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Fundamental and Molecular Mechanisms of Mutagenesis

The molecular basis of nucleotide excision repair syndromes

D. Bootsma *, J.H.J. Hoeijmakers

Medical Genetics Centre – Department of Cell Biology and Genetics, Institute of Genetics, Erasmus University Rotterdam, The Netherlands

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1. Introduction

Under the supervision of Frits Sobels I (D.B.) performed my first experiments to study the response of organisms and cells to DNA-damaging agents by feeding *Drosophila* flies formaldehyde. This treatment resulted in the induction of crossing-over and lethal mutations in *Drosophila* males and led to my first contribution to science in the official literature (Sobels, Bootsma and Tates, 1959). In the following 35 years the field of radiation genetics and chemical mutagenesis expanded impressively, not least by the stimulating ideas and leadership of Frits Sobels.

In the early 1960s the first concepts on DNA repair of DNA damage were formulated (Boyce and Howard-Flanders, 1964; Setlow and Carrier, 1964). The biological relevance of these processes in the human situation became apparent in 1968 after Cleaver's discovery of a DNA repair defect in xeroderma pigmentosum (XP) patients (Cleaver, 1968). Today, as discussed in this review, a number of genes controlling DNA repair pathways in mammalian cells have been identified. Their function in maintaining genetic integrity is becoming clear, yielding unexpected insights into the complex molecular basis of DNA repair syndromes.

2. Nucleotide excision repair (NER)

Various single enzyme and complex multi-step repair systems are operational in the cell to ensure proper functioning and faithful transmission of genetic information. Examples of the latter class of repair pathways are nucleotide excision repair (NER), base excision repair, post-replication repair and recombination repair. These processes have, in general, a broad lesion specificity. For example NER recognizes and eliminates a wide spectrum of structurally unrelated lesions such as UV-induced photoproducts, (bulky) chemical adducts and certain types of crosslinks, whereas recombination repair is required for the removal of X-ray-induced double-strand breaks and interstrand crosslinks.

The most extensively studied repair system is NER. In most, if not all, organisms two NER sub-pathways operate. One deals with the rapid

* Corresponding author.

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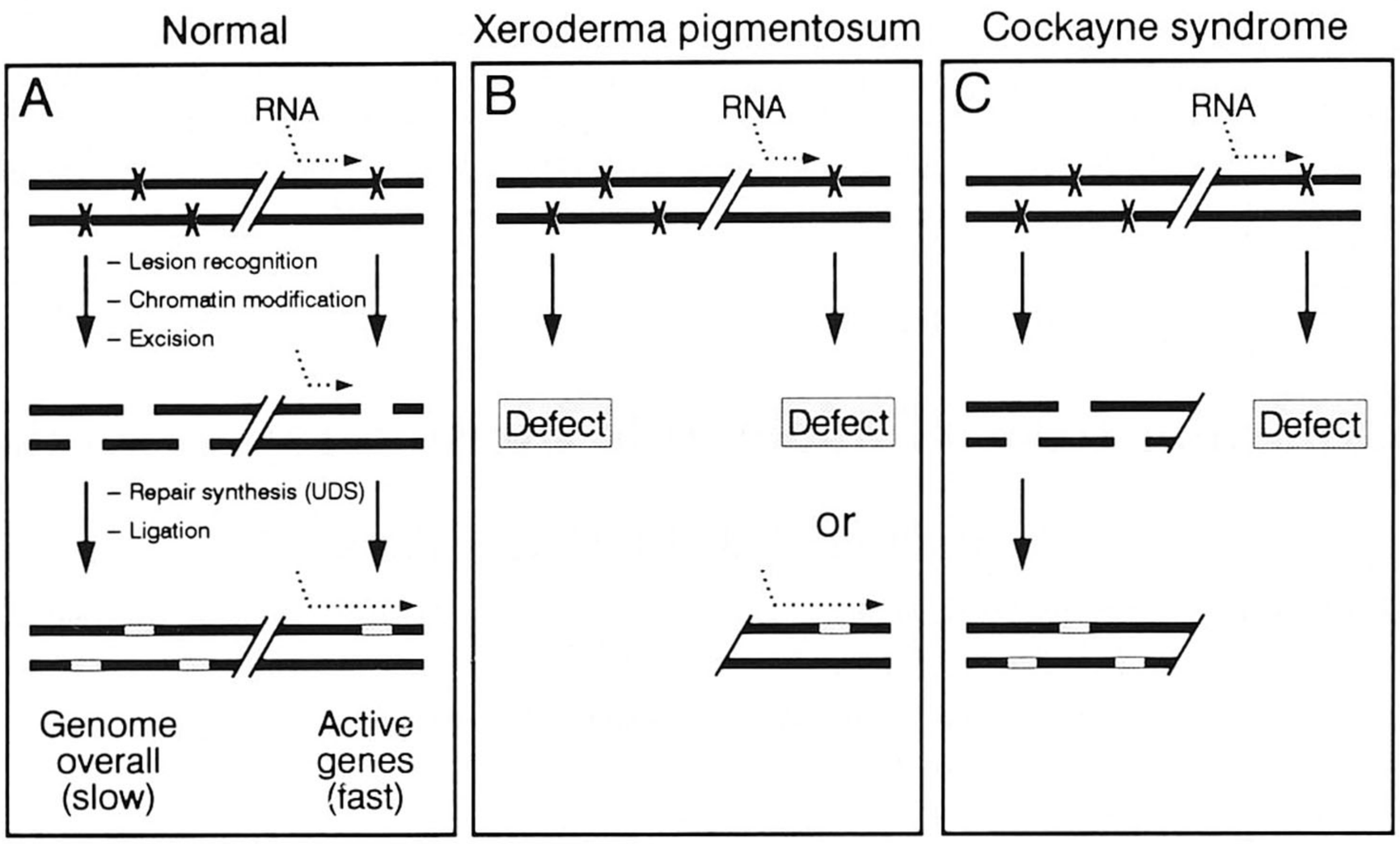

Fig. 1. Model of nucleotide excision repair (A) and the defects in xeroderma pigmentosum (B) and Cockayne syndrome (C) Two sub-pathways can be distinguished. One is the slow and less efficient repair of the bulk DNA, including the non-transcribed strand of active genes (genome overall repair) The other is the rapid and efficient repair of the transcribed strand of active genes (transcription-coupled repair). In xeroderma pigmentosum both sub-pathways are affected in complementation groups A, B, D, E, F and G; in XP-C the defect is limited to overall genome repair (B). In Cockayne syndrome only transcription-coupled repair is defective (C).

and efficient removal of lesions that block ongoing transcription and thus need to be eliminated urgently (transcription-coupled repair, Bohr et al., 1985). The other accomplishes the slower and less efficient repair of bulk DNA, including the non-transcribed strand of active genes (genome overall repair). A simple scheme of these pathways is presented in Fig. 1A. The NER pathway in *E. coli*, which is known in great detail (Liu and Sancar, 1992), is adopted as a model for the molecular mechanism of NER in eukaryotic cells (Hoeijmakers, 1993a). In the generally accepted scheme for this process the initial detection of DNA injury is thought to involve some sort of tracking system similar to the $UvrA_2B$ function in *E. coli* NER (Grossman and Thiagalingam, 1993). After lesion recognition a dual incision is introduced in the damaged strand 27–29 nucleotides apart (Huang et al., 1992). It is likely that these steps involve chromatin remodelling and include the cooperative action of a large number of proteins. Excision of the lesion-containing oligonucleotide is completed by the action of DNA helicase(s), and one or more single-

Table 1
Eukaryotic NER deficiency mutants

Origin	Mutant	Genes
Human genetic diseases	Xeroderma pigmentosum (XP) (incl. combined XP/CS)	*XPA–G*
	Cockayne syndrome (CS)	*CSA–B*
	Trichothiodystrophy (TTD) (PIBIDS)	*TTDA–B, XP-D*
Rodent cell lines	UV-sensitive mutants	*ERCC1–11*
Yeast	RAD3 epistasis group	*RAD1–4, 7, 10, 14, 16, 23, 25*, a.o.

stranded binding proteins (Coverley et al., 1991). The gap is subsequently filled in by polymerase δ and/or ϵ in a PCNA-dependent reaction (Shivji et al., 1992).

Biological relevance of NER

Most of the insight gained into the molecular mechanism of NER is derived from mutants defective in NER (Table 1). Typically, they display sensitivity to UV light and numerous chemicals as well as elevated levels of induced mutagenesis. The collection of yeast NER mutants currently includes at least 11 members, comprising the *RAD*3 epistasis group. Most of the genes involved have been cloned (Hoeijmakers, 1993a). Similarly, a large number of mammalian NER mutants have been generated in the laboratory using rodent cell lines. Cell fusion has identified a minimum of 11 complementation groups. By transfection cloning a series of complementing human NER genes have been retrieved (Hoeijmakers, 1993b). These genes were designated *ERCC* (for *e*xcision *r*epair *c*ross *c*omplementing) genes followed by the number of the rodent complementation group corrected by the human gene.

The phenotypic consequences of a NER defect in man are apparent from several rare, inborn disorders. In fact three distinct syndromes have been recognized to be associated with NER deficiencies. They are characterized by hypersensitivity of the skin to sun (UV)-light and a remarkable clinical and genetic heterogeneity (Hoeijmakers, 1993b) (Table 2). These are the prototype repair syndrome XP (seven genetic complementation groups, designated XP-A–G), Cockayne syndrome (two groups: CS-A and CS-B) and PIBIDS, a peculiar photosensitive form of the brittle hair disease trichothiodystrophy (TTD, at least two groups: TTD-A and TTD-B, and one equivalent to XP-D). Repair deficiency is most severe in XP groups A, B and G. In these groups and in XP-D, E and F the deficiency affects both overall genome repair and preferential repair of active genes. In XP group C the excision defect is limited to the overall genome sub-pathway (Venema et al., 1990a) (Fig. 1B). In PIBIDS both sub-pathways are affected whereas in CS-A and CS-B the NER defect is limited to preferential repair of the transcribed strand of active genes (Venema et al., 1990b) (Fig. 1C). The overall genome repair system is still functional. This explains the nearly normal levels of repair DNA synthesis (UDS) in CS cells after UV exposure. The preferential repair defect prevents a rapid recovery of RNA synthesis and this may cause the increased UV sensitivity of CS. XP shows, in addition to sun sensitivity, other cutaneous manifestations including pigmentation abnormalities and an over 2000-fold elevated frequency of skin cancer, often accompanied by progressive neurological degeneration. A different type of neurologic dysfunction is seen in CS. In this disorder it is associated with dysmyelination of neurons, which could be due to poor expression of one of the myelin proteins. In addition this disease is characterized by poor physical and sexual development. PIBIDS manifests essentially all of the CS symptoms and in addition two hallmarks of TTD: ichthyosis and brittle hair and nails. The latter is due to a reduced content of a cysteine-rich matrix protein. CS and PIBIDS individuals do not appear to be cancer-prone. Thus a remarkable clinical heterogeneity is found to be associated with NER impairment. It has been shown that defects in one gene can give rise to XP, combined XP/CS or PIBIDS symptoms. This holds for XP-D and to a lesser extent for XP-B, XP-G and TTD-A and

Table 2
Main features of the NER syndromes XP, CS and PIBIDS

	XP	CS	PIBIDS
(A) Clinical:			
Sun sensitivity	+/++	+	+
Pigmentation abnormalities	+	−	−
Skin cancer	+	−	−
Ichthyosis	−	−	+
Brittle hair/nails	−	−	+
Neurological abnormalities:			
accelerated degeneration	−/+		
dysmyelination	−	+	+
Short stature	−/±	+	+
Decreased fertility	−	+	+
Dental caries	−	+	+
(B) Biochemical:			
Overall genome repair	−	+	−
Transcription coupled repair	−/+ [a]	−	−

[a] XP-C.

TTD-B and suggests that the syndromes involved are different manifestations of a much broader clinical entity (Hoeijmakers, 1993b; Bootsma and Hoeijmakers, 1993). Obviously, it is difficult to adequately explain the entire spectrum and unusual combination of clinical features on the sole basis of a NER defect. As discussed below, recent findings have placed this notion in a new perspective.

3. NER genes and proteins

A series of DNA repair genes have been cloned by transfection correction of rodent mutants, or by evolutionary walking based on sequence conservation with isolated yeast repair genes. In the Medical Genetics Centre we cloned the NER genes *ERCC1* (Westerveld et al., 1984), *ERCC3* (Weeda et al., 1990), *ERCC6* (Troelstra et al., 1992), two human genes *HHR6A* and *6B* homologous to the yeast post-replication repair gene *RAD6* (Koken et al., 1991), and recently two human homologues of the yeast NER gene *RAD23* (Masutani et al., 1994). Subsequent analysis of these genes has provided clues to their function and disclosed a remarkable conservation between the human and yeast DNA repair pathways. Furthermore, a considerable overlap between human and rodent mutants was revealed with the finding that *ERCC3* is involved in XP-B (Weeda et al., 1990), *ERCC6* in CS-B (Troelstra et al., 1992), *ERCC2* in XP-D (Flejter et al., 1992) and *ERCC5* in XP-G (Scherly et al., 1993; O'Donovan and Wood, 1993). A summary of the cloned human NER genes is presented in Table 3. For a recent review see Hoeijmakers, 1993b. The *ERCC2* and *ERCC3* genes will be discussed in more detail here.

The dual role of ERCC2 and ERCC3

The *ERCC2* gene – first cloned by Weber et al. (1990) by transfection correction of rodent group 2 mutants – appeared also to be implicated in XP group D (Flejter et al., 1992) (Table 3). This group displays a remarkable heterogeneity as it is comprised of classical XP, combined XP/CS and PIBIDS patients all due to (different?) mutations in the same *ERCC2* gene. The yeast homologue, *RAD3*, encodes a $5' \rightarrow 3'$ DNA and DNA/RNA helicase (Bailly et al., 1991) and is found to be essential for yeast viability (references in Park et al., 1992). Previously, we have noted surprising parallels between ERCC2 and ERCC3 that prompted us to postulate that both proteins fulfil very similar or complementary functions and may interact with each other (Weeda et al., 1990).

The *ERCC3* gene compensates for the NER defect of rodent group 3 and cells from XP group B (Weeda et al., 1990). This complementation group is very rare (in total three individuals) and – like XP-D – shows the exceptional combination of XP and CS symptoms (Weeda et al., 1990;

Table 3
Cloned human NER genes

Gene	Phenotype	Homology to	Reference
ERCC1	?	RAD10; UvrA and C	van Duin et al., 1986
ERCC2	XP-D	RAD3	Weber et al., 1990
			Flejter et al., 1992
ERCC3	XP-B	RAD25	Weeda et al., 1990
ERCC5	XP-G	RAD2	Mudgett and MacInnes, 1990
			Scherly et al., 1993
			O'Donovan and Wood, 1993
ERCC6	CS-B	ERCC6^{sc}	Troelstra et al., 1992
			van Gool, unpublished results
XPA	XP-A	RAD14	Tanaka et al., 1990
			Bankmann et al., 1992
XPC	XP-C	RAD4 (?)	Legerski and Peterson, 1992

Vermeulen et al., 1994). From the amino acid sequence we predicted that the gene specifies a DNA helicase. The homologous gene in yeast (designated by us *RAD25*) has – like RAD3 – an additional function in a process essential for yeast viability (Gulyas and Donahue, 1992; Park et al., 1992). Very surprising and unexpected discoveries were made with respect to this gene during the last 2 years. It has appeared up independently in different laboratories working in unrelated fields. First Gulyas and Donahue (1992) identified *ERCC3* in yeast in a search for suppressors that alleviate a block of expression imposed upon the *HIS4* gene by an artificial strong stem-loop structure in its 5′UTR that hampers translation. This suggested but did not prove that the protein is implicated in initiation of translation which is quite unexpected for a gene already associated with NER. A specific mutation in the yeast gene mimicking that found by Weeda et al. (1990) in the original XP-B patient caused UV sensitivity as revealed by S. and L. Prakash in collaboration with us (Park et al., 1992). Secondly, Mounkes et al. (1992) uncovered *ERCC3* as the gene responsible for the 'haywire' mutants of *Drosophila*. These mutants exhibit various defects including male sterility (that is thought to be caused by reduced expression of the *B2t* tubulin gene required for normal spermatogenesis), abnormalities in the central nervous system and UV sensi-

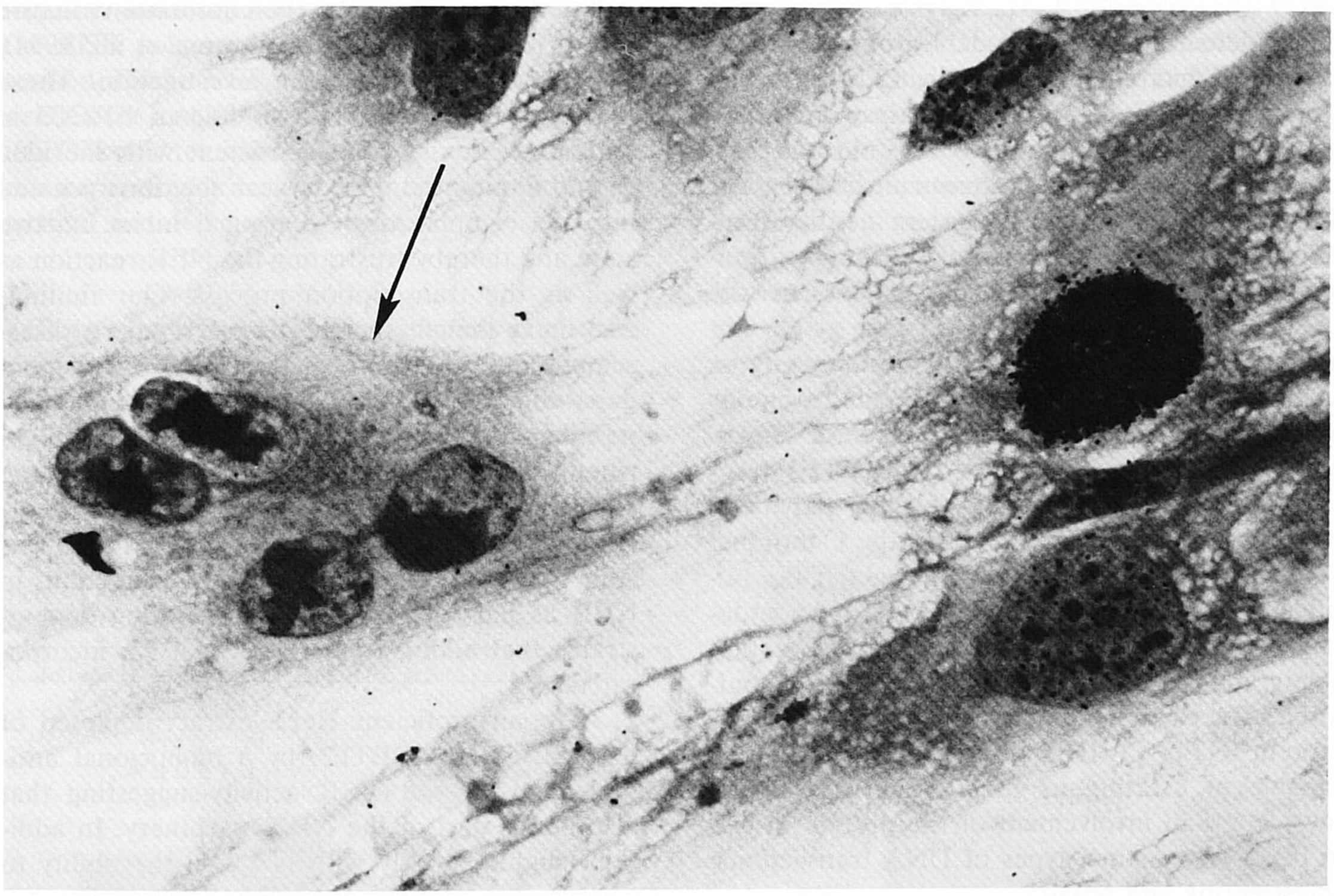

Fig. 2. Effect of K → R ERCC3 mutant on excision repair. Micrograph of a control (wild-type) fibroblast injected with *ERCC3* cDNA encoding the K → R mutated protein. Cells were UV-irradiated (15 J/m^2) 48 h after injection and incubated for 2 h in culture medium containing 10 μCi/ml ^{3}H-thymidine followed by autoradiography. The absence of autoradiographic grains above the nuclei of the injected polykaryon (arrow) demonstrates a complete inhibition of nucleotide excision repair. Normal UDS levels are seen in the non-injected fibroblasts and one fibroblast in S-phase of the cell cycle is heavily labeled. The dominant negative effect of the mutated ERCC3 also induced a chromatin collapse in the injected cell.

tivity. As in yeast some alleles were found to be lethal. Finally, the group of J.-M. Egly (Strasbourg) discovered that the *ERCC3* gene product is as one of the components of the human basal transcription factor BTF2/TFIIH, is required for a late step in the initiation of transcription of class II genes, thus defining the mysterious vital function of the protein (Schaeffer et al., 1993). In view of this finding the puzzling observations of Gulyas and Donahue (1992) as well as those of Mounkes et al. (1992) could be reinterpreted in terms of effects affecting the transcription levels of the respective genes.

The role of BTF2 / TFIIH in NER

Very recently, we have examined the ability of the highly purified BTF2/TFIIH transcription factor (provided by J.-M. Egly) to correct the repair defect of XP-B or rodent group 3 mutants in vivo by microinjection into living cells as well as in vitro by the cell-free repair assay developed by Wood et al. (1988) (see van Vuuren et al., 1994). Clear correction was seen in both assays. Since in the in vitro NER system neither transcription nor translation can take place, this finding rules out the formal possibility that the ERCC3 protein is not a NER factor at all, but only a transcription factor, whose activity is specifically required for the expression of one or more NER genes. Furthermore, the XP-B correcting activity nicely followed the BTF2 transcription stimulatory profile, helicase activity of the complex and kinase activity of the C-terminal domain of RNA polymerase, throughout the purification scheme of BTF2. On HAP chromatography as well as glycerol gradient sedimentation comigration of all these activities could be monitored. Thus ERCC3 in the context of the multiprotein BTF2/TFIIH transcription factor is fully capable of exerting its function in NER. This reveals a dual involvement of the protein in two in itself very distinct types of DNA transactions: transcription and repair.

Since the role of BTF2 in transcription has only been demonstrated in an in vitro system and for a limited set of RNA polymerase II promoters it was of interest to verify its role in vivo and to find out whether it is involved in basal transcription of all structural genes. To this aim we have investigated the effect of microinjection of our polyclonal ERCC3 antiserum on NER and on general transcription in repair-competent cells. A clear reduction (to approximately 50%) in UV-induced UDS and overall transcription was observed. Furthermore, in collaboration with LiBin Ma and Van der Eb of the University of Leiden a conservative K → R substitution was introduced in the nucleotide binding domain of ERCC3 which is predicted to inactivate the ATPase and consequently the helicase activity of the protein. The inactive mutant protein was overexpressed by injection of the gene into normal fibroblasts and found to completely paralyze NER (Fig. 2) as well as transcription. Concomitant with these dominant negative effects very peculiar abnormalities were induced in the chromatin structure of the nucleus (Fig. 2; van Vuuren et al., 1994), which are presently under investigation. These findings establish the in vivo role of ERCC3 in both processes and are consistent with the idea that the mutated protein can be incorporated into the complex thus freezing it in an inactive state and thereby frustrating the NER reaction as well as the transcription process. Our findings also imply that the complex must regularly disassemble and reassemble at least in vivo. Independent evidence for this came also from other experiments: microinjection of the free partially purified ERCC3 protein from overproducing *E. coli* cells was able to induce a specific transient correction of the NER defect of XP-B fibroblasts (van Vuuren et al., 1994). If ERCC3 functions in NER as part of a complex this finding demonstrates that added free ERCC3 can be incorporated.

A repair-proficient HeLa extract depleted of p62, a subunit of BTF2, by a monoclonal antibody had lost all NER activity-suggesting that also p62 is part of the NER machinery. In addition, simultaneously with p62 also the ability to complement extracts from ERCC3 and ERCC2 mutants appeared to be lost but not that for ERCC5. This indicates that the ERCC2 and ERCC3 activity available for the NER function resides in a complex with p62 (van Vuuren, 1994; unpublished observations). Most likely this is

BTF2/TFIIH. Moreover, this confirms our early idea (Weeda et al., 1990) that ERCC2 and ERCC3 might interact with each other.

What can be the role of BTF2 in NER and in transcription? It is most likely that the function of the complex in both processes is the catalysis of a similar step in the context of an otherwise different process. We can envisage several scenarios. One possibility is that the (presumed) helicase activity of ERCC2 and ERCC3 in BTF2 is required for translocation of the RNA polymerase or the NER damage recognition complex along the DNA template (Fig. 3A, B). This suggests that the BTF2 complex may be a separate DNA translocation carrier which can be coupled to the transcription as well as to the NER machinery. Alternatively, or in addition, it is possible that local denaturation is an obligatory step in loading the RNA polymerase onto the strand to be transcribed. In the NER reaction such a step may be needed for assembling the scanning complex onto the DNA or for inducing a specific DNA conformation around a lesion for the incision complex to bind and make a dual incision.

4. DNA repair and DNA transcription syndromes

Recently we have found by microneedle injection and in vitro repair that the highly purified BTF2/TFIIH complex in fact contains at least three NER proteins all associated with distinct human NER syndromes (Vermeulen et al., submitted). An intriguing observation is that all three fall into a specific subclass of NER disorders which displays the exceptional combination of XP and CS (XPB/ERCC3 and XPD/ERCC2) or the peculiar PIBIDS syndrome (XPD/ERCC2 and one newly identified very rare PIBIDS complementation group TTD-A, Stefanini et al., 1993). These findings support the notion that the entire TFIIH complex is recruited for repair purposes. Since the ERCC2, ERCC3, and TTD-A proteins also participate directly in transcription it is plausible that at least part of the unusual symptoms associated with the corresponding disorders are related to this other function of the complex. This sheds a new light onto the clinical features of XP, XP/CS and PIBIDS that were difficult to rationalized on the sole basis of a NER defect (Bootsma and Hoeijmakers, 1993). These include the neurological abnormalities associated with neurodysmyelination (which can be due to poor expression of one of the myelin genes), the brittle hair symptoms (which could be related to a low expression of a cysteine-rich matrix protein) and the severe growth defect (dwarfism, microcephaly and immature sexual development). In conclusion, we believe that the findings mentioned above reveal a novel type of syndrome caused by a

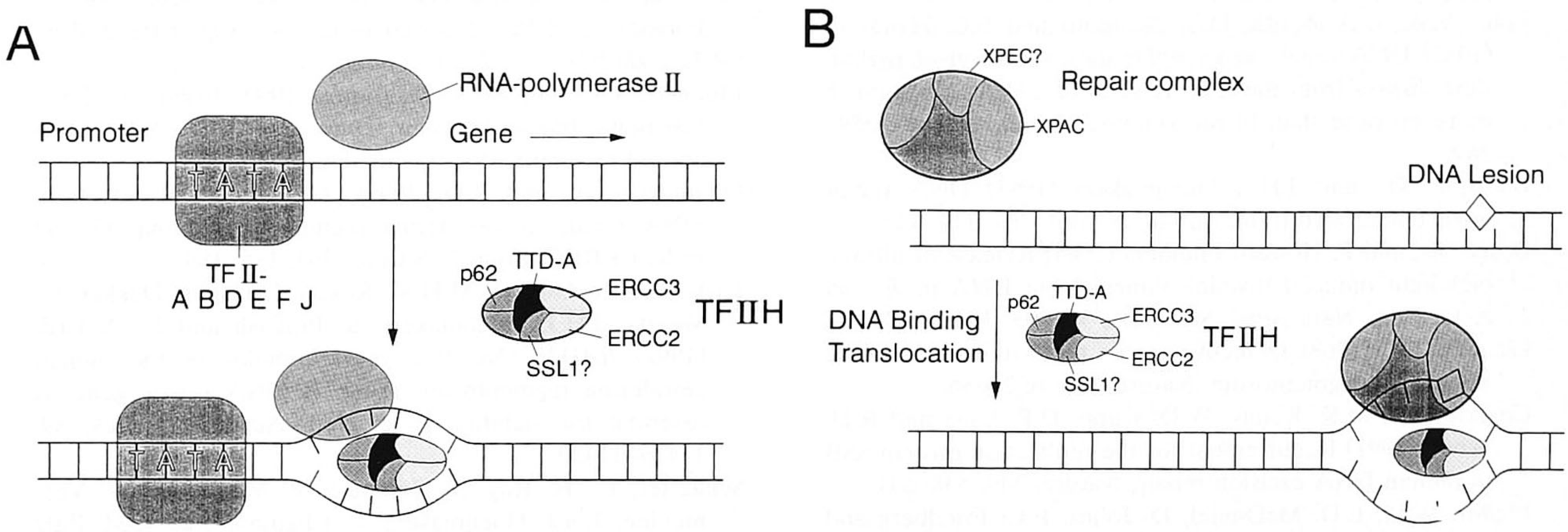

Fig. 3. Possible role of BTF2/TFIIH in transcription (A) and DNA repair (B). The complex may be required to position RNA polymerase II on the template and to translocate it along the DNA by locally melting the double helix. In DNA repair the complex may play a similar role in translocation and in positioning the specific NER proteins at the site of the DNA lesions.

subtle defect in general transcription that also occurs in the absence of a repair deficiency (such as in the form of trichothiodystrophy without photosensitivity). In this syndrome BTF2 plays a crucial role.

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