Mammalian nucleotide excision repair and syndromes

W. Vermeulen*, J. de Boer*, E. Citterio*, A. J. van Gool*, G. T. J. van der Horst*, N. G. J. Jaspers*, W. L. de Laat*, A. M. Sijbers*, P. J. van der Spek*, K. Sugasawa*, G. Weeda*, G. S. Winkler*, D. Bootsma*, J.-M. Egly† and J. H. J. Hoeijmakers*

*Department of Genetics, Medical Genetics Centre, Erasmus University, P.O. Box 1738, 3000DR Rotterdam, The Netherlands and †IGMBC, Université Louis Pasteur, Illkirch, France

Introduction

A variety of endogenous and environmental events threaten the integrity of genetic material. The intrinsic instability of DNA, along with metabolites, natural and man-made chemicals, X-rays and UV light may all alter the structure of the DNA molecule. Persistent DNA damage can disrupt vital cellular processes, such as DNA replication and transcription. To counteract the deleterious effects of DNA damage, several DNA-repair mechanisms have evolved [1]. One of the most versatile DNA-repair mechanisms is nucleotide excision repair (NER) [2]. This repair pathway is able to remove a broad spectrum of structurally unrelated DNA lesions in a multi-step fashion requiring the action of a large number of nuclear activities.

The overall mechanism of NER has been conserved during evolution, with structural and functional conservation being observed even between yeast and man. The process can be divided into roughly three distinct steps: (1) damage recognition and demarcation; (2) removal of the damage by a dual incision (excision) in the damaged strand on either side of the lesion; (3) gap filling, using the opposite undamaged strand as a template, and turnover of the excision complex.

Cloning of the mammalian NER genes and biochemical characterization of the gene products using *in vitro* [3] and *in vivo* [4] DNA-repair assays has resulted in the development of a detailed model of the reaction mechanism.

NER-deficient syndromes

A collection of NER-deficient mutants from *Saccharomyces cerevisiae* and laboratory-induced repair mutants of Chinese hamster ovary cells have been instrumental in the cloning and analysis of eukaryotic NER genes. In addition, a panel of well-characterized cells from hereditary syndromes with a NER defect facilitated the further investigation of mammalian NER [1,2].

The biological relevance of NER is apparent from the existence of severe autosomal recessive DNA-repair disorders, including xeroderma pigmentosum (XP), Cockayne's syndrome (CS) and trichothiodystrophy (TTD) [5]. These conditions are characterized by photosensitive skin, which is the result of the repair defect of UV-induced DNA lesions. Typical features of the prototype repair syndrome, XP (summarized in Table 1), are pigmentation abnormalities and a dry parchment appearance of the sun-exposed areas of the skin and an over 2000 times increased risk of the development of skin cancer. A broad clinical variation has been observed among different individuals with XP. A subgroup of such patients is associated with neurological abnormalities, caused by progressive neuron degeneration, and retarded physical development [5]. Greatly reduced DNA-repair synthesis (the gap-filling step), expressing the NER defect, has been observed *in situ* in cultured cells from patients with XP by autoradiographically moni-

Table 1

Main clinical symptoms of NER syndromes

There are also patients with CS and TTD without photosensitivity (and NER defect).

Clinical symptoms	XP	XP/CS	CS	TTD
Photosensitivity	++	++	+	+
Abnormal pigmentation	++	+	−	−
Skin cancer	++	+	−	−
Neuron degeneration	−/+	+	?	?
Neuron dysmyelination	−	++	++	+
Wizened facies	−	+	+	+
Growth defect	−/+	+	+	+
Hypogonadism	−	+	+	+
Brittle hair and nails	−	−	−	+
Scaling of skin	−	−	−	+

Abbreviations used: CS, Cockayne's syndrome; ERCC, excision repair cross complementing; HA, haemagglutinin; IF7, incision factor 7; NER, nucleotide excision repair; RPA, replication protein A; RNAPII, RNA polymerase II; XP, xeroderma pigmentosum; TFIIH, transcription factor IIH; TTD, trichothiodystrophy.

toring the incorporation of radioactive nucleotides.

