol and stored at −80°C.

The reaction mixture (50 μl) contained 250 ng of both damaged and non-damaged plasmid DNA, 45 mM HEPES−KOH (pH 7.8), 70 mM KCl, 7.4 mM $MgCl_2$, 0.9 mM DTT, 0.4 mM EDTA, 20 μM each of dCTP, dGTP and TTP, 8 μM dATP, 74 kBq of [α-^{32}P]dATP, 2 mM ATP, 40 mM phosphocreatine, 2.5 μg creatine phosphokinase, 3.45% glycerol, 18 μg bovine serum albumin and 200 μg of cell-free extract. The reaction was incubated for 3 h at 30°C, afterwards the plasmid DNAs were isolated, linearized and separated on an agarose gel electrophoresis. Data were analysed via autoradiography and quantified by scintillation counting of excised DNA bands.

For microinjection and antibody depletion experiments, the following antisera were used: (i) a rabbit polyclonal anti-ERCC1 antiserum raised against a ubiquitin−ERCC1 fusion protein and characterized as previously described (van Vuuren *et al.*, 1993); (ii) a polyclonal antiserum raised against a GST−ERCC3 fusion protein containing an internal part (amino acids 82−480) of ERCC3; (iii) a monoclonal antibody (Mab3C9) against the 62 kDa polypeptide, a component of BTF2, used in depletion experiments was published earlier (Fischer *et al.*, 1992).

To deplete 150 μg of repair-proficient HeLa extract, anti-p62 antibodies (3 μl of ascites fluid) were immobilized on protein A−Sepharose CL-4B beads; after incubation with HeLa extract and centrifugation the supernatant was used as a depleted Hela extract and tested for repair activity *in vitro* as detailed above (van Vuuren *et al.*, 1993).

Overproduction and purification of recombinant ERCC3 protein

ECCR3 cDNA cloned in pGEX2T was transferred to *E.coli* strain BL21 and gene expression was induced during 3 h by IPTG. Cells were homogenized in PBS (containing 2 mM PMSF and 15% glycerol) by sonication and extracts were cleared by centrifugation. Fusion protein was purified by passing the cell homogenate through a glutathione−agarose containing column, proteins were eluted with 5 mM reduced glutathione (in 50 mM Tris, pH 8.0). Alternatively recombinant ERCC3 was cleaved from the GST part by incubating the immobilized fusion protein (on the glutathione−agarose beads) with thrombin (1−2 ng/μg protein) for 45 min at 20°C.

Site-directed mutagenesis

Mutations in the *ERCC3* cDNA sequence were made using the oligonucleotide directed, uracil−DNA method (Kunkel *et al.*, 1987). An internal fragment of *ERCC3* was inserted in a M13 vector, and after mutation induction used to replace the wild-type fragment in the parental plasmid pE3-WT. The desired mutation was verified by sequence analysis.

Acknowledgements

We are grateful to J.van Kampen and A.Bosch for help in some of the experiments, J.L.Weickert for cell culture, Y.Lutz for antibody production and M.Chipoulet for technical assistance. M.Kuit, R.Koppenol and T.de Vries Lentsch are acknowledged for photography. This work was supported in part by grants from the Netherlands Foundation for Chemical Research (SON), the Dutch Cancer Society (EUR 92-118), J.A.Cohen Inter-university Research Institute for Radiopathology and Radiation Protection (IRS), the INSERM, the CNRS, the Ministère de la Recherche et de l'Enseignement Supérieur and the Association pour la Recherche sur le Cancer.

References

Azzaria,M., Schurr,E. and Gros,P. (1989) *Mol. Cell. Biol.*, **9**, 5289−5297.

Bailly,V., Sommers,C.H., Sung,P., Prakash,L. and Prakash,S. (1992) *Proc. Natl Acad. Sci. USA*, **89**, 8273−8277.

Bardwell,L., Cooper,A.J. and Friedberg,E.C. (1992) *Mol. Cell. Biol.*, **12**, 3041−3049.

Biggerstaff,M., Robins,P., Coverley,D. and Wood,R.D. (1991) *Mutat. Res.*, **254**, 217−224.

Biggerstaff,M., Szymkowski,D.E. and Wood,R.D. (1993) *EMBO J.*, **12**, 3685−3692.

Bootsma,D. and Hoeijmakers,J.H.J. (1993) *Nature*, **363**, 114−115.

Cleaver,J.E. and Kraemer,K.H. (1989) *Xeroderma pigmentosum. The Metabolic Basis for Inherited Disease.* Vol. II. McGraw-Hill Book Co., New York, pp.2949−2971.

Conaway,R.C. and Conaway,J.W. (1989) *Proc. Natl Acad. Sci. USA*, **86**, 7356–7360.
Coverley,D., Kenny,M.K., Lane,D.P. and Wood,R.D. (1992) *Nucleic Acids Res.*, **20**, 3873–3880.
De Jonge,A.J.R., Vermeulen,W., Klein,B. and Hoeijmakers,J.H.J. (1983) *EMBO J.*, **2**, 637–641.
Drapkin,R., Merino,A. and Reinberg,D. (1993) *Curr. Biol.*, **5**, 469–476.
Feaver,W.J., Gileadi,O. and Kornberg,D. (1991) *J. Biol. Chem.*, **266**, 19000–19005.
Fischer,L., Gerard,M., Chalut,C., Lutz,Y., Humbert,S., Kanno,M., Chambon,P. and Egly,J.-M. (1992) *Science*, **257**, 1392–1395.
Flores,O., Lu,H. and Reinberg,D. (1992) *J. Biol. Chem.*, **267**, 2786–2790.
Gerard,M., Fischer,L., Moncollin,V., Chipoulet,J.-M., Chambon,P. and Egly,J.-M. (1991) *J. Biol. Chem.*, **266**, 20940–20945.
Gill,G. and Tjian,R. (1992) *Curr. Opin. Gen. Dev.*, **2**, 236–242.
Grossman,L. and Thiagalingam,S. (1993) *J. Biol. Chem.*, **268**, 16871–16874.
Gulyas,K.D. and Donahue,T.F. (1992) *Cell*, **69**, 1031–1042.
Hanawalt,P. and Mellon,I. (1993) *Curr. Biol.*, **3**, 67–69.
Hoeijmakers,J.H.J. (1993a) *Trends Genet.*, **9**, 173–177.
Hoeijmakers,J.H.J. (1993b) *Trends Genet.*, **9**, 211–217.
Huang,J.C., Svoboda,D.L., Reardon,J.T. and Sancar,A. (1992) *Proc. Natl Acad. Sci. USA*, **89**, 3664–3668.
Kunkel,T.A., Robetts,J.D. and Zakour,R.A. (1987) *Methods Enzymol.*, **154**, 367–382.
Landegent,J.E., Jansen in de Wal,N., Baan,R.A., Hoeijmakers,J.H.J. and Van der Ploeg,M. (1984) *Exp. Cell Res.*, **153**, 61–72.
Lehmann,A.R. (1987) *Cancer Rev.*, **7**, 82–103.
Lu,H., Zawel,L., Fisher,L., Egly,J.-M. and Reinberg,D. (1992) *Nature*, **358**, 641–645.
Madura,K. and Prakash,S. (1986) *J. Bacteriol.*, **166**, 914–923.
Manley,J.L., Fire,A., Samuels,M. and Sharp,P.A. (1983) *Methods Enzymol.*, **101**, 568–582.
Mounkes,L.C., Jones,R.S., Liang,B.-C., Gelbart,W. and Fuller,M.T. (1992) *Cell*, **71**, 925–937.
Nance,M.A. and Berry,S.A. (1992) *Am. J. Med. Genet.*, **42**, 68–84.
Nichols,A.F. and Sancar,A. (1992) *Nucleic Acids Res.*, **20**, 2441–2446.
O'Donovan,A. and Wood,R.D. (1993) *Nature*, **363**, 185–188.
Park,E., Guzder,S., Koken,M.H.M., Jaspers-Dekker,I., Weeda,G., Hoeijmakers,J.H.J., Prakash,S. and Prakash,L. (1992) *Proc. Natl Acad. Sci. USA*, **89**, 11416–11420.
Peserico,A., Battistella,P.A. and Bertoli,P. (1992) *Neuroradiology*, **34**, 316–317.
Popko,B., Puckett,C., Lai,E., Shine,H.D., Readhead,C., Takahashi,N., Hunt,S.W.,III, Sidman,R.L. and Hood,L. (1987) *Cell*, **48**, 713–721.
Reinstein,J., Brune,M. and Wittinghofer,A. (1990) *Biochemistry*, **29**, 7451–7459.
Roeder,R.G. (1991) *Trends Biochem. Sci.*, **16**, 402–408.
Sambrook,J., Fritsch,E.F. and Maniatis,T. (1989) *Molecular Cloning. A Laboratory Manual*. 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Sancar,A. and Tang,M.-S. (1993) *Photochem. Photobiol.*, **57**, 905–921.
Sawadogo,M. and Sentenac,A. (1990) *Annu. Rev. Biochem.*, **59**, 711–754.
Schaeffer,L., Roy,R., Humbert,S., Moncollin,V., Vermeulen,W., Hoeijmakers,J.H.J., Chambon,P. and Egly,J.-M. (1993) *Science*, **260**, 58–63.
Scherly,D., Nouspikel,T., Corlet,J., Ucla,C., Bairoch,A. and Clarkson,S.G. (1993) *Nature*, **363**, 182–185.
Schiestl,R.H. and Prakash,S. (1990) *Mol. Cell. Biol.*, **10**, 2485–2491.
Scott,R.J., Itin,P., Kleijer,W.J., Kolb,K., Arlett,C. and Müller,H.J. (1993) *J. Am. Acad. Dermatol.*, **29**, 883–889.
Serizawa,H., Conaway,J.W. and Conaway,R.C. (1993a) *Nature*, **363**, 371–374.
Serizawa,H., Conaway,R.C. and Conaway,J.W. (1993b) *J. Biol. Chem.*, **268**, 17300–17308.
Shivji,M.K.K., Kenny,M.K. and Wood,R.D. (1992) *Cell*, **69**, 367–374.
Stanway,C.A. (1993) *Bioessays*, **15**, 559–560.
Stefanini,M., Lagomarsini,P., Arlett,C.F., Marinoni,S., Borrone,C., Crovato,F., Trevisan,G., Cordone,G. and Nuzzo,F. (1986) *Hum. Genet.*, **74**, 107–112.
Stefanini,M. *et al.* (1993) *Am. J. Hum. Genet.*, **53**, 817–821.
Sung,P., Higgins,D., Prakash,L. and Prakash,S. (1988) *EMBO J.*, **7**, 3263–3269.
Tijan,G., Yan,H., Jiang,R.-T., Kishi,F., Nakazawa,A. and Tsai,M.-D. (1990) *Biochemistry*, **29**, 4296–4304.
van Vuuren,A.J., Appeldoorn,E., Odijk,H., Yasui,A., Jaspers,N.G.J., Bootsma,D. and Hoeijmakers,J.H.J. (1993) *EMBO J.*, **12**, 3693–3701.
Venema,J., Mullenders,L.H.F., Natarajan,A.T., Van Zeeland,A.A. and Mayne,L.V. (1990) *Proc. Natl Acad. Sci. USA*, **87**, 4707–4711.
Vermeulen,W., Stefanini,M., Giliani,S., Hoeijmakers,J.H.J. and Bootsma,D. (1991) *Mutat. Res.*, **255**, 201–208.
Vermeulen,W., Jaeken,J., Jaspers,N.G.J., Bootsma,D. and Hoeijmakers, J.H.J. (1993) *Am. J. Hum. Genet.*, **53**, 185–192.
Vermeulen,W., Scott,R.J., Potger,S., Müller,H.J., Cole,J., Arlett,C.F., Kleijer,W.J., Bootsma,D., Hoeijmakers,J.H.J. and Weeda,G. (1994) *Am. J. Hum. Genet.*, **54**, 191–200.
Weeda,G., Van Ham,R.C.A., Vermeulen,W., Bootsma,D., Van der Eb,A.J. and Hoeijmakers,J.H.J. (1990) *Cell*, **62**, 777–791.
Wood,R.D., Robins,P. and Lindahl,T. (1988) *Cell*, **53**, 97–106.

Received on November 18, 1993; revised on December 23, 1993