NUCLEOTIDE EXCISION REPAIR: ERCC1 AND TFIIH COMPLEXES

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NUCLEOTIDE EXCISIE HERSTEL: ERCC1 EN TFIIH COMPLEXEN

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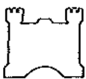

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Chapter 1

Introduction

1. General introduction

DNA is the carrier of genetic information in living organisms. The information stored in the nucleotide sequence of DNA is transmitted to the offspring by generating identical copies of the parental DNA molecules. Damage in DNA can cause loss of genetic information. Nevertheless, the DNA is continuously subject to alterations, and its instability is likely one of the major factors in mutagenesis. The structure of DNA can be modified spontaneously by hydrolysis, oxidation, or by environmental factors such as ultra-violet (UV) light, X-rays, or numerous chemical agents. Replication of unrepaired DNA can cause genetic changes, which may affect proper functioning of proteins encoded by that DNA. As a result cellular malfunction, onset of carcinogenesis, inborn defects, or even cell death can occur. In addition, DNA damage can interfere with other essential metabolizing processes, like recombination or transcription, with deleterious consequences for the cell.

In order to maintain the integrity of DNA, all organisms have evolved a complex network of mechanisms to prevent or repair DNA damage. The different pathways known are able to handle distinct classes of DNA damage.

1. *Direct repair of the damaged base:* Two examples of this mode of repair are the light-dependent enzyme photolyase (Phr), which monomerizes specifically cyclobutane pyrimidine dimers, a lesion induced by UV radiation, and the methyltransferase reaction, in which the methyl group of a methylated guanine is transferred to O^6-methylguanine-DNA-methyltransferase.

2. *Base excision repair*: Spontaneous DNA lesions which are caused by oxidation and hydrolysis, or lesions induced by X-rays and alkylating agents, are examples of lesions that are removed by base excision repair (BER). The damaged base is excised by a glycosylase, cleaving the N-glycosylic bond between the damaged base and its sugar group. The remaining sugar-phosphate is hydrolysed by an abasic endonuclease activity. The main pathway of BER replaces a single nucleotide and requires a deoxyribophosphodiesterase to remove the 5' terminal deoxyribophosphate residue, a DNA polymerase for gap-filling, and a DNA ligase for sealing the newly synthesized nucleotide to the pre-existing strand. An alternative, but minor, BER pathway exists, in which a repair patch of two to five nucleotides is displaced and DNA synthesis occurs using the non-damaged strand as template, like in nucleotide excision repair (Dianov and Lindahl, 1994).

3. *Nucleotide excision repair*: This mechanism recognizes a wide variety of structurally unrelated DNA lesions, including various UV-induced photoproducts, cyclobutane pyrimidine dimers (CPD) and pyrimidine (6-4) pyrimidone photoproducts (6-4 photoproducts), bulky chemical adducts, and certain types of crosslinks. These lesions destabilize severely the helical structure of the DNA. Globally, five steps are involved in the nucleotide excision

repair (NER) process: recognition of the DNA damage, incision of the damaged strand on both sites of the lesion, removal of the damage-containing oligonucleotide, gap-filling by DNA synthesis, and finally ligation of the remaining nick (Friedberg, 1985; Hoeijmakers, 1993a; Hoeijmakers, 1993b). For a subset of lesions, like CPDs, cisplatin intrastrand cross-links, and benzo[a]pyrene adducts, two subpathways are recognized in NER: the rapid transcription-coupled repair (TCR) of expressed genes, directed to the transcribed strand and repair at a slower rate of the genome overall, which is designated here as 'global' genome repair (GGR) (Bohr, 1991; Hanawalt and Mellon, 1993). However, the extent of discrimination between transcriptionally active and inactive regions varies between species. Transcription-coupled repair is more pronounced in rodent cells than in human, since the small amount of CPD removal in rodent cells is almost completely targeted to the transcribed strand of active genes.

4. *Post-replication daugther strand gap repair*: When the replication machinery is blocked by a DNA lesion in the template, replication can restart 3' of the injury, depending on whether the lesion occurs in leading or lagging strand. To overcome a blocking lesion in the leading strand, replication initiation from a downstream origin can occur. Alternatively, translesion synthesis at the expence of increased mutagenesis can solve the problem. In *E.coli*, the single-stranded gaps, that are left in the newly synthesized DNA strand, are repaired by recombinational strand exchange, using the daughter strand as a template, allowing complete replication of the DNA. In mammalian cells, this mechanism has not yet been elucidated.

5. *Mismatch repair*: Another post-replication correction mechanism is the mismatch repair pathway. Replication errors occur, due to occasional insertion of a mismatched base, or slippage of the DNA replication machinery, in regions of simple di- or trinucleotide repeat sequences. In *E.coli*, the MutS protein binds the mismatched site, then a dimer of MutL/MutH cleaves the non-methylated DNA strand, which is newly synthesized. The DNA containing the mismatch is degraded by a single strand-specific exonuclease, followed by gap-filling using DNA polymerase III and joining by DNA ligase. Mismatch repair is conserved from bacteria to man; MutS and MutH homologues have been identified in human cells (Fishel et al., 1993; Leach et al., 1993; Parsons et al., 1993). A remarkable observation was the localisation of the human MutS gene to chromosome 2, in the same region where the locus responsible for hereditary nonpolyposis colorectal cancer (HNPCC) has been mapped. These tumour cells show marked instability of simple DNA repeat sequences, like mismatch-deficient mutants in *E.coli* and yeast. The strongest evidence that the mutation responsible for HNPCC affects mismatch repair, is obtained by the fact that extracts of tumour cells are deficient in this type of repair (Lindahl, 1994).

6. *Recombinational repair*: Double strand breaks and interstrand crosslinks, induced by X-rays and several crosslinking agents, are severe types of damage, since repair requires both DNA strands and the presence of homologous duplex DNAs. The mechanism is intensively studied in *E.coli*. Each end of the double strand break is degraded by an exonuclease, which

leaves single-stranded ends. RecA protein binds to these free ends and initiates strand exchange with the homologous duplex DNA molecule, forming two adjacent recombination junctions. The remaining gaps are filled by DNA polymerase. Then both recombination joints are resolved, yielding two intact duplexes. Many other enzymes are required for recombinational repair, including RecBCD, RecE, RecF, RecG, RecQ, RuvA, RuvB, and RuvC, but these will not be discussed here (for review, see West, 1992).

This introduction focusses on the nucleotide excision repair (NER) pathway. First evidence that NER plays a significant role in human health was provided in the late sixties by Cleaver, who described a defective NER pathway in patients with the human hereditary disease xeroderma pigmentosum (XP) (Cleaver, 1968). XP patients show hypersensitivity to ultraviolet light and have a predisposition to develop skin cancer in sun-exposed areas of the skin. Unravelling of the molecular mechanism of NER requires information about the genes involved, and the functions of the encoded proteins in this repair process. NER-deficient mutants in various organisms have played an essential role in the elucidation of the NER mechanism. Presumably, insights in the NER process shed light upon skin cancer induction in general. In the last twenty years, an increased incidence of various types of skin cancer has been observed in fair-skinned people. A major reason might be exposure of the skin to solar UV-light by sunbathing, especially in the developed countries. Reduction of the ozone layer may also enhance the risk of skin cancer, since more harmful ultraviolet-B radiation will reach the earth's surface.

2. Nucleotide excision repair

2.1 Excision repair in *Escherichia coli*

The NER pathway has been characterized in most detail in *Escherichia coli* (van Houten, 1990; Visse, 1994). The key NER proteins are UvrA, UvrB, UvrC, UvrD, DNA polymerase I, DNA ligase, and two auxiliary factors: photolyase (Phr) and Mfd, the product of the *m*utation *f*requency *d*ecline gene (also called transcription repair couplings factor, TRCF). The first step in the repair process is dimerisation of UvrA molecules in the presence of ATP, followed by the association of one UvrB molecule forming a damage recognition complex (Lin et al., 1992). The $UvrA_2B$ complex binds to DNA and scans for lesions by a weak helicase activity (Grossman and Yeung, 1990). A wide spectrum of structurally unrelated DNA lesions, ranging from thymine glycols to bulky adducts as well as inter- and

intrastrand crosslinks, are recognized by this $UvrA_2B$ complex. Therefore, it is believed that the recognition is based on detection of a distortion in the DNA helix, rather than on direct sensing of the actual damage. After recognition of the lesion, UvrB is linked to DNA (preincision complex), whereas $UvrA_2$ is released (Orren and Sancar, 1989). UvrC binds to the preincision complex and induces a dual incision in the damaged strand. The incision 5' of the injury is catalyzed by UvrC, while the 3' incision is made by UvrB (Lin and Sancar, 1992; Sancar and Tang, 1993). A 12-13 mer oligonucleotide containing the adduct together with UvrC are released by the action of the UvrD helicase. DNA polymerase I fills the single-stranded gap and releases UvrB from the DNA (Orren et al., 1992). Finally, the newly synthesized DNA is joined to the pre-existing strand by DNA ligase I. The scheme depicted in Figure 1 represents the NER reaction *in vitro*, using naked DNA as a substrate and the purified proteins as mentioned above.

The basic rate of repair can be modulated and regulated by auxiliary factors. The regulatory SOS mechanism mediated by the LexA and RecA gene products promotes the expression of a number of NER genes when the cell is confronted with DNA damage. Another example of a NER modulator is Phr, which specifically stimulates the repair of CPDs by UvrABC endonuclease. Probably the binding of Phr to the CPD results in a more efficient recognition of this lesion (Sancar and Smith, 1989).

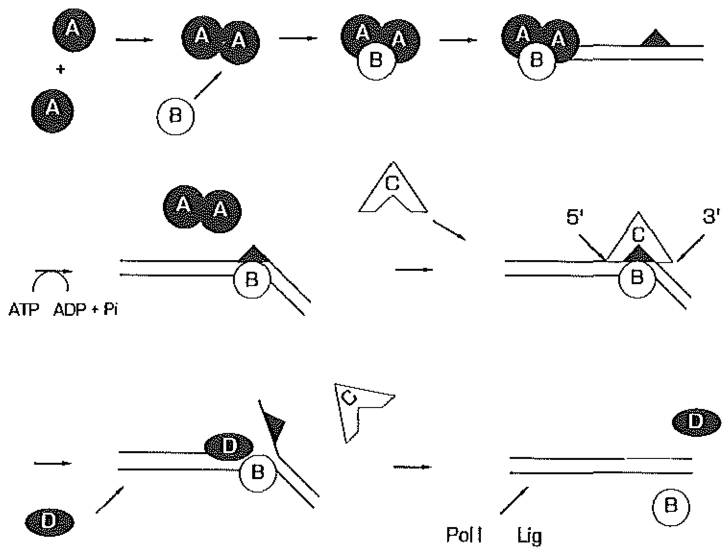

Figure 1. A model mechanism for nucleotide excision repair in *Escherichia coli*.

Strand-specific repair of active genes has been discovered in *E.coli* both *in vivo* and *in vitro*, after identification of such a preferential repair in mammalian cells (Bohr, 1991; Mellon and Hanawalt, 1989; Selby and Sancar, 1991). Strand-specific repair achieves fast and complete elimination of lesions that cause a block in transcription, implying a priority for repair of the expressed fraction of the genome compared to non-transcribed regions. Strand-specific repair in *E.coli* requires at least an additional factor for communication between the RNA polymerase and nucleotide excision repair. Recently, this transcription-repair coupling factor (TRCF) has been identified as the product of the *mfd* gene (*m*utation *f*requency *d*ecline) (Selby et al., 1991). When RNA polymerase is stalled at the lesion, it will be released from the template by Mfd. The proposed model is that the Mfd protein remains bound to DNA and recruits $UvrA_2B$ to accomplish fast repair of the transcribed strand (Selby and Sancar, 1993).

It is obvious that NER in *E.coli* is a complex process, which requires many repair proteins. In eukaryotes, chromatin structure and dynamics, genome complexity, or cell cycle regulation, might further complicate the NER pathway. As a first step on the evolutionary ladder from *E.coli* and man, NER in yeast will be discussed below. The budding yeast *Saccharomyces cerevisiae* has served as a model organism, because of its relatively low genome complexity in comparison to higher eukaryotes.

2.2 Excision repair in yeast

An extensive collection of repair-deficient yeast mutants has been identified due to the versatile genetic system of *Saccharomyces cerevisiae*. Three epistasis groups, defective in DNA repair, have been characterized in yeast: *RAD3*, *RAD6* and *RAD52* (Friedberg, 1988). Each group is called after one of their representants. The *RAD6* group is required for post-replication repair and is involved in damage-induced mutagenesis, whereas the *RAD52* group is implicated in recombinational processes. The *RAD3* group represents nucleotide excision repair. In the *RAD3* epistasis group at least thirteen complementation groups (listed in Table 1) have been identified so far (Hoeijmakers, 1993a; Prakash et al., 1993). In the distantly related fission yeast *Schizosaccharomyces pombe* also a large set of repair genes have been identified, many of which were found to be homologous to members of the *RAD3* epistasis group of the budding yeast *Saccharomyces cerevisiae* (McCready et al., 1993). These homologous genes suggest a conservation of the repair pathway at least in yeast.

Rad1, *2*, *3*, *4*, *10*, *14*, and *rad25* mutants are highly sensitive to UV-light and completely deficient in NER. These gene products are indispensable for the incision step. Other mutants, including *rad7*, *16*, *23*, *24*, *rad26*, *ssl1*, and *mms19*, are moderately or even not sensitive to UV-light. These mutants are only partially defective in NER, suggesting that the encoded proteins serve as accessory factors or act in a specific subpathway of repair, for instance in preferential repair of transcribed genes.

NER in *E.coli* and yeast share all the basic features: recognition, dual incision, removal

of the damage-containing patch, DNA synthesis and ligation. In both cases, a wide spectrum of unrelated lesions is substrate for the repair mechanism. Furthermore, the two subpathways of repair, transcription-coupled and global genome repair, are present. Although the NER mechanism is conserved during evolution, *E.coli* NER genes appear not to have served as ancestors for the eukaryotic repair genes, since repair in yeast and *E.coli* is exerted by non-homologous proteins. The properties of the different yeast repair proteins are discussed in relation to the five basic steps in NER and briefly summarized in Table 1.

RAD14 is a hydrophilic protein containing a zinc-finger domain. It binds preferentially 6-4 photoproducts in UV-damaged DNA, but also other types of lesions (Guzder et al., 1993). On the basis of these binding properties, it is likely that RAD14 is required for the damage recognition step. However, the existence of additional proteins, that recognize DNA damage, cannot be excluded.

Incision in yeast requires at least two endonuclease activities, carried out by RAD2 and by the RAD1/RAD10 complex. Expression of the *RAD2* gene is induced by UV-irradiation and the protein shows a single strand-specific DNA nuclease activity, independent of the lesion (Habraken et al., 1993). The RAD1/RAD10 complex (Bailly et al., 1992; Bardwell et al., 1992) shows low endonuclease activity on both single-stranded and on supercoiled double-stranded DNA (Sung et al., 1993; Tomkinson et al., 1994).

Presumably, the DNA around the injury must be locally unwounded to make it accessible for the repair proteins, which are involved in incision. Candidates for this role are RAD3 and RAD25, since both are helicases. RAD3 has a 5'→ 3' helicase activity on both double-stranded DNA and DNA-RNA hybrids, but RAD3 is not able to unwind RNA duplexes (Sung et al., 1987; Bailly et al., 1991; Naegeli et al., 1992b). The direction of the helicase activity of RAD25 is the reverse of RAD3 (Park et al., 1992). In addition, these helicases might be involved in recognition of the damage, when the helicases scan the DNA for lesions, like the $UvrA_2B$ complex in *E.coli*. Inhibition of the movement of DNA polymerases or helicases, which translocate along the DNA, might be important for efficient recognition of the damage. For instance, the helicase activity of RAD3 is strongly reduced by DNA damage and alterations in the strand to which RAD3 is bound (Naegeli et al., 1992a). When RAD3 remains bound at or near the lesion, it may enhance recognition by other repair proteins. However, the possibility cannot be excluded, that these helicases are involved in the strand displacement of the damage-containing patch, like UvrD in *E.coli*.

As in *E.coli*, two NER subpathways exist in *S.cerevisiae*: transcription-coupled repair and global genome repair. *Rad7* and *rad16* mutants are deficient in repair of non-transcribed DNA, whereas transcribed strands of active genes are repaired at a rate similar to wildtype cells (Terleth et al., 1990; Verhage et al., 1994). The sequence of RAD16 predicts a helicase domain, which demonstrates remarkable sequence homology to a subfamily of putative helicases (Bang et al., 1992; Mannhaupt et al., 1992). Generally, helicases can affect opening of the DNA helix. In case of RAD16, it is believed that this protein serves to make the DNA accessible for the repair proteins (Winston and Carlson, 1992). RAD7 interacts with SIR3,

a protein that is probably involved in the packaging of DNA into transcriptionally silent chromatin (Paetkau et al., 1994). The function of RAD7 might be remodelling of the chromatin structure, to allow NER proteins to remove DNA lesions from these non-transcribed regions of the genome.

In contrast to *rad7* and *rad16* mutants, the *rad26* mutant is defective in transcription-coupled repair and not in global repair. The *RAD26* gene has been characterized only recently, based on sequence homology to its human counterpart CSB (Huang et al., 1994). The *rad26* mutant is not sensitive to UV-light or X-rays, explaining why it was not identified as a repair-deficient mutant before (van Gool et al., 1994). RAD26 has also helicase motifs of the same putative helicase subfamily as RAD16. The proteins of this subfamily exhibit various functions in repair- and transcription regulation: for example RAD54 (recombinational repair), RAD5 (post-replication repair), RAD26 and human CSB (transcription-coupled repair), and SNF2 and MOT1 (transcription regulators) (Clark et al., 1992; Johnson et al., 1992; Laurent et al., 1992; Schild et al., 1992; Troelstra et al., 1992).

SSL1, TFB1, RAD3, and RAD25 are part of the transcription initiation factor b (Feaver et al., 1993) and will be discussed in the context of the homologous human transcription factor TFIIH. Several *ssl1* and *tfb1* mutants display UV-sensitivity, which suggests that these mutants are also members of the *RAD3* epistasis group (Yoon et al., 1992; Wang et al., 1994b). It appears that NER requires proteins which have additional functions in other DNA metabolizing processes in the cell. A dual function of RAD3 and RAD25 has been predicted previously, as 'null' mutations in these genes were not viable (Higgins et al., 1983; Naumovski and Friedberg, 1983; Park et al., 1992). Another example of a dual role is presented by RAD1 and RAD10; both are involved in NER and mitotic recombination (Schiestl and Prakash, 1988; Schiestl and Prakash, 1990). Little is known about the function of proteins encoded by *RAD4, RAD23, RAD24* and *MMS19.*

The next question to be answered is to what extent NER in yeast can be related to repair in higher eukaryotes, like mammals. The nuclear organisation in mammals is different from yeast, this is due to a larger number of chromosomes and the presence of introns in the DNA. Presumably, the diversity of tissues in higher organisms can affect the rate of repair. Two classes of mammalian mutants have been identified: rodent cell mutants and naturally occurring human disorders, in which patients show a genetic defect in NER. Both classes will be discussed in the following paragraphs.

2.3 Mammalian excision repair

2.3.1 DNA repair-deficient rodent mutants

A large number of NER mutants has been obtained from cultured rodent cell lines.

Table 1: NER genes in yeast and their properties (the RAD3 epistasis group)

Gene	Homologs		Function/Activity		Further characteristics
	human	*S.pombe*	NER	Additional	
RAD1	*ERCC4*[◆]	*rad16*[*]	incision	recombination	complexed with RAD10, endonuclease activity
RAD2	*XPG*	*rad13*	incision		expression induced by DNA damage, ss-DNA endonuclease activity
RAD3	*XPD*	*rad15*	helix unwinding	transcription	5'→3' helicase, subunit of factor b
RAD4	*XPC*				ss DNA binding?
RAD7			repair of non-transcribed DNA		gene expression induced by DNA damage
RAD10	*ERCC1*	*swi10*	incision	recombination	complexed with RAD1, endonuclease activity
RAD14	*XPA*		damage recognition		preferential binding to damaged DNA
RAD16			repair of non-transcribed DNA		RAD16-subfamily of putative DNA helicases
RAD23	*HHR23A,B*				ubiquitin-like N-terminal domain
RAD25[*]	*XPB*	*ERCC3*^{sp}	helix unwinding	transcription	3'→5' helicase activity, factor b association
RAD26	*CSB*		transcription-coupled repair		RAD16-subfamily of putative DNA helicases
SSL1	*p44*			transcription	subunit of factor b, mutant is UV^s
TFB1	*p62*			transcription	subunit of factor b, mutant is UV^s

* *rad16* is also designated as *swi9*; * *RAD25* is also designated as *SSL2*

◆ RAD1 has sequence homology with ERCC4 (L. Thompson, personal communication)

Eleven different complementation groups have been classified by complementation analysis based on cell fusion experiments (Busch et al., 1989; Riboni et al., 1992; Collins, 1993). Representatives of the groups 1-5 and 11 are highly sensitive to DNA-damaging factors, like UV-irradiation, and deficient in one of the early steps of NER. Groups 6-10 show only a moderate UV-sensitivity. Cross-sensitivity to chemical agents, that produce bulky DNA adducts, is a feature of all complementation groups. Mutants of rodent groups 1 and 4 are unique, since they display an extreme sensitivity to mitomycin C (MMC), a crosslinking agent (Busch et al., 1989). Recently, it was found that not all representants of these groups are highly sensitive to MMC. Several mutants of group 1 do not exhibit such an extreme MMC-sensitivity, whereas the UV sensitivity is retained, and one representative of group 4 (UV140) has been isolated with a moderate response to UV and MMC (Busch, manuscript in preparation).

Six human NER genes have been cloned by transfection of the human genomic DNA into the rodent repair mutants. A practical reason for this approach is that transfection to rodent cells is much easier than to human cells. The correcting genes are designated *e*xcision *r*epair *c*ross *c*omplementing genes (ERCC), *ERCC1* (Westerveld et al., 1984), *ERCC2* (Flejter et al., 1992), *ERCC3* (Weeda et al., 1990b), *ERCC4* (Thompson et al., 1994) *ERCC5* (O'Donovan and Wood, 1993; Scherly et al., 1993) and *ERCC6* (Troelstra et al., 1992). With the exception of *ERCC1* and *ERCC4*, these cloned genes are also involved in human NER disorders. Their functions and mode of action will therefore be discussed along with the human syndromes.

2.3.2 Human nucleotide excision repair disorders

Three rare, inherited human disorders are known, where a defective NER mechanism is involved. These are xeroderma pigmentosum (XP), Cockayne's syndrome (CS), and a photosensitive form of trichothiodystrophy (TTD) also designated as PIBIDS. Of all TTD patients approximately 50% exhibit photosensitivity. Patients suffering from the DNA repair disorder XP show marked sun(UV)sensitivity, pigmentation abnormalities, and a strong predisposition to skin cancer restricted to sun-exposed areas of the skin. Several XP patients exhibit also accelerated neurological degeneration (Cleaver and Kraemer, 1994). Both CS and TTD patients display a broad range of clinical features distinct of XP: including a relatively mild UV-sensitivity, mental retardation, dysmyelination of the neurons, poor physical and sexual development and dental caries (Johnson and Squires, 1992; Nance and Berry, 1992). In addition, TTD patients show several specific hallmarks such as brittle hair and nails, due to a reduced synthesis of a class of cystine-rich matrix proteins, and ichthyosis (scaling of the skin), and do not exhibit an increased risk for developing skin tumours (Lehmann, 1987; Peserico et al., 1992). However, many parallels have recently been observed between CS and TTD. For instance, CS patients show thin hair and ichthyosis, which was found to be a pronounced feature of TTD (Nance and Berry, 1992). TTD patients may exhibit

neurodysmyelination, bird-like facies, cataract and dental caries, which are typical CS symptoms (Peserico et al., 1992; McCuaig et al., 1993). These observations suggest that both CS and TTD are part of one broader clinical spectrum. Many of these clinical features of CS and TTD, besides UV-sensitivity, are remarkable since these symptoms are hard to explain on the basis of a deficiency in NER.

Genetic heterogeneity in these human repair disorders is extensive. At least eleven complementation groups have been identified and the characteristics of them are listed in Table 2. Seven excision-deficient groups exist in XP, designated XP-A to XP-G (Vermeulen et al., 1991). An eighth XP-group (called XP-variant) is not defective in NER, but impaired in daughter-strand repair (Lehmann et al., 1975). Two groups have been observed in CS (CS-A and CS-B). In TTD, three complementation groups have been characterized, one is called TTD-A (Stefanini et al., 1993), and two other groups coincide with XP-D and XP-B (Stefanini et al., 1992, and Chapter 5). Finally, several individuals show combined features of XP and CS. These patients fall into XP group B, D, and G (Johnson and Squires, 1992; Vermeulen et al., 1993; Vermeulen et al., 1994), suggesting that the disorders XP and CS might be related or that both diseases are part of a much broader clinical phenotype. The XP groups A-G are defective in NER, although to a variable extent. Most XP groups are deficient in both subpathways of NER: in transcription-coupled - as well as global genome repair. Both CS-A and CS-B are only deficient in preferential repair of the transcribed strand of active genes (Venema et al., 1990a). The reverse is found in XP group C, in which the repair defect is limited to the global genome pathway (Venema et al., 1990b and 1991). The high incidence of skin cancer associated with XP-C and the absence of this risk in CS suggests that the efficiency to remove lesions from the genome might be the main determinant for the induction of skin tumours. Several XP and CS complementation groups display considerable overlap with Chinese hamster mutants. The *ERCC2* gene corrects the UV-sensitivity of XP-D fibroblasts (Flejter et al., 1992), while *ERCC3* compensates the repair defect of XP-B cells (Weeda et al., 1990b). Recently, *ERCC5* has been found to be responsible for XP-G (Scherly et al., 1993), and finally *ERCC6* corrects the repair defect of CS-B (Troelstra et al., 1992). This extensive overlap stresses the value of the rodent mutants for understanding the molecular basis of NER defects in man; repair-deficient rodent cells can be very useful to isolate genes, in which mutations are not compatible with life or proper embryonic development in humans. For instance, *ERCC1* is not affected in any of the known human complementation groups of XP, CS or TTD(PIBIDS) (van Duin et al., 1989b, and unpublished results).

As already indicated in Table 1, nearly all the cloned mammalian NER genes have a yeast counterpart. This strong evolutionary conservation between yeast and man suggests that no principal differences exist between low and higher eukaryotes. Different versions of the same genes in the evolutionary diverse organisms *Saccharomyces cerevisiae* or *Schizosaccharomyces pombe* might help to isolate the mammalian homologs by characterization of conserved domains. By such an evolutionary walking two human RAD6

Table 2: Characteristics of the human NER-deficient complementation groups

Group	Clinical characteristics			Repair properties		Remarks
	skin cancer	neurological abnormalities	relative incidence	UV[s]	residual UDS	
XP-A	+	++	high	+++	<5%	
XP-B	+/-	+++/+	very rare	++/+	<10%/30-40%	combined XP/CS and TTD patients
XP-C	+	-	high	+	15-30%	defective in global genome repair
XP-D	+/-	++/+/-	intermediate	++/+	15-50%	XP, combined XP/CS and TTD patients
XP-E	+/-	-	rare	±	>50%	
XP-F	+/-	-	rare	+	15-30%	slow repair but prolonged
XP-G	+/-	++/+	rare	++	<10%	XP and combined XP/CS patients
CS-A	-	++	rare	+	100%	defective in transcription-coupled repair
CS-B	-	++	high	+	100%	defective in transcription-coupled repair
TTD-A	-	+	very rare	+	~10%	typical TTD patient, not the same as XP-B or XP-D

homologs (HHR6A and HHR6B) have been cloned using *S.pombe* and *Drosophila melanogaster* as intermediates (Koken et al., 1991a; Koken et al., 1991b). The *RAD6* gene plays a role in postreplication repair, damage-induced mutagenesis, and sporulation. As an example in NER: sequence conservation permitted the isolation of *haywire*, the *Drosophila* counterpart of XPB. It appears that NER genes of yeast and higher eukaryotes have the same ancestor.

The DNA repair capacity is most severely reduced in fibroblasts of XP groups A, B, and G. XP group A represents one quarter of all the XP cases and displays the severe clinical phenotype with both skin tumours and symptoms of accelerated neurological degeneration (Cleaver and Kraemer, 1989). The severe phenotype in patients cannot be related to a severe type of mutation, such as a large deletion, in *XPA*. For instance, the characterization of one of the mutations, which has been found in several Japanese XP-A patients, has shown that only a single base substitution in the 3' splice acceptor site of the third intron causes mRNA instability (Satokata et al., 1990). In the rare group B, four patients have been documented. Three of these XP-B patients display simultaneously features of XP and CS, whereas one individual manifests the clinical characteristics of TTD (Robbins et al., 1974; Vermeulen et al., 1994 and Chapter 5). Complementation group C represents also a quarter of the total number of XP patients. XP-C patients have a high risk for skin cancer, such as in XP group A, but they rarely display neurological abnormalities. (Cleaver and Kraemer, 1989). Complementation group D is the most heterogeneous, including XP patients with neurological problems, rare cases with combined XP/CS features, and individuals with TTD (PIBIDS) (Vermeulen et al., 1991; Johnson and Squires, 1992; Stefanini et al., 1992). Most individuals showing the photosensitive form of TTD belong to XP group D. Patients assigned to XP-E display a mild clinical phenotype of XP, which correlates with the high residual repair synthesis (50 - 60% in comparison to normal individuals) measured in fibroblasts of these patients. XP group F mainly consists of Japanese patients with relatively mild symptoms. Although repair in XP-F cells is rather slow, it can reach completion since it continues for a longer time (Zelle et al., 1980). Finally, three XP-G patients demonstrate the combined XP/CS phenotype (Vermeulen et al., 1993). The patient, representing the complementation group TTD-A, shows typical TTD symptoms such as hair-shaft abnormalities with reduced sulfur content, ichthyosis, and short stature. Apart from sun sensitivity, no clinical features associated with XP patients have been found. Pigmentary changes or skin tumours have not been observed.

All the XP fibroblasts show a NER deficiency to a various extent. In these cells cyclobutane pyrimidine dimers can be removed by introduction of a CPD-specific endonuclease from bacteriophage T4 or *Micrococus luteus*. When the incision is made by these exogenous enzymes, the job of repair can be finished, resulting in nearly normal levels of CPD repair (Tanaka et al., 1977; De Jonge et al., 1985). Therefore, it is suggested that NER defects in XP cells are related to early steps of the reaction: recognition or incision.

2.3.3 Functions of nucleotide excision repair proteins

Recognition of the DNA damage. The XPA protein has been purified from NER-proficient HeLa cells (Robins et al., 1991). The protein contains a DNA-binding zinc-finger domain (Miyamoto et al., 1992) and has high affinity to bind DNA, damaged by UV or by cisplatin (Jones and Wood, 1993). Its yeast homolog is RAD14, which preferentially binds 6-4 photoproducts in UV-damaged DNA (Guzder et al., 1993). This implicates a possible role for XPA in recognition of the lesions, which is one of the early steps in nucleotide excision repair. The main properties of the human NER genes and their encoded products are summarized in Table 3 and illustrated in Figure 2.

Another candidate to recognize DNA adducts is XPE. A polypeptide of 125 kDa was purified based on its affinity for UV-damaged oligonucleotides, and an associated 41 kDa protein was found in these preparations. A subset of XP-E patients lack a DNA binding activity (Chu and Chang, 1988), and microinjection of the purified protein (complex) into these XP-E fibroblasts restores DNA repair synthesis to normal levels (Keeney et al., 1994). This suggests that the protein (complex) is responsible for correction of the repair defect in XP group E. However, mutations in XP-E patients still have to be identified, which is possible because both genes encoding the 125 kDa and 41 kDa subunits have been cloned (Takao et al., 1993; Keeney et al., 1994). No indications for the function of the encoded proteins nor any homology to known yeast genes could be found in the sequences. The mild repair defect in XP-E cells, even when the binding activity is completely lacking, suggests that the DNA damage binding protein has a role as stimulatory or accessory factor. Both XPA and XPE proteins show a much higher affinity for (6-4) photoproducts than for CPDs, which could be an explanation for the more efficient, about 10-fold, repair of (6-4) lesions in humans (Szymkowski et al., 1993).

DNA unwinding. Both XPB and XPD exhibit helicase activities; each in opposite direction, XPB 3'→5' and XPD 5'→3' (Schaeffer et al., 1994), like their yeast counterparts RAD25 and RAD3. The possible functions of these helicases in the NER process have already been discussed (see paragraph 2.2). In XPB both the helicase domains and the putative DNA-binding motif are needed as indicated by site-specific mutagenesis (Ma et al., 1994). The discovery that XPB and XPD are subunits of the basal transcription factor TFIIH is highly important, because it links two distinct processes: DNA repair and basal transcription (Schaeffer et al., 1993; Schaeffer et al., 1994). TFIIH is required for transcription initiation (discussed in detail later) of genes transcribed by RNA polymerase II, suggesting that the complex has a vital function in the cell. Mutation analysis of several TTD patients deficient in the *XPD* gene revealed also an additional function for XPD, besides its role in NER. When excision repair activity is abolished >80%, the growth kinetics remain normal in these cell strains (Broughton et al., 1994). This result is in complete agreement with mutations found in the yeast *RAD3* gene, especially in the ATP-binding domain, in which both helicase and repair activities were fully impaired, but not the viability of the cells

Table 3: Main properties of human NER genes

Group	Cloned gene*	Function	Further characteristics
XP-A	*XPA*	recognition of lesions	binds preferentially damaged DNA, different from rodent group 1-7
XP-B	*XPB/ERCC3*	transcription and repair	3'→ 5' helicase, subunit of TFIIH
XP-C	*XPC*	global genome repair	complexed with HHR23B, binds strongly ss-DNA
XP-D	*XPD/ERCC2*	transcription and repair	5'→ 3' helicase, subunit of TFIIH
XP-E	-	recognition of lesions?	binds damaged DNA?
XP-F	-	5' incision and mitotic recombination?	complexed with ERCC1, 4 and 11, both *ERCC4* and *ERCC11* are candidates for deficiency in XP-F
XP-G	*XPG/ERCC5*	incision 3' of the lesion	conserved domains with FEN1, a structure-specific ss-DNA endonuclease
CS-A	-	transcription-coupled repair	
CS-B	*CSB/ERCC6*	transcription-coupled repair	helicase domains of RAD16 subfamily
TTD-A	-	transcription and repair	
-	*ERCC1*	5' incision and mitotic recombination?	complexed with ERCC4, 11 and XPF
-	*HHR23A,B*	genome overall repair?	ubiquitin-like N-terminal domain, HHR23B complexed with XPC

* The old ERCC-designation indicates the complementation groups of rodent mutants where the gene is defective

(Sung et al., 1988). These findings indicate that the helicase activity of RAD3 and probably also of XPD are not essential for their vital function in cells.

Incision on both sites of the lesion. XPG shows limited but significant homology with the yeast RAD2 protein (Scherly et al., 1993) and with the human FEN1 protein (Harrington and Lieber, 1994). Although FEN1 is smaller than XPG, it shares all the domains conserved between XPG and RAD2. FEN1 is a structure-specific endonuclease and cleaves a DNA substrate with a 5' single stranded flap (Harrington and Lieber, 1994). In addition, RAD2 has single-stranded DNA cleaving properties. XPG demonstrates endonuclease activity on a Y-shaped DNA structure, which contains a duplex region and single-stranded tails. XPG cleaves specifically the DNA strand with a 5' single-stranded tail and the incision is made at the branch point a few bases into the duplex region (O'Donovan et al., 1994). These results suggest that XPG is responsible for the 3' incision in the human NER pathway. The conservation between XPG, FEN1 and yeast RAD2 implicates the existence of a subfamily of endonucleases specific for branched DNA.

Strong evidence exists for a human protein complex carrying ERCC1, and the complementing activities of XP-F and the rodent complementation groups 4 and 11 (Chapter 2, and Biggerstaff et al., 1993). The ERCC1 complex might be responsible for incision of the damaged DNA, and will be discussed in detail in paragraph 2.3.5.

DNA synthesis and ligation. The final events in the NER reaction are refilling of the gap by DNA synthesis and ligation to the pre-existing DNA strand. RP-A (hSSB), which binds the non-damaged single-strand DNA template, proliferating cell nuclear antigen (PCNA), polymerase δ and/or polymerase ϵ, all previously discovered as replication proteins, are also required for the repair synthesis reaction *in vitro* (Coverley et al., 1991; Shivji et al., 1992). Furthermore, *in vivo*, PCNA can be detected in repair sites and in replication sites. (Jackson et al., 1994). DNA ligase 1 is responsible for the final closure of the remaining gap after DNA repair synthesis.

NER genes with a so far unknown function. The *XPC* gene has been cloned, but since the predicted amino acid sequence did not reveal any known enzymatic domains, no clues could be obtained for the role of XPC in repair (Legerski and Peterson, 1992). The XPC protein is found to be tightly associated with HHR23B, one of the two human homologs of the yeast RAD23 protein (Masutani et al., 1994). The complex binds preferentially ss-DNA and this binding specificity probably resides in XPC, since no DNA-binding activity could be measured using HHR23A or HHR23B alone (van der Spek, unpubl. results) The predicted role of the complex is likely to be restricted to the subpathway of global genome repair (Venema et al., 1990). It is not known whether other repair factors, in addition to the XPC/HHR23B complex, are specifically involved in the slower repair of lesions in the non-transcribed part of the genome. However, a fully inactivated *RAD4* gene, which is the presumed yeast homolog of *XPC* based on the sequences of both genes, results in a defect in both subpathways (Legerski and Peterson, 1992). In yeast, functional homologs of XPC might be RAD7 or RAD16, because the corresponding mutants are only affected in the repair

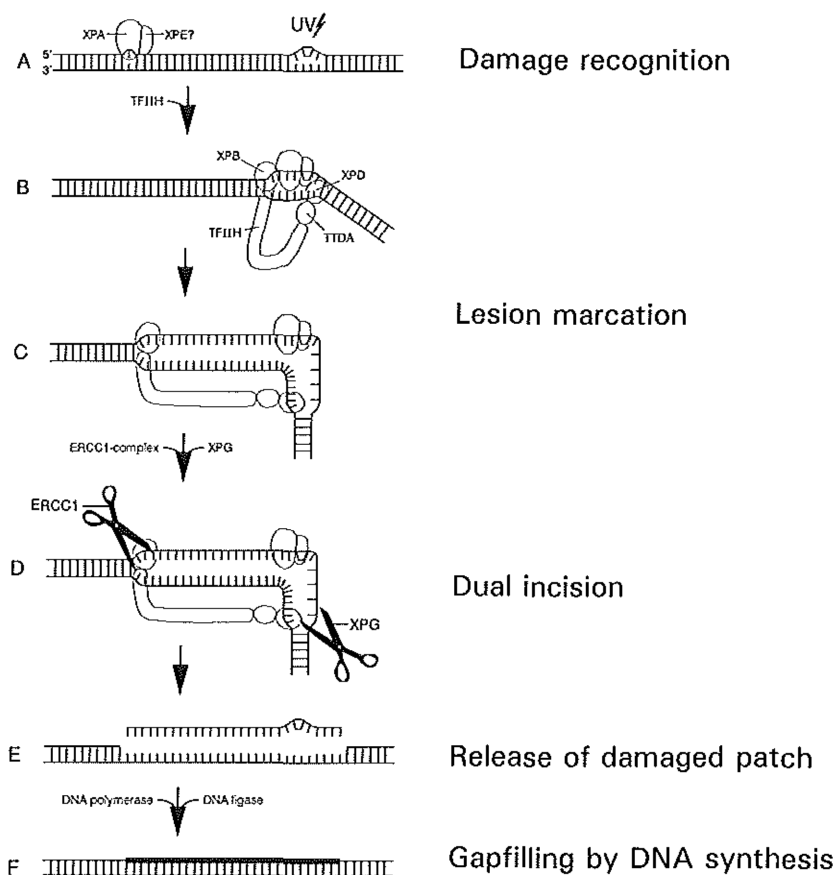

Figure 2. Model for human nucleotide excision repair.

of non-transcribed DNA (Verhage et al., 1994). It is possible that this reflects a principle difference between the NER processes in yeast and man, which may be related to genome organisation of both organisms.

Mutations in *CSB* affect the mechanism of strand-specific repair. The protein contains the putative helicase domains, characteristic of the RAD16 subfamily (Troelstra et al., 1992). CSB might be the factor that specifically couples NER and transcription, like the Mfd protein in *E.coli*. However, CSB is not found to be associated with any of the well-known transcription factors so far, and there is no sequence homology with Mfd (Bootsma and

Hoeijmakers, 1993).

2.3.4 DNA repair *in vitro*

Information about the genes and the gene products involved in NER in mammalian cells is accumulating very rapidly. Two fruitful assays to detect repair of DNA damage are microneedle injection and cell-fusion. Complementation analysis have been performed using cell-fusion experiments, resulting in the identification of at least 10 different human- and 11 rodent complementation groups involved in NER. Proteins, antibodies, and DNA can easily be introduced into fibroblasts by microinjection. The effect on the repair capacity of the cells is measured by UV-induced unscheduled DNA synthesis (UDS) (Vermeulen et al., 1993). Both repair assays reflect the entire NER process *in vivo*. Therefore, these procedures are not very useful to study the biochemistry of excision repair. A powerful *in vitro* DNA repair assay as developed by Wood et al. (1988) and independently by Sibghat-Ullah et al. (1989) might help to investigate the components involved, and to dissect the NER reaction into discrete steps.

The *in vitro* DNA repair assay is based on defined damaged DNA substrates and cell-free extracts of rodent or human cell lines. These cell-free extracts have already been used in *in vitro* transcription experiments, suggesting that proteins (complexes) still remain active in this type of extract (Manley et al., 1983). In the reaction mixture, the repair enzymes incise the damaged DNA on both sites of the lesion and the damage-containing patch is removed. The remaining gap is filled by DNA polymerase while [^{32}P]-labelled nucleotides are incorporated in the newly synthesized patch. As an internal control non-damaged DNA of a different size is included in each reaction. Then DNAs are isolated and linearized by restriction enzyme digestion prior to gel electrophoresis and autoradiography. The repair synthesis is ATP-dependent and requires Mg^{2+} and K^{+}. Extracts of cells representing the different XP complementation groups are repair-deficient in this *in vitro* assay. Two mixed extracts from distinct complementation groups correct the repair activity to normal levels, indicating that the assay can mimic the *in vivo* cell fusion complementation experiments.

The *in vitro* assay removes not only UV-induced damage like CPDs and 6-4 photoproducts, but also psoralen and cisplatin adducts. However, the efficiency of repair is not equal for all types of lesions. The repair of 6-4 photoproducts is 10-fold faster than CPD repair (Szymkowski et al., 1993). This relates to the situation *in vivo* in which the less abundant UV-induced 6-4 photoproducts are removed far more rapidly and completely from the genome than the main UV-induced CPD lesions. A problem is observed using UV-irradiated DNA as substrate, since, in addition to CPDs and 6-4 photoproducts, pyrimidine hydrates are induced as minor lesions. These pyrimidine hydrates are efficiently recognized by a NER-independent endonuclease activity present in all extracts of mammalian cell types. As expected, nicks in the DNA induce an aspecific incorporation of labelled nucleotides; therefore, it is important that the DNA has its circular closed form before adding to the

reaction mixture. The DNA substrate can be cleaned from these pyrimidine hydrates by treatment with *E.coli* Nth protein (Chapter 2, and Wood et al., 1988). Another method to damage the DNA, without such a nuclease problem, is treatment with the chemical agents N-acetoxy-2-acetyl-aminofluorene (AAF), which induces almost specific N-(guanine-8-yl) adducts (Chapter 2). *In vitro*, AAF lesions are repaired with an efficiency comparable to 6-4 photoproducts (Szymkowski et al., 1993). The *in vitro* repair mechanism can easily be manipulated by adding antibodies or purified factors. The repair assay represents bona fide nucleotide excision repair, although there are some limitations. For instance, the fact that approximately 1% of the damaged DNA molecules undergo a repair event, and only global- and no transcription-coupled repair can be detected in this *in vitro* procedure.

The *in vitro* repair assay has been used for several approaches: testing purified repair factors for their repair capacity, the identification of some replication proteins to be involved in repair, and elucidation of the size of the damage-containing patch. In such a correction assay purified repair proteins from repair-proficient extracts were found to increase the repair synthesis to normal levels in extracts of XP group A (Robins et al., 1991), XP group G (O'Donovan and Wood, 1993), and in XP group C. Purification of the XPC/HHR23B complex permitted the cDNA cloning of both components (Masutani et al., 1994). In this case the *in vitro* assay was modified using UV-irradiated SV40 minichromosomes, a more chromatin like structure as substrate instead of naked DNA. RP-A (hSSB), proliferating cell nuclear antigen (PCNA), and the polymerases δ and ϵ, previously isolated as part of the replication pathway, turned out to be essential for repair *in vitro* (Coverley et al., 1991; Shivji et al., 1992), illustrating an overlap between these distinct processes: replication and repair. An excision assay with four defined CPDs in one DNA molecule clearly demonstrates that mammalian NER induces a dual incision in the damaged strand, like in *E.coli*. The incision is made 4-6 base pairs 3' and 21-23 nucleotides 5' of the lesion. An 27-30 mer oligonucleotide, containing the damage, is excised (Huang et al., 1992), which is larger than the 12-13 mer found in *E.coli*.

The experimental work described in this thesis is centred around this *in vitro* repair assay. Firstly, it was used in complementation analysis of extracts of different rodent complementation groups, resulting in the discovery that ERCC1 is part of a functional protein complex together with ERCC4, ERCC11, and XPF, and characterization of this complex (Chapters 2-3). Secondly, the assay appeared to be crucial for the unexpected discoverly that at least three NER proteins are subunits of the transcription initiation factor TFIIH. These NER proteins, XPB, XPD and TTDA, function in both transcription and repair, suggesting that the entire TFIIH complex plays a role in these two DNA metabolizing processes (Chapters 4-6).

2.3.5 ERCC1 resides in a protein complex

The first human NER gene was cloned in 1984 (Westerveld et al., 1984). The gene,

designated *ERCC1* corrected the repair defect of rodent complementation group 1 after genomic transfection. The gene was isolated and characterized; the encoded protein showed sequence homology with the *S.cerevisiae* RAD10 and the *S.pombe* swi10 proteins. The C-terminal part of ERCC1 has homology with a UvrA segment and with the C-terminus of the UvrC protein (van Duin et al., 1988). So far, *ERCC1* is the only NER gene, having homology with *E.coli* repair genes, but the UvrA/C homology of ERCC1 was not found in the *S.cerevisiae* counterpart. The C-terminal part of *ERCC1* is essential for repair, in contrast to the N-terminus, since transfection of a cDNA with an N-terminal deletion of approximately 1/3 of *ERCC1*, still shows correction of the repair defect in rodent complementation group 1. ERCC1 is not associated with a defect in any of the known human disorders, since direct transfer of *ERCC1* cDNA to cells of all XP, CS or TTD complementation groups failed to restore the repair defect (van Duin et al., 1988, Vermeulen, unpublished results).

In yeast, RAD10 forms a tight complex with RAD1. This complex is required in both NER and mitotic recombination (Schiestl and Prakash, 1988; Schiestl and Prakash, 1990), suggesting that also ERCC1 might play an additional role in recombination. The RAD1/RAD10 complex displays endonuclease activity on both single-stranded and supercoiled double-stranded DNA (Sung et al., 1993; Tomkinson et al., 1994). Recently, evidence has been observed that the complex is a structure-specific nuclease (Bardwell et al., 1994).

The first indication that ERCC1 also resides in a protein complex was deduced from complementation studies using the *in vitro* DNA repair assay. Unexpectedly, mixtures of cell-free extracts of the rodent complementation groups 1, 4, 11, or the human XP group F, show no restoration of the repair activity to normal levels. These data are in contrast to cell hybridization experiments *in vivo*, in which repair activity was observed when two mutants of distinct complementation groups were fused. However, cell-free extracts of rodent groups 2, 3, 5, and human XP-A are able to complement the NER deficiency of all other complementation groups (Chapter 2, and Biggerstaff et al., 1993). The absence of correction is not due to trivial problems of the *in vitro* repair assay, because other complementation groups do increase the repair synthesis of extracts of rodent groups 1, 4, 11, and human XP-F to normal levels. Lack of correction has been observed using extracts of several independent mutants of rodent groups 1 and 4, so allele-specific behaviour is unlikely. This observed pattern of non-complementation of NER defects *in vitro* between a specific subset of complementation groups suggests that the corresponding proteins affect each other. This may occur when these proteins form a stable complex, of which the components cannot be exchanged under these *in vitro* conditions. On the basis of this complementation analysis of rodent groups 1, 4, 11, and human XP-F, one may conclude that the *in vitro* DNA repair assay cannot replace cell fusion experiments to identify new NER-deficient complementation groups.

Further evidence for a protein complex concerning ERCC1, 4, 11, and XPF has been

obtained from immunodepletion studies. Anti-ERCC1 antibodies deplete a repair-proficient HeLa extract not only of the ERCC1 repair capacity, but also of the repair-correcting activities of ERCC4, ERCC11, and XPF. ERCC1 protein is significantly reduced in extracts of rodent groups 4, 11, and human XP-F compared to extracts of other XP groups or wild-type cells (Chapter 3, Biggerstaff et al., 1993, and R.D. Wood, personal communication). This indicates that complex formation is needed for the stability of its subunits.

Transfection experiments have already ruled out a direct involvement of *ERCC1* in the NER deficiency of XP group F (van Duin et al., 1988 and Vermeulen, unpubl. results). In addition to *ERCC1*, only *ERCC4* has been cloned as the complementation group 4 correcting gene (Thompson et al., 1994). Unfortunately, sequence data have not yet been published. Partial data have revealed homology to the yeast *RAD1* gene (Thompson, personal communication). Therefore, both *ERCC4* and *ERCC11* remain candidates for the gene, which is affected in XP-F cells.

Despite extensive studies (Chapter 3) the composition of the protein complex has not been completely elucidated. A tentative subunit of 120 kDa was immunoprecipitated specifically from ^{35}S-labelled HeLa extract and not from a labelled XP-F extract by anti-ERCC1 antiserum. This mammalian subunit might be the human homolog of the yeast RAD1 protein, based on the size of both proteins: 120 kDa for the subunit, compared to 126 kDa for RAD1. The yeast *rad1* and *rad10* mutants are defective both in NER and mitotic recombination (Schiestl and Prakash, 1988; Schiestl and Prakash, 1990). Rodent *ERCC1* and *ERCC4* mutants demonstrate also a remarkable similarity. Generally, these rodent mutants display hypersensitivity to cross-linking agents, and these lesions are thought to be repaired by a recombinational event. Rodent group 11 and human XP-F cells do not show such an extreme sensitivity to MMC, which is maybe due to subtle mutations in these genes. In non-denaturing gels the protein complex has a molecular mass of ~250 kDa, which is much larger than the ~120 kDa previously found in glycerol gradient sedimentation (Chapter 2, Biggerstaff et al., 1993). The larger size of the protein complex found by gel-electrophoresis would better accommodate the presence of ERCC1, a protein with a predicted weight of 33 kDa, a 120 kDa subunit, which is an ERCC4 candidate, and at least one unidentified protein in the complex.

Purification of the ERCC1 complex to homogeneity could not be achieved so far using classical chromatography columns. One complication to purify the ERCC1 complex is the low expression level (van Duin et al., 1989a). The ERCC1 complex shows partially co-elution with the transcription initiation factor TFIIH over four chromatography columns, but this co-elution is incidental, since no co-depletion of ERCC1 and TFIIH was found by anti-ERCC1 antiserum or anti-p62 antiserum, a subunit of TFIIH. In addition, immunodepletion of a repair-proficient extract using anti-ERCC1 antiserum does not remove the repair capacity to correct rodent groups 2 and 3 extracts (Chapter 2), while both correcting proteins reside in TFIIH (Chapters 4 and 5). Although an interaction between ERCC1 and XPA was recently described (Li et al., 1994a; Park and Sancar, 1994) association studies failed to disclose a

stable interaction between both proteins, but transient affinities during the NER process cannot be excluded. Presumable, another approach via a tagged ERCC1 protein might be necessary to purify the ERCC1 complex.

It is expected that the ERCC1 complex is the human homolog of the yeast RAD1/RAD10 complex, based on the homology of ERCC1 and ERCC4 with RAD10 and RAD1 respectively. The ERCC1 complex might have an additional function in recombination, like the yeast counterpart. Very recent data showed that the yeast RAD1/RAD10 complex cleaves *in vitro* a splayed-arm DNA structure, at the border of the single-stranded and double-stranded area, only in the strand with a single 3' end (Bardwell et al., 1994). When this is also true *in vivo*, the RAD1/RAD10 complex is likely to be responsible for the 5' incision in NER. By analogy to yeast, the ERCC1 complex is predicted to exhibit incision activity, which would be in line with the additional function of the ERCC1 complex in recombination, since both processes imply a DNA incision step in their reaction mechanisms. However, the incision activity of the ERCC1 complex still has to be confirmed. Recently, XPG was found to display endonuclease activity on similar splayed-arm DNA structures with 5' single-stranded tails, implicating that XPG is required for the 3' incision (O'Donovan et al., 1994).

No human disorder has been observed yet to be due to an *ERCC1*-deficiency. Perhaps the additional involvement of ERCC1 in recombination is responsible for clinical symptoms in patients that are not directly associated with NER. Presumably, a mouse model can give more indications of an ERCC1-deficiency in man. Mice have been generated in which both *ERCC1* alleles were inactivated (McWhir et al., 1993, and Weeda, unpublished results). The homozygous ERCC1-deficient mice (-/-) show retarded growth in comparison to the heterozygote (+/-) or the wild-type (+/+) litter mates, and die at an age of 4 to 10 weeks. In addition to growth deficiency, liver and kidney abnormalities have been observed in (-/-) mice. Homozygous mice are underrepresented, suggesting that these mice might have a decreased viability. Embryonic fibroblasts of homozygotes display both UV-sensitivity and hypersensitivity to the cross-linking agent MMC, confirming the expected NER-deficiency.

If a human disorder due to an ERCC1-deficiency exists, and if the phenotype is related to these homozygous mice, patients may die very young due to liver problems before they can be clinically recognized as having a defect in NER. Pigmentation abnormalities or UV-sensitivity are often not noted in very young XP patients. Alternatively, a defect in *ERCC1* might not be viable in man, since no expected segregation of ERCC1-deficient alleles has been observed in mice. More subtle mutations in *ERCC1*, rather than a complete inactivation of the gene, will hopefully result in a longer lifetime of the (-/-) mice. This will allow to study the role of ERCC1 in carcinogenesis, since skin tumour induction by carcinogenic agents usually requires several months. A series of mouse models lacking different excision repair genes will be generated in the future, which is important to study the clinical aspects of a NER-deficiency in more detail.

3. DNA repair: link to the process of transcription

3.1 Transcription initiation by RNA polymerase II

One of the basal initiation factors, TFIIH, is required for transcription initiation and is involved in nucleotide excision repair. Therefore, the process of transcription initiation by RNA polymerase II (pol II) will be discussed briefly. In eukaryotes, transcription of protein-encoding genes is carried out by RNA pol II. Two classes of transcription factors are observed: general initiation factors, essential for initiation of a basal level of transcription from many core promoters, and regulatory factors, mediating the action of activators, silencers, and enhancers.

Transcription initiation by RNA pol II requires at least six basal transcription initiation factors. The assembly of these transcription factors on the promoter has been extensively studied *in vitro* (Conaway and Conaway, 1993). Promoters recognized by pol II often have a TATA-box, which is located ~30 bp upstream of the transcription start site, and an initiation motif at position +1. As an initial step the TATA box-binding protein (TBP), a subunit of TFIID, binds to the promoter. However, a subset of promoters lacking this TATA element is also recognized by the protein. In addition to TBP, TFIID contains several TBP-associated factors (TAFs), which are probably mediating transcriptional activation. Subsequently, TFIIA binds TBP, thereby stabilizing the TFIID-DNA interaction and acting as a binding site for TFIIB. TFIIF appears to associate with RNA pol II and delivers it to the pre-initiation complex.

Eukaryotic pol II is composed of 10 different subunits. The largest subunit of pol II contains an unusual C-terminal domain (CTD) comprised of multiple repeats of the heptamer consensus sequence Tyr-Ser-Pro-Thr-Ser-Pro-Ser. This CTD is specific for eukaryotic pol II enzymes. The length of the heptapeptide repeats correlates with the genomic complexity of the organism; it occurs 26-27 times in yeast, 42-44 times in *Drosophila*, and 52 times in mouse and man (Young, 1991). As a result of this high content of serine and threonine residues, the CTD can be extensively phosphorylated. The function of CTD phosphorylation has been analyzed: the nonphosphorylated (IIA) form of the polymerase stably associates with the preinitiation complex, whereas the phosphorylated (IIO) form is isolated from actively elongating complexes. The conversion of IIA to IIO occurs prior to the formation of the first phosphodiester bond (Laybourn and Dahmus, 1990). Phosphorylation of the CTD might be the trigger for transition from the initiation- to the elongation phase.

The association of the basal factors TFIIE and TFIIH completes the assembly of the pre-initiation complex (Zawel and Reinberg, 1993). An ATP-dependent step is required during the initiation step of transcription (Sawadogo and Sentenac, 1990). So far, TFIIH is the only

factor displaying known catalytic activities. Its activity phosphorylates the large subunit of RNA pol II. In addition, TFIIH shows DNA-dependent ATPase and helicase activities (Lu et al., 1992; Schaeffer et al., 1993). Most of the transcription initiation factors are multisubunit protein complexes, and these factors have been characterized by purification, after which the corresponding genes could be isolated.

Transcription *in vitro* from the adenovirus major late promoter (AdML) requires all the described general initiation factors, but only a subset of basal factors is necessary for transcription of the immunoglobulin heavy chain (IgH) promoter. The transcription appears to be dependent on the topology of the DNA; negatively supercoiled DNA obviates the need for the factors TFIIE and TFIIH. Probably, melting of the DNA for the formation of an open complex is facilitated by negative superhelical twists (Parvin and Sharp, 1993; Timmers, 1994), suggesting that the configuration of the DNA template is an important parameter determining initiation *in vitro*. There is evidence that TFIIH has a stimulatory effect on transcription of supercoiled templates. The effect is more pronounced on HIV type 1 than on the AdML promoter, implying a possible promoter specificity. Whether this is the case *in vivo* remains to be proven, since all data have been obtained using *in vitro* run-off assays and a limited number of promoters. Only a 'minimal initiation complex' containing TBP, TFIIB, TFIIF, and pol II, is sufficient for the production of transcripts up to 70 nucleotides (Tyree et al., 1993).

It is not yet clear how many subunits reside in the TFIIH complex. In the most purified fraction five predominant subunits are observed, including p89, p62, p44, p41 and p34, and at least three more loosely associated components: p80, p55 and p38 (Gerard et al., 1991). The function of the p62 protein is unknown, although the gene was cloned some years ago. Suprisingly, the p89 subunit was found to be identical to the XPB protein described previously by Weeda (Weeda et al., 1990a; Schaeffer et al., 1993). Both 3'→5' helicase activity and at least part of the ATPase activity appear to be associated with the XPB subunit of TFIIH (Schaeffer et al., 1993; Roy et al., 1994b). The p80 subunit has been identified as the equivalent of the repair protein XPD, and a 5'→3' helicase activity is attributed to this subunit (Schaeffer et al., 1994). The p80 subunit could hardly be detected in the most purified TFIIH fraction, suggesting that either a small portion of the complex is in an active conformation or the different subunits are not available in stoichiometric amounts in TFIIH. In addition to XPB, XPD and p62, the genes encoding the subunits p44, the homolog of the yeast SSL1, and p34 have been cloned. Putative Zn-finger domains suggest that both proteins are involved in DNA binding (Chapter 6). The subunit responsible for the kinase activity is recently identified as MO15, a cyclin-dependent kinase, which is involved in cell cycle regulation (Feaver et al., 1994; Roy et al., 1994a). This exciting discovery will be discussed in more detail in paragraph 4.

In addition to human cells, transcription initiation factors have also been isolated from yeast and rat liver cells. The yeast factor b, homologue of TFIIH, consists of five polypeptides: p85 (RAD3), p75 (TFB1), p55, p50 (SSL1), and p38. Although, the less

tightly associated component of p105 (RAD25/SSL2) stimulates the transcription activity *in vitro* (Feaver et al., 1993) and indications have been found for a holocomplex in yeast showing three additional subunits p47, p45 and p33 (Svejstrup et al., 1994). Previously SSL1 and SSL2 have been identified as suppressors of an artificial stemloop structure in the 5'UTR of His4 mRNA, which blocks translation initiation (Gulyas and Donahue, 1992; Yoon et al., 1992), one assumes tacitly that both proteins are subunits of the transcription initiation factor b. In factor δ, the rat homolog of TFIIH, at least eight subunits have been identified (Conaway and Conaway, 1989). These transcription initiation factors from man, rat and yeast and their subunits are summarized in Table 4. The distinct composition of TFIIH in diverse organisms is probably due to different purification schemes to isolate these transcription initiation factors, and variable dissociation of more loosely associated components during purification.

3.2 Role of TFIIH in transcription.

The function of TFIIH in transcription is not completely clear. The initiation factors TFIIE and TFIIH are not required for transcription of certain promoters on supercoiled DNA templates, while these factors are necessary in the case of the same promoters on linear templates (Parvin and Sharp, 1993; Timmers, 1994). The p56 subunit of TFIIE mediates the recruitment of TFIIH by binding the XPB(p89) subunit (Serizawa et al., 1994); *in vitro* this interaction is sufficient to purify transcriptionally active TFIIH complexes (Maxon et al., 1994). In addition, TFIIE interacts also with pol IIA, TBP, TFIID, and TFIIF. TFIIE stimulates the TFIIH-associated kinase activity in the presence of DNA at a late stage of the assembly of the preinitiation complex. TFIIH phosphorylates the CTD of RNA pol II and, in addition, several other factors, like $TFIID_{\tau}$ (TBP), $TFIIE_{\alpha}$ (p56), and $TFIIF_{\alpha}$ (RAP74) can be phosphorylated (Ohkuma and Roeder, 1994). Probably TFIIE regulates the phosphorylation activity of TFIIH, resulting in conversion into the active initiation complex.

Recently, data have been obtained that TFIIH is involved in promoter clearance, rather than in the initiation or the elongation phase of transcription (Goodrich and Tjian, 1994). Association of TFIIE and TFIIH to the minimal initiation complex completes the formation of an active transcription complex. The complex requires ATP to produce extended transcripts, suggesting an intermediate phase between initiation and elongation. TFIIH is not necessary for open complex formation, because initiation can occur without TFIIH on supercoiled DNA templates and in abortive initiation assays, wherein a trinucleotide abortive transcript is formed. Probably the helicase activity of TFIIH extends the melted region of the DNA, when this region reaches a sufficient length the RNA pol II looses contact with the promoter and enters the elongation phase. TFIIE and TFIIH are not required during elongation (Goodrich and Tjian, 1994). However, it cannot be excluded that TFIIH is also involved in early elongation, because transcriptional pause sites in 5' regions of a subset of *Drosophila* heat-shock genes are found to be associated with pol II (A) molecules (Ohkuma

Table 4: Human transcription initiation factors and their homologs in rat and *Saccharomyces cerevisiae*.

Human	Subunits	Rat	Subunits	Yeast	Subunits
TFIIA[1]	p34, p19, p14				
TFIIB	p33	α	p35	e	
TFIID	p38(TBP) and TAF's	τ		d	p27(TBP)
TFIIE	p34, p56	ϵ	p58, p34	g[§]	p105, p54, p30
TFIIF	p30, p74	ßγ	p67, p31	a[§]	p66, p43
TFIIH	p89(XPB), p62, p44, p41, p34, [p80(XPD), p50, p38, p32]	δ	p94, p85, p68, p46, p43, p40, p38, p35	b	p85(RAD3), p75(TFB1), p55, p50(SSL1), p38, [p105(SSL2), p47, p45, p33]

1) The human TFIIA has been purified as a subunit of 43 kD, as 38 kD and as a heterotrimer composed of the subunits 34, 19 and 14 kD (Conaway and Conaway, 1993).

§ It is not clear yet how these factors are related to mammalian initiation factors

[ ] indicates more loosely associating components

and Roeder, 1994). This suggests that pol II has to be re-phosphorylated before efficient readthrough of a pause site.

Chromatin structures, like loops, decondensed, and condensed domains, are important, since these can influence the processing of transcription, replication and recombination. How these structures affect gene expression is largely unknown. Transcription may require at least a transient unwrapping of the nucleosome-bound DNA. The proteins of the high mobility group (HMG), chromatin-associated proteins, are known to bind and bend linker DNA (Travers, 1994). It appears that the HMG2 protein represses basal transcription *in vitro* after linkage of the second and before addition of the fifth nucleotide. The effects of HMG2 are completely relieved by adding purified TFIIH fractions, suggesting an essential role for TFIIH in removing DNA-bound repressors (Stelzer et al., 1994). Fine-tuning of different regulatory mechanisms, like transcription activation, promoter specificity or chromatin structure has to be elucidated.

3.3 Role of TFIIH in repair

The identification of XPB as a subunit of TFIIH suggests a possible function of this transcription factor in repair. Chapter 4 presents data showing that purified fractions of TFIIH correct the XPB-deficiency *in vivo* as well as *in vitro*. In addition, microinjection of the same TFIIH fractions into XP-D and TTD-A fibroblasts induces repair synthesis to normal levels. These results could be confirmed *in vitro* by adding these fractions to extracts of rodent complementation group 2 or human TTD-A cells: again a clear correction of the repair defect was observed (Chapter 5). Immunodepletion studies, using antibodies raised against the subunits p89 or p62, show the removal of the repair-correcting activities of XPB, XPD and TTDA from a repair-proficient extract. In contrast, repair-correcting activities of XPA and XPG remain in the depleted extract. These results suggest that all three subunits reside in a TFIIH configuration. The subunit responsible for the correction of the TTD-A defect is not yet known. The three complementation groups XP-B, XP-D and TTD-A are defective in both subpathways of NER: global genome - and transcription-coupled repair. The requirement of XPB(p89) and XPD(p80) in transcription initiation was deduced from *in vitro* results (Schaeffer et al., 1993; Schaeffer et al., 1994). To verify that both subunits XPB and XPD are also directly involved in transcription *in vivo*, antibodies against these proteins were injected into normal fibroblasts. The NER activity as well as RNA synthesis were significantly decreased. In contrast, anti-ERCC1 antiserum reduces only the repair activity, whereas RNA synthesis remains normal. The dual functions of XPB and XPD in NER as well as in transcription strongly suggest that the entire TFIIH complex is essential for both DNA metabolizing processes. Evidence for this multifunctional role of TFIIH is extended by data observed in yeast. The *SSL1* and *TFB1* mutants show hypersensitivity to UV and the NER defect of these mutants can be corrected by purified factor b (Wang et al., 1994b).

Evidence that XPC is associated with TFIIH as described previously (Drapkin et al.,

1994) is difficult to reconcile with the notion that XPC is implicated only in the transcription independent mechanism of global genome repair. The connection between XPC and TFIIH could not be confirmed in our experiments. In the first chromatography step the repair activities of XPB and XPD, both subunits of TFIIH, and XPC are already separated (Vermeulen, unpubl. results). However, the global genome repair requires most of the repair proteins, which are also involved in the transcription-coupled repair pathway. These include the damage-recognition proteins, as well as the incising and helix-unwinding components. A transient interaction between TFIIH and XPC/HHR23B cannot be excluded during the repair process in the non-transcribed genome.

Transcription is impaired by DNA damage, since RNA pol II is arrested at the site of the lesion in the transcribed DNA template. Pol II covers the lesion, because photolyase can no longer cleave a CPD that blocks the DNA polymerase (Donahue et al., 1994). Thus the polymerase has to be displaced from the lesion to allow access for repair enzymes. Presumably, this is accomplished by a backward movement of pol II and cleavage of six nucleotides from the 3' end of the nascent transcript, because the 3' incision in NER is made 6 base pairs upstream of the lesion (Huang et al., 1992). Both the polymerase and the nascent transcript remain stably associated with the DNA (Donahue et al., 1994). This stability contrasts with the situation in *E.coli*, where the transcription repair coupling factor (TRCF) removes the polymerase from the DNA. TRCF remains bound as a signal to recruit the $UvrA_2B$ complex (Selby and Sancar, 1993). In this way the damage is rapidly repaired by the transcription-coupled repair process. When the eukaryotic polymerase is displaced and the repair proteins are recruited, the TFIIH factor might unwind the DNA helix around the lesion by its helicase-associated activity. After the lesion has been repaired, the transcription factor TFIIS may probably help to restart elongation using the nascent transcript. Normally TFIIS assists the process of elongation through transcriptional pause sites, which are sequence-specific sites, where transcription can be arrested (Reines et al., 1989).

The CSA and CSB proteins also play a role in transcription-coupled repair, although it is not known whether these proteins interact with one of the transcription factors. Perhaps, CSB functions in scanning the transcribed DNA strand for lesions and is involved in the recruitment of the repair proteins. Alternatively, the developmental abnormalities found in CS patients might be the result of a repair defect in expressed genes rather than a deficiency in transcription. Ionizing radiation induces lesions through oxidation by free radicals that damages the DNA. Normally free radicals are produced as by-products of oxidative metabolism in the cell, and the damage generated by these radicals is repaired mainly by base excision repair (BER). CS patients are also deficient in transcription-coupled repair of X-ray induced lesions. This suggests that certain lesions induced by free radicals are repaired by NER, or that other repair pathways, such as BER, might be coupled to transcription. Cells that proliferate rapidly during development or cells with a high metabolic activity produce a high level of free radicals. This might be the reason that these cells are very sensitive to defects in transcription-coupled repair (Hanawalt, 1994). More detailed studies are necessary

to elucidate this complex mechanism of transcription-coupled repair and its biological relevance.

3.4 Clinical consequences of mutations in subunits of TFIIH.

The disorders related to mutations in subunits of TFIIH show a very broad spectrum of clinical symptoms. XP group D displays three classes of patients. Patients with a classical XP phenotype, patients with combined XP/CS features, and several individuals with TTD symptoms. In contrast, XP group B is very rare and displays two classes of patients, XP/CS and TTD. Within groups B and D, the severity of the biochemical NER defect cannot be related to the clinical symptoms in patients. A higher level of residual repair activity is observed in XP-D cells compared to XP-B, but the clinical features are similar in both groups. Even within one complementation group differences in the clinical phenotype occur. Two patients of XP-B: XP11BE and XP1BA, display the same low level of residual repair activity ($\leq 10\%$). However, patient XP11BE is severely affected, showing skin tumours at early age, while only mild skin lesions at an age of >40 years and no skin tumours have been observed in XP1BA (Vermeulen et al., 1994). Several peculiar symptoms of CS and TTD, like mental retardation due to dysmyelination of neurons, impaired sexual development and brittle hair and nails, can hardly be explained on the basis of a NER-deficiency. Probably transcription is to some extent affected in these patients, in addition to the NER defects. The defects in transcription must be subtle, because severe deficiency in transcription is probably not compatible with life. These defects might occur when mutations in TFIIH subunits affect only the transcription of a subset of genes or when overall transcription is slightly decreased. The effect might be that the level of mRNA or translated proteins is below a certain critical threshold. The reduced cysteine content in brittle hair in TTD patients may be due to a decreased synthesis of a group of high sulphur proteins. Since transcription of the myelin basic protein is rate-limiting (Readhead et al., 1987), its reduced transcription may cause dysmyelination of the neurons in CS and TTD. Combined XP/CS features have also been recognized in patients of XP group G (Johnson and Squires, 1992; Vermeulen et al., 1993), suggesting that possibly XPG affects basal transcription as well. Perhaps XPG influences RNA pol II, that has to move backwards to make the DNA accessible for the 3' incision in case of a lesion in the DNA.

If the hypothesis that mutations in TFIIH subtly affect transcription is correct, it should be possible to detect transcriptional defects without a repair involvement. Indeed, patients have been identified displaying clinical characteristics of TTD and CS, but no evidence of photosensitivity or a NER deficiency (Lehmann, 1987; Lehmann et al., 1993). The hupothesis would be strengthened when patients exhibiting mutations in transcription factors, that do not affect NER, can be identified. The non-photosensitive TTD patients are good candidates, harbouring partial mutations in *XPB* or *XPD* genes, which only disturb the transcription function of TFIIH. This has to be established by further experimental studies.

Presumably, a much wider class of human disorders can be regarded as " transcription syndromes". A search in the OMIM database (McKusick, 1992) reveals several syndromes, like Rothmund-Thompson, OTD, IFAP, ICE, CAM(F)AK, Sjögren-Larsson, RUD and KID. All these syndromes share at least four pronounced clinical features of CS or TTD, and even some of them display the occurrence of skin cancer.

4. Evidence for interactions between DNA repair and cell cycle regulation

Interactions between different DNA metabolizing processes like repair, transcription, and replication have been observed. Remarkably, the process of cell cycle control can also affect NER. The tumorsuppressor gene *p53* plays an important role in stressed cells. Its expression is significantly increased in response to a variety of DNA damaging agents. As a consequence, the cells are arrested in their cell cycle until the damage is repaired (Vogelstein and Kinzler, 1992). The importance of its ability to arrest cell growth is shown by the fact that alterations of the *p53* gene appear to be involved in the majority of human malignancies. In response to UV irradiation P53 has at least one additional function, it initiates the process of apoptosis, also named programmed cell death. Apoptosis might prevent the development of skin cancer, because unwanted cells are eliminated, for instance those that have an increased amount of mutations. P53 is also a transcription factor, which regulates the activity of other genes. Enhanced p53 levels stimulate the expression of *p21* and *GADD45*, which causes growth arrest. P21 is an inhibitor of the cyclin-dependent kinases (cdk), key enzymes needed for cell cycle progression. The p21 induces a G1 arrest to allow repair of DNA damage before replication. A remarkable observation is the fact that p21 inhibits specifically the role of PCNA in replication, but does not block the PCNA-dependent NER reaction (Li et al., 1994b). Gadd45 is found to be associated with PCNA, it enhances the repair activity *in vitro* and inhibits cells to enter the S phase (Smith et al., 1994). It is not clear yet whether Gadd45 has a direct role in repair, or that Gadd45 suppresses cell growth through another mechanism. In addition to the fact that p53 indirectly stimulates repair through Gadd45, there is evidence for a direct role of p53 in repair, since p53 might be associated with XPB *in vitro* (Wang et al., 1994a). However, very little is known about how p53 binds XPB and affects NER. These data suggest that the arrest of cell cycle progression regulates NER before the cell enters S or M phase.

More evidence in favour of a correlation between cell cycle regulation and NER is

recently found by the isolation of the gene that encodes the kinase activity of TFIIH both in human and yeast (Feaver et al., 1994; Roy et al., 1994a). These genes are homologous to the previously isolated *Xenopus MO15*. MO15 has been identified as the catalytic subunit of a CDK-activating kinase (CAK), a cyclin-dependent protein kinase that phosphorylates $p34^{cdc2}$ and $p33^{cdk2}$, which has a key function in cell cycle control (Poon et al., 1993; Solomon et al., 1993; Fesquet et al., 1993). MO15 requires cyclin H as its partner, which enhances the kinase activity (Mäkelä et al., 1994). In addition to MO15, cyclin H could be observed in the most purified TFIIH fractions. MO15 was shown to interact with XPD, a subunit of TFIIH. Phosphorylation of the CTD of RNA pol II requires not only the presence of MO15, but also certain key subunits of TFIIH. Presumably the cell uses the CAK activity in two independent processes: cell cycle regulation and transcription. It appears that there are forms of TFIIH complexes, that differ in their subunit composition. This suggests that the various functions of TFIIH in transcription, repair, and possibly in cell cycle control need different TFIIH compositions.

5. Conclusions and future perspectives

Information about the excision repair pathway increased rapidly in the last few years. Genetic analysis using repair-deficient mutants have revealed nearly all the repair genes corresponding to the different complementation groups in rodents and human. Properties of the encoded gene-products have been found by purification and biochemical studies of most enzymes. Very soon it will be possible to reconstitute the entire NER process *in vitro* using the purified and/or recombinant repair factors. Details concerning the interactions of the distinct repair proteins with each other or with DNA have to be elucidated, and mechanisms regulating DNA repair response are largely unknown.

The first indication that transcription and repair were linked to each other has been provided by the discovery of a mechanism designated as transcription-coupled repair. Certain types of DNA lesions, for instance CPD, are repaired by such a pathway. This preferential repair of expressed genes is very complex, since differences in repair exist between rodent and human cells. In rodent cells CPDs are rapidly removed from the transcribed strand of active genes, although this represents only a small fraction of the total amount of CPDs in the genome. In contrast, human cells repair CPDs very efficiently both from expressed genes and the genome overall. In spite of this difference in repair efficiency of total genomic DNA rodent and human cells are equally resistant to UV. This paradox might be explained by the fact that rodents remove dimers from regions that are immediately essential for UV-survival.

Alternatively, CPDs are not as mutagenic as other lesions, although the high rate of spontaneous transformations in rodent cells might be explained by the presence of many CPDs in the genome overall.

Another link between the processes of repair and transcription was found by the remarkable observations that the repair enzymes: XPB, XPD, and TTDA are subunits of the transcription initiation factor TFIIH, and function in both repair and transcription. Many clinical symptoms in CS and TTD patients, which might not be related to a NER-deficiency, can be explained as subtle defects in transcription, suggesting the existence of 'transcription syndromes' in which patients suffer from transcriptional defects without any NER deficiency, but this hypothesis has to be confirmed. An alternative explanation as suggested by Hanawalt (Hanawalt, 1994) that growth abnormalities found in CS and TTD patients are probably due to the accumulation of free radical damage in expressed genes, thus by a DNA repair defect, cannot be excluded. Perhaps highly proliferating or metabolic active cells are very sensitive to deficiencies in transcription-coupled repair.

The influence of chromatin structure on DNA metabolizing processes like replication, transcription, and repair is far from understood. Loosening of the chromatin structure is necessary to allow transcription. In these open chromatin domains, not only active genes are repaired more rapidly, but this rapid repair extends beyond the entire region. The nucleosomes in the DNA structure have to be removed before transcription can be initiated, since RNA polymerase II complex is even larger than the nucleosomes. Presumably, this nucleosome removal promotes also excision repair of DNA damage. The observation that preferential repair varies between genes, even when the same lesion is studied, might be explained by the high stability of some nucleosomes inside transcribed regions.

The very exciting results recently observed that cell cycle control might affect excision repair and probably also transcription, require more detailed studies to elucidate the precise role of proteins like MO15 in these processes. Is MO15 the trigger to initiate cell cycle arrest, after transcription is blocked by DNA lesions? Is the same kinase used in both distinct processes: transcription and cell cycle regulation? Does the possible interaction between MO15 and XPD indicate that MO15 is involved in sensing DNA lesions or changing the configuration mode of TFIIH from transcription into repair? Several other genes are induced in response to X-ray irradiation, like *p53*, *GADD45* and *p21*, enhancing directly or indirectly excision repair.

In the future, NER defects in humans will be mimicked in animal models by gene replacement of the gene of interest. The generation of repair-deficient mice would provide more insight in the distinct clinical features observed in NER-deficient patients and in the processes of mutagenesis and carcinogenesis. Mice defective in the genes *ERCC1, XPC*, or *XPA* have already been developed (McWhir et al., 1993; G. Weeda and E. Friedberg, both personal communication; Tanaka, submitted). *XPC*-deficient mice might give clues about the role of global genome repair in carcinogenesis, whereas CSB-deficient mice will shed light on the contribution of transcription-coupled repair to the clinical symptoms observed in these

NER syndromes. The example of *XPA*-deficient mice (Tanaka, submitted), which closely imitate the phenotype of XP-A patients, is very promising in this respect.

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Chapter 2

Evidence for a repair enzyme complex involving ERCC1 and complementing activities of ERCC4, ERCC11 and xeroderma pigmentosum group F

Evidence for a repair enzyme complex involving ERCC1 and complementing activities of ERCC4, ERCC11 and xeroderma pigmentosum group F

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Nucleotide excision repair (NER), one of the major cellular DNA repair systems, removes a wide range of lesions in a multi-enzyme reaction. In man, a NER defect due to a mutation in one of at least 11 distinct genes, can give rise to the inherited repair disorders xeroderma pigmentosum (XP), Cockayne's syndrome or PIBIDS, a photosensitive form of the brittle hair disease trichothiodystrophy. Laboratory-induced NER-deficient mutants of cultured rodent cells have been classified into 11 complementation groups (CGs). Some of these have been shown to correspond with human disorders. In cell-free extracts prepared from rodent CGs 1–5 and 11, but not in a mutant from CG6, we find an impaired repair of damage induced in plasmids by UV light and *N*-acetoxy-acetylaminofluorene. Complementation analysis *in vitro* of rodent CGs is accomplished by pairwise mixing of mutant extracts. The results show that mutants from groups 2, 3, 5 and XP-A can complement all other CGs tested. However, selective non-complementation *in vitro* was observed in mutual mixtures of groups 1, 4, 11 and XP-F, suggesting that the complementing activities involved somehow affect each other. Depletion of wild-type human extracts from ERCC1 protein using specific anti-ERCC1 antibodies concomitantly removed the correcting activities for groups 4, 11 and XP-F, but not those for the other CGs. Furthermore, we find that 33 kDa ERCC1 protein sediments as a high mol. wt species of ~120 kDa in a native glycerol gradient. These results strongly suggest the presence of a pre-existing enzyme complex in mammalian cell extracts, harbouring at least the products of the *ERCC1* and *ERCC4* genes. This complex also carries complementing activites of XP group F and rodent CG11. We postulate that the complex, like the one in *Saccharomyces cerevisiae* involving the RAD1 and RAD10 proteins (the latter being the homologue of ERCC1), functions in both NER and recombinational repair.

Key words: Chinese hamster mutant/complementation analysis/damaging agents/nucleotide excision repair/repair complex

Introduction

Nucleotide excision repair (NER) is a DNA repair system that has evolved in all living organisms for the removal of lesions inflicted on DNA by mutagenic radiations (notably UV light) or numerous chemical agents. It is a multi-enzyme process involving specific recognition and dual incision of the damaged strand, followed by lesion removal, gap-refilling and strand ligation (Grossman and Yeung, 1990; Selby and Sancar, 1990; Hoeijmakers, 1991). In man, genetic defects in NER of UV-induced DNA damage give rise to the severe disorders xeroderma pigmentosum (XP), Cockayne's syndrome (CS) or PIBIDS. Besides hypersensitivity of the skin to sunlight and progressive neurological dysfunction, a dramatically increased risk of skin cancer (XP) or developmental abnormalities (CS) entail the clinical hallmarks of these disorders (Cleaver and Kraemer, 1989; Nance and Berry, 1992). PIBIDS is a photosensitive form of the brittle hair disorder trichothiodystrophy (Lehmann, 1987). Somatic cell hybridization studies have revealed extensive genetic heterogeneity: in XP, at least seven NER-defective complementation groups (named A to G) exist whereas in CS two (CS-A and CS-B) have been identified so far (Lehmann, 1982; Vermeulen *et al.*, 1991) and two complementation groups have been found in PIBIDS patients (Stefanini *et al.*, 1992, 1993).

In addition to these naturally occurring mutants, a large collection of laboratory-induced, UV-sensitive, NER-defective mutants have been obtained from cultured rodent cells. By restoration of UV resistance in cell hybrids, 11 complementation groups (CGs) were discerned among these mutant cell lines (for recent overviews see Thompson *et al.*, 1988; Busch *et al.*, 1989; Stefanini *et al.*, 1991; Zdzienicka *et al.*, 1991; Riboni *et al.*, 1992; Collins, 1993). Cross-sensitivity to chemicals producing bulky DNA lesions is a feature of the UV-sensitive mutants in all groups. However, representatives from groups 1 and 4 are unique with regard to the property of extreme sensitivity of DNA crosslinking agents, such as mitomycin C (Busch *et al.*, 1989).

A number of excision repair cross-complementing (*ERCC*) genes, capable of correcting the NER defects in the rodent CGs have been isolated using gene transfer strategies. Characterization has provided clues to the function of their gene products and their involvement in human disease (for reviews see Hoeijmakers and Bootsma, 1990; Friedberg, 1992; Hoeijmakers, 1993). For instance, based on sequence motifs, both the genes *ERCC2* and *ERCC3* are expected to encode proteins with helicase activity. These genes are now known to be involved in XP-D and XP-B, respectively (Weeda *et al.*, 1990; Flejter *et al.*, 1992; Weber, cited in Lehmann *et al.*, 1992). A third putative helicase, encoded by *ERCC6*, is required for preferential NER of actively transcribed DNA segments and is defective in CS group B (Troelstra *et al.*, 1992). The *ERCC5* gene was recently found to be responsible for XP-G (O'Donovan and Wood, 1993; Scherly *et al.*, 1993). The ERCC1 protein is not associated with the defect in any of the XP, CS or PIBIDS groups (Van Duin *et al.*, 1989). On the other hand, the XPAC protein is affected in XP-A (Tanaka *et al.*, 1989), but in none of

the rodent CGs 1–7 or CG 11 (our unpublished results).

As another outcome of the characterization of NER genes it has become clear that they are highly conserved in evolution. Thus, homologues for most cloned mammalian NER genes could be identified in lower eukaryotes, particularly in yeast. As examples, the *ERCC1, 2, 3* and *5* genes share extensive sequence homology at the protein level with the *RAD10, RAD3, RAD25/SSL2* and *RAD2* genes of *Saccharomyces cerevisiae*, respectively (Van Duin *et al.*, 1986; Weber *et al.*, 1990; Gulyas and Donahue, 1992; Park *et al.*, 1992; Scherly *et al.*, 1993). Therefore, it is likely that the basic features of the NER mechanism are conserved from yeast to man as well and that the homologues have functional equivalence. Both yeast and mammalian studies indicate that some NER genes play a role in other cellular processes. For instance, *RAD1* and *RAD10* are required for mitotic recombination (Schiestl and Prakash, 1988, 1990) and recently a direct involvement of the ERCC3 protein in transcription initiation was found (Schaeffer *et al.*, 1993).

A cell-free *in vitro* assay of mammalian NER is another important tool to elucidate the functions of the NER gene products at the enzymological level. The *in vitro* system measures repair synthesis in UV-irradiated plasmid DNA mediated by isolated human cell extracts (Wood *et al.*, 1988). The assay can mimic *in vitro* complementation analysis of XP extracts (Hansson *et al.*, 1991; Coverley *et al.*, 1992) and can detect repair of bulky lesions induced by chemical agents (Hansson *et al.*, 1989; Sibghat-Ullah *et al.*, 1989; Sibghat-Ullah, 1990). It has served to assess the topology of the early endonucleolytic step of human NER (Huang *et al.*, 1992), the involvement in NER of additional protein factors such as PCNA and SSB/RP-A (Coverley *et al.*, 1991, 1992; Nichols *et al.*, 1992; Shivji *et al.*, 1992) and the purification of XPAC protein from calf thymus (Robins *et al.*, 1991).

Here, we report the results of a systematic study of NER in UV-sensitive mutants of various rodent CGs using the *in vitro* assay. In attempting *in vitro* complementation analysis, we encounter selective absence of correction, when cell extracts from representatives of groups 1, 4 and 11 are mixed. Results from antibody depletion experiments and sedimentation analysis indicate that these specific patterns of *in vitro* non-complementation can be explained by the presence of a high mol. wt repair enzyme complex, comprising at least the products of the *ERCC1* and *ERCC4* genes. In addition, the factors able to complement XP group F and CG11 are associated with this complex.

Results

In vitro repair activity in Chinese hamster cell extracts

Two cell-free extracts, prepared from wild-type Chinese hamster cells (CHO9) and from a NER-deficient strain belonging to CG1 (43-3B), were compared with respect to their ability to mediate repair synthesis *in vitro* using UV-irradiated plasmid DNA (450 J/m^2) as a substrate. As shown in Figure 1A, UV-dependent repair synthesis was observed *in vitro*, with only a very slight incorporation of ^{32}P-labelled dATP in the non-damaged plasmid. With these substrates no significant difference in repair synthesis was detected between a wild type (CHO9) and a CG1 mutant extract (43-3B).

Besides pyrimidine dimers and 6-4 photoproducts UV

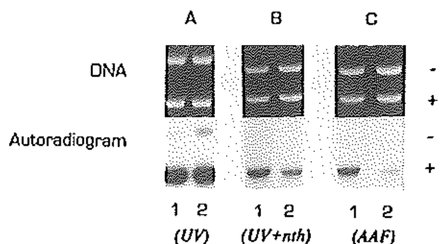

Fig. 1. Damage-dependent incorporation of [^{32}P]dATP into various DNA substrates. Plasmid DNAs were incubated with 200 μg of wild-type (CHO9, lanes 1) or CG1 (43-3B, lanes 2) extract in the repair assay. After incubation isolated plasmids were linearized and separated on agarose gels. Upper panels show ethidium bromide-stained gels and lower panels are autoradiograms of the dried gels. The positions of non-damaged and damaged substrates are indicated by a minus (−) and a plus (+). (A) Plasmids exposed to 450 J/m^2 of UV light; (B) plasmids exposed to 450 J/m^2 UV and further processed using Nth protein; (C) plasmids exposed to AAF.

Table I. Residual activity of the NER mutants *in vitro*

CG	Cell strain	Residual activity (% of wild type)[a]	
1	43-3B	27 ± 4	(*n* = 13)
	UV85	22 ± 4	(*n* = 10)
2	UV5	21 ± 3	(*n* = 11)
3	27-1	23 ± 3	(*n* = 20)
4	UV41	28 ± 7	(*n* = 5)
	UV47	17 ± 3	(*n* = 6)
5	UV135	24 ± 4	(*n* = 9)
6	14-60-23B	116 ± 19	(*n* = 3)
11	UVS1	24 ± 4	(*n* = 6)
XP-A	XP12RO-SV	15 ± 5	(*n* = 5)
XP-F	XP2YO	16 ± 9	(*n* = 3)

[a]Mean ± SEM (*n* = number of experiments).

induces oxidative thymine species as a minor UV lesion, which is efficiently recognized by a NER-independent endonuclease activity present in human extracts. Plasmids can be cleared from such damage by treatment with the *Escherichia coli* Nth protein (Wood *et al.*, 1988). Using a 'cleaned' UV-irradiated substrate [UV(*nth*)] a clear difference between the repair activities of wild-type CHO9 and mutant 43-3B extracts became apparent (Figure 1B). Similar results were detected with plasmids damaged by *N*-acetoxy-2-acetyl-aminofluorene (AAF) (Figure 1C), which causes almost exclusively *N*-(guanin-8-yl) adducts (Landegent *et al.*, 1984). Apparently, the endonuclease-triggered activity is very potent in CHO extracts, necessitating the use of AAF or UV(*nth*) substrates to measure NER activity. The observed levels of repair synthesis of human and Chinese hamster repair-proficient protein extracts, when expressed as fmol ^{32}P incorporated per ng plasmid, were in the same range and linearly dependent on the amount of extract added (data not shown).

As in CG1, a clear defect in *in vitro* NER is present in the tested rodent CG2, 3, 4, 5 and 11, as well as in XP-A and XP-F (Table I), in agreement with their inability to incise UV damage *in vivo*. In an extract derived from CG6 a normal response was observed, in line with the near normal

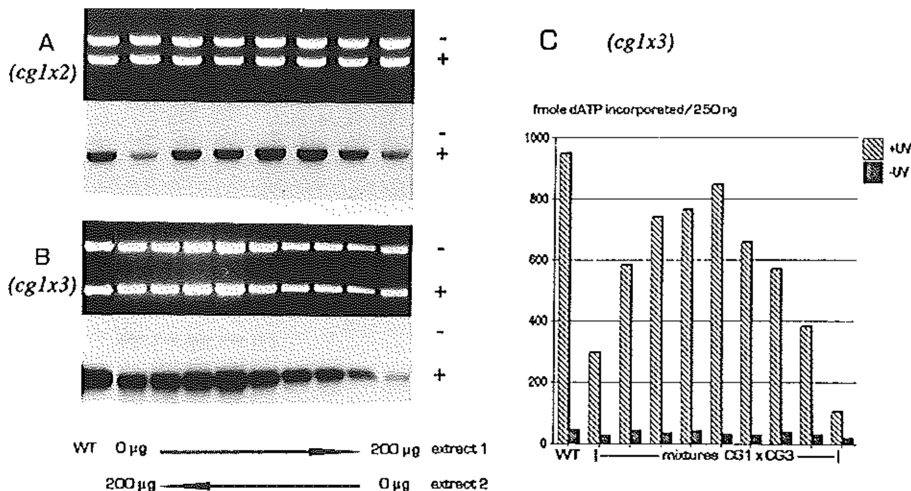

Fig. 2. Complementation of repair activity in mixtures of CG1 versus CG2 (A) and CG1 versus CG3 (B and C). In both mixtures UV(*nth*) damaged plasmids were used. Extracts were mixed in different ratios totalling 200 µg of protein. Increments were 25 and 30 µg in (A) and (B/C), respectively. Cell extracts used are in (A) 43-3B (CG1) and UV5 (CG2) and in (B/C) 43-3B (CG1) and 27-1 (CG3), extracts 1 and 2, respectively. First lanes in each panel contain 200 µg of CHO9 extract (WT). Because the amounts of recovered DNA in (B) are not completely equal in all lanes, a bar graph of this experiment is shown in (C), representing normalized data obtained after liquid scintillation counting of the ^{32}P incorporation and scanning of the DNA fluorograms.

level of unscheduled DNA synthesis and incision in cultured cells (Thompson *et al.*, 1988). Some variation of residual activity in separate extracts of these NER-deficient mutants was found, ranging from 15 to 30% of the wild-type response. We conclude that NER can be detected readily *in vitro* using rodent cell-free extracts and that this repair requires the active presence of the products of the *ERCC1, 2, 3, 4, 5* or *11* genes, in addition to those of *XPAC* and *XPFC*.

In vitro complementation analysis

Since *in vitro* complementation with human extracts can be achieved by mixing representatives of different CGs of XP (Wood *et al.*, 1988), it is expected that mutant extracts from various rodent CGs should complement each other as well. To determine the complementation patterns of the various Chinese hamster CGs in more detail, CG1, 2, 3, 4, 5 and 11 were tested, together with human CGs XP-A and XP-F. In view of the normal levels of repair activity *in vitro*, CG6 could not be used for analysis.

First, extracts from CG1 and 2 or CG1 and 3 were combined in various proportions and *in vitro* NER was measured. A substantial, ratio-dependent increase of repair synthesis was found with maximal repair activity around a mixing ratio of 1:1 (Figure 2). Restoration of repair synthesis was observed in the damaged plasmids only. Some variation in different experiments was experienced, but the presence of correction was unequivocal.

Then, proportional mixing experiments were conducted with other CGs. The results from a number of typical tests are shown in Figure 3 and the complete data set is summarized in Table II. Clear complementation was observed in any mutual combination of CG2, 3, 5 and XP-A and in combination with extracts from CG1, CG4 and CG11. Unexpectedly, pairwise mixing of representatives of CG1, CG4 or CG11 did not yield levels of repair synthesis significantly different from those in the deficient extracts alone (Figure 3). Similarly, XP-F extracts failed to complement CG1, 4 and 11, whereas clear correction was observed with CG2 and CG3. This pattern of non-complementation was consistently observed using independent isolates and with different representatives of CG1 and CG4 (Table II). These results indicate absence of correction in all combined extracts of CG1, CG4, CG11 or XP-F.

Apparent molecular weight of ERCC1 protein on glycerol gradients

The consistent pattern of non-complementation *in vitro* between groups 1, 4, 11 and XP-F suggests that the corresponding proteins somehow affect each other; the presence of a defect in one has an effect on the activities of the others. Such a situation may occur, for instance, when these gene products form a protein complex *in vivo*, of which the subunits cannot exchange under the conditions of the *in vitro* assay. To investigate this possibility, a NER-proficient HeLa extract was size-fractionated on a 10–30% glycerol gradient. To minimize effects of buffer and salt the gradient was run in the conditions of the repair assay together with internal mol. wt markers. The presence of ERCC1 protein in gradient fractions was monitored by SDS–PAGE and immunostaining with a specific anti-ERCC1 antiserum (for characterization of the antiserum see below). ERCC1 runs on SDS gels with an apparent mol. wt of 39 kDa, which is larger than the calculated mol. wt of 33 kDa. This is probably due to the proline-rich N-terminus, since truncated ERCC1 missing the N-terminal segment migrates at the

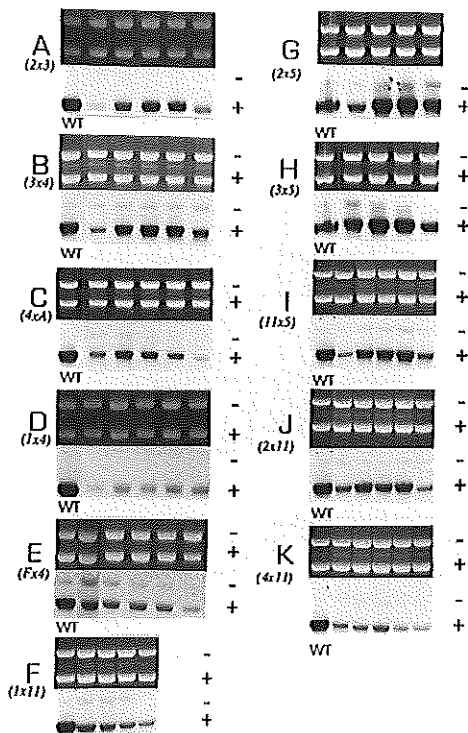

Fig. 3. *In vitro* complementation analysis. Substrate plasmids are damaged (+) with AAF (B, C and F–K) or with UV(*nth*) (A, D and E). Panels consist of an ethidium bromide fluorogram (top) and an autoradiogram (bottom). Each first lane (left) represents wild-type extract, either HeLa (E) or CHO9 (other panels), taken as an internal control. Second and last lanes are mutant extracts alone (200 μg). The lanes in between are mixtures of extracts in different ratios of extract 1 (left) and extract 2 (right), with a total of 200 μg of protein. Extracts 1 versus 2 are: (A) UV5 (CG2) versus 27-1 (CG3); (B) 27-1 (CG3) versus UV47 (CG4); (C) UV41 (CG4) versus XP12RO (XP-A); (D) UV85 (CG1) versus UV41 (CG4); (E) XP2YO (XP-F) versus UV47 (CG4); (F) UV85 (CG1) versus UVS1 (CG11); (G) UV5 (CG2) versus UV135 (CG5); (H) 27-1 (CG3) versus UV135 (CG5); (I) UVS1 (CG11) versus UV135 (CG5); (J) UV5 (CG2) versus UVS1 (CG11); (K) UV41 (CG4) versus UVS1 (CG11).

predicted position (data not shown). The profile of the glycerol gradient depicted in Figure 4 showed that, under native conditions, the protein sediments as a uniform band between the 67 and 149 kDa mol. wt markers, peaking on the position corresponding to ~120 kDa. Although non-specific aggregation of the ERCC1 protein cannot be completely excluded, the overall protein patterns analysed on SDS–PAGE did not show any indication of this. These results are consistent with the idea that ERCC1 resides in a complex.

ERCC1 protein produced by E.coli fails to correct CG1 extracts in vitro

Human ERCC1, overproduced in *E.coli* as a ubiquitin–ERCC1 fusion protein, was tested for *in vitro* complementation. Details of the overexpression constructs will be published elsewhere (M.H.M.Koken, H.Odijk, M.Van Duin, M.Fornerod and J.H.J.Hoeijmakers, in preparation). The ubiquitin moiety can be removed either by a specific ubiquitin lyase (UBP1 protein) from *S.cerevisiae* (Tobias and Varshavsky, 1991) or by ubiquitin-specific lyase activity present in Manley-type extracts incubated under the conditions of the repair assay (Figure 5A, lanes 1–6). The free full-length ERCC1 protein migrated at 39 kDa in SDS–PAGE (Figure 5A) at the same position as ERCC1 protein synthesized in a reticulocyte lysate *in vitro* (Figure 5C, lane 6).

Purified cleaved ERCC1 protein or fusion protein (either crude or partially purified) was added in excess to CG1 extracts. In none of these cases was significant correction observed. The presence of inhibiting factors in the ERCC1 protein preparation was ruled out by adding these proteins also to repair-competent extracts. It appears that in these *in vitro* conditions ERCC1 protein on its own cannot participate in the repair reaction.

Characterization of anti-ERCC1 antibodies

To study the possibility that ERCC1 is involved in a complex, we needed to do experiments at the enzymological level. Therefore a polyclonal antiserum was raised in rabbits against the ubiquitin–ERCC1 protein and affinity-purified on a column carrying immobilized fusion protein. The antisera were characterized in the following ways. First, Western blot analysis visualized a protein band with the exact mol. wt of ERCC1 on SDS–PAGE in crude lysates and Manley-type extracts of HeLa cells (Figure 5D, lanes 4, 5, 7 and 8). This band was not recognized by the preimmune serum (Figure 5D, lanes 1 and 2) and the reaction could be competed by partially purified ERCC1 fusion protein.

Table II. Summary of *in vitro* complementation data[a]

CG→		XP-F	XP-A	11	5	4		3	2
I	Strain	XP2YO	XP12RO	UVS1	UV135	UV41	UV47	27-1	UV5
1	43-3B	–	+	–	+	–	–	+	+
	UV85	–	+	–	+	–	–	+	+
2	UV5	+	nd	+	+	+	+	+	
3	27-1	+	+	+	+	+	+		
4	UV47	–	nd	–	+				
	UV41	nd	+	–	nd				
5	UV135	nd	+	+					
11	UVS1	–	nd						
XPA	XP12RO	+							

[a] +, complementation; –, non-complementation; nd, not determined.

No cross-reaction was observed with ubiquitin or with the ERCC1 protein from bovine or Chinese hamster origin (Figure 5D, lanes 6 and 9). Secondly, the antiserum was able to immunoprecipitate ^{35}S-labelled ERCC1 protein, synthesized *in vitro* by reticulocyte lysate (Figure 5C, lanes 2–5). Finally a complete abolition of UV-induced unscheduled DNA synthesis was observed after micro-

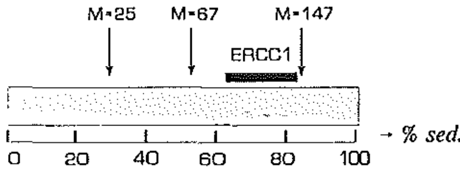

Fig. 4. Mol. wt determination of ERCC1 by sedimentation analysis. Active HeLa extract was layered, together with mol. wt standards, on a non-denaturing glycerol gradient and sedimented. The markers are chymotrypsinogen A (25 kDa), albumin (67 kDa) and aldolase (147 kDa). Gradient fractions were analysed on SDS–PAGE to determine the marker positions and immunoblotted to assess the position of ERCC1.

injection of the antibodies into repair-proficient human fibroblasts, whereas the preimmune serum did not have any effect (data not shown). These results indicate that the antibodies are able to react specifically with ERCC1 *in vivo* as well as *in vitro*.

ERCC1 antiserum depletes a repair-competent HeLa extract from ERCC1, 4, 11 and XPFC

In view of the species specificity of the antiserum, a NER-competent extract from HeLa cells was used to study the effect of removal of ERCC1 protein in the *in vitro* repair reaction. To this end antiserum was immobilized on protein A–Sepharose beads prior to incubation with the HeLa extract. After removal of the beads by centrifugation the supernatant exhibited a significantly decreased *in vitro* NER activity (Figure 6A). No such effect was detected when preimmune serum was used even at a 50× higher concentration (Figure 6B). We conclude that ERCC1 protein can be removed from a human wild-type extract using specific antibodies, resulting in a lowered repair activity comparable with the level of a CG1 mutant.

The HeLa extract depleted of ERCC1 (HeLa^{-ERCC1}) was

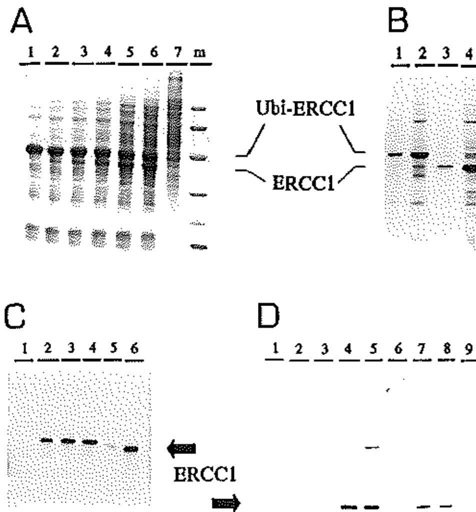

Fig. 5. Production of ubiquitin–ERCC1 fusion protein and characterization of anti-ERCC1 antibodies. (**A**) SDS–PAGE analysis of crude soluble lysate of ERCC1-overproducing *E.coli* incubated with increasing amounts of HeLa Manley-type extract. Lanes 1–6, lysate reacted for 2 h with 0, 15, 30, 45, 60 or 75 μg of HeLa extract; lane 7, 75 μg of HeLa extract alone; lane m, mol. wt markers: phosphorylase b (97 kDa), albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20 kDa) and α-lactalbumin (14 kDa). (NB the released ubiquitin of 8 kDa has run off the gel.) (**B**) SDS–PAGE analysis of lysate from ERCC1 fusion protein, partially purified by 33% saturated ammonium sulfate precipitation. Lanes 1–2, not treated with lyase, 0.7 and 35 μg of lysate; lanes 3–4, pre-treated with purified UBP1-lyase, 0.7 and 35 μg of lysate. (**C**) SDS–PAGE and autoradiography of immune-precipitated [^{35}S]ERCC1 translated *in vitro*. Lane 1, preimmune serum, undiluted; lanes 2–5, crude immune serum, diluted 1×, 10×, 100× and 1000×, respectively; lane 6, input amount of [^{35}S]ERCC1. (**D**) Specificity of ERCC1 antiserum on immunoblots of different cell extracts. Lanes 1–3, preimmune serum; lanes 4–6, crude immune serum; lanes 7–9, affinity-purified anti-ERCC1 antibodies; amounts used are equivalent to lanes 4–6. The extracts analysed are total HeLa cell sonicate (lanes 1, 4 and 7), Manley-type extracts of HeLa (lanes 2, 5 and 8) or CHO9 (lanes 3, 6 and 9).

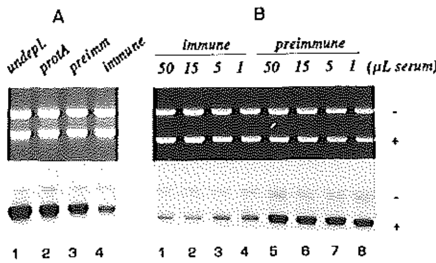

Fig. 6. Depletion of HeLa extract using anti-ERCC1 antibodies. Serum was immobilized on protein A−Sepharose beads; after incubation with repair-competent HeLa extract and centrifugation, the supernatant was tested for repair activity *in vitro*. (A) 10 μl of serum was used for depletion of 150 μg HeLa extract. Lane 1, undepleted extract (100 μg); lane 2, extract treated with empty protein A beads (90 μg); lane 3, extract depleted with pre-immune serum (80 μg); lane 4, extract depleted with immune serum (110 μg). (B) Various concentrations of sera were used to deplete 150 μg of HeLa extract. Lanes 1−4, anti-ERCC1 serum (50, 15, 5 and 1 μl respectively); lanes 5−8, pre-immune serum (50, 15, 5 and 1 μl respectively).

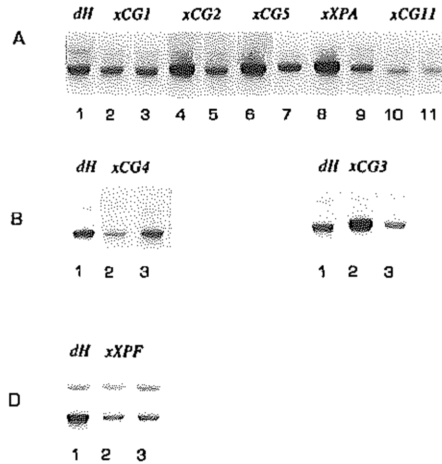

Fig. 7. Complementation analysis with ERCC1-depleted HeLa extract. 7.5 μl of anti-ERCC1 serum used for depletion. Different panels represent independent experiments. Plasmid recoveries are similar within 10% in each experiment. (A) Lane 1, $HeLa^{-ERCC1}$ alone; lane 2, 1:1 mix with UV85 (CG1); lane 3, UV85 alone; lane 4, mix with UV5 (CG2); lane 5, UV5 alone; lane 6, mix with UV135 (CG5); lane 7, UV135 alone; lane 8, mix with XP12RO (XPA); lane 9, XP12RO alone; lane 10, mix with UVS1 (CG11); lane 11, UVS1 alone. (B) Lane 1, $HeLa^{-ERCC1}$ alone; lane 2, 1:1 mix with UV47 (CG4); lane 3, UV47 alone. (C) Lane 1, $HeLa^{-ERCC1}$ alone; lane 2, 1:1 mix with 27-1 (CG3); lane 3, 27-1 alone. (D) Lane 1, $HeLa^{-ERCC1}$ alone; lane 2, 1:1 mix with XP2YO (XP-F); lane 3, XP2YO alone. In (B) and (C) controls for positive complementation taken along in these experiments are not shown, but gave results identical to lanes A4 and D2.

then tested for the ability to correct NER-deficient Chinese hamster mutants in mixing experiments. As expected, no complementation was detected when $HeLa^{-ERCC1}$ was mixed with a representative of CG1. However, when an extract of CG2, CG3, CG5 or XP-A was added to $HeLa^{-ERCC1}$ a significantly higher repair synthesis was observed (Figure 7A and C), indicating that the antibodies cannot remove the correcting activities of these groups. In contrast, $HeLa^{-ERCC1}$ failed to restore repair in mixtures with extracts of CG4, CG11 and XP-F (Figure 7B, A and D), showing that in addition to ERCC1 protein, correcting activities of CG4, CG11 and XP-F are concomitantly removed from the wild-type extract. Both crude and affinity-purified antisera were equally capable of depleting HeLa extract from ERCC1, 4, 11 and XPFC.

Discussion

In vitro repair studies in Chinese hamster NER-deficient mutants

In agreement with findings of others (Biggerstaff and Wood, 1992) we have observed that extracts isolated from cultured Chinese hamster cells can support NER of DNA damage *in vitro*. DNA substrates with a well-defined and narrow damage spectrum can be used in this assay, provided they are extensively purified. Clearance of UV-damaged plasmids from oxidative thymine species by pretreating with bacterial Nth protein proves to be an absolute requirement when Chinese hamster cell extracts are used, due to an apparently high endonuclease III-like activity in these cells.

The NER deficiency of CG1, 2, 3, 4, 5 and 11 *in vitro* agrees with the low unscheduled DNA synthesis levels (Van Duin *et al.*, 1988) and low incision capabilities of these cell strains in culture. In CG6, which is only moderately sensitive to UV, the repair activity is in the range of NER-proficient extracts. The ERCC6 gene is defective in CS-B (Troelstra *et al.*, 1992), where preferential repair of actively transcribed strands is impaired, but overall genome repair, which is responsible for the bulk of repair synthesis, is unaffected (Venema *et al.*, 1990). The normal *in vitro* repair rates are likely to reflect overall repair, since transcription is not taking place in the reaction conditions. Our finding that, *in vitro*, human and hamster repair activities are similar, contrasts with the low activity of overall repair in cultured rodent cells in comparison with human cells (Bohr and Hanawalt, 1987; Lommel and Hanawalt, 1991). It follows that the factors responsible for this difference do not play a role in the *in vitro* reaction, perhaps because they are related to chromatin structure.

Extracts of CG2, 3 and 5 are able to complement each other and representatives of all other CGs tested. This suggests that the proteins which are missing or defective in these CGs are available in the complementing extracts and can freely interact to ensure restoration of normal repair activity. The findings that XP-F can correct CG2 and CG3 and that XP-A complements CG3 and CG5 show that human and Chinese hamster repair proteins are sufficiently conserved and interchangeable *in vitro*. This is in line with the correction *in vivo* by transfection of human genes into the Chinese hamster mutants.

Evidence for a repair enzyme complex

Mixtures of CGs 1, 4, 11 and XP-F do not show restoration of repair activity *in vitro*, whereas they do *in vivo* after cell hybridization. Since non-complementation occurred with independent mutants from CG1 and CG4, this unexpected phenomenon is unlikely to be related to an allele-specific behaviour. Our data indicate that the normal gene products needed for correction are either unable to exchange *in vitro* or are not present in the mixtures. Such a situation can occur

when these correcting proteins are part of a pre-existing stable complex. Depletion of a wild-type extract using specific anti-ERCC1 antibodies not only results in the loss of CG1 complementing activity, but concomitantly removes the activity which is capable of correcting CG4 and CG11 or even XP-F. This strongly suggests that such a complex does exist in wild-type extracts. This is further supported by the finding that human ERCC1 protein, which has an apparent mol. wt of 39 kDa in denaturing SDS gels, sediments in glycerol gradients as a much larger species.

The absence of complementation in some combinations indicates that the *in vitro* assay cannot replace classical cell fusion as a system for complementation analysis. To explain these non-complementation patterns *in vitro*, several mechanisms can be envisaged. (i) Reconstitution of a complex from its components may not be possible under *in vitro* conditions. An inactive complex harbouring a tightly bound defective protein in one mutant extract will be unable to complement another mutant, in which the same complex is crippled by the presence of another impaired component. (ii) Alternatively, or in addition, dependent on the type of mutation in the cell strain, the complex may be required for stability of its components *in vivo*. In that case cell-free extracts from CG1 lacking ERCC1 would be also deficient in ERCC4 and the other proteins, and vice versa. Indirect support for this idea comes from studies with 41D, a CG1 cell strain containing an amplified correcting human *ERCC1* gene. Whereas at DNA and mRNA levels *ERCC1* is amplified in this strain 100- to 1000-fold, the amount of ERCC1 protein is enhanced only by a factor of three and many degradation products become apparent on immunoblots with anti-ERCC antibodies. In addition, micro-injection into human fibroblasts of an excess of ERCC1 protein, over-produced in *E.coli*, results in unusually rapid (<1 h) degradation *in vivo* as judged by immunofluorescence (our unpublished results). These data suggest that ERCC1 protein on its own may not be very stable in the cell, which is in agreement with our observation that addition of an excess of purified ERCC1 protein fails to restore repair activity in CG1 extracts.

Involvement of the xeroderma pigmentosum group F gene product

Extracts from a human XP-F cell strain fail to complement Chinese hamster extracts of CG1, CG4 and CG11 *in vitro*. This pattern of non-complementation is group specific, as efficient correction of repair is found with other CGs. In addition, the XP-F extract is not able to correct the ERCC1-depleted HeLa extract, indicating that the XPF-correcting activity is also part of the postulated complex. Well-controlled cell fusions *in vivo* between human XP-F cells and Chinese hamster NER-deficient mutants, being complicated by poor interspecies fusion and chromosome loss in the resulting hybrids, have not been done so far, so there is no *in vivo* correlation of our *in vitro* results. Direct involvement of ERCC1 in XP-F is ruled out by the inability of the *ERCC1* gene to correct XP-F cells *in vivo* (Van Duin *et al.*, 1989). Therefore, ERCC4 and ERCC11 are possible candidates for the gene defective in XP-F. This would represent another overlap between Chinese hamster mutants and human repair disorders. Alternatively, XPFC might also encode a distinct protein, which may be less likely in view of gradient sedimentation data.

The relation between ERCC4 and ERCC11

The complex between the ERCC1 and ERCC4 proteins also contains the correcting activities of CG11 and XP-F. The latter two mutants do not show the extreme sensitivity to DNA crosslinking agents characteristic for representatives of CG1 and 4. However, absence of mitomycin C sensitivity has also been noted in some ERCC1 mutants, whereas the sensitivity to UV has been retained (J.Van de Berg and J.H.J.Hoeijmakers, unpublished results). It is possible that such relatively mild mutations are present in CG11 and XP-F as well. Since the strain UVS1 is the sole representative of CG11 isolated so far, one has to consider also the theoretical possibility that the complementation in cell hybrids of CG11 with CG1 or CG4 mutants is of the intra-allelic type. Our (unpublished) observation that transfected *ERCC1* cDNA is unable to correct UVS-1 cells definitely rules out involvement of *ERCC1*. On the other hand, complementation has been shown after cell fusion with UVS1 and the two independent CG4 mutants UV47 (Hata *et al.*, 1991) and UV41 (D.Busch, personal communication), which makes intra-allelic complementation less likely. Although such patterns of complementation have never been observed in the numerous fusions between either Chinese hamster or human (XP) repair-deficient mutant cells, a definitive answer must await the isolation of the *ERCC4* or *ERCC11* gene and its transfection into the reciprocal mutants.

Possible functions of the complex

The homologue of *ERCC1* in *S.cerevisiae* is the *RAD10* gene (Van Duin *et al.*, 1986). This protein can form a very stable complex with the *RAD1* gene product, both *in vivo* and *in vitro* (Bailly *et al.*, 1992; Bardwell *et al.*, 1992), explaining the similar phenotypes of the yeast *RAD1* and *RAD10* mutants, having a defect in NER as well as in mitotic recombination (Schiestl and Prakash, 1988, 1990). Also *ERCC1* and *ERCC4* mutants share unique features, such as extreme sensitivity to crosslinking agents. Therefore, it is conceivable that the RAD1–RAD10 complex and the mammalian repair complex identified here are equivalent and have similar functions. The studies in yeast do not exclude the possibility that additional proteins are part of the RAD1–RAD10 complex, which is relevant in view of our findings that ERCC11 and/or XPFC correcting activities also appear to be present in the mammalian complex. In this idea, the yeast *RAD1* gene becomes a good candidate for being the homologue of one of the mammalian genes *ERCC4*, *ERCC11* or *XPFC*.

Combining these data, we postulate that the mammalian enzyme complex has a dual function, as has its homologue in yeast. Deficiency of the NER function results in a ~5- to 10-fold increased sensitivity to UV light and other chemical agents inducing bulky DNA adducts. Such a sensitivity is also found in XP-A cells, where NER is fully impaired due to the complete inactivation of the XPAC protein (Tanaka *et al.*, 1990). On top of the moderate sensitivity for crosslinks (such as found in XP-A cells), the additional participation of ERCC1 and ERCC4 proteins in a recombinational repair pathway might explain the extreme (80- to 100-fold) sensitivity of the corresponding mutants to crosslinking agents. Obviously, interstrand crosslinks may require additional recombination steps for their removal.

Note added

In the course of manuscript preparation we became aware of recent results obtained by M.Biggerstaff, D.E.Szymkowski and R.D.Wood, indicating an association between CG1, CG4 and XP-F correcting activities and showing non-complementation in extracts mixtures obtained from these CGs (Biggerstaff *et al.*, 1993).

Materials and methods

Plasmid DNA

Plasmids pBluescript KS$^+$ (pBKS, 3.0 kb), pTZ19R (3.0 kb), pHM14 (3.7 kb) and pSLM (4.3 kb) were isolated from *E.coli* RecA$^-$ hosts by alkaline lysis and purified twice on a CsCl gradient. pBKS and pTZ19R were either exposed to 450 J/m^2 of UV light of predominantly 254 nm or treated with 0.1 mM *N*-acetoxy-2-acetylaminofluorene (AAF) (a kind gift of R.Baan, TNO, Rijswijk), inducing *N*-(guanin-8-yl) AAF adducts. pBKS plasmid was treated with Nth protein from *E.coli* (generously provided by C.J.Jones, ICRF, London) after UV irradiation and extensively purified as closed circular DNA on neutral sucrose gradients as described by Wood *et al.* (1988) and Biggerstaff *et al.* (1991).

AAF-modified plasmids were collected by repeated di-ethyl-ether extractions and ethanol precipitation (Landegent *et al.*, 1984) and also repurified on a sucrose gradient. In both cases pHM14 or pSLM plasmids were mock-treated in parallel. The average numbers of lesions per damaged plasmid molecule were 10–12 pyrimidine dimers and 15–20 AAF–guanine adducts, respectively.

Cell lines and extracts

Mutant Chinese hamster cells, hypersensitive to UV irradiation or other mutagenic agents, were isolated from either the wild-type CHO strains AA8 (Busch *et al.*, 1980) or CHO9 (Wood and Burki, 1982). These mutants have been assigned to complementation group 1 (43-3B, UV85), group 2 (UV5), group 3 (27-1), group 4 (UV41, UV47), group 5 (UV135) (Busch *et al.*, 1989), group 6 (14-60-23B) (D.Busch, personal communication) and group 11 (UVS1) (Hata *et al.*, 1991; Riboni *et al.*, 1992; Numata *et al.*, 1993). XP12RO(SV40) and XP3YO(SV40) are SV40-transformed lines belonging to XP CGs A and F, respectively. HeLa and CHO9 cells served as repair-competent controls.

Cells were cultured in 850 cm^2 plastic roller bottles or in 265 cm^2 glass Petri dishes in a 1:1 mixture of Ham's F10 and DMEM medium (Gibco) supplemented with 10% fetal calf serum and antibiotics. Cells were harvested by trypsinization and washed with phosphate-buffered saline (PBS). Extracts were prepared from 2–5 ml of packed cell pellet by the method of Manley *et al.* (1983) as modified by Wood *et al.* (1988), dialysed in buffer A and stored at −80°C. Buffer A contained 25 mM HEPES/KOH pH 7.8, 0.1 M KCl, 12 mM $MgCl_2$, 1 mM EDTA, 2 mM dithiothreitol (DTT) and 17% (v/v) glycerol.

In vitro repair assay

The standard reaction mixture (50 µl) contained 250 ng damaged pBKS and 250 ng pHM14 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 mixtures were incubated for 3 h at 30°C. Plasmid DNAs were isolated, linearized by restriction and electrophoresed on an agarose gel. Data were analysed by autoradiography and quantified by scintillation counting of DNA bands excised from dried gel.

Anti-ERCC1 antibodies and immunoblotting

Human ERCC1 protein was overproduced as a ubiquitin–ERCC1 fusion protein in *E.coli*. The ubiquitin part is thought to protect the N-terminal part of the ERCC1 protein against degradation. The pETUBI..ERCC1 vector contained a strong inducible T7 promoter and was transformed to the bacterial host strain BL21(DE3)LysS (M.H.M.Koken *et al.*, submitted).

A polyclonal anti-ERCC1 antiserum was raised in rabbits by injection of gel-purified ubiquitin–ERCC1 fusion protein. For immunoblot analysis protein samples were separated on 11% SDS–polyacrylamide gel and transferred to a PVDF membrane in 25 mM Tris–HCl, pH 8.3 containing 20% methanol and 0.2 M glycine. The membranes were treated with non-fat milk containing 0.1% Tween 20 and sodium azide for at least 1 h and then incubated for 16 h with 1000× diluted antiserum at 4°C. Then the blots were washed with PBS containing 0.5% Tween 20 and incubated for 2 h at room temperature with alkaline phosphatase-conjugated goat anti-rabbit IgG. The blots were washed again and developed with the coloured substrates nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate.

The ERCC1 antiserum was affinity-purified using a protein A–Sepharose column for isolating the IgG fraction of the serum and an Affigel® 10 column (Bio-Rad) carrying immobilized purified fusion protein.

In vitro translation and immunoprecipitation

ERCC1 protein was translated *in vitro* using a rabbit reticulocyte lysate system as described by the manufacturer (Promega) using 50 µCi of [^{35}S]methionine (1 mCi/mmol). Antiserum was incubated with ERCC1 protein for 2 h at 4°C in 100 mM NaCl, 50 mM Tris–HCl pH 7.5, 5 mM Na_2EDTA and 0.5% Triton (NETT buffer). The 10% protein A–Sepharose beads in NETT buffer containing 2% BSA and 0.02% sodium azide were added and tumbled for 1 h at 4°C. After centrifugation and washing four times in NETT buffer the immunoprecipitate was separated on 11% SDS–PAGE and the gel was dried and exposed to X-ray film.

Glycerol gradient sedimentation

The molecular weight of ERCC1 was determined on a 10–30% non-denaturing glycerol gradient in buffer A. NER-proficient HeLa extract (650 µg) was loaded, together with three markers: aldolase (147 kDa), albumin (67 kDa) and chymotrypsinogen A (25 kDa) and sedimented in a Beckman SW41 rotor at 280 000 *g* at 4°C for 24 h. After fractionation the presence of ERCC1 protein was monitored on immunoblots using anti-ERCC1 antibodies and horseradish peroxidase-conjugated second antibody, using a phosphorescence detection method.

Antibody depletion of NER-proficient extract

HeLa cell extract was treated with polyclonal anti-ERCC1 antiserum. Protein A–Sepharose CL-4B beads (protA) (70 µg) were washed three times in PBS and incubated with 10 µl anti-ERCC1 antibodies or preimmune serum for 15 min at 0°C. Then the beads were washed three times in buffer A and added to a repair-competent HeLa extract for 30 min at 0°C. The supernatant obtained after spinning down the beads was used as a depleted HeLa extract and tested in the *in vitro* repair assay.

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Chapter 3

Partial characterization of the DNA repair protein complex, containing the ERCC1, ERCC4, ERCC11 and XPF correcting activities

Partial characterization of the DNA repair protein complex, containing the ERCC1, ERCC4, ERCC11 and XPF correcting activities

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Abstract

The nucleotide excision repair (NER) protein ERCC1 is part of a functional complex, which harbours in addition the repair correcting activities of ERCC4, ERCC11 and human XPF. ERCC1 is not associated with a defect in any of the known human NER disorders: xeroderma pigmentosum, Cockayne's Syndrome or trichothiodystrophy. Here we report the partial purification and characterization of the ERCC1 complex. Immunoprecipitation studies tentatively identified a subunit in the complex with an apparent MW of ~120 kDa. The complex has affinity for DNA, but no clear preference for ss, ds or UV-damaged DNA substrates. The size of the entire complex determined by non-denaturing gradient gels (~280 kDa) is considerably larger than previously found using size-separation on glycerol gradients (~120 kDa). Stable associations of the ERCC1 complex with other known repair factors (XPA, XPC, XPG and TFIIH complex) could not be detected.

Keywords: nucleotide excision repair; ERCC1 protein complex; 120 kDa subunit; immunoprecipitation; purification

1. Introduction

Environmental agents such as UV and X-rays or chemical compounds can induce a wide range of lesions in the DNA. Persisting lesions can interfere with essential processes like transcription or replication, which might cause cell death or lead to mutations. To protect the genetic information in the cell a network of DNA repair mechanisms has evolved in all living organisms. In nucleotide excision repair (NER), a major repair pathway, five steps can be identified: recognition of the DNA injury, dual incision on the damaged strand (Huang et al., 1992), removal of the damaged-containing patch, gap refilling by DNA synthesis and finally strand ligation. (Grossman and Thiagalingam, 1993; Hoeijmakers, 1993a; Sancar and Tang, 1993).

A NER deficiency can give rise to three genetically heterogeneous human disorders: xeroderma pigmentosum (XP) (seven genetic complementation groups: XP-A to XP-G), Cockayne's syndrome (two groups: CS-A and CS-B), and a photosensitive form of the brittle hair disease trichothiodystrophy(TTD)(Hoeijmakers, 1993b). Patients suffering from this subtype of TTD have been classified in three complementation groups: TTD-A (Stefanini et al., 1993), XP group D (Stefanini et al., 1992), and one TTD family is assigned to XP group B (Vermeulen et al., 1994). In addition to cell lines of these patients, a large collection of UV-sensitive mutants has been obtained from rodent cell cultures, in which eleven complementation groups have been identified. A number of correcting human genes has been isolated by gene transfer experiments, designated as *e*xcision *r*epair *c*ross-*c*omplementing (*ERCC*) genes (Busch et al., 1989; Riboni et

al., 1992; Collins, 1993). Most of these *ERCC* genes have been found to be involved in the human NER disorders as well: *ERCC2* is identical to *XPD* (Flejter et al., 1992), *ERCC3* is *XPB* (Weeda et al., 1990), *ERCC5* is *XPG* (O'Donovan and Wood, 1993; Scherly et al., 1993) and *ERCC6* is identical to *CSB* (Troelstra et al., 1992). However, *ERCC1* is not affected in any of the known human complementation groups (van Duin et al., 1989).

The eukaryotic NER genes are highly conserved during evolution. As an example, *ERCC1* shares sequence homology with the *Saccharomyces cerevisiae* NER gene *RAD10*. The RAD10 gene product forms a stable complex with the yeast RAD1 protein (Bailly et al., 1992; Bardwell et al., 1992), which is required for both NER and mitotic recombination (Schiestl and Prakash, 1988; Schiestl and Prakash, 1990). The complex may perform the 5' incision in the NER process, since it has a structure-specific endonuclease activity that cleaves a splayed arm DNA structure only in the strand with a 3' single end (Bardwell et al., 1994b). Mammalian ERCC1, like its yeast homolog, is also part of a protein complex, which harbours the correcting activities of the rodent groups 1, 4, 11 and human XP group F, as observed by co-purification and co-immunodepletion of these repair activities (Biggerstaff et al., 1993; van Vuuren et al., 1993).

Cosmid clones harbouring the functional *ERCC4* gene have been recently described, (Thompson et al., 1994), although no information is available on the encoded gene product. Also the *ERCC11* and *XPF* genes have not yet been cloned. Therefore, an enzymological approach has been chosen to characterize the ERCC1-complex. Here we report its partial purification from HeLa cells, the tentative identification of a 120 kDa subunit, and the possible associations with other repair factors.

2. Material and Methods

Gel-electrophoresis, immunoblotting and restriction enzyme digestion were performed according to standard procedures (Sambrook et al., 1989). The purification scheme was prepared according to Gerard et al. (1991).

2.1 Cell lines and extracts

Repair-competent cell lines: HeLa and Chinese hamster ovary (CHO9) as well as rodent NER mutants of complementation groups 1 (43-3B, UV20), 2 (UV5), 3 (27.1, UV24), 4 (UV41, UV47), 5 (UV135), and 11 (UVS1) and human XP group F (XP3YOSV) were cultured in a mixture of Ham's F10 and DMEM medium (1:1), with 10% fetal calf serum and antibiotics (streptomycin and penicillin). 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) dialysed in buffer A and stored at - 80°C. Buffer A contains 25 mM Hepes-KOH pH 7.8, 0.1 M KCl, 12 mM $MgCl_2$, 1 mM EDTA, 2 mM DTT and 17% glycerol. Primary fibroblasts of XP-F (XP3YO) and XP-C (XP21RO) were cultured for microinjection in Ham's F10 medium supplemented with 12% serum and antibiotics.

2.2 In vitro DNA repair

Plasmid pBKS was treated with 0.1 mM N-acetoxy-2-acetylaminofluorene (AAF) and closed circular forms were isolated as described earlier (van Vuuren et al., 1993). Each reaction contained 250 ng of AAF-modified plasmids and 250 ng of non-damaged pHM14 plasmid as a control in 45 mM HEPES-KOH (pH7.8), 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 [^{32}P]αdATP, 2mM ATP, 40 mM phosphocreatine, 2.5 μg creatine phosphokinase, 3.5% glycerol, 18 μg bovine serum albumin, and 100 or 200 μg protein of the cell-free extracts. Samples were incubated 3 h at 30°C, then the DNA was isolated, linearized by BamH1 digestion and separated by electrophoresis on 0.8% agarose gel. Incorporation of [^{32}P]dATP in damaged and undamaged plasmids was detected by autoradiography and quantified using a B&L Phosphor-Imager and a LKB Densitometer.

2.3 Microinjection

Microinjection of XP fibroblasts was performed as previously described (Vermeulen et al., 1986). After injection cells were irradiated with UV-light (254 nm, 15 J/m^2), incubated for 2 h in [^{3}H]-thymidine-containing culture medium (10 μCi/ml; s.a.: 50 Ci/mmol) and fixed. Repair activity was detected by autoradiography. The number of silver grains above the nuclei is a measure for the level of unscheduled DNA synthesis (UDS).

2.4 Antibodies

Polyclonal antibodies were raised in rabbits against an *E.coli* overproduced human ubiquitin-ERCC1 fusion protein (Koken et al., 1993; van Vuuren et al., 1993) and against a C-terminal peptide of XPA. Anti-ERCC1 antiserum was purified on protein A-sepharose followed by an Affi-Gel 10 column carrying purified ubiquitin-ERCC1 fusion protein. Immunoblots were incubated with either crude anti-ERCC1 antiserum, crude anti-XPA antiserum, affinity purified anti-ERCC1 antiserum or with monoclonal antibodies (M3C9) against the p62 subunit of TFIIH. As a second antibody horse radish peroxidase- or alkaline phosphatase-conjugated goat anti-rabbit or goat anti-mouse Ig were used. These antibodies were detected by a chemiluminescent substrate ECL (Amersham) or nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate.

2.5 Immunoprecipitation experiments

HeLa cells were cultured 1 h in DMEM medium lacking methionine, supplemented with fetal calf serum (dialysed against phosphate-buffered saline (PBS)), and labelled with [^{35}S]-methionine (20μCi/ml; s.a. 1 mCi/mMol) for 16-20 h. Cells were harvested and washed twice with PBS and stored at -80°C. For immunoprecipitation the pellet was dissolved in IPB7 buffer (20 mM Triethanolamine pH 7,8, 0.7 M NaCl, 0.5% NP40 and 0.2% natrium deoxycholaat) and the solution was centrifuged for 15'. Afterwards the supernatant was incubated with anti-ERCC1 antibody beads for 16 h at 4°C with agitation. The beads were extensively washed in IPB7 buffer and PBS, dissolved in loading buffer, heated for 5' and the soluble proteins were separated on a 11% SDS-polyacrylamide gel. Protein bands were visualized by autoradiography of the dried gel.

2.6 Purification of ERCC1 complex

Chromatographic materials were tested batch-wise or in columns. The following materials were used: Heparin-Ultrogel A4R, DEAE-Spherodex M, Phosphor Ultrogel-P A6R, and ss DNA Ultrogel A4R (IBF); Sulfopropyl SP-5PW, and TSK gel Heparin 5PW, and TSK gel HA-100 Hydroxylapatite (TosoHaas); Phenyl Sepharose, Octyl Sepharose, Sepharose CL-4B, Sephacryl-S300, and Q-sepharose Fast Flow (Pharmacia); ds DNA Cellulose, and ATP Sepharose (Sigma); Red Sepharose CL-4B, Blue Sepharose CL-4B, and UV-irradiated-DNA cellulose; Cellulose phosphate P11 (Whatman); Hydroxylapatite Spheroidal beads (Fluka). Hela extracts or partially purified ERCC1 fractions (Hep 10 or Sulfopropyl) were loaded.

2.7 Non-denaturing gel-electrophoresis

A 4-15% polyacrylamide gradient gel in TBE buffer and 12% glycerol was prepared (TBE: 90 mM Tris, 80 mM boric acid and 2.5 mM EDTA). The gel was prerun for 30 min at 70 V, loaded with samples and run for 2 h at 70 V and for 16-20 h at 150 V. Proteins included as molecular weight markers were ferritin (440 kDa), catalase (232 kDa), lactate dehydrogenase (140 kDa), and albumin (67 kDa). HeLa extracts or partially purified ERCC1 fractions were loaded and immunoblotted. The Westernblot was stained with Ponceau-S to visualize the molecular weight markers for determination of the apparent MW of the ERCC1 complex. There was a good relationship between the migration and the known MW of the markers. The ERCC1 comples was detected by antibodies. The ERCC1-containing band was cut out from the gel and incubated 30 min in 0.1% SDS, 50 mM boric acid pH 8.0, and electro-eluted for 16 h in 30 mM Tris and 200 mM glycine. The eluted proteins were concentrated on a Centricon-10 filter at 4 °C.

2.8 Endonuclease assay

Partially purified ERCC1 fractions were incubated with different DNA substrates: 200 ng ss-DNA (M13), ds-DNA (pHM14) or damaged DNA (pUR288-AAF) under *in vitro* repair conditions for 30 min at 37° C. Heparin 5PW fraction 10 with or without prior depletion of ERCC1 by immunobeads was used in a range of 1 to 30 μg protein. After the reaction, samples were treated with proteinase K, SDS and phenol extracted to de-proteinize. The DNA was isolated and separated on 0.8% agarose gels.

3. Results

3.1 Immunoprecipitation studies

Affinity-purified anti-ERCC1 antiserum described previously (van Vuuren et al., 1993) recognizes ERCC1 as the only band on a Western blot of a HeLa cell-free extract, (prepared according to Manley et al., 1983) (Figure 1D, lane 7). Antibodies were immobilized on protein A-sepharose beads and washed prior to incubation with a total [^{35}S]-methionine labelled HeLa extract. A protein band, migrating at the position of *in vitro* translated ERCC1 protein (39 kDa in SDS-PAGE), was precipitated by crude or affinity-purified anti-ERCC1 antiserum among a number of aspecific polypeptides, but not by preimmune serum (Figure 1A). ERCC1 appeared as a minor band on the autoradiogram, presumably due to its low expression level (van Duin et al., 1986) and the low specific labelling due to the presence of only two internal methionines in the protein. Background bands could not be reduced significantly by pretreatment of the antibodybeads with a non-labelled HeLa extract (Figure 1C; lane 1). In addition to ERCC1, at least one polypeptide with an apparent molecular weight of ~120 kDa was co-precipitated by the ERCC1 immunobeads (Figure 1A; lane 2 and 3 indicated with *). This band as well as the ERCC1 band disappeared when the immunobeads were preincubated with an excess of purified ubiquitin-ERCC1 fusion protein overproduced in *E.coli* (Figure 1B; lane 2). Further evidence in favour of the 120 kDa protein being a subunit of the ERCC1 complex

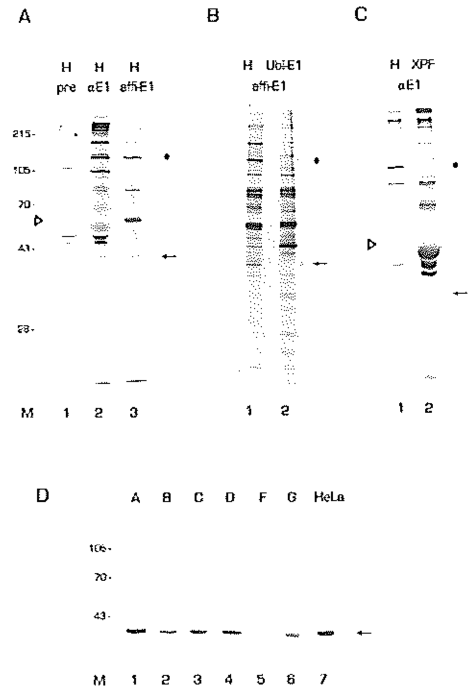

Fig. 1. Immunoprecipitation using anti-ERCC1 antibodies of a total [^{35}S]-labelled protein extract of HeLa cells and detection of ERCC1 in different XP cell strains. A: HeLa extract was precipitated with pre-immune serum (pre), with crude anti-ERCC1 antibodies (αE1) or with affinity-purified anti-ERCC1 antiserum (affi-E1). Both lane 1 (pre) and 2 (αE1) show a migration effect of the large amount of IgG in the samples, the heavy chain is indicated by an arrowhead. The ERCC1 protein is indicated as an arrow, the presumed 120 kDa subunit with an asterix. B: Competition experiment using affi-E1 beads which were pretreated with non-labelled recombinant ubiquitin-ERCC1 fusion protein (lane 2) in comparison to affi-E1 immunoprecipitation of HeLa extract (lane 1). C: Immunoprecipitation with labelled HeLa using αE1 competed with non-labelled HeLa extract to reduce aspecific binding (lane 1). Lane 2 shows a [^{35}S]-labelled XP-F extract that was precipitated with ERCC1-immunobeads. D: 10 μg protein of the cell-free extracts of respectively XP-A, XP-B, XP-C, XP-D, XP-F, XP-G and HeLa cells were analyzed for the presence of ERCC1 protein on immunoblot using affinity-purified anti-ERCC1 serum. Protein staining with Coommassie Brilliant Blue (CBB) indicated that similar amounts of protein were loaded in each lane (not shown).

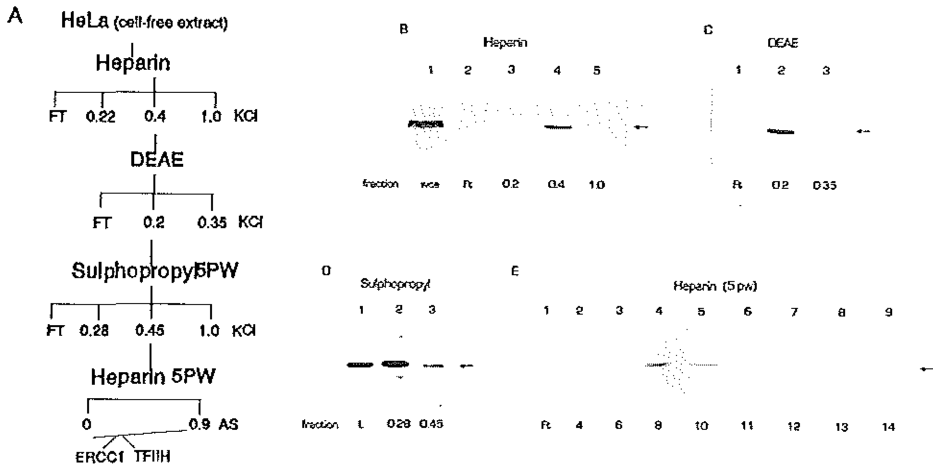

Fig. 2. Co-elution of the ERCC1 complex with transcription initiation factor TFIIH over the first chromatography columns. ERCC1 is visualized by immunoblot analysis. A: Purification scheme of TFIIH (Gerard et al., 1991). In panels B - E 30 μl of each fraction was loaded or 10 μg protein when the protein concentration is known. B: Heparin column, ERCC1 is present in the 0.4 M KCl elution fraction, C: DEAE column, ERCC1 is found predominantly in the 0.2 M KCl elution fraction, a minor amount in the 0.35 M fraction D: Sulfopropyl 5PW column, ERCC1 is detected in both 0.28 and 0.45 M KCl elution fractions (TFIIH is present in 0.45 M KCl fraction), E: Heparin 5PW column, the peak of ERCC1 is observed in fraction 8-10, whereas the peak of TFIIH is found in fraction 10-14.

was obtained by analysis of an XP-F extract. Immunoblot analysis of cell-free extracts from XP complementation groups A to D and G showed that ERCC1 is significantly reduced only in XP-F (Figure 1D). These results are in agreement with earlier findings (Biggerstaff et al., 1993). After precipitation of a [^{35}S]-labelled XP-F extract using ERCC1 immunobeads both ERCC1 and the 120 kDa band were no longer detectable, whereas the aspecific bands remained (Figure 1C; lane 2). These data suggest a close association between ERCC1 and the 120 kDa protein and might identify the latter as a subunit of the complex.

3.2 Partial purification of the ERCC1 complex.

An elaborated purification scheme, designed for isolation of active protein complexes, has been developed for the purification of mammalian basal transcription factors, including the multisubunit TFIIH complex (Gerard et al., 1991). TFIIH accommodates the repair-correcting activities of XPB, XPD and TTD-A (Drapkin et al., 1994; Schaeffer et al., 1994; van Vuuren et al., 1994; Vermeulen et al., 1994), illustrating that this procedure can be used for the purification of (repair) complexes. From this purification scheme (Figure 2A), which starts with a large amount of repair-competent HeLa extract, fractions of several chromatography steps were tested for the presence of ERCC1. Immunoblot analysis indicated that ERCC1 and TFIIH eluted in the same fractions on the first two chromatography columns: Heparin-Ultrogel (0.4 M KCl) and DEAE-Spherodex (0.2 M KCl) (Figure 2B-C). On Sulfopropyl 5PW ERCC1 was detected in the 0.28 and 0.45 M KCl fractions (Figure 2D). The latter contained the TFIIH complex and was loaded on a Heparin 5PW column. Gradient elution achieved an incomplete separation of

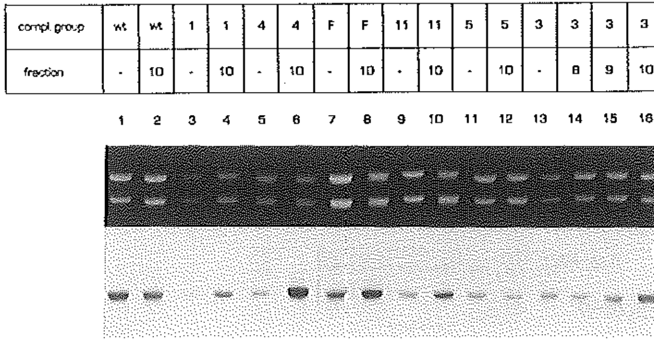

Fig. 3. Correction of the repair defect by ERCC1-containing Heparin 5PW fractions using an *in vitro* DNA repair assay. In all cases 6.2 μg of Heparin fraction 10 was added to 100 μg cell-free extract of rodent group 1, 4, 11, human XP group F or HeLa (even numbered lanes), no fractions were added in odd numbered lanes. The same amount of Heparin fraction 8, 9 or 10 was added to a rodent group 3 extract (lane 14-16). AAF-damaged plasmid is indicated as '+' and the non-damaged plasmid as '-'. The upper panel shows the ethidium bromide stained gel and the lower panel the autoradiogram.

ERCC1 and TFIIH. The peak of ERCC1 resides in fractions 8 to 10, just in front of the TFIIH-containing fractions 10 to 14 (Figure 2E), see also (Gerard et al., 1991).

The biological activity of the ERCC1-containing fractions was investigated utilizing the *in vitro* DNA repair assay based on cell-free extracts (Wood et al., 1988). The Sulfopropyl 0.28 M KCl elution containing ERCC1 but no significant fraction of TFIIH, induced complementation of the repair defect in extracts of rodent group 11 and human XP-F. Correction of the repair defect was also observed after administration of Heparin 5PW fractions 8-10 to extracts of rodent groups 1, 4, 11 and human XP-F, but not when added to a group 5 extract (Figure 3, showing the results of fraction 10). Addition of these fractions to a repair-proficient extract did not influence its NER activity, demonstrating that the stimulation of repair observed in the above mentioned extract was not due to an aspecific effect. Furthermore, the level of correction achieved was similar to that observed in the above mentioned extracts was obtained when the same extracts were mixed with a rodent complementation group 2 extract (data not shown, see van Vuuren et al., 1993). A small part of the TFIIH activity (known to peak in fraction 12 and 13 (Gerard et al., 1991)) was detected in fraction 10 and to a lesser extent in fraction 9, as measured by the ability to correct extracts of rodent group 3 (Figure 3, lane 14-16). These data were in agreement with immunoblot analysis and indicate again that ERCC1 and TFIIH complexes become separated in this stage

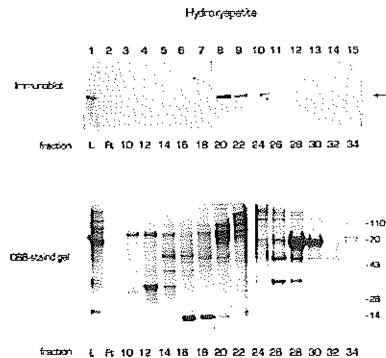

Fig. 4. Hydroxylapatite chromatography of ERCC1 containing Heparin 5PW fraction 10. The upper panel shows the presence of ERCC1 in fractions 20-24 (0.26 M phosphate buffer) by immunoblot analysis. The lower panel shows the protein profile of a CBB-stained gel.

of the purification, but an overlap of both activities was found in fraction 10. As an independent test for functionality *in vivo*, the Heparin 5PW fractions 8-10 and the 0.28 KCl fraction of Sulfopropyl were microinjected into XP fibroblasts.

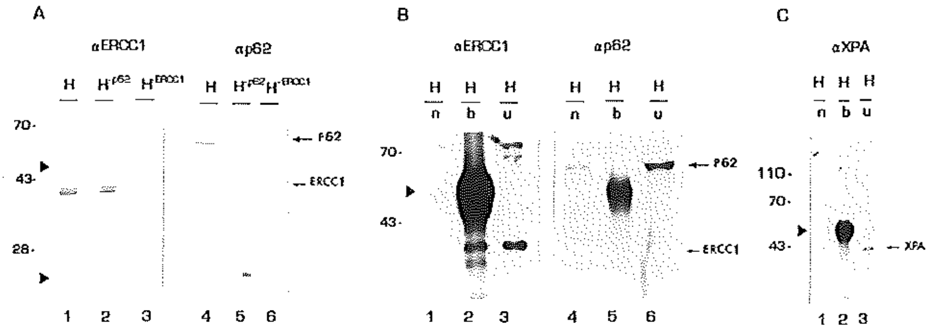

Fig. 5. Absence of a physical association between ERCC1 and TFIIH or XPA. A: HeLa extract (lane 1 and 4), HeLa extract depleted for p62 (H^{-p62}) (lane 2 and 5) and HeLa extract depleted for ERCC1 (H^{-E1}) (lane 3 and 6) were loaded on SDS-PAGE, immunoblotted and analyzed with anti-ERCC1 antiserum (lane 1-3) or with anti-p62 antiserum (lane 4-6). Arrowheads indicate IgG bands. B: HeLa extract was depleted for ERCC1 and the non-bound fraction (n), protein fraction bound to the ERCC1-immunobeads (b), and untreated HeLa extract (u) were analyzed by immunoblotting with anti-ERCC1 antiserum (lane 1-3) or with anti-p62 antibodies (lane 4-6). ERCC1 is bound to the anti-ERCC1 immunobeads (lane 2), while binding of p62 was not detectable (lane 5). Note: the samples in lane 1 and 4 were 10x diluted compared to lane 3 and 6. C: HeLa extract tested for the presence of XPA protein after ERCC1 depletion: lane 1, non-bound fraction; lane 2, proteins bound to the ERCC1-immunobeads (b) and lane 3 untreated HeLa extract (u) as a control and analyzed by polyclonal antiserum against XPA.

Correction of the repair defect was clearly observed in XP-F cells as an increase in unscheduled DNA synthesis (UDS) to wild-type levels within 2 h after injection. No correction was found in XP-C fibroblasts, included as a negative control. These results indicate that at this stage of purification both *in vitro* and *in vivo* active ERCC1 complex is present and that the repair-correcting activities of ERCC1, ERCC4, ERCC11 and XPF remain associated.

The Heparin fractions still contain many proteins as detected on Coommassie Brilliant Blue (CBB) stained SDS-polyacrylamide gels. Therefore, the behaviour of the ERCC1 complex on a wide range of chromatographic mate-rials was analyzed, using extracts of HeLa cells as well as partially purified Sulfopropyl 5PW and Heparin 5PW eluates. A strong hydrophobic interaction with Phenylsepharose and Octylsepharose caused irreversible binding of ERCC1, which could only be eluted using a denaturing detergent solution (0.5 % SDS). Application to the weakly hydrophobic unsubstituted sepharose carrier resulted in binding in the presence of 40% ammoniumsulphate solution, but ERCC1 eluted with the bulk of proteins between 35-20% ammoniumsulphate. ERCC1 was found to bind at low ionic strength to the column materials: Q-, Blue-, Red-, Heparin-sepharose, ATP-agarose, Phosphocellulose, DEAE, Sephacryl S-300, ss-DNA agarose, ds-DNA cellulose, UV-irradiated DNA cellulose and Hydroxylapatite. Generally, the complex eluted between 0.2 and 0.4 M salt. However, with none of these column materials a significant additional purification of the ERCC1 complex could be achieved. The best purification was observed using Hydroxylapatite. ERCC1 eluted at 0.26 M phosphate buffer (fractions 20-24), but the CBB-stained profile still showed many protein bands (Figure 4). Neither CBB-nor silver-stained protein profiles revealed prominent SDS-PAGE bands at he MW of 39 or 120 kDa, indicating that the ERCC1 complex is present as a minor component at this stage of purification. However, the elution fractions of the Hydroxylapatite column, containing ERCC1, still induced potent correction indicating that they are biologically active.

To investigate DNA-binding activity of the ERCC1 complex Heparin 5PW fraction 10 was loaded on ss-DNA agarose, on ds-DNA cellulose and on UV-irradiated DNA cellulose. In all three

cases ERCC1 eluted between 0.2 and 0.4 M KCl, indicating that the ERCC1 complex binds to DNA without pronounced preference for ss-, ds- or damaged DNA. This observation together with the finding that the homologous yeast RAD1/RAD10 complex exhibits ss-specific endonuclease activity (Sung et al., 1993; Tomkinson et al., 1994), may imply that the ERCC1 complex has such an activity as well. Heparin fraction 10 contained nuclease activity for both single- and double-stranded DNA under the conditions used in the *in vitro* repair assay, but the fraction was not pure enough to unequivocally demonstrate an association of a nuclease with the ERCC1-containing protein complex.

3.3 Possible interactions with other repair factors

Since NER requires many enzymes during the reaction, the possibility of specific association of ERCC1 and other NER factors was investigated. At first, TFIIH seemed a good candidate, because ERCC1 co-eluted at least partially with TFIIH in the initial four purification steps. However, separation occurred in Sulfopropyl and Heparin 5PW chromatography, arguing against such an interaction. This result was confirmed by antibody depletion studies. Monoclonal antiserum against the p62 subunit of TFIIH and polyclonal anti-ERCC1 antiserum were immobilized on protein A sepharose beads prior to incubation with repair-proficient cell-free extracts of HeLa cells. After removal of the beads, the non-bound fraction (supernatant) displayed a strong decrease in NER activity measured in the *in vitro* repair assay, consistent with the removal of at least one essential NER component (van Vuuren et al., 1993; van Vuuren et al., 1994). HeLa extracts depleted of ERCC1 ($HeLa^{-ERCC1}$) or p62 ($HeLa^{-p62}$) were loaded on SDS-page and immunoblotted. A clear reduction of ERCC1 was found in $HeLa^{-ERCC1}$, whereas no significant decrease in the amount of ERCC1 was observed in the $HeLa^{-p62}$ (Figure 5A, lane 2 and 3). The reverse pattern was detected in $HeLa^{-p62}$: a decreased amount of p62 was found in this extract in comparison to normal levels of p62 in the $HeLa^{-ERCC1}$ extract (Figure 5A, lane 5 and 6). To exclude the possibility that only a small fraction of TFIIH is associated with ERCC1, the proteins bound to the ERCC1-immunobeads were analyzed. This bound fraction showed clearly ERCC1 as expected, while no p62 could be observed (Figure 5B, lane 2 and 5). It appears that no significant stable association between the ERCC1 and the TFIIH complexes exists in repair-competent extracts of HeLa cells.

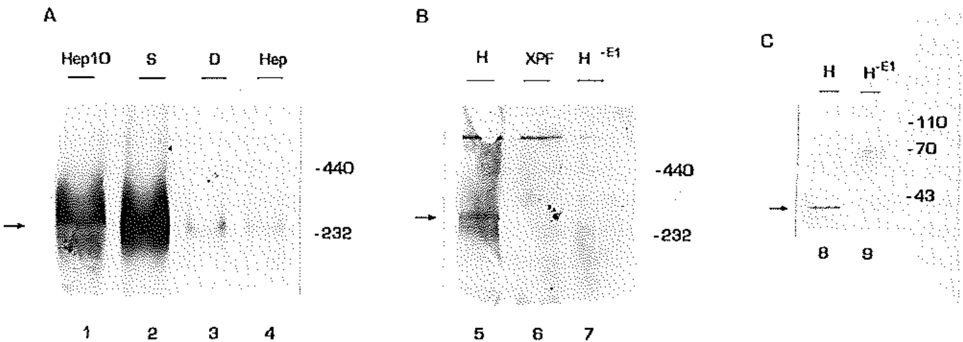

Fig. 6. The size of the ERCC1 complex determined by non-denaturing gel-electrophoresis. The immunoblots were analyzed with anti-ERCC1 antiserum. A: lane 1: Heparin 5PW fraction 10 (100 μg), lane 2: Sulphopropyl 5PW fraction 0.45 M KCl (120 μg), lane 3 DEAE fraction 0.2 M KCl (10 μg), lane 4 Heparin Ultrogel 0.4 M KCl. (20) B: lane 5 Hela extract, lane 6 XP-F extract, and lane 7 ERCC1-depleted HeLa extract (H^{-E1}), in all cases 100 μg protein was loaded. C: the region corresponding to the position of the ERCC1 complex was cut out of the non-denaturing gel, the proteins were electro-eluted and analyzed by SDS-PAGE and Western blotting: lane 8 HeLa extract and lane 9 ERCC1-depleted HeLa extract (H^{-E1}).

In the first chromatography step ERCC1 co-eluted neither with XPC nor with CSB, whereas XPA and XPG were found in the same Heparin fraction as ERCC1 (van der Spek, van Gool, A.P.M.E., unpub. res.). XPG correcting activity became separated from ERCC1 on the second DEAE column using the *in vitro* NER assay (data not shown). This is consistent with our previous findings in which no close association of XPG with TFIIH was found (van Vuuren et al., 1994). Interaction of ERCC1 with XPA was recently suggested by Li et al. (1994) and Park and Sancar (1994). After depletion of HeLa extract by ERCC1-immunobeads the presence of XPA was analyzed in the different fractions using XPA specific antiserum. The XPA protein was found in the ERCC1 depleted HeLa extract, whereas no XPA was detected in the fraction bound to the ERCC1-immunobeads (Figure 5C, lane 1 and 2), indicating that XPA was not co-depleted by anti-ERCC1 antiserum.

3.4 MW of the ERCC1 complex under non-denaturing conditions

Previously, the size of the ERCC1 complex was estimated to be approximately 120 kDa using size-separation on glycerol gradients (Biggerstaff et al., 1993; van Vuuren et al., 1993). This size is quite small considering the fact that the combined MW of ERCC1 (33 kDa) and the ERCC1-associated factor (~ 120 kDa) tentatively identified in this paper exceeds 150 kDa even without a third possible subunit. Therefore we examined the size of the ERCC1 complex by an independent method using non-denaturing 4-15% polyacrylamide gradient gel-electrophoresis. To investigate whether during purification the size of the complex had changed due to dissociation of loosely bound factors, the apparent MW of the complex was analyzed in unfractionated HeLa extract and in partially purified ERCC1 fractions (see Figure 2A). After immunoblotting ERCC1 was found to migrate at a position corresponding with an apparent MW of 280 kDa ± SD 36 (Figure 6A and B, lane 5). This signal was absent when HeLa^{-ERCC1} or XP-F extracts were analyzed (Figure 6B, lane 6 and 7). To further establish that the detected protein band is ERCC1, part of the gel containing ERCC1 was cut out and the polypeptides were electro-eluted and analyzed using SDS-PAGE. After immunoblotting the anti-ERCC1 antiserum clearly detected the ERCC1 protein as a 39 kDa band. In contrast, the HeLa^{-ERCC1} extract did not contain any detectable ERCC1 after electro-elution and blotting (Figure 6C, lane 8 and 9). No XPA could be observed by polyclonal anti-XPA antibodies in this eluted protein fraction (data not shown), confirming the results described above that under our conditions XPA is not detectably associated with ERCC1.

4. Discussion

4.1 Composition and size of the ERCC1 complex

The ERCC1 complex contains the correcting activities of ERCC1, ERCC4, ERCC11 and XPF, although the latter might be identical to either ERCC4 or ERCC11 (van Vuuren et al., 1993). These findings support the idea that the ERCC1 complex contains a minimum of three subunits, ERCC1 (predicted molecular weight of 33 kDa), a subunit of 120 kDa and at least one unidentified subunit. Immunoprecipitation studies revealed co-precipitation of the 120 kDa component. This is not due to cross-reaction of the antiserum: competition experiments prevent the precipitation of both ERCC1 and the 120 kDa protein. ERCC1 is specifically reduced in XP-F extracts and after immunoprecipitation neither ERCC1 nor this 120 kDa protein band could be observed. These data strongly suggest that the 120 kDa protein represents a component of the ERCC1 complex and that the complex is probably required for stability of ERCC1. The latter conclusion is consistent with the findings of Biggerstaff et al., who also noted reduced ERCC1 amounts in rodent group 4 and 11 (Biggerstaff et al., 1993 and R.D. Wood, pers. comm.).

The yeast *RAD1* and *RAD10* mutants show similar phenotypes distinct from other RAD3 group mutants, both have a defect in NER and, in addition, in mitotic recombination (Schiestl and Prakash, 1988; Schiestl and Prakash, 1990).

Mammalian *ERCC1* and *ERCC4* mutants share a unique characteristic as well; unlike mutants of other complementation groups they are extremely sensitive (50-100x) to crosslinking agents. Repair of these crosslinks is thought to require recombinational events. Based on the fact that ERCC1 is the homologue of the yeast RAD10 one might suggest that the human *ERCC4* gene is the counterpart of the yeast *RAD1*. The MWs of the mammalian subunit (~120 kDa), found in the immunoprecipitation studies, and RAD1 (126 kDa) fit well in this prediction. These data suggest that the 120 kDa subunit might be ERCC4. No such extreme sensitivity for crosslinking agents has been observed in *ERCC11* or *XPF* mutants, possibly due to partial mutations in these genes.

It appears that at least one subunit has escaped detection in the immunoprecipitation studies and remains to be identified. It is possible that the ERCC1 antibodies competes with binding sites for ERCC4 or ERCC11. This explanation seems unlikely, in view of the immunodepletion studies (van Vuuren et al., 1993), but cannot be completely ruled out. A second possibility is that the complementation found between mutants of groups 1, 4 and 11 is due at least in one of the combinations to intragenic complementation. However, transfection of functional *ERCC1* cDNA and gene to mutants of rodent group 4 and 11 as well as to XP group F have excluded a possible involvement of this gene in any of these mutants (van Duin and Hoeijmakers, 1989 and W. Vermeulen, J. de Wit and J.H.J.H. unpubl. res.). Furthermore, intragenic complementation between the sole representative (UVS1) of rodent complementation group 11 and several independent mutants of groups 1 or 4 is rather unlikely, since correction of the repair defect was found in all cases in different laboratories (Hata et al., 1991; Busch et al., 1994). In addition, cosmid clones containing the *ERCC4* gene failed to complement the repair defect in group 11 (L. Thompson, pers. comm.). The most likely explanation is that the missing subunit has escaped detection due to a low methionine content or because it is hidden behind background bands.

These arguments further support the idea that the ERCC1 complex consists of at least three subunits: ERCC1, ERCC4 and ERCC11. Experiments in yeast concerning the RAD1/RAD10 complex (Bailly et al., 1992; Bardwell et al., 1992) have not excluded the presence of additional proteins.

Non-denaturing gel electrophoresis shows an ERCC1 complex with an apparent molecular weight (MW) of ~280 kDa both in Hela extracts and in partially purified ERCC1 fractions, much larger than the ~120 kDa previously observed by glycerol gradient sedimentation experiments (Biggerstaff et al., 1993; van Vuuren et al., 1993). The larger MW obtained in non-denaturing gels would better accommodate the presence of ERCC1 (predicted as 33 kDa), a subunit of 120 kDa and at least one additional polypeptide. In general, MW estimations from these two methods must be taken with caution, and may strongly depend on the conformation of the complex.

4.2 Purification and activity of the ERCC1 complex

Purification of the ERCC1 complex to homogeneity from mammalian cells is very difficult by classical methods. After initial purification most of the chromatographic materials showed elution of ERCC1 together with the majority of the remaining proteins. In our experience the Hydroxylapatite column yielded the best purification, but even after this step ERCC1 could not be unequivocally identified on protein-stained gels. The protein complex must be present in relatively small amounts in this stage of purification. Unfortunately, immunodetection of ERCC4, ERCC11 or XPF during the different chromatography steps is impossible at the moment, since antibodies are not available yet. The ERCC1 complex has affinity for DNA but no strong preference for binding ss, ds, or UV-irradiated DNA. This suggests that the ERCC1 complex does not have a direct role in recognition of the DNA lesion, like XPA and XPE, which preferentially bind 6-4 photoproducts and other NER lesions (Jones and Wood, 1993; Reardon et al.,

1993). Very recently, the RAD1/RAD10 complex has been found to cleave a splayed-arm DNA structure (Bardwell et al., 1994b). By extrapolation the ERCC1 complex might be involved in the 5' incision, whereas XPG has been shown to be responsible for the 3' incision (O'Donovan et al., 1994). However, no ss or ds endonuclease activity specific for the ERCC1 complex could be demonstrated due to contaminating nucleases. Absence of ERCC1 endonuclease activity was found by Park and Sancar (1994). Further purification of the complex and the use of structure-specific DNA substrates (such as splayed-arm or bubble-containing DNA molecules) may be required for disclosing the enzymatic function of the ERCC1 complex.

4.3 Association of ERCC1 complex with other NER factors

A coordinated action of many repair proteins is required in the NER process. This might indicate binding affinities between different repair factors. For instance, evidence has been reported that XPC associates with TFIIH (Drapkin et al., 1994). In addition, RAD2 and RAD4, yeast homologs of XPG and XPC res-pectively, were claimed to interact with factor b, the yeast equivalent of TFIIH (Bardwell et al., 1994a). In case of the ERCC1 complex no specific stable interactions are observed with any of the known repair proteins (complexes). Depletion of a repair-proficient extract with antibodies against the p62 subunit of TFIIH or against ERCC1 failed to reveal co-depletion. Also XPA did not show a stable association with the ERCC1 complex in unfractionated HeLa cell-free extracts. An ERCC1-depleted HeLa extract still contains the repair-correcting activities for XPB, XPD (both subunits of TFIIH) and XPA, while the activities to complement the defect of ERCC1, ERCC4, ERCC11 and XPF are lost (van Vuuren et al., 1993). Another argument against a strong association is the fact that in gel-filtration experiments purified XPA yielded a MW consistent with the expected MW of a free XPA monomer and did not provide evidence for a stable XPA-containing complex (Eker et al., 1992). An interaction between XPA and ERCC1 was previously detected by two methods: using the two hybrid system (which is capable of picking up very weak interactions) and by binding to immobilized protein (in which case a vast excess of one of the proteins was necessary to demonstrate this interaction) (Li et al., 1994; Park and Sancar, 1994). In the first purification step (Figure 2A) CSB and XPC elute in different fractions than ERCC1, while XPG separates from ERCC1 on the second column. These association studies fail to disclose stable interaction between ERCC1 and TFIIH, XPA, XPC, XPG, and CSB and do not support the presence of detectable quantities of one large repair complex in mammalian cells. It is possible that repair factors have transient affinities for each other during the NER reaction. This interaction might be weak or depend on the recruitment of specific proteins or on binding to DNA.

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Chapter 4

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

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

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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.

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

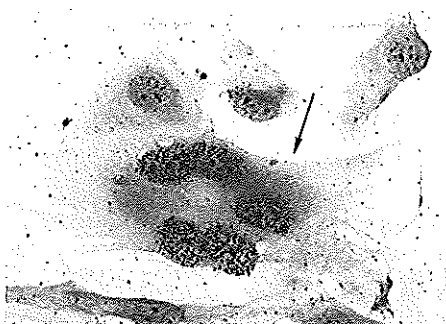

Fig. 1. Assessment of XP-B correcting activity by BTF2 using microneedle injection. (A) Copurification of XP-B repair correction and transcription stimulation of BTF2. Peak fractions of transcription stimulatory activity of each BTF2 purification step (for details on the BTF2 purification see Gerard *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.

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

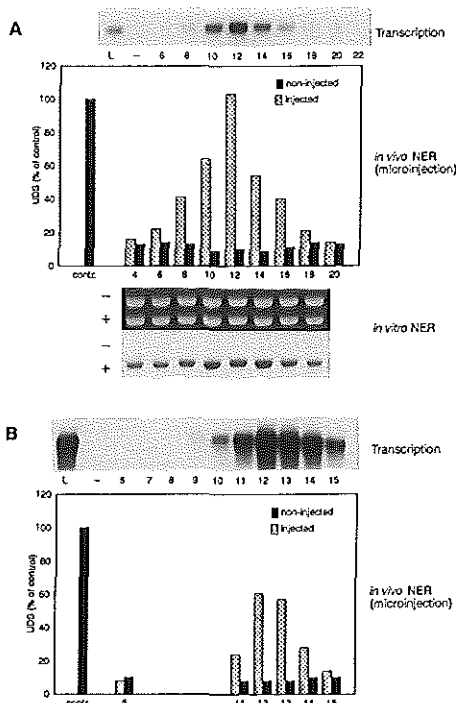

Fig. 2. Cochromatography and cosedimentation of BTF2 transcription stimulating and XP-B/ERCC3 repair-correcting activities. The transcriptionally active fractions that were eluted from the phenyl-5PW column were successively applied on a HAP column and a 15–35% glycerol gradient. All fractions were tested 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 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.

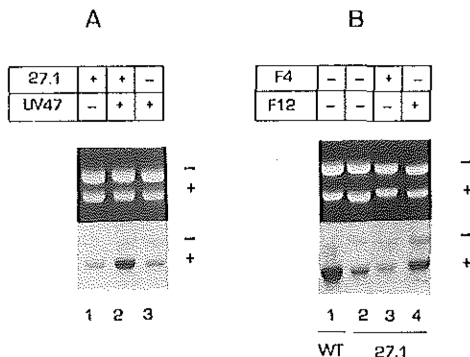

Fig. 3. BTF2 corrects NER defect of rodent complementation group 3 *in vitro*. (A) *In vitro* complementation of NER defect in CHO mutants of rodent complementation groups 3 (mutant 27-1) and 4 (mutant UV47). Reactions contained a damaged plasmid (pBKS, 3.0 kb) and a non-damaged internal control plasmid (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.

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)

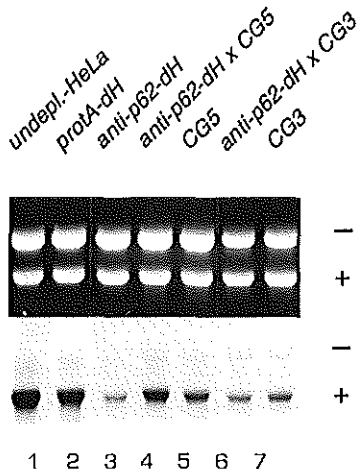

Fig. 4. Monoclonal antibodies against p62 deplete a repair-competent HeLa extract from repair and ERCC3 activity. HeLa cell-free extract (150 μg protein) was incubated with Mab3C9 against the p62 BTF2 subunit immobilized on protein A—sepharose CL-4B beads. After centrifugation to remove the bound proteins 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).

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

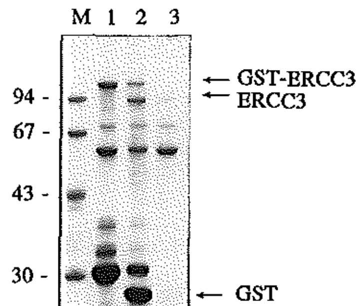

Fig. 5. SDS—PAGE analysis of recombinant-produced ERCC3 fusion protein. Recombinant-produced fusion protein of GST—ERCC3 (using vector pGEX2T) was partly purified on glutathione—agarose beads and eluted with 5 mM reduced glutathione (lane 1). Lane 2, eluted proteins, after digestion with thrombin; lane 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.

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

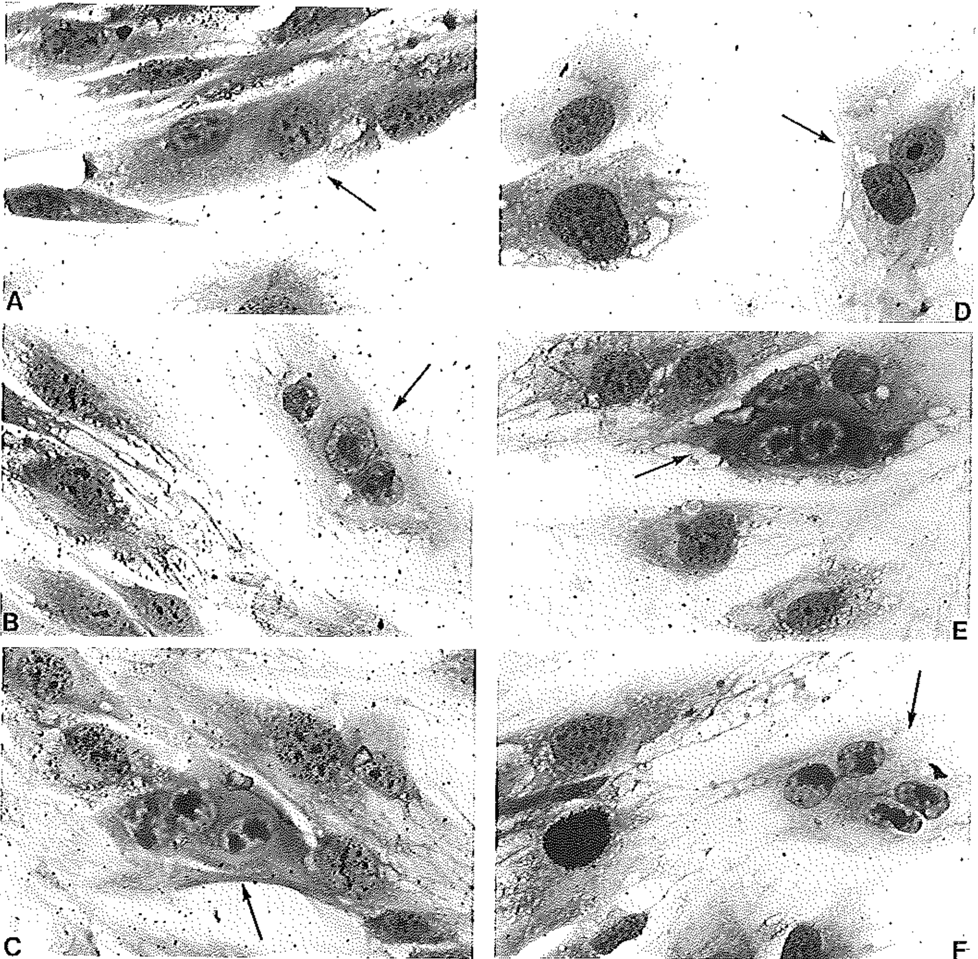

Fig. 6. Effect of K346 → R ERCC3 mutant on transcription and NER. Micrographs A, B and C show the time dependent effect on RNA synthesis, assayed by pulse labelling with [^{3}H]uridine, in control (wild-type) fibroblasts injected with the *ERCC3* cDNA encoding the K346 → R mutated protein. Micrographs 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.

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Chapter 5

Three unusual repair deficiencies associated with transcription factor BTF2(TFIIH). Evidence for the existence of a transcription syndrome.

Three unusual repair deficiencies associated with transcription factor BTF2(TFIIH). Evidence for the existence of a transcription syndrome.

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To counteract the deleterious effects of DNA damage a sophisticated network of DNA repair systems has evolved, which is essential for genetic stability and prevention of carcinogenesis. Nucleotide excision repair (NER), one of the main repair pathways can remove a wide range of lesions from the DNA by a complex multi-step reaction (for a recent review see Hoeijmakers, 1993). Two subpathways are recognized in NER: a rapid 'transcription-coupled' repair and the less efficient global genome repair (Bohr 1991; Hanawalt and Mellon 1993). The consequences of inborn errors in NER are highlighted by the prototype repair syndrome xeroderma pigmentosum (XP), an autosomal recessive condition, displaying sun (UV) sensitivity, pigmentation abnormalities, predisposition to skin cancer and often progressive neurodegeneration (Cleaver and Kraemer 1994). Two other excision repair disorders have been recognized, Cockayne syndrome (CS) and trichothiodystrophy (TTD), which present different clinical features. These are, besides sunsensitivity, neurodysmyelination, impaired physical and sexual development, dental caries (in CS and TTD), ichthyosis and sulphur-deficient brittle hair and nails (in TTD)(Lehmann 1987; Nance and Berry 1992), which are difficult to rationalize on the basis of defective NER only. The NER syndromes are genetically heterogeneous and comprise at least 10 different complementation groups: 7 in XP (XP-A to XP-G), 5 in CS (CS-A, CS-B, XP-B, XP-D and XP-G) and 2 for TTD (TTDA and XP-D) (Hoeijmakers 1993; Stefanini et al. 1993b). Thus, considerable overlap and clinical heterogeneity are associated with a selected subset of complementation groups, of which XP-D is the most extreme, harboring patients with XP only, or combined XP and CS, or TTD (Johnson and Squires 1992).

Recently it was discovered that the DNA repair helicase encoded by the *ERCC3* gene (Roy et al. 1994), which is mutated in XP-B (Weeda et al. 1990), is identical to the p89 subunit of the transcription factor BTF2/TFIIH (Schaeffer et al. 1993). ERCC3 in the context of TFIIH, is directly involved in NER and transcription *in vitro* as well as *in vivo* (van Vuuren et al. 1994). Furthermore, another repair helicase, XPD/ERCC2 was recognized to be associated with TFIIH (Schaeffer et al. 1994), and it was shown that a partially purified TFIIH fraction is able to correct the NER-deficiency of XP-D *in vitro* (Drapkin et al. 1994). These results reveal a link between two distinct DNA-metabolizing processes: repair and transcription.

Initiation of transcription is believed to require the formation of an elongation-competent protein complex in a highly ordered cascade of reactions.

A preinitiation (DAB) complex is formed by the binding of the multisubunit TFIID factor to the TATA box element of core promoters, stabilized by TFIIA and followed by the association of TFIIB. The DAB intermediate stimulates the entry of RNA polymerase II mediated by TFIIF. Initiation is completed by the ordered association of TFIIE, TFIIH/BTF2 and TFIIJ (Drapkin et al. 1993; Gill and Tjian 1992; Roeder 1991). T-FIIH/BTF2 is thought to be involved in the conversion of a closed to an open initiation complex by local melting of the transcriptional start site and phosphorylation of the C-terminal repeat of the large subunit of RNA polymerase II (Lu et al. 1992; Schaeffer et al. 1993; Serizawa et al. 1993), either at the preinitiation stage, or at a step between initiation and elongation, referred to as promotor clearance (Goodrich and Tjian 1994).

Here we report that defects in the XPB/-ERCC3 subunit define a new TTD complementation group extending the clinical heterogeneity associated with ERCC3. Furthermore, we demonstrate that probably the entire TFIIH complex has a dual role in transcription and repair, as it harbors now at least three NER proteins, all associated with the TTD and CS symptoms. The dual function of TFIIH, and the link between mutations in this complex and the pleiotropic features of TTD and CS strongly support the idea that part of the clinical manifestations arise from defects in the transcription function of TFIIH. This provides for the first time a molecular explanation for the seemingly unrelated symptoms of these disorders and introduces a novel clinical entity, a heterogenous 'transcription syndrome' complex, that may include many more inherited conditions.

METHODS

Purification of BTF2/TFIIH. The purification of BTF2 starting from HeLa whole cell extract and involving sequential chromatography on Heparin-Ultrogel, DEAE-Spherodex, SP-5PW sulfopropyl, and -after ammonium sulphate precipitation- Phenyl-5PW Sepharose and hydroxyapatite was essentially as described earlier (Gerard et al. 1991). In some fractions an additional purification step using heparin chromatography was inserted in the standard protocol before the Phenyl-5PW column. The *in vitro* assay for following the transcriptional stimulation activity of BTF2 on a AD2MLP promoter containing template involving purified RNA polymerase II and all transcription fators except BTF2/-TFIIH is described in detail before (Gerard et al. 1991).

Cell lines and extracts. The human cell lines used for microinjection and for complementation analysis were XP25RO and XP11PV (both XP group A), XPCS1BA and XPCS2BA (XP-B), XP21RO (XP-C), XP1BR, XP3NE and TTD8PV (all XP-D), XP2RO (XP-E), XP126LO (XP-F), XP2BI (XP-G) and TTD1BR (TTD-A) and TTD6VI (Stefanini et al. 1993b; Vermeulen et al. 1986; Vermeulen et al. 1994). The primary fibroblasts were cultured in Ham's F10 medium supplemented with antibiotics and 10-15% fetal calf serum.

For preparing cell-free extracts utilized for the *in vitro* repair assay the following cell lines were used: a SV40-transformed line belonging to TTD-A (TTD1BRSV), human repair-proficient HeLa cells, mutant Chinese hamster cells 43-3B, UV5, 27.1, UV41 and UV135 assigned to complementation groups 1, 2, 3, 4 and 5 respectively (Busch et al. 1989). The cells were grown in a 1:1 mixture of F10 and DMEM medium, antibiotics and 10% fetal calf serum were added. After harvesting and washing with phosphate-buffered saline (PBS), cell-free extracts were prepared as described by others (Manley et al. 1983; Wood et al. 1988), dialysed against a buffer containing 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.

In vitro DNA repair assay. Plasmid pBluescript KS^+ (3.0 kb) was damaged by treatment with 0.1 mM N-acetoxy-2-acetylaminofluorene (AAF) (a kind gift of R. Baan, TNO, Rijswijk).

As a non-damaged control plasmid pHM14 (3.7 kb) was used. Circular closed forms of both plasmids were isolated and extensively purified as described by (Biggerstaff et al. 1991; van Vuuren et al. 1993). The reaction mixture contained 250 ng each of damaged and non-damaged control plasmids, 45 mM HEPES/KOH pH 7.8, 70 mM KCl, 7.4 mM $MgCl_2$, 0.4 mM EDTA, 0.9 mM DTT, 2 mM ATP, 40 mM phosphocreatine, 2.5 μg creatine phosphokinase, 3.5% glycerol, 18 μg bovine serum albumin, 20 μM each of dCTP, dGTP and TTP, 8 μM dATP, 74 kBq of α-^{32}PdATP and 200 μg of cell-free extract for the rodent extracts and 100 μg for HeLa. After 3 h incubation at 30°C, plasmid DNAs were purified from the reaction mixture, linearized by restriction and separated by electrophoresis on an 0.8% agarose gel. Results were quantified using a LKB Densitometer and B&L Phospho-Imager.

Anti-p62 antibody depletion of a repair-proficient extract. Protein A sepharose beads in PBS were incubated with monoclonal antibodies against the p62 subunit of BTF2 (Mab3C9, (Fischer et al. 1992)) or against p89 for 1 h at 0°C. The beads were washed with dialysis buffer and added to a repair-competent Hela extract or a (partially) purified heparin or hydroxyapatite BTF2 fraction for 1 h at 0°C (van Vuuren et al. 1993). The bound proteins were removed by centrifugation and the supernatant was tested in the *in vitro* repair assay or for microneedle injection. Westernblot analysis following SDS polyacrylamide gel electroforesis was carried out according to standard protocols. (Sambrook et al. 1989)

Microinjection. Microinjection of XP homopolykaryons was preformed as described earlier (Vermeulen et al. 1994). Repair activity was determined after UV irradiation (15 J/m^2), ^{3}H-Thymidine incubation (10 μCi/ml; s.a.: 50 Ci/m

Complementation analysis by cell hybridization. Fibroblasts of each fusion partner, were labeled with latex beads (0.8 μm or 2.0 μm), 3 days prior to fusion by adding a suspension of beads to the culture medium. Cell fusion was performed with the aid of inactivated Sendai virus or using Poly ethylene glycol, cells were seeded onto coverslips and assayed for UV-induced UDS as described in detail by Vermeulen et al. (Stefanini et al. 1993a; Vermeulen et al. 1993).

Other procedures. Isolation of DNA, subcloning, and *in vitro* transcription and translation were done using established procedures (Sambrook et al. 1989).

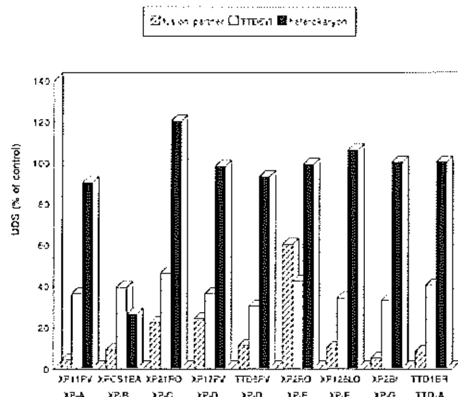

Fig. 1. Complementation analysis of TTD6VI cells. Fibroblasts of TTD6VI (labeled with 0.8 μm beads) were fused with representatives of different XP and TTD complementation groups (labeled with 2.0 μm beads). After performing the UV-induced UDS assay, the average number of autoradiographic grains above the nuclei were determined. DNA repair synthesis was expressed as the percentage of control UDS (vertical-axis), observed in normal fibroblasts assayed in parallel. The relative standard errors of the mean are in all cases less than 5%. The different types of binuclear cells (homodikaryons of TTD6VI and of the different fusion partners, and heterodikaryons) are recognized by their different bead content and indicated as such in the figure.

RESULTS

Identification of further genetic heterogeneity within TTD

In an effort to assess the genetic heterogeneity within the class of repair-deficient TTD patients we have conducted a systematic complementation study of a large number of photosensitive TTD families by cell fusion and microneedle injection of cloned repair genes. This resulted recently in the identification of a second complementation group among NER-deficient TTD patients (designated TTD-A) (Stefanini et al. 1993b). Figure 1 shows the results of an exhaustive complementation analysis of fibroblasts from one of two siblings (TTD6VI and TTD4VI) with relatively mild clinical features of TTD and moderately impaired NER characteristics (detailed clinical description to be presented elsewhere). Full complementation of the repair defect was seen when fibroblasts of patient TTD6VI were fused with representants of the known TTD complementation groups: XP group D (3 cell lines tested) and with TTD-A fibroblasts (Fig.1) (Stefanini et al. 1993a; Stefanini et al. 1993b). This demonstrates that this family defines a new TTD complementation group. To see whether this group is genetically identical to one of the other XP groups not previously associated with TTD further cell hybridization experiments were carried out. No restoration of the deficient UV-induced unscheduled DNA synthesis (UDS) was found when TTD6VI cells were fused with a XP-B representant (XPCS1BA), whereas normal complementation was observed with the other XP groups (Fig. 1). This indicates that the repair defect in this family resides in the *XPB/ERCC3* gene. Although TTD6VI cells appeared exceptionally sensitive to nuclear microneedle injection of DNA, still a few cells could be found corrected by *ERCC3* cDNA, in agreement with the assignment by cell fusion. Defects in the TFIIH subunit ERCC3 thus extend to the disorder TTD, increasing the already observed clinical heterogeneity among patients carrying mutations in

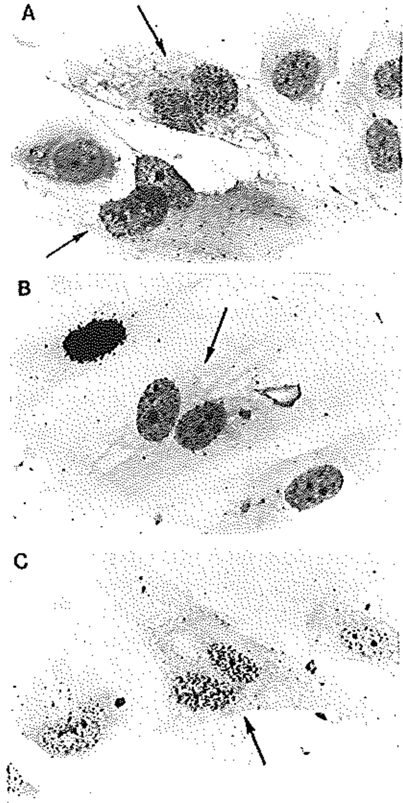

Fig. 2. Microneedle injection of BTF2 in XP-D, TTD-A and XP-B fibroblasts. **A**, **B** and **C**, are micrographs showing the effect of purified BTF2 (HAP-fraction 12, containing the highest transcriptional activity (Schaeffer et al. 1993)) on NER-activity of injected XP-D (cell line XP1BR), TTD-A (TTD1BR) and XP-B (TTD6VI) fibroblasts respectively. The injected cells (binuclear fibroblasts obtained by cell fusion prior to injection) are indicated by arrows. As apparent from the number of silver grains above their nuclei they exhibit a high (wild type) level of UV-induced UDS when compared to the non-injected surrounding cells, which express residual UDS levels typical for these complementation groups. The heavily labelled fibroblast in panel **B** is a cell performing S-phase replicative DNA-synthesis at the moment of the UDS-assay.

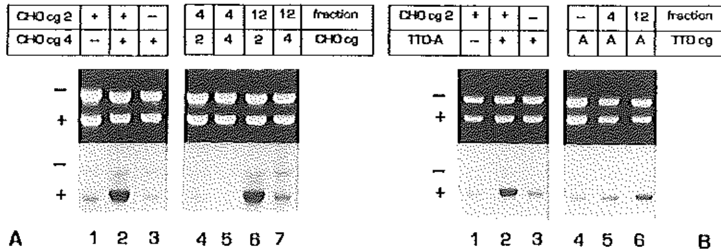

Fig. 3. *In vitro* correction of NER defect by purified BTF2. BTF2 HAP-fraction 12 was added to an extract of rodent complementation group 2 (mutant UV5, equivalent to XP-D)(A), and to a human TTD group A lysate (TTD1BRSV)(B), and tested for its capacity to restore the NER defect using an *in vitro* cell-free repair assay (Wood et al. 1988). The level of correction of NER activity using purified BTF2 is of the same order (lanes 6, Fig.2A and B) as the level of complementation reached when the extract of group 2 is mixed with a group 4 extract (Fig.2A, lane 2) or with the TTD-A extract (Fig.2B, lane 2). No significant correction is observed when the purified BTF2-fraction is added to rodent complementation group 4 (mutant UV47) extract (Fig.2A, lane 7). The upper panel shows the ethidium-bromide stained DNA gel, the lower panel the autoradiogram of the dried gel, the presence of ^{32}P-dATP indicates repair synthesis. The positions of AAF-damaged and non-damaged DNA substrates, 250 ng each, are indicated by plus (+) and min (-) respectively. Lanes 1-3 contain in total 200 μg of cell-free extract (in complementations 100 μg of each extract was used), the others lanes contain 100 μg, the protein contribution by the BTF2 fraction is negligible.

ERCC3 (Vermeulen et al. 1994).

Systematic screening of TFIIH for NER proteins

To examine whether besides XPB/ERCC3 (p89) additional NER factors are hidden in the TFIIH/BTF2 complex, we have systematically screened the existing human and rodent NER mutants for complementation by BTF2. The final fractions of TFIIH purification show at least 5 tightly associated proteins, including p89 (XPB/-ERCC3), p62, p44, p41, p34 (Gerard et al. 1991; Schaeffer et al. 1993). A purified BTF2-fraction (hydroxyapatite chromatography, gel pattern and properties see (Gerard et al. 1991)) was inserted by microneedle injection into fibroblasts of all known excision-deficient XP, XP/CS and TTD complementation groups. The injected cultures were exposed to UV and incubated with tritiated thymidine to permit visualization of the repair synthesis step of NER by *in situ* autoradiography (unscheduled DNA synthesis, UDS). The results summarized in Table 1, show that 3 out of 8 NER-deficient human complementation groups are corrected by the purified BTF2 transcription factor. Figure 2A, B and C show micrographs of BTF2 injected multi-nucleated fibroblasts of XP group D, TTD-A and the new XP group B patient with TTD symptoms. The repair activities in injected cells reach the levels of normal fibroblasts assayed in parallel, indicating full correction of the excision defect (see also Table 2).

The restoration of NER could also be explained by correction of expression of one or more critical repair genes whose transcription is impaired by mutations in BTF2 subunits. Therefore, purified BTF2 was checked for its repair capacity in an *in vitro* repair assay (van Vuuren et al. 1993; Wood et al. 1988), where neither transcription nor translation can occur, because ribonucleotides and aminoacids are lacking. The results in Fig. 3 and Table 1 show that the BTF2 fraction not only corrects the repair defect in rodent group 3 (the equivalent of XP-B), as reported earlier (van Vuuren et al. 1994) but also the rodent group equivalent to XP-D (CHO group 2, defective in ERCC2) (Flejter et al.

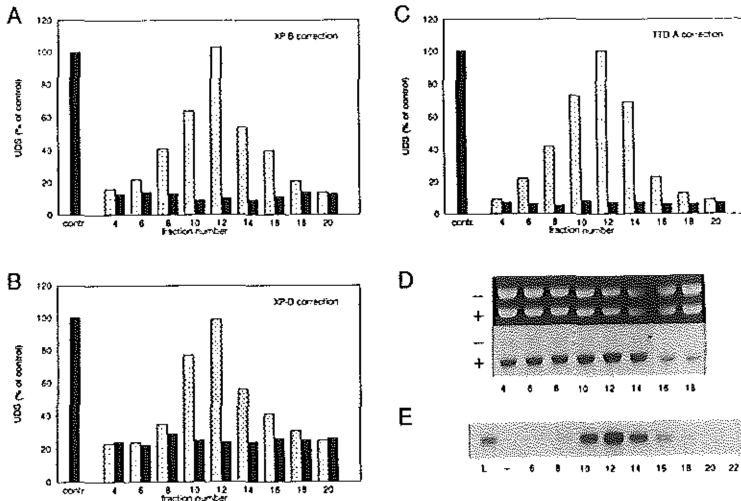

Fig. 4. Correlation between transcription activation and correction of the NER defect in XP-D/ERCC2 and TTD-A mutant cells using different fractions from the HAP chromatography column. BTF2-derived *in vivo* NER-correcting activity, was quantitatively determined, after microinjection, by counting the grains above the nuclei (UV-induced UDS) of injected cells and expressed as % of the UDS-level of repair-proficient control cells (C5RO) assayed in parallel (left black bar) (Vermeulen et al. 1986). The hatched bars indicate the average UDS level (determined by counting grains above 50 nuclei) of multikaryons injected with BTF2. The black bars represent UDS in non-injected neigbouring cells. The *in vivo* NER-activity profiles for respectively: XP-B (XPCS1BA) (A), XP-D (XP1BR) (B) and TTD-A (TTD1BR) (C) are compared to the *in vitro* repair capacity, for rodent complementation group 2 (*ERCC2* mutant UV5) extracts (D) and to the transcription activity, as determined in a BTF2-dependent *in vitro* transcription run-off assay using the Adenovirus late promoter (E). Note that the UV5 (group 2) extract in panel D (like the XP-D cells in panel B) has a considerable residual repair. The lower activity seen in the higher salt fractions (lanes 16-18) is attributed to a inhibition of repair incorporation by increased salt (Wood et al. 1988).

1992) and an extract from a SV40-transformed TTD1BR cell line (TTD group A). In contrast, no significant NER restoration is observed in extracts of UV-sensitive mutants from groups 1, 4 and 5 (defective in ERCC1, ERCC4 and XPG/ERCC5 respectively). These findings extend the specificity of the repair-correcting activity of TFIIH, and confirm the *in vivo* results obtained by microinjection. Furthermore, they demonstrate that besides XPB/ERCC3, also XPD/ERCC2 and TTDA must have a direct involvement in NER. The *in vitro* and *in vivo* correction of *ERCC2* mutants confirmed the *in vitro* data of Drapkin et al. who used a partially purified TFIIH preparation (Drapkin et al. 1994). However, the TFIIH/BTF2 purified fractions used in this study do not contain a XP-C correcting activity, in contrast to the observations made by these authors.

Relationship of XPD/ERCC2 and TTDA with the core of TFIIH

To further strengthen the link between NER and the TFIIH complex, the full elution profile of hydroxyapatite chromatography, the final purification step of BTF2 (Gerard et al. 1991), was quantitatively screened for NER-activity by microinjection as well as by *in vitro* complemen-

tation. The XP-D and TTD-A correcting activities coelute with the activities of XP-B correction and transcription initiation (Fig.4). Identical results were obtained after cosedimentation in glycerol gradients (van Vuuren et al. 1994 and unpl. results).

Independent evidence for a physical association of the XPD/ERCC2, XPB/ERCC3 and TTDA proteins with each other and with other components in the TFIIH complex can be obtained from antibody depletion experiments. A crude repair-proficient HeLa extract was incubated with a monoclonal antibody against the p62 TFIIH subunit (Fischer et al. 1992), immobilized on protein A sepharose beads, and after centrifugation to remove bound proteins, the extract was tested for remaining repair capacity.

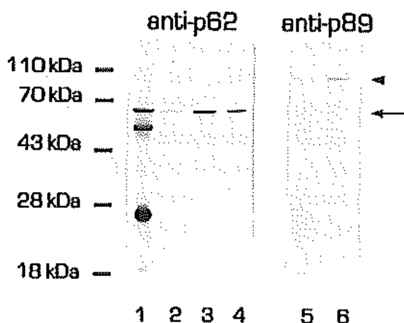

Fig. 5. Western blot analysis of p62 and p89 depletion of a repair-proficient HeLa extract. Monoclonal antibody (Mab3C9) against the p62 subunit of BTF2(-TFIIH) coupled to protein-A sepharose beads was incubated with a repair-proficient extract prepared from HeLa cells. To verify the removal of p62 and simultaneous removal of other components of BTF2 the remaining cell-free extract and the bound fraction were analysed by immunoblotting using monoclonal antibodies against p62 itself (lanes 1-4) and against the p89 (XPB/ERCC3) subunit of BTF2 (lanes 5-6). Lane 1 contains the protein fraction bound to the anti-p62 beads, released by SDS (the strong bands at 55 and 25 kDa represent the heavy and light chains of the antibodies released from the beads). Lanes 2 and 5 HeLa whole cell extract treated with the anti-p62 beads. Lanes 3 and 6: HeLa whole cell extract treated with protein-A beads alone (control). Lane 4 untreated HeLa whole cell extract. In lanes 2-6 equal amounts of sample were loaded.

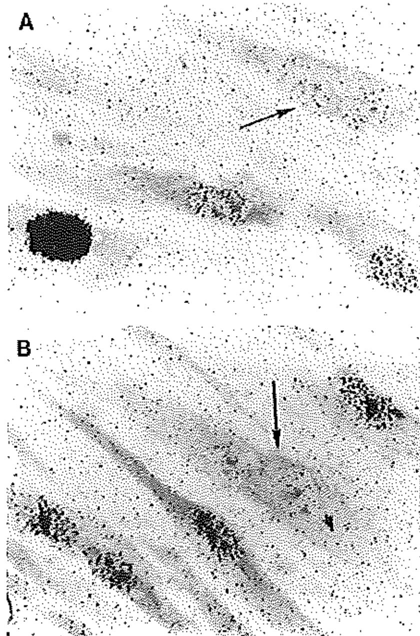

Fig. 6. Effect of injection of antibodies against XPD/ERCC2 on transcription and repair of normal cells. Micrograph A demonstrates the effect of injection of ERCC2 antibodies on UV-induced UDS of normal fibroblasts, assayed by a 2 h incubation in ^{3}H-thymidine immediately following UV-irradiation. Micrograph B shows the effect of injection of XPD/-ERCC2 antiserum on RNA synthesis of control (wild-type) fibroblasts assayed by a 1 h pulselabelling with ^{3}H-Uridine. The UDS and RNA synthesis assays were performed 20 h after injection of the antibodies. The strong reduction of autoradiographic grains above the nuclei of injected dikaryons (arrows) compared to non-injected cells indicates a virtually complete inhibition of RNA and repair synthesis. The heavily labelled nucleus in A is from a cell in S-phase at the time of incubation. Note that these preparations have a higher background labelling than the micrographs shown in Fig.1.

Western blot analysis verified that the amount of p62 was strongly reduced (Fig.5). In the *in vitro* correction assay, the $p62^{depleted}$ HeLa extract had lost most of its repair activity when compared with the repair level of mutant extracts alone and a mock-treated extract (Table 2, footnote). To examine whether other NER factors were co-depleted the treated extract was tested for its com

Table 1. Involvement of BTF2 in different human and rodent NER-deficient complementation groups

NER-deficient complementation groups		Correction of NER defect by BTF2
human mutants		microneedle injection
XP25RO	XP-A	-
XPCS1BA	XP-B	+
XP21RO	XP-C	-
XP1BR	XP-D	+
XP2RO	XP-E	-
XP126LO	XP-F	-
XP2BI	XP-G	-
TTD1BR	TTD-A	+
		in vitro repair assay
TTD1BRSV	TTD-A	+
rodent mutants		
43.3B	group 1	-
UV 5	group 2	+
27.1	group 3	+
UV 41	group 4	-
UV 135	group 5	-

plementing capacity using both the *in vitro* assay and the microneedle injection. Previously, we have demonstrated that XPB/ERCC3, but not XPG/ERCC5, is simultaneously removed with p62 (van Vuuren et al. 1994). Table 2 shows that most XPD/ERCC2 and TTDA activities are also removed, whereas XPA, ERCC1, ERCC4 and XPG/ERCC5 are not significantly eliminated (Table 2 and unp. res.). This is confirmed by the Westernblot analysis revealing depletion of XPB/ERCC3(p89) at the protein level (Fig 5, lanes 5-6). The same co-depletion patterns were observed with a monoclonal antibody against p89 (XPB/ERCC3)(table 2). Furthermore, depletion experiments using monoclonal antibodies against two other components of TFIIH, p44 (the human homolog of yeast SSL1) and p34, revealed tight association with repair (Humbert et al. 1994 and results not shown). As with crude HeLa extracts, antibody depletion of purified BTF2 (Hydroxy apatite and heparin purification fractions) using p62 monoclonal antibodies resulted in simultaneous removal of the XPB/ERCC3, XPD/ERCC2 and TTDA factors (Table 2). The depletion and correction experiments provide strong evidence that 3 repair factors are physically associated with 3 different TFIIH subunits and, thus, likely constitute an integral part of transcription factor BTF2.

Table 2 also shows that small amounts of correcting activities persist in the Anti-p62-treated extract. This is largely due to incomplete removal of p62 (as shown by the Western blot analysis in Fig.5). However, it is not fully excluded that a small fraction of the ERCC2, ERCC3, and TTDA molecules exist dissociated from (the p62 part of) TFIIH, or reside in forms of TFIIH lacking p62. The same holds for the anti-p89 experiments. A somewhat looser association between ERCC2 and the p62-ERCC3 core of TFIIH is also derived from independent dissociation studies by Schaeffer and coworkers (Schaeffer et al. 1994). Further indications for exchange of various TFIIH subunits can be deduced from the *in vitro* complementation between ERCC2- and TTDA-deficient extracts shown in Fig 3*a* (lanes 1-3), each of these extracts being defective in a different component of the same complex. In line with this also extracts defective in XPD/ERCC2 and XPB/-ERCC3 exhibit complementation *in vitro* (van Vuuren et al. 1993) and microinjection of free (recombinant) ERCC3 protein is able to induce a partial but clear correction of the repair defect of XP-B cells *in vivo* (van Vuuren et al. 1994). We conclude that a complex containing at least XPD/ERCC2, XPB/ERCC3, TTDA and p62 and possibly also p44 (SSL1) and p34 is implicated in NER and that subunits of this complex can exchange *in vivo* and to some extent also *in vitro*. It is plausible that this complex represents the entire multisubunit TFIIH transcription factor.

Is *TTDA* identical to any of the cloned TFIIH subunits?

A prediction of the findings reported above is that one of the components of BTF2 is responsible for the repair defect in TTD-A for which no repair gene has been isolated yet. Therefore, cDNAs encoding the cloned TFIIH subunits p62, p44 and p34 (Humbert et al. 1994) and *XPD-/ERCC2*(p80) and *XPB/ERCC3*(p89) were inserted into a mammalian expression vector and injected into the nucleus of TTD-A fibroblasts. Prior to injection the cDNA-containing vectors

were checked for their ability to specify proteins of the predicted size. None of these genes (*p62, p44, p34*), nor any of the cloned NER genes (*ERCC1, XPD/ERCC2, XPB/ERCC3, CSB/-ERCC6, XPA, XPC,* and the genes *HHR23A* and *B* (Masutani et al. 1994)) were able to exert correction of the repair defect, whereas micro-injected *ERCC2* and *ERCC3* were able to restore UDS to XP-D and XP-B cells, respectively, in the same experiments. It appears that the TTDA factor is yet another non-cloned component of TFIIH.

Evidence for direct involvement of XPD/-ERCC2 in transcription *in vivo*.

The data described thus far tightly link the repair protein XPD/ERCC2 with transcription factor TFIIH/BTF2, however, they do not demonstrate that the protein is directly involved in transcription. Recently, Schaeffer and coworkers (Schaeffer et al. 1994) have shown that a p80 protein associated with TFIIH is identical to XPD/ERCC2 and that this protein stimulates the TFIIH-dependent *in vitro* transcription reaction. In addition Drapkin and coworkers have shown that an antibody directed against ERCC2 is able to inhibit the *in vitro* transcription reaction (Drapkin et al. 1994). To verify whether the *in vitro* results can be extrapolated to the *in vivo* situation we have conducted antibody microinjection experiments into living normal cells. As shown in Figure 6A and quantitatively in Table 3 introduction of antibodies against XPD/ERCC2 causes a strong inhibition of UV-induced UDS, consistent with the direct role of the protein in NER (Fig.2 and 3) and similar to the effect observed using antibodies against another repair protein ERCC1 that is not detectably associated with purified BTF2 (Table 3). In addition, a strong inhibition of general transcription (as measured by a 1h tritiated uridine labeling) is found (Fig.6B), which is absent in the injections with ERCC1 antiserum (Table 3). Similar, but less pronounced effects on transcription and UDS are exerted by a less powerful serum against the XPB/ERCC3 component of TFIIH (Table 3, see also: (van Vuuren et al. 1994)). These results indicate that XPD/ERCC2 like XPB/ERCC3 is involved in transcription *in vivo*.

Table 2. Effect of immunodepletion of BTF2 components on NER activity

Repair assay	Immunodepleted material	Tested cells/extracts (compl. group)		NER activity (% of control)			
				protA depl.	anti p62 depl.	anti p89 depl.	residual[1]
microneedle injection	crude HeLa lysate	XP25RO	(XP-A)	99	100	94	2
		XPCS2BA	(XP-B)	103	39	33	13
		XP1BR	(XP-D)	98	41	39	21
		TTD1BR	(TTD-A)	102	29	23	8
microneedle injection	BTF2 (Heparin 5PW, fraction 12)	XPCS2BA	(XP-B)	92	37	35	12
		TTD6VI	(XP-B)	100	42	44	44
		XP1BR	(XP-D)	94	37	38	23
		TTD1BR	(TTD-A)	100	35	35	8
in vitro repair	crude HeLa lysate[2]	27.1	(group3)	100	35	ND[3]	34
		UV-5	(group2)	100	32	ND	24
		TTD1BR	(TTD-A)	100	32	ND	27

[1] residual NER activity: amount of repair exhibited by mutated cells/extracts.
[2] p62-depleted crude extract exhibits a residual incorporation of 35% compared to a protA-treated control extract, which is close to incorporation by most mutant extracts.
[3] ND: not determined.

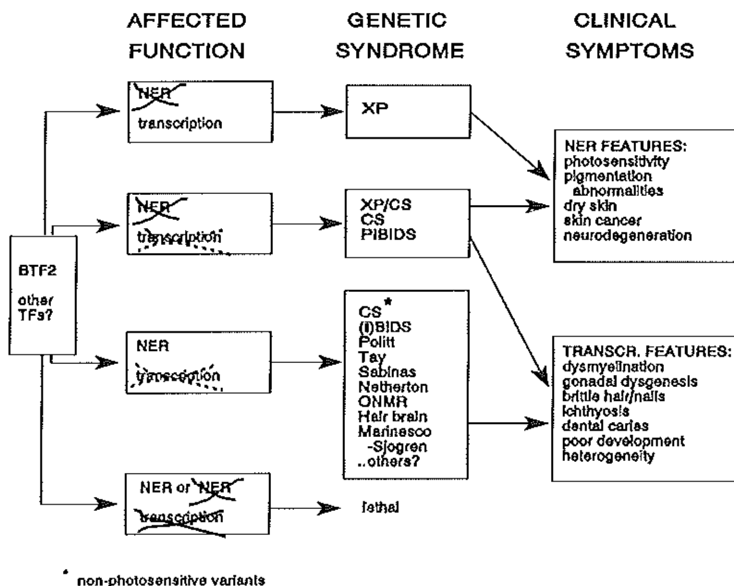

Fig. 7. Model for involvement of defects in transcription and repair in human disease. (see text for explanation).

DISSCUSION

The findings reported here have implications in several directions. At the molecular level the identification of at least three repair factors in TFIIH endows this complex with a dual functionality and extends the functional overlap between two quite different processes, basal transcription and NER. At the clinical level the identification of a link between TTD and XP/CS group B extends the association between TFIIH subunits and TTD and supports the notion that CS, TTD and some forms of XP represent different manifestations of one large clinical continuum. The connection between TFIIH and TTD provides new clues to understand the basis of the complex clinical features displayed by these disorders and potentially a number of other, strikingly related syndromes.

Mechanistic implications for transcription and repair.

A minimum of three NER factors is found to be associated with transcription factor TFIIH. In addition to XPB/ERCC3 (Schaeffer et al. 1993), we demonstrate that XPD/ERCC2 and TTDA reside in this complex. We provide evidence for the involvement of the XPD/ERCC2 helicase in transcription *in vivo*. Circumstantial data support the idea that the transcription proteins p62, p44 and p34 are also implicated in NER (Humbert et al. 1994; van Vuuren et al. 1994), (this paper). As previously anticipated (Bootsma and Hoeijmakers 1993) the human homolog of SSL1, a yeast repair factor which was linked to translation before (Yoon et al. 1992) was recently identified as the p44 subunit of TFIIH (Humbert et al. 1994). Table 4 summarizes the current evidence for the involvement of different human BTF2/

TFIIH and yeast factor b subunits in repair. It is possible that the TTDA protein is identical to the (non-cloned) p41 BTF2 subunit. Together these data suggest that most, if not all, components of the transcription complex participate in excision repair, converting this complex into a functional unit participating in at least two processes.

It is still difficult to estimate how many polypeptides constitute TFIIH. Because of the absence of XPD/ERCC2 as a major protein in the most pure BTF2 preparation (Schaeffer et al. 1994, Drapkin, 1994), it is likely that the five predominant bands present in the final stages of BTF2 purification (Gerard et al. 1991) are derived from the most tightly associated protein fraction constituting the core of the complex. Protein profiles of the rat homolog (factor δ) suggest the presence of at least eigth polypeptides (Conaway and Conaway 1989). Thus BTF2 could well be considerably larger *in vivo* and form a "supercomplex" of which the more loosely bound factors have a tendency to (partly) dissociate during purification. This could also explain the absence of a XPC-correcting activity in our TFIIH purifications, whereas others found association of XPC with TFIIH, but not in the final purification fraction (Drapkin et al. 1994). Alternatively, it is possible that the XPC-correcting activity represents a spurious copurification with TFIIH. Association of XPC with TFIIH is somewhat unexpected, since XPC is thought to be selectively involved in the global genome (transcription-independent) NER pathway (Venema et al. 1990).

BTF2 possesses a bidirectional unwinding activity involving XPB/ERCC3 and XPD/ERCC2 (Schaeffer et al. 1994), which may promote transition from a closed to an open initiation complex. It is likely that TFIIH can be utilized as an independent unit in the context of transcription and NER; e.g., for loading and/or translocation of the preinitiation complex or a NER scanning/incision complex or for the repair synthesis step. The TFIIH mutants XP-B, XP-D, and TTD-A display defects in both the "transcription-coupled" as well as the "global genome" NER subpathways, so the complex is likely to play a role in the core of the NER reaction mechanism (Sweder and Hanawalt 1993).

Clinical heterogeneity and pleiotropy associated with mutations in TFIIH.

The spectrum of diseases linked with TFIIH is heterogeneous and pleiotropic, including seemingly unrelated symptoms, such as photosensitivity, brittle hair and nails, neurodysmyelination, impaired sexual development, ichthyosis, and dental caries. Both the rare XP group B and the more common group D present pronounced clinical heterogeneity: classical XP (only in group D), atypical combination of XP and CS, and TTD (Johnson and Squires 1992; Vermeulen et al. 1994; this paper). The occurrence of patients displaying TTD symptoms within XP group B, extends the parallels between XP group B and D and their respective gene products noted before (Weeda et al. 1990). Clinical variability in TTD is even observed within families and also apparent from the close association with at least 7 disorders (shown in Fig.7) appearing in the OMIM database (McKusick 1992). The occurrence of TTD in 3 NER-deficient complementation groups argues against a chance association between genetic loci separately involved in NER and in brittle hair. Consistent with this notion, mutations in the *XPD/ERCC2* gene have been detected recently in TTD(XP-D) patients (Broughton et al. 1994).

How can we rationalize the pleiotropy and clinical heterogeneity in the above mentioned conditions? The symptoms associated with a sole NER defect are displayed by the most common XP groups, A (totally deficient in NER) and C (defective in the 'genome overall' repair subpathway). Patients in these groups present a relatively uniform clinical picture involving photosensitivity, pigmentation abnormalities, predisposition to skin cancer and, in the case of XP-A, accelerated neurodegeneration (which is not associated with neurodysmyelination), but no CS and TTD symptoms. The gene products affected in these groups are not vital and therefore do not appear to be essential for basal transcription.

Obviously, the hallmarks of a pure NER

Table 3. Effect of antibody injection on repair and transcription

Injected antiserum	% inhibition of NER[1]	inhibition of transcription[2]
rabbit anti ERCC1[3]	97	0
rabbit anti ERCC3[3]	43	48
rabbit anti ERCC2	87	85
preimmune rabbit serum	2	7

[1] compared to UDS level observed in uninjected cells on the same slide.
[2] compared to transcription level (assayed by 1h pulse labelling with ^{3}H-uridine) observed in uninjected cells on the same slide.
[3] van Vuuren et al. (1994).

deficiency do not include the salient features of CS and TTD. It is tempting to link these with the additional transcription-related function. Indeed, it would be highly unlikely when all mutations in the three subunits of this bifunctional complex would only affect the repair function and leave the inherent transcriptional role entirely intact. This interpretation is supported by the *haywire* phenotype of the Drosophila *ERCC3* mutant, involving UV sensitivity, central nervous system abnormalities, and impaired sexual development, as found in XP-B (Mounkes et al. 1992). Spermatogenesis in Drosophila is very sensitive to the level of ß2 tubulin (Kemphues et al. 1982). Mutations in the Drosophila *ERCC3* gene seem to affect ß tubulin expression, causing male sterility (Mounkes et al. 1992). In mammals, ß-tubulin mRNA is selectively regulated by a unique cotranslational degradation mechanism (Theodorakis and Cleveland 1992). It is possible that this renders ß-tubulin expression particularly sensitive to the level of transcription and thereby to subtle mutations in BTF2, resulting in the immature sexual development found in TTD and CS. Similarly, reduced transcription of genes encoding ultrahigh sulfur proteins of the hairshaft may account for the observed reduced cysteine content in the brittle hair of TTD patients (Itin and Pittelkow 1990). Low expression of the myelin basic protein, whose transcription is known to be rate-limiting in mouse (Readhead et al. 1987), may cause the characteristic neurodysmyelination of CS and TTD (Peserico et al. 1992; Sasaki et al. 1992). A comparable explanation is proposed for the poor enamelation of teeth in CS and TTD (McCuaig et al. 1993; Nance and Berry 1992). The skin abnormalities typical of TTD often involve ichthyosis. Various classes of ichthyoses show abnormalities in the production of filaggrin (Fleckman and Dale 1993). Thus, mutations in TFIIH which subtly disturb its transcription function may affect a specific subset of genes whose functioning critically depends on the level or fine-tuning of transcription. Recent studies indicate that the requirement for basal transcription factors may vary from promoter to promoter depending on the sequence around the initiation site, the topological state of the DNA, and the local chromatin structure (Parvin and Sharp 1993; Stanway 1993; Timmers 1994). These mechanisms can easily explain the pronounced clinical heterogeneity even within families.

Upon close inspection (Bootsma and Hoeijmakers 1993), many parallels can be found between CS and TTD. To a varying degree, CS patients exhibit features prominent in TTD, such as thin, dry hair and scaly skin (Nance and Berry 1992). TTD has recently been recognized to include neurodysmyelination (Peserico et al. 1992), bird-like facies, dental caries, and cataract (McCuaig et al. 1993), hallmarks normally associated with CS. This suggests that CS and TTD are manifestations of a broad clinical continuum, consistent with the notion that mutations in different subunits of the same (TFIIH) complex give rise to a similar set of phenotypic features. In this proposition, defects in the classical CS genes *CSA* and *ERCC6/CSB* (Troelstra et al. 1992), as well as *XPG/ERCC5* (Bootsma and Hoeijmakers 1993; Vermeulen et al. 1993), are expected to somehow affect basal transcription as well.

Model for involvement of transcription and repair in CS and TTD: deduction of the existence of transcription syndromes.

A tentative model proposed for the aetiology of the defects in the conglomerate of CS, TTD, and related disorders is shown in Fig.7. In this model, mutations in BTF2 factors inactivating only the NER function result in a XP phenotype as observed in the classical XP patients of XP

Table 4. The NER connection of BTF2/TFIIH

NER proteins associated with BTF2	NER proteins with yeast factor b	Polypeptides identified in SDS-PAGE
XPB/ERCC3	**RAD25/SSL2**[2]	p89
XPD/ERCC2	**RAD3**[3]	p80
TTDA	?	?
P62[1]	TFB1[4]	p62
p44[1]	SSL1[4]	p44
?	?	p41[5]
?	?	p34

Factors for which the involvement in NER is unequivocally demonstrated are in boldface.
[1] NER function based on inference from (presumed) NER involvement of yeast homologs.
[2] references: Park (1992); Feaver (1993).
[3] reference: Feaver (1993).
[4] some alleles are UV sensitive, suggesting that they are deficient in NER.
[5] the relationship between p41 and TTDA is not yet known.

group D. If, in addition, the transcription function is subtly affected, the photosensitive forms of combined XP/CS and TTD are found. Theoretically, mutations causing a (still viable) transcription problem without NER impairment are predicted. The notion, that the new TTD XP-B members have only a mild repair defect (see table 2), but nevertheless display TTD features not exhibited by the more repair-deficient original XP-B cases, fits perfectly into this reasoning. Indeed, a significant proportion of TTD patients, as well as clinically characteristic CS patients, is not noticably photosensitive and has normal NER (Lehmann 1987; Lehmann et al. 1993; Nance and Berry 1992). These findings extend the implications to non-repair-defective disorders. Therefore, and in view of the pronounced heterogeneity inherent to the model, we propose that also the Sjögren-Larsson (270200), RUD (308200), ICE (146720), OTD (257960), IFAP (308205), CAM(F)AK (214550), Rothmund-Thompson (268400), and KID (242150) syndromes (for references, see Baden 1991; McKusick 1992) also fall within this category. Interestingly, some of these diseases show occurrence of skin cancer.

In conclusion, our findings provide evidence for the presence of a wide class of disorders that we propose to designate collectively as "transcription syndromes". A prediction from our model is that these patients carry mutations in transcription factors, that do not affect the NER process. This proposition is testable. The explanation put forward here for this class of disorders would introduce a novel concept into human genetics. It can be envisaged that similar phenomena are associated with subtle defects in translation, implying the potential existence of "translation syndromes" (as suggested earlier on completely different grounds; Fisher et al. 1990).

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Chapter 6

P44 and p34 subunits of the BTF2/TFIIH transcription factor havehomologies with SSL1, a yeast protein involved in DNA repair

p44 and p34 subunits of the BTF2/TFIIH transcription factor have homologies with SSL1, a yeast protein involved in DNA repair

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The human BTF2 (TFIIH) transcription factor is a multisubunit protein involved in transcription initiation by RNA polymerase II (B) as well as in DNA repair. In addition to the previously characterized p62 and p89/ERCC3 subunits, we have cloned two other subunits of BTF2, p44 and p34. The gene encoding p44 appeared to be the human counterpart of *SSL1*, a gene involved in translation and UV resistance in yeast. Interestingly, the p34 subunit also has homology with a domain of SSL1, suggesting that it corresponds to an as yet unidentified protein involved in DNA repair. Both p44 and p34 possess zinc finger domains that may mediate BTF2 binding to nucleic acids.

Key words: BTF2 (TFIIH)/DNA repair/SSL1/transcription factor

Introduction

BTF2 (TFIIH) is an essential class II transcription factor that has recently been shown to play a role in nucleotide excision repair (NER) (Schaeffer *et al.*, 1993). This basal factor participates in the formation of the preinitiation transcription complex, which includes the TFIIA, TFIID, TFIIB, TFIIE, and TFIIF basal transcription factors and RNA polymerase II (B) onto a minimal promoter (containing the TATA box and cap site) (Conaway and Conaway, 1991; Zawel and Reinberg, 1992). BTF2, like its homologs the rat factor δ and the yeast factor b, is absolutely required for the basal *in vitro* transcription of most protein coding genes. Little is known about the role of BTF2 in the transcription reaction, although a kinase activity which is capable of phosphorylating the carboxy-terminal domain (CTD) of the large subunit of RNA polymerase II, and a DNA-dependent ATPase activity have been found to be associated with BTF2 and its rat and yeast homologs (Bunick *et al.*, 1982; Sawadogo and Roeder, 1984; Conaway and Conaway, 1988; Feaver *et al.*, 1991; Dahmus and Dynan, 1992; Lu *et al.*, 1992; Serizawa *et al.*, 1992). These two activities may be responsible for the obligate ATP hydrolysis required for completing transcriptional initiation.

A role of BTF2 in NER was recently suggested when one of the subunits (the 89 kDa polypeptide; p89) was identified as the *ERCC3* gene product, a presumed helicase associated with DNA repair (Weeda *et al.*, 1990; Schaeffer *et al.*, 1993; van Vuuren *et al.*, 1994). Mutations in this gene confer sensitivity to sunlight (UV) and a predisposition to skin cancer manifested by xeroderma pigmentosum group B (XP-B), a severe form of this repair syndrome, which also exhibits the clinical hallmarks of another DNA repair disorder, namely Cockayne's syndrome. Further experiments, using both an *in vivo* microinjection repair assay and an *in vitro* NER system based on cell-free extracts (Wood *et al.*, 1988), confirmed that the p89/ERCC3 subunit of BTF2 is directly involved in the excision repair reaction (van Vuuren *et al.*, 1994). More recent results have shown that the ERCC2 80 kDa polypeptide is also associated with the BTF2 complex, although not as tightly as p89 (Schaeffer *et al.*, 1994).

The dual function of BTF2 in these two important, but otherwise quite distinct, mechanisms of DNA metabolism has recently generated some controversy as to whether BTF2 is indeed a transcription repair coupling factor, or whether components of BTF2 function independently in each of the two processes (Sweder *et al.*, 1993). A means to resolve this disparity lies in the cloning of all subunits of BTF2, which will permit the elucidation of their individual roles and that of the entire complex in DNA repair and transcription. As a step toward this goal, we report here the cloning of two additional subunits of this multifunctional complex. Analysis of these components provides indications that BTF2 may be involved in yet another basic cellular process: translation.

Results

Cloning and expression of the p44 and p34 polypeptides of BTF2/TFIIH

The BTF2 (TFIIH) transcription factor was purified as previously described (Gérard *et al.*, 1991). A concentrated hydroxyapatite fraction was subjected to SDS−PAGE and electrotransferred onto a PVDF membrane. The 44 kDa (p44) and the 34 kDa (p34) polypeptides were then individually digested with trypsin, before being resolved by reversed phase chromatography. Amino acid sequences obtained from tryptic digests of p44 or p34 were used to synthesize degenerate oligonucleotides for screening a HeLa λZAPII cDNA library. Multiple positive clones were obtained, and sequencing revealed that for each, one clone contained the entire open reading frame (ORF). The p44 and p34 cDNAs (Figure 1) possess ORFs of 1185 and 909 bp respectively that encode proteins of 395 and 303 amino acids, with calculated mol. wts of 44 451 and 33 920 Da respectively, which is in good agreement with the previously estimated mol. wt of the purified protein on an SDS gel (43 and 35 kDa respectively; Gérard *et al.*, 1991). Moreover, when overexpressed in *Escherichia coli*, both

```
A
p44    1 MDEEPERTKR................WEGGYERTWEILKEDESGSLKATI
SSL1  50 PDKHVQRKKKKRLSNRNLQGSNGGYAWEDEIKRSWDLVKVDDEGDMASLV

      35 EDILFKAKRKRVFEHHGQVRLGMMRHLYVVVDGSRTMEDQDLKPNRLTCT
     100 ASIVEARKKRTAKKNIIPYERGIIRSLILTLDCSEAMLEKDLRPNRHAMI

      85 LKLLEYFVEEYFDQNPISQIGIIVTKSKRAEKLTELSGNPRKHITSLKKA
     150 IQYAIDFVHEFFDQNPISQMGIIIMRNGLAQLVSQVSGNPQDHIDALKSI

     135 VDMTCHGEPSLYNSLSMAMQTLKHMPGHTSREVLIIFSSLTTCDPSNIYD
     200 RKQEPKGNPSLQNALEMARGLLLPVPAHCTREVLIVFGSLSTTDPGDIHQ

     185 LIKTLKAAKIRVSVIGLSAEVRVCTVLARET....GGTYHVILDESHYKE
     250 TIDSLVSEKIRVKVLGLSAQVAICKELCKATNYGDESFYKILLDETHLKE

     231 LLTHHLSP.PPASSSSECSLIRMGFPQHTIASLSDQDAKPSFSMAHLDGN
     300 LFNEAVTPLPVNKINKGFTLVKMGFPTRIF......EDTPTFCSCH....

     280 TEPGLTLGGYFCPQCRAKYCELPVECKICGLTLVSAPHLARSYHHLFPLD
     340 ..SKLVYGGYFCPNCHSKVCSLPTVCPCCDLMLILSTHLARSYHHLMPLK

     330 AFQEIPLEEYNGERFCYGCQ............GELKDQHVYVCAVCQNVF
     388 TFAEVPTTEKFRSEDCFSCQSRFPILKNHKNGKLLTSSRYRCEDCKQEF

     368 CVDCDVFVHDSLHCCPGCIHKIPAPSGV 395
     437 CVDCDVFIHEILHNCPGCESKPVIT 461
B
p34    1 MVSDEDELNLLVIVVDANPIWWGKQALKESQFTLSKCIDAVMVLGNSHLF

      51 MNRSNKLAVIASHIQESRFLYPGKNGRLGDFFGDPGNPPEFNPLGSKDGK

     101 YELLTSANEVIVEEIKDLMTKSDIKGQHTETLLAGSLAKALCYIHRMNKE

     151 VKDNQEMKSRILVIKAAEDSALQYMNFMNVIFAAQKQNILIDACVLDSDS

     201 GLLQQACDITGGLYLKVPQMPSLLEYLLWVFLPDQDQRSQLILPPPVHVD

     251 YRAACFCHRNLIEIGYVCSVCLSIFCNFSPICTTCETAFKISLPPVLKAK

     301 KRN
C
SSL1 332 PTFCSCH......SKLVYGGYFCPNCHSKVCSLPTVCPCCDLMLILS
p34  252 RAACFCH......RNLIEIGYVCSVCLSIFCNFSPICTTCETAFKIS
p44  269 PSFSMAHLDGNTEPGLTLGGYFCPQCRAKYCELPVECKICGLTLVSA
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Fig. 1. Sequences of p44 (A) and p34 (B) BTF2 polypeptides, and alignments with SSL1 (A and C). Positions of the amino acids are indicated on the left. The peptides that were microsequenced from the purified 44 and 34 kDa polypeptides are underlined. The zinc finger motifs in p44 and p34 are boxed or in bold characters (see text). The SSL1 sequence has been aligned with the p44 sequence and homologies are indicated by | (threshold 1), : (threshold 0.5) or . (threshold 0.1). The nucleotide sequences of p44 and p34 subunits can be found in the GenBank database under the accession numbers Z30094 and Z30093, respectively.

recombinant polypeptides (rp44 and rp34) exhibit the same electrophoretic mobility on SDS−PAGE as the endogeneous BTF2 p44 and p34 polypeptides according to immunoblotting experiments depicted in Figure 2A (compare lane 1 with lanes 2 and 3 respectively). In both cases the microsequenced peptides were observed in the deduced amino acid sequences confirming the identity of the clones (Figure 1A and B).

Searches in DNA and protein databases revealed a significant overall homology between p44 and the yeast protein SSL1 (Figure 1A). SSL1 is a yeast zinc finger protein previously identified as the product of a stem−loop suppressor gene associated with initiation of translation (Yoon *et al.*, 1992). At the amino acid level, p44 and SSL1 have a 58% similarity and a 40% identity. p44 as well as SSL1 contains the motif Tyr-X-Cys-X_2-Cys-X_3-Phe-

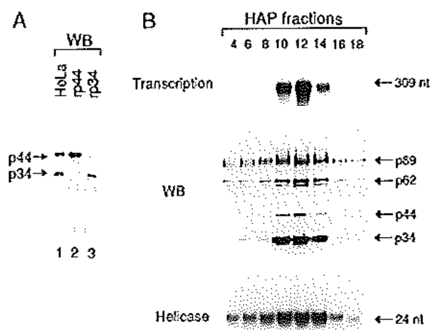

Fig. 2. Western blot analysis of endogenous and recombinant p44 and p34 (A) and copurification of BTF2 polypeptides with transcription and helicase activities over the hydroxyapatite gradient (B). (A) An SDS gel was loaded with either 2 μl of a heparin HPLC fraction (lane 1), or 0.01 μl of the pelleted fraction of either rp44 (lane 2) or rp34 (lane 3). After transfer to nitrocellulose, the polypeptides were detected using monoclonal antibodies raised against each subunit. (B) The BTF2 hydroxyapatite fractions were tested in a standard run-off transcription assay lacking BTF2 (2 μl), in Western blot (10 μl) for the presence of the four cloned subunits of BTF2 (p89, p62, p44 and p34) and in a helicase assay (2 μl). The arrows on the right indicate respectively the 309 nucleotide transcript (Transcription), the BTF2 polypeptides (WB) or the oligonucleotide (24 nt) displaced from the single-stranded DNA (Helicase).

X_8-His-X_2-Leu-His (amino acids 358−380) characteristic of TFIIIA-like zinc finger proteins that have been demonstrated to interact with DNA (Jacobs, 1992; Berg, 1993). We also noticed in p44 as well as SSL1, a repeat of the motif Cys-X_2-Cys-X_4-Cys/His-X_5-Cys-X_2-Cys interrupted by a Cys-X_2-Cys-X_{11}/X_{22}-Cys-X_2-Cys domain. Surprisingly, the first type is also present in p34. Alignment of the three sequences in this region (Figure 1C) highlights the conservation of the cysteine residues among the three polypeptides, which also suggests that p44 and p34 must share some common function, e.g. in the interaction with the DNA template. Preliminary results suggest that p44 indeed interacts with the DNA promoter (unpublished results). No other known motifs were found, but we noticed that the p34 is rich in leucine and isoleucine residues (20% over the entire sequence) which confers a very hydrophobic character to the polypeptide.

The p44 and p34 polypeptides are bona fide subunits of BTF2

When overexpressed in *E.coli*, the p44 and p34 polypeptides remained in an insoluble form (up to 98%) and were therefore subjected to denaturation−renaturation with guanidium hydrochloride. In this condition a small part of the proteins was recovered in a soluble form that was used in the following experiments.

The recombinant polypeptides p44 and p34 did not substitute for the BTF2 activity when added either alone or in combination with the two other bacterially expressed BTF2 subunits (p62 and p89) in a BTF2-dependent *in vitro* transcription system (data not shown). Indeed, this was expected since our purification evidenced at least an

additional polypeptide in BTF2 (Gérard *et al.*, 1991).

Human BTF2 (TFIIH), the rat factor δ and the yeast b factor have been found to contain several activities including a DNA-dependent ATPase (Roy *et al.*, 1994), a CTD kinase capable of phosphorylating the CTD of the largest subunit of RNA polymerase II (Feaver *et al.*, 1991; Lu *et al.*, 1992; Serizawa *et al.*, 1992) and a helicase (Schaeffer *et al.*, 1993), thus suggesting the presence of putative helicase motifs, ATP-binding sites, or kinase motifs in the various BTF2 subunits. None of the above motifs was detected in either p44 or p34 and no kinase, ATPase or helicase activities could be found associated with p44 or p34 when tested in the three enzymatic assays under the conditions used for the native BTF2 complex (data not shown). Furthermore, no stimulation of any of these activities was observed upon addition of p44 or p34.

Despite our inability to detect any of the aforementioned biological activities associated with the two polypeptides, several lines of evidence indicate that both p44 and p34 are indeed subunits of BTF2 (TFIIH). First, monoclonal antibodies raised against the p44 (Ab-p44) and p34 (Ab-p34) polypeptides recognized the 44 and 34 kDa polypeptides (Figure 2B, middle panel) that cofractionated with BTF2 transcription activity (upper panel) and the helicase activity (lower panel) through the HAP chromatography (sixth step of the purification; Gérard *et al.*, 1991) as well as on the glycerol gradient sedimentation step (data not shown; see also Figure 3B, upper panel). Furthermore, both p44 and p34 perfectly coelute with the previously described p62 and p89 (lanes 10–14) that have previously been shown to belong to BTF2 transcription factor (Fischer *et al.*, 1992; Schaeffer *et al.*, 1993); the CTD kinase and the DNA-dependent ATPase activities were also found to coelute with the four BTF2 subunits (unpublished results; Roy *et al.*, 1994). Identical results were also observed with the phenyl fractions (fifth step of our purification procedure; Gérard *et al.*, 1991). Thus, in the three last steps of the purification scheme (using three different separation techniques based either on the hydrophobic character, the charge or the sedimentation constant of the BTF2 complex), p44 and p34 are always found tightly associated with the various activities and polypeptides characteristic of BTF2. In addition, when the BTF2 multisubunit complex is treated with 1 M KCl before glycerol gradient sedimentation, the four polypeptides cosediment in a region corresponding to ~200–250 kDa (unpublished results).

Second, to confirm that p44 and p34 belong to the BTF2 multisubunit complex, two additional sets of experiments were performed: an immunoprecipitation (Figure 3A) and a glycerol gradient shift (Figure 3B) in which either of the two previously described monoclonal antibodies was used to precipitate or to shift all the BTF2 subunits. A partially purified BTF2 fraction eluted from the heparin column (Gérard *et al.*, 1991), was incubated either with protein A–agarose beads alone (– Ab, lanes 3 and 7) or with protein A–agarose beads to which anti-p44 (lane 2) or anti-p34 (lane 6) antibodies or an antibody against an unrelated polypeptide (Ab control, lanes 4 and 8) were bound. After extensive washing with a buffer containing 0.15 M KCl, the remaining proteins were analyzed by SDS–PAGE (Figure 3A). The anti-p44 or anti-p34 antibodies immunoprecipitate both p34 and p44 in addition to p62 and p89 (lanes 2 and 6, respectively), while the protein A–agarose, either alone

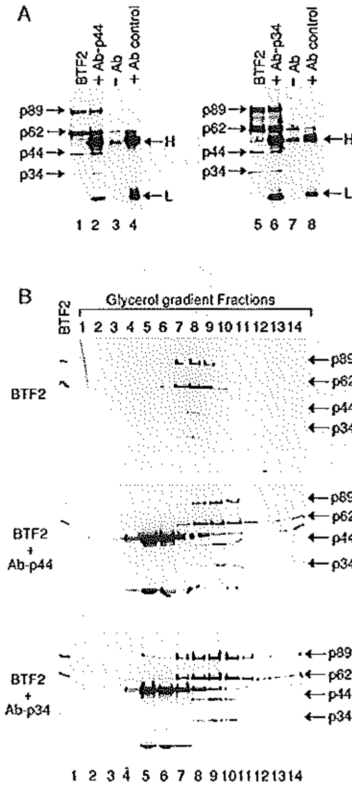

Fig. 3. The monoclonal antibodies raised against p44 or p34 immunoprecipitate BTF2 (A) and displace this complex in a glycerol gradient (B). (A) 50 μl of the BTF2 heparin HPLC fraction were incubated for 2 h at 4°C with either no antibody (lanes 3 and 7), anti-p44 antibody (lane 2), anti-p34 antibody (lane 6), or an unrelated control antibody (lanes 4 and 8). These mixtures were incubated with protein A–Sepharose. The beads were washed and loaded onto an SDS–polyacrylamide gel. After electrophoresis and transfer onto nitrocellulose, the various BTF2 subunits (as indicated by arrows on the left) were detected with the corresponding antibody. Lanes 1 and 5: 2 μl of BTF2 heparin HPLC fraction. (B) 100 μl of heparin HPLC BTF2 fraction were incubated for 2 h at 4°C with either no antibody (upper panel), anti-p44 antibody (middle panel) or anti-p34 antibody (lower panel). After centrifugation for 12 h at 300 000 g on a 10–30% glycerol gradient, 15 fractions were collected and analyzed by Western blotting using anti-p62, anti-p44 and anti-p34 antibodies. The arrows indicate the position of the four BTF2 subunits. The heavy (H) and light chain (L) subunits of the antibody are also illuminated by the second anti-mouse antibody.

or coupled to the control antibody, precipitated none or negligible amounts of p89, p62, p44 and p34 polypeptides (lanes 3, 4, 7 and 8). Thus, both of the antibodies raised against either p44 or p34 are able to coprecipitate p62 as well as p89 in addition to their corresponding antigenic p44 and p34 subunits. No transcription activity was recovered either from the protein A–agarose beads on which Ab-p44 or Ab-p34 were immobilized or from their corresponding

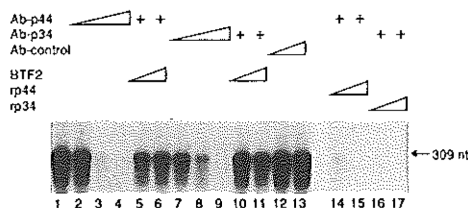

Fig. 4. Inhibition of *in vitro* transcription by anti-p44 or anti-p34 antibodies. The BTF2 heparin HPLC fraction (10 μl) was preincubated for 2 h at 4°C with either no antibody (lane 1), increasing amounts of anti-p44 (lanes 2–4), anti-p34 (lanes 7–9) or control (lanes 12 and 13) antibodies, or with a 100% inhibitory amount of anti-p44 (lanes 5 and 6, and 14 and 15) or anti-p34 (lanes 10 and 11, and 16 and 17) antibodies. The reaction was then complemented with the other basal transcription factors, RNA polymerase II, DNA template and as indicated, increasing amounts of either BTF2 hydroxyapatite fraction (lanes 5 and 6, and 10 and 11), rp44 (lanes 14 and 15) or rp34, (lanes 16 and 17). After 15 min of incubation at 25°C, the nucleotides were added and transcription was allowed to proceed for 45 min at 25°C. The transcripts were analyzed as previously reported. The arrow indicates the 309 nucleotide long specific transcript.

supernatants due to the inhibitory effect of the antibody (see also below).

In another set of experiments, BTF2 was preincubated either alone (BTF2) or with either Ab-p44 (BTF2 + Ab-p44) or Ab-p34 (BTF2 + Ab-p34) before glycerol gradient centrifugation (Figure 3B). In both cases, and thus independent of the nature of the antibody, p44 and p34 were shifted from fractions 7–9 in the absence of antibody (upper panel) to fractions 8–11 in the presence of either antibody (middle and lower panels). The p62 subunit as well as the p89 subunit (see Figure 3B) followed p44 and p34, thus demonstrating that the antibodies were able to displace the other subunits of BTF2. Altogether, these data demonstrate that p44 and p34 are strongly associated with the previously characterized p62 and p89 BTF2 subunits as well as with all the enzymatic activities exhibited by BTF2.

Finally, the antibodies were tested for their ability to prevent BTF2 transcription activity in an *in vitro* transcription assay (Figure 4). Increasing amounts of either purified Ab-p44, Ab-p34 or Ab-control were incubated for 1 h at 4°C, with a fixed amount of partially purified BTF2 (heparin HPLC fraction; Gérard *et al.*, 1991). The mixture was then added to the *in vitro* transcription system containing RNA polymerase II, the Ad2 MLP template and all basal transcription factors except BTF2, before addition of nucleotides. As shown in Figure 4, transcription was reduced as a function of the concentration of Ab-p44 and Ab-p34, whereas transcription was not inhibited when increasing amounts of the control antibody were added (compare lanes 2–4 and lanes 7–9 with lanes 12–13, respectively). To establish further that inhibition resulted from the specific interaction between the two antibodies and BTF2, increasing amounts of BTF2 were added to an *in vitro* transcription reaction that was previously 100% inhibited after addition of each of the two antibodies. In both cases, we were able to restore the transcription activity as shown by the synthesis of a specific transcript of 309 nucleotides (see lanes 5 and 6 and lanes 10 and 11, respectively). Increasing amounts of rp44 or rp34 did not restore BTF2 transcription activity (lanes 14–17). In conclusion all of the above data unambiguously demonstrate the participation of both p44 and p34 in BTF2 transcription activity.

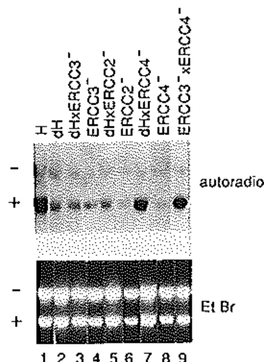

Fig. 5. Inhibition of *in vitro* NER activity. Monoclonal antibodies against p34, deplete a repair-proficient HeLa extract from repair capacity. A cell-free HeLa extract was incubated with anti-p34 antibody immobilized on protein A–Sepharose CL-4B beads. After removal of the proteins bound to the antibody by centrifugation, the supernatant was tested in an *in vitro* NER assay for repair and complementation activities (Wood *et al.*, 1988). The *in vitro* NER assay contained 250 ng AAT-damaged plasmid (+) and an equal amount of non-damaged plasmid (–). The upper panel (autoradio) detected the [α-^{32}P]dATP incorporation that indicates the repair activity and the lower panel (EtBr) shows the ethidium bromide stained DNA gel. Lane 1: HeLa extract (H) treated with protein A beads as a non-depleted control. Lane 2: HeLa extract depleted with Ab-p34 (dH). The depleted HeLa extract was mixed (1:1) with extracts of rodent complementation group 3 (ERCC3⁻; lane 3), group 2 (ERCC2⁻; lane 5) or group 4 (ERCC4⁻; lane 7). The extracts were also tested in the absence of HeLa extract, group 3 (lane 4), group 2 (lane 6) and group 4 (lane 8). Lane 9: detection of complementation activity using two unrelated extracts (groups 3 and 4).

Inhibition of in vitro NER activity by anti-p44 or anti-p34 antibodies

Since the antibodies were able to immunoprecipitate the BTF2 complex, we have tested their effects on NER activity. Antibody depletion experiments were thus performed using an *in vitro* NER system (Wood *et al.*, 1988; van Vuuren *et al.*, 1993). Repair-proficient HeLa whole cell extracts were incubated with anti-p34 antibody that was immobilized on protein A–Sepharose beads. After removal of the beads by centrifugation, the supernatant was tested for its repair capacity. The anti-p34 depleted HeLa extract (Figure 5, lane 2) showed a clear reduction in repair activity in comparison with the non-depleted HeLa extract (lane 1). Furthermore, Western blot analyses confirmed the depletion of p44 and p34 (data not shown). In order to determine whether additional NER factors were simultaneously removed, the treated extract was mixed with rodent repair-deficient extracts of complementation group 2, 3 or 4. The depleted HeLa extract had lost the ability to restore repair activity for ERCC2 (lane 5) and ERCC3 (lane 3) but not ERCC4 (lane 7). These findings indicate that the anti-p34 antibody not only removed p89 (ERCC3), but also ERCC2, a 80 kDa polypeptide that we recently found associated with BTF2 (Schaeffer *et al.*, 1994), which is in agreement with immunoprecipitation experiments using the heparin fraction

(van Vuuren *et al.*, 1994). Similar results were obtained using the anti-p44 antibody (data not shown).

Discussion

Here, we report the cloning and characterization of two additional subunits of BTF2 that appear to be intimately associated with the p62 subunit which shares homology with the 74 kDa subunit (TFB1) of yeast transcription factor b (Gileadi *et al.*, 1992) and the p89 subunit that corresponds to the product of the *ERCC3* gene (Schaeffer *et al.*, 1993). All our data demonstrate unequivocally that both polypeptides are subunits of BTF2. First, p44 and p34 copurified not only with the other identified subunits of BTF2 (p89 and p62) but also with the previously characterized enzymatic activities of BTF2 (helicase, DNA-dependent ATPase and CTD kinase). Second, all subunits can be immunoprecipitated or shifted on a glycerol gradient with monoclonal antibodies raised against p44 or p34 (or p62; not shown). Third, the latter antibodies inhibit an *in vitro* transcription assay, and this inhibition can be relieved by addition of purified BTF2.

Preliminary experiments to reconstitute the transcription activity using the four recombinants subunits of BTF2 (p89, p62, p44 and p34) either alone or in combination were not successful. This may be due to the lack of correct folding of the protein in *E.coli*, but also, and most likely, to the absence of some other component(s) of the BTF2 factor. It also remains possible that additional polypeptides that are not strongly associated with BTF2 (three other polypeptides were found to coelute with the five polypeptides of BTF2; Fischer *et al.*, 1992) can modulate the function of the 'core' BTF2 to select its implication in either transcription or NER but it is still unclear which ones constitute the transcriptional 'core' or the repair function of BTF2. In a similar context, we also noticed that the most purified fraction of the δ factor, the rat homolog of BTF2, contains at least seven polypeptides (Conaway and Conaway, 1989). Although we have demonstrated that the recombinant ERCC3, the p89 subunit of BTF2, contains a DNA helicase activity (Schaeffer *et al.*, 1993), we were unable to detect any enzymatic activity such an ATPase, or CTD kinase associated with p44 or p34. In fact, none of the characteristic motifs for such function were obvious when considering their amino acid sequence. Instead, both proteins contain various zinc finger motifs, such as Cys-X_2-Cys-X_{12}-His-X_3-His, or Cys-X_2-Cys-X_{10}-Cys-X_2-Cys, that may mediate the binding of BTF2 to the DNA template. More interesting is the observation that there is some common zinc finger to both p44 and p34 proteins that may suggest some similarity in their function. It will be interesting to determine which of the identified zinc fingers is reponsible for the DNA binding and if so, if such a polypeptide displays sequence specificity.

The dual role of BTF2 in NER as well as in transcription initially suggested by the identification of ERCC3 as one subunit of BTF2, is further strengthened by the following observations. First, antibodies against p44 or p34 were able to deplete both the *in vitro* NER system and *in vitro* transcription. This strongly suggests that p44 and/or p34 are either directly involved or are associated with one or more polypeptides of the BTF2 protein complex that are absolutely required for either of the two reactions. Secondly, p44 has significant homology (58% similarity) with SSL1, a protein that has been shown to play a role in UV resistance in yeast as well as translational initiation (Yoon *et al.*, 1992). Thus, p44 is most likely the human homolog of SSL1.

The relationship between p44 and the function of SSL1 is difficult to rationalize with the observations reported by Yoon *et al.* (1992) who strongly assert that SSL1 acts at the translation initiation level. SSL1 was identified in a search for screening for suppressors of an artificial stem–loop in the 5'UTR of the selectable HIS4 mRNA that is assumed to block translation. The role of SSL1 in translation was further confirmed by the fact that an extract of ts^- SSL1 suppressor strains presents a decrease of protein synthesis from exogenously added mRNA in a cell-free *in vitro* translation system. In the same search for suppressors, they isolated SSL2, the yeast counterpart of ERCC3/p89 (Gulyas and Donahue, 1992). Thus, our findings link a second presumed translation regulating protein with the basal transcription factor BTF2. This is consistent with the genetic cross studies of Gulyas and Donahue (1992) who showed that SSL1 and SSL2 interact with each other. This interaction likely occurs in the yeast homolog of BTF2, the transcription factor b, which also contains the TFB1 subunit (Gileadi *et al.*, 1992; p62 BTF2 homolog), and suggests that SSL1 is a component of the yeast factor b. The reported role of the SSL1/p44 protein in translation (Yoon *et al.*, 1992) and the claimed involvement of the SSL2/p89 subunit (Gulyas and Donahue, 1992) in this process, when correct, would extend the multiple functioning of BTF2 from repair and transcription to protein synthesis. BTF2 was shown to contain ERCC3, a 3'–5' helicase, and to be associated with the ERCC2 5'–3' helicase (Schaeffer *et al.*, 1993, 1994). This bidirectional unwinding capacity would allow reading of the coding strand in transcription initiation, removal of the damaged sequence in DNA repair and ribosomal binding/scanning of the mRNA in translation. In light of all these data, it appears now that at least two subunits of BTF2, i.e. p89 (ERCC3) and p44 (SSL1), may be involved in several essential functions of the cell such as transcription, DNA repair and translation. The further characterization of BTF2 and the reconstitution of the whole complex will represent a major step towards the understanding of its role in eukaryotic gene expression.

Materials and methods

Isolation and sequence of cDNA clones

Microsequencing of the polypeptides, screening of the HeLa cDNA library constructed in the λZAPII vector (Stratagene) and sequencing of the positive clones were as described (Fischer *et al.*, 1992). The 1.2 kb p44 cDNA was generated by PCR using the following oligonucleotides: 5'-TGAAACATATGGATGAAGAACCTGTAAAGAACT-3' and 5'-GACCGGATCCTCAAACACCTGAAGGAGCTGGA-3'. The resultant PCR fragment was inserted into the *Nde*I–*Bam*HI site of pET11a (Novagene).

To subclone the p34 ORF, the pET3d plasmid (Novagene) cut by *Nco*I–*Bam*HI was ligated to an *Nco*I–*Bst*EII–*Bam*HI adaptor. The 1038 bp *Nco*I–*Bst*EII fragment containing the entire ORF of p34 was cloned in the modified pET3d digested by *Nco*I and *Bst*EII.

Expression of recombinant p44 and p34

E.coli strain BL21(DE3) (Novagene) containing the pET11a-p44 or pET3d-p34 plasmids were grown in LB medium supplemented with ampicillin (100 μg/ml) at 37°C. Cultures were induced with isopropyl-β-D-thiogalactopyranoside (0.4 mM) at an optical density at 600 nm of 0.6. After 2 h at 37°C, the cells were collected by centrifugation and the pellet was resuspended in 50 mM Tris–HCl pH 8, 1 mM EDTA pH 8, 100 mM KCl. The cells were then frozen, thawed and lysed by sonication on ice.

Soluble and non-soluble fractions were collected after centrifugation. Renaturation of the recombinant polypeptides was performed as described (Hager and Burgess, 1980).

Production of antibodies

The antibodies against rp44 (1H5) and rp34 (2B1) were produced as described (Fischer *et al.*, 1992). The p44 antibody was raised to a polypeptide from amino acids 1 to 17. For p34, the entire protein that was overexpressed in *E.coli* was used. The anti-p62 and anti-p89 monoclonal antibodies were as described in Fischer *et al.* (1992) and Schaeffer *et al.* (1993) respectively. The monoclonal antibodies were purified from ascite fluids by caprilic acid and ammonium sulfate precipitations.

Immunoprecipitation

The BTF2 heparin fraction was incubated for 2 h at 4°C with the antibodies and then for 1 h with protein A–Sepharose FF (Pharmacia) in TG10EK150 (50 mM Tris–HCl pH 7.9, 10% glycerol, 0.1 mM EDTA, 150 mM KCl) containing 1 mg/ml BSA. Beads were washed twice with TG10EK150 containing 0.1% NP40 and 1 mg/ml BSA and once with TG10EK150 containing 0.15% NP40, and resuspended in SDS–PAGE loading buffer. After SDS–PAGE and transfer to nitrocellulose, the various polypeptides were detected with the corresponding antibodies.

Other techniques

The purification of BTF2 and all the basic transcription factors required for the transcription run-off assay were as previously described (Gérard *et al.*, 1991). The *in vitro* NER assay was as described (Weeda *et al.*, 1990). Mutants 27.1 (*ERCC3*$^-$) and UV140 (*ERCC4*$^-$) are as described by Wood and Burki (1982) and Busch *et al.* (1994) respectively.

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Summary/Samenvating

Summary

A network of DNA repair processes protects the genetic information of organisms from accumulation of deleterious mutations. Alterations in DNA can be due to chemical instability intrinsic to the DNA itself or to exposure to genotoxic agents. Nucleotide excision repair (NER) is one of the most important repair mechanisms in the cell that removes a broad range of structurally unrelated lesions, for instance lesions induced by UV-light. If not repaired, lesions may disturb essential cellular processes as transcription, recombination, replication, or lead to mutations. This might result in malfunction of the cell, inborn defects or even onset of carcinogenesis. The consequences of a NER-defect in man are illustrated by three hereditary disorders: xeroderma pigmentosum (XP), Cockayne's syndrome (CS), and a photosensitive form of trichothiodystrophy (TTD). A wide variety of clinical symptoms are associated with these disorders. XP patients show an extreme sensitivity to sun(UV)light and an elevated risk to develop skin cancer. CS and TTD patients display a milder sensitivity to UV-irradiation than XP patients and do not exhibit a predisposition to skin tumours. In addition, they show distinct features as growth abnormalities, mental retardation, and, a specific hallmark of TTD, brittle hair and nails. Genetic heterogeneity has been defined by identification of at least 10 different complementation groups defective in NER, seven in XP (XP-A to XP-G), two in CS (CS-A and CS-B), and one in TTD (TTD-A). In rodent cell lines at least 11 groups have been found, which show extensive overlap with the human groups. The clinical picture is even more confusing, since certain XP patients belonging to group B, D, and G show combined features of XP and CS, while some TTD patients have been classified into XP groups B and D.

To understand in more detail the complex molecular mechanism of NER, it is necessary to isolate and identify the genes involved and their encoded products. The work described in this thesis focuses on the characterization of different repair proteins. The development of a 'bona fide' *in vitro* NER assay has been essential to study the function and/or interaction of distinct repair components. The assay is based on damaged DNA substrates and protein extracts of rodent or human cells. The repair enzymes recognize the damage and remove the damage-containing patch, and the remaining gap is filled by *de novo* DNA synthesis. This last step can be detected by autoradiography when [^{32}P]-labelled nucleotides are included in the reaction mixture. The assay can be used to purify biologically active repair factors; for example XPA, XPG, and XPC/HHR23B complex have been isolated from extracts of repair-proficient cells.

The first evidence of a mammalian repair protein complex is described in Chapter 2. Complementation groups have been defined as correction of the repair defect when two different cells were fused *in vivo*. Analysis *in vitro* of repair-deficient extracts revealed results, which do not correlate with data observed *in vivo*. When two extracts of different

rodent complementation groups 1, 4, 11, or human XP-F were mixed, they could not restore the repair synthesis to normal levels. However, when they are mixed with extracts of rodent groups 2, 3, 5, or human XP-A, correction to wild-type levels is found. This is the first indication that repair proteins ERCC1, ERCC4, ERCC11, and XPF affect each other. A possible explanation for the differences between the *in vivo* and *in vitro* findings is, that proper complex formation is only possible *in vivo*, while *in vitro* subunits of the complex cannot be exchanged. A repair-proficient HeLa extract depleted for ERCC1 also lost the activity to correct extracts of rodent groups 4, 11 and human XP-F, but not the activity to complement the repair defects of rodent groups 2, 3, 5, and human XP-A. The immunodepletion studies confirm the hypothesis of such a repair complex. Since the *ERCC4* and *ERCC11* genes have not been cloned yet, it cannot be excluded that one of these genes corrects the human XP-F deficiency.

Chapter 3 describes the partial characterization of the ERCC1/4/11/XPF complex. A subunit of approximately 120 kDa could specifically be immunoprecipitated, suggesting that at least one additional subunit of the ERCC1 complex still has to be identified. The ERCC1 complex displays DNA-binding affinity without pronounced preference for single-stranded, double-stranded, or UV-damaged DNA. Using non-denaturing gradient gels the molecular mass of the complex was measured as ~280 kDa, much larger than previously observed by glycerol gradient sedimentation studies (~120 kDa). The size of 280 kDa for the complex would better accommodate the presence of ERCC1 (33 kDa), the precipitated subunit (120 kDa), and at least one additional component.

An unexpected link between NER and transcription initiation is reported in Chapters 4 and 5. The experiments were done in collaboration with the group of Dr. J.M. Egly (Strasbourg). Purified fractions of basal transcription initiation factor TFIIH were found to correct the repair defects of complementation groups XP-B, XP-D, and TTD-A, both in an *in vivo* microinjection repair assay and in an *in vitro* NER assay. Immunodepletion studies suggest that probably the entire TFIIH complex is directly implicated in excision repair. Expression of an *ERCC3* mutant containing a Lys→Arg replacement in the ATP-binding domain, which is expected to have lost all helicase activity, displays a dominant-negative effect on DNA repair as well as on transcription, after injection into repair-proficient fibroblasts. Antiserum against XPB and XPD injected into normal cells causes also a strong inhibition of both repair and transcription activities. These data indicate that XPB and XPD are involved in transcription *in vivo*, extending the evidence for involvement of these subunits in transcription *in vitro* as observed by Dr. J.M. Egly and coworkers. Furthermore, cell fusion experiments established the existence of a new TTD complementation group, since the repair defect in patient TTD6VI is identified as a defect in XP group B. The specific association between at least three TFIIH subunits and TTD supports the idea that TTD, and those forms of XP with combined XP/CS features belong to one broad clinical entity. Probably some clinical symptoms that are atypical for a NER defect, such as neurodysmyeliniaton, impaired sexual development, brittle hair, and growth abnormalities,

can be explained by subtle mutations, that affect the transcriptional role of TFIIH.

Two other subunits, p44 and p34, of TFIIH have been cloned (Chapter 6). The gene encoding p44 appeared to be the human homolog of yeast *SSL1*. Both proteins p44 en p34 contain certain zinc-finger domains, which might bind TFIIH to the DNA template. Antibodies against p44 and p34 are able to precipitate p62 and XPB, other subunits of TFIIH, and inhibit the transcription reaction *in vitro*. Again immunodepletion of repair-proficient extracts reduces repair activity to mutant levels. This depleted extract cannot complement the repair defect of rodent groups 2 and 3, which are the equivalents of human groups XP-D and XP-B respectively.

The dual role of TFIIH in DNA repair and transcription might explain the diverse phenotypes found, when a subunit of TFIIH is impaired. For instance, mutations can influence only the NER function, resulting in a classical XP-phenotype as observed in some patients of XP group D. If in addition the transcriptional role of TFIIH is subtly affected, combined XP/CS features and photosensitive forms of TTD can be expected. These findings support the hypothesis that mutations can cause viable transcriptional problems without any NER impairment. Indeed, several TTD and CS patients with no photosensitivity and normal NER have been described, providing evidence for the existence of a new class of disorders that can be designated as 'transcription syndromes'. However, this proposition has to be confirmed in the future.

Samenvatting

Een netwerk van DNA herstelprocessen beschermt de genetische informatie van organismen tegen de accumulatie van schadelijke mutaties. Veranderingen in het DNA kunnen veroorzaakt worden door de chemische instabiliteit van het DNA zelf of door blootstelling aan genotoxische agentia. Nucleotide excisie herstel (verder afgekort als NER afkomstig van de Engelse uitdrukking "Nucleotide excision repair") is een zeer belangrijk herstelproces in de cel en verwijdert een variatie aan lesies, bijvoorbeeld lesies geïnduceerd door ultraviolet licht. Wanneer lesies in DNA niet hersteld worden, kunnen ze mutaties veroorzaken of andere essentiële processen in de cel verstoren, zoals transcriptie, recombinatie of replicatie, met als mogelijk gevolg afwijkingen bij de geboorte of de ontwikkeling van kanker. De consequenties van een hersteldefect in de mens worden geïllustreerd door drie erfelijke ziektebeelden: xeroderma pigmentosum (XP), Cockayne's syndroom (CS) en trichothiodystrophy (TTD). Naast TTD-patiënten die overgevoelig zijn voor ultraviolet licht bestaat er een groep van TTD-patiënten die niet sensitief is voor licht. Deze laatste groep heeft geen defect in het NER-proces. Een brede variatie aan klinische symptomen wordt geassocieerd met deze ziektebeelden. XP patiënten vertonen een zeer extreme gevoeligheid voor ultraviolette straling en hebben een sterk verhoogde kans op de ontwikkeling van huidkanker. CS en TTD patiënten zijn in tegenstelling tot XP veel minder gevoelig voor ultraviolet licht en hebben geen verhoogde kans op huidtumoren, maar zij vertonen andere kenmerken zoals groei- en ontwikkelingsstoornissen. Specifieke symptomen van TTD patiënten zijn broze haren en nagels. Tenminste 10 verschillende genen betrokken bij DNA herstel zijn tot nu toe geïdentificeerd in de mens: 7 in XP (*XPA* tot *XPG*), 2 in CS (*CSA* en *CSB*) en 1 in TTD (*TTDA*). In knaagdieren zijn al 11 herstelgenen gevonden, waarvan sommige identiek zijn aan de humane genen. Het klinisch beeld is echter ingewikkelder dan geschetst, omdat sommige XP patiënten ingedeeld in groep B, D en G gecombineerde XP/CS kenmerken vertonen en er TTD patiënten zonder een verhoogd risico op huidkanker bestaan met een hersteldefect in *XPB* of *XPD*.

Om dit complexe mechanisme van DNA herstel te begrijpen is het noodzakelijk om alle genen, betrokken bij dit proces, en de eiwitprodukten afgeschreven van deze genen te isoleren. Het werk beschreven in dit proefschrift concentreert zich op het karakteriseren van verschillende DNA hersteleiwitten. De ontwikkeling van een 'bona fide' *in vitro* herstelmethode heeft belangrijk bijgedragen aan de analyse van de functies en/of interacties van de verschillende herstelfactoren. Deze methode is gebaseerd op het gebruik van een beschadigd DNA substraat en eiwitextracten uit gekweekte knaagdier- of menselijke cellen. De hersteleiwitten herkennen de schade in het DNA en verwijderen het stukje DNA met de lesie. Daarna wordt de enkelstrengs opening opgevuld door normale DNA synthese. Dit nieuw gesynthetiseerde DNA kan met behulp van autoradiografie gedetecteerd worden, indien radio-actief gelabelde nucleotiden aanwezig zijn in het reactiemengsel. Deze *in vitro* methode

is ook erg nuttig bij het zuiveren van biologisch aktieve herstelfactoren uit normale cellen. Op deze manier zijn XPA, XPG en het XPC/HHR23B eiwitcomplex geïsoleerd.

De eerste aanwijzing dat hersteleiwitten een complex vormen in zoogdieren wordt beschreven in Hoofdstuk 2. Wanneer cellen uit twee verschillende patiënten na fusie elkaars defect kunnen corrigeren *in vivo*, worden ze gedefinieerd als verschillende complementatie groepen. Analyse van herstel-deficiënte extracten toont in bepaalde combinaties een verschil in de *in vivo* en *in vitro* respons aan. Wanneer twee extracten van de complementatie groepen 1, 4, 11 of XP-F gemengd worden kunnen ze elkaars defect niet corrigeren. Al deze extracten kunnen echter wel het defect repareren van de groepen 2, 3, 5 en XP-A. Dit is de eerste aanwijzing dat de hersteleiwitten ERCC1, ERCC4, ERCC11 en XPF elkaar beïnvloeden, waarschijnlijk door de vorming van een complex *in vivo* waarin de individuele componenten niet uitgewisseld kunnen worden *in vitro*. Na verwijdering van ERCC1 uit een extract van herstel-competente HeLa cellen is dit extract niet meer in staat tot correctie van groepen 1, 4, 11 en XP-F, terwijl de aktiviteit om extracten van groepen 2, 3, 5 en XP-A te complementeren nog steeds aanwezig is. De aanwijzing dat hersteleiwitten een complex vormen wordt versterkt door de resultaten van deze immuundepletie studies. Aangezien de *ERCC4* en *ERCC11* genen nog niet gekloneerd zijn, is het niet uitgesloten dat één van deze genen het defect in XP groep F kan complementeren.

Hoofdstuk 3 beschrijft de partiële karakterisering van het ERCC1/4/11/XPF complex. Eén component met een massa van ~120 kDa kan specifiek geprecipiteerd worden. Het feit dat *ERCC4* of *ERCC11* identiek zou kunnen zijn aan *XPF* suggereert dat ten minste nog één component van het ERCC1-complex geïdentificeerd moet worden. Het ERCC1-complex kan binden aan DNA zonder voorkeur voor enkelstrengs, dubbelstrengs of UV-beschadigd DNA. Met behulp van een niet-denaturende gradient gel is de massa van het complex bepaald op ~280 kDa; dit is echter groter dan het eerder gevonden resultaat met glycerol gradient sedimentatie (~120 kDa). De massa van 280 kDa zou beter de aanwezigheid van ERCC1 (33 kDa), de hier geïdentificeerde component (120 kDa) en ten minst nog één additionele component kunnen herbergen.

Een onverwachte relatie tussen DNA herstel en transcriptie wordt besproken in de Hoofdstukken 4 en 5. Deze experimenten zijn uitgevoerd in samenwerking met de onderzoeksgroep van Dr. J.M. Egly (Straatsburg). Gezuiverde fracties van de basale transcriptie initiatie factor TFIIH corrigeren het defect van de complementatie groepen XP-B, XP-D en TTD-A, zowel in de *in vitro* methode als in een *in vivo* methode door middel van microinjectie in cellen. Immuundepletie studies suggereren dat mogelijk het gehele TFIIH-complex direct betrokken is bij excisie herstel. Expressie van een *ERCC3* mutant, waarin het aminozuur lysine is vervangen door arginine in het ATP-bindings domein met als gevolg verlies van helicase aktiviteit, vertoont een dominant negatief effect zowel op DNA herstel als op transcriptie na microinjectie in normale fibroblasten. Injectie van antiserum gericht tegen XPB of XPD remt in sterke mate beide processen, DNA herstel en transcriptie, in normale cellen. Deze gegevens geven een indicatie dat beide eiwitten XPB en XPD betrokken

zijn bij transcriptie *in vivo*, terwijl deze betrokkenheid *in vitro* al gevonden is door Dr. Egly en medewerkers. Verder bevestigen celfusie experimenten het bestaan van een nieuwe complementatie groep voor TTD patiënten. Het hersteldefect in patiënt TTD6VI is geïdentificeerd als een defect in XP groep B. De relatie tussen tenminste drie componenten van het TFIIH-complex, namelijk XPB, XPD en TTDA, en TTD versterkt de veronderstelling dat zowel TTD als de XP vormen met gecombineerde XP/CS kenmerken tot één breed klinisch ziektebeeld behoren. Sommige klinische symptomen, die ongewoon zijn voor een hersteldefect, zoals de afbraak van de myeline schede hetgeen neurologische afwijkingen in CS patiënten veroorzaakt, en groei- en ontwikkelingsafwijkingen, zijn mogelijk geassocieerd met mutaties die de functie van TFIIH in transcriptie subtiel beïnvloeden.

Twee andere componenten van TFIIH, p44 en p34 zijn gekloneerd (Hoofdstuk 6). Het gen dat codeert voor p44 blijkt de humane homoloog te zijn van *SSL1* in gist. Beide eiwitten p44 en p34 bevatten bepaalde zink-vinger domeinen, die mogelijk belangrijk zijn bij de binding van TFIIH aan DNA. Antilichamen gericht tegen p44 en p34 zijn in staat andere componenten van TFIIH, p62 en XPB, te precipiteren en remmen de transcriptie *in vitro*. Normale extracten waaruit p44 of p34 verwijderd zijn met antilichamen, vertonen een duidelijke reductie van de herstelaktiviteit tot het niveau van herstel-deficiënte cellen. Tegelijkertijd heeft dit extract de mogelijkheid verloren om de knaagdier complementatie groepen 2 en 3 te complementeren, welke homoloog zijn aan XP-D en XP-B.

De functie van TFIIH in zowel DNA herstel en transcriptie verklaart mogelijk de diverse fenotypes, die gevonden worden als een component van dit complex defect is. Mutaties kunnen bijvoorbeeld enkel de functie in DNA-herstel beïnvloeden met als gevolg een klassiek XP fenotype zoals gevonden in sommige patiënten van XP groep D. Wanneer tegelijkertijd de rol van TFIIH in transcriptie subtiel is aangetast kunnen gecombineerde XP/CS kenmerken en lichtgevoelige vormen van TTD verwacht worden. Deze bevindingen versterken de hypothese dat mutaties subtiele problemen in transcriptie kunnen veroorzaken zonder dat het DNA-herstelproces wordt beschadigd. Inderdaad zijn er TTD en CS patiënten beschreven, die niet gevoelig zijn voor ultraviolet licht en lesies in het DNA normaal kunnen herstellen. Het klinisch beeld van deze patiënten verschaft aanwijzingen voor het bestaan van een nieuwe klasse van ziektebeelden, die aangeduid kan worden als 'transcriptie syndromen', maar het bewijs voor deze veronderstelling moet nog gevonden worden.

Abbreviations

AAF	N-acetoxy-2-acetyl-aminofluorene
ATP	adenosine triphospate
cdk	cyclin dependent kinase
CG	complementation group
CHO	Chinese hamster ovary
CPD	cyclobutane pyrimidine dimer
CS	Cockayne's syndrome
CTD	carboxy terminal domain
DNA	deoxyribonucleic acid
E.coli	*Escherichia coli*
ERCC	excision repair cross complementing
HAP	hydroxyapatite
kb	kilobase
kDa	kilodalton
MMC	mitomycin C
MW	molecular weight
NER	nucleotide excision repair
PCNA	proliferating cell nuclear antigen
Phr	photolyase
pol II	RNA polymerase II
RNA	ribonucleic acid
RP-A (hSSB)	human single strands binding protein
(6-4) photoproduct	pyrimidine (6-4) pyrimidone photoproduct
S.cer	*Saccharomyces cerevisiae*
S.pombe	*Schizosaccharomyces pombe*
TCR	transcription-coupled repair
TFIIH (BTF2)	transcription initiation factor IIH
TRCF	transcription repair couplings factor
TTD	trichothiodystrophy
UDS	unscheduled DNA synthesis
UV	ultraviolet light
XP	xeroderma pigmentosum

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