The sensitivity to sun of the two other NER disorders, CS and TTD, is accompanied by neurological complications (caused by dysmyelination) and impaired physical and sexual development [6,7]. Patients with CS and TTD do not exhibit pigmentation abnormalities and are not predisposed to cutaneous tumours. Individuals with TTD are marked by sulphur-deficient brittle hair and nails and ichthyosis [7]. Most of the clinical features of CS and TTD are difficult to rationalize solely on the basis of an NER defect. Like XP, the clinical spectrum of CS and TTD is also heterogeneous. The genetic complexity within the NER syndromes [seven genes are involved in XP (*XPA* to *XPG*), five in CS (*CSA*, *CSB*, *XPB*, *XPD* and *XPG*) and three in TTD (*XPB*, *XPD* and *TTDA*)] indicates the molecular intricacy of mammalian NER. A considerable genetic overlap between the three conditions is evident for the *XPB*, *XPD* and *XPG* genes. Moreover, a remarkable clinical heterogeneity is apparent in XP groups B and D including combined XP/CS and TTD. Recently, we have identified three additional patients who exhibit mainly CS features; they belong to XP group G. Mutational analysis of these patients is in progress (S. Clarckson, University Medical Centre, Geneva, Switzerland).

A large group of patients with TTD are not photosensitive and do not display NER deficiency. In addition, we recently thoroughly analysed patients who exhibit the main clinical hallmarks of CS, but do not show any sign of an NER defect. These findings suggest that some of the CS and TTD symptoms are not based on an NER defect, but have another molecular origin. The possible reason for these additional, not NER-based features, will be discussed below.

Model of NER

For quite some time the reaction mechanism of *Escherichia coli* NER has served as a model for mammalian NER. Recently, the outlines of the mammalian reaction have emerged. With the help of purified and recombinant proteins, the mechanism can be reconstituted *in vitro* using damaged DNA substrates [8]. A model for the reaction is presented in Figure 1. Recent biochemical studies suggest that most of the NER activities reside in one or more large complexes. In terms of efficiency, the idea of a preassembled 'repairosome' is conceptually elegant; however, evidence for its existence is tentative and subject to discussion. Unravelling the individual steps of NER requires biochemical analysis of the individual components rather than of a complex of activities.

Damage recognition and demarcation

An obvious first step in the reaction is the recognition of the lesion. This is likely to be performed by the XPA protein, which has affinity for a number of different types of DNA lesion [9]. XPA also plays an important role in the assembly of the excision complex and in determining the orientation of the specific incisions, since it has binding sites for replication protein A (RPA), excision repair cross complementing (ERCC1)-containing complex and transcription factor IIH (TFIIH). The multiprotein-containing complex, TFIIH, was first described as an essential factor, required for the transcription of RNA polymerase II (RNAPII) genes [10]. This complex contains the DNA helicases XPB (ERCC3) [11] and XPD (ERCC2) [12], with an unwinding polarity of respectively $3'\rightarrow5'$ and $5'\rightarrow3'$. Microinjection and *in vitro* repair experiments with purified TFIIH, as well as antibody depletions, reveal that the entire complex is an integral part of the NER reaction. The bidirectional helicase activity of this complex may melt the duplex DNA around the lesion. The native composition of TFIIH is under debate, and seems to depend on the cell lysate preparation. The group of J.-M.E. first described the purification and characterization of TFIIH. In co-operation with this group we further dissected and analysed the different components, and investigated their function in NER and transcription. The complex consists of six components, which have been cloned and characterized: XPB (p89, helicase), XPD (p80, helicase), p62, p44 (two Zn fingers), p34 (Zn finger) and the recently cloned p52 (WD repeat). The specific kinase activity of native TFIIH resides in an associated subcomplex consisting of CDK7, cyclin H and MAT1. We have also recently developed an alternative TFIIH-purification protocol, using haemagglutinin (HA)-tagged XPB. Immunoaffinity-purified tagged TFIIH is active in NER and transcription and contains all the known components, as judged from immunoblots. Using this method we are also able to analyse the possible associated activities. Moreover, the effect of specifically introduced mutations on biochemical activities can be analysed. We did not find any evidence of copurification of other

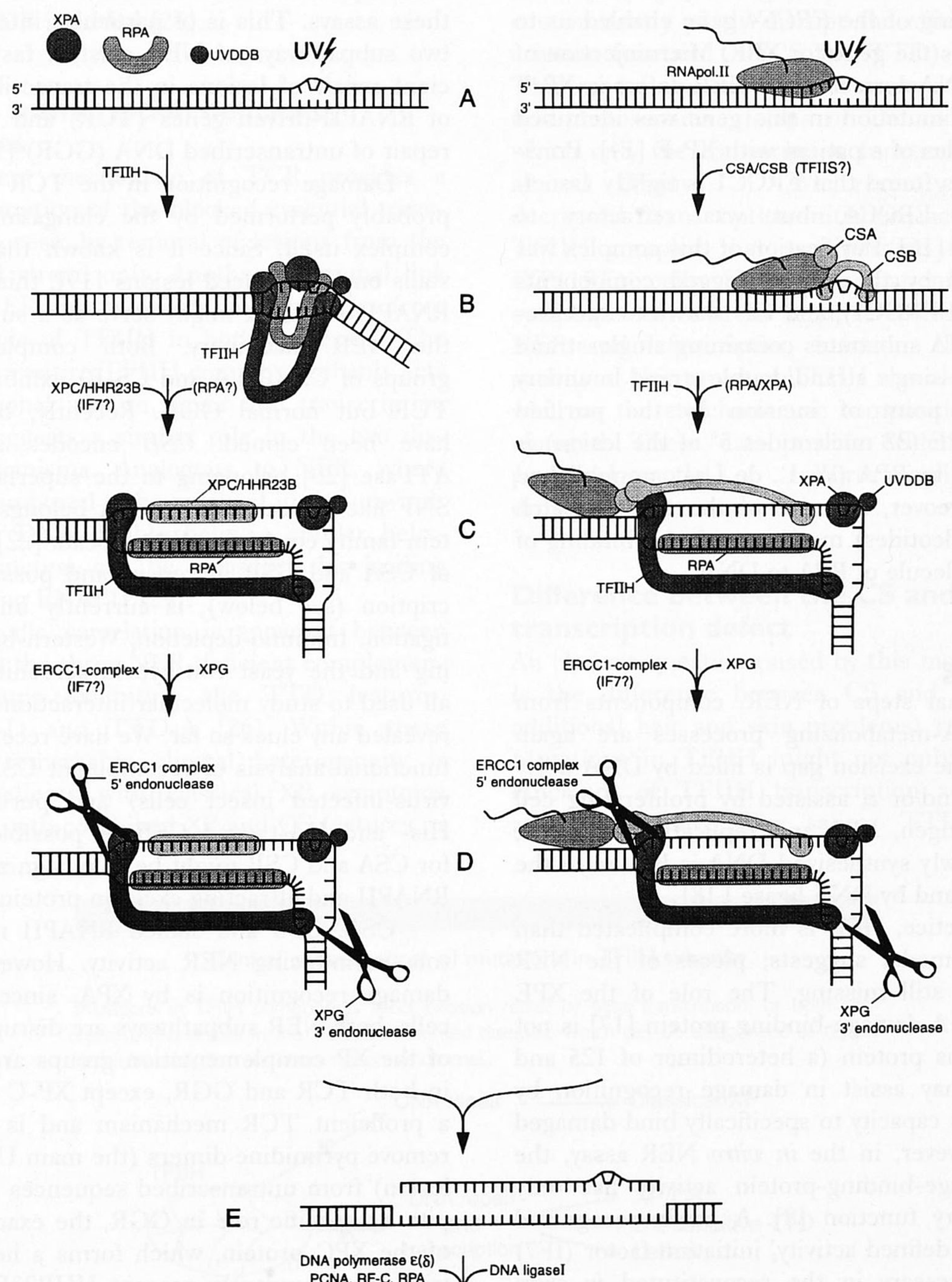
NER and basal transcription activities, using this mild purification protocol for TFIIH.

RPA (consisting of three polypeptides, 70, 34 and 11 kDa) is required for the incision step

Figure 1

Scheme of mammalian NER mechanism

Models of the two NER pathways, global genome repair (GGR) and transcription-coupled repair (TCR) are shown. For an explanation of the mechanisms, see the text.



[13]. RPA, which has a high affinity for single-stranded DNA and XPA, probably stabilizes partly melted duplex DNA.

Excision of damaged DNA

The two identified NER incision factors, ERCC1–XPF complex (5′ incision) and XPG (3′ incision), both act as structure-specific endonucleases at the boundary of single-stranded to double-stranded DNA (Figure 1) [14,15]. The recent cloning of the *ERCC4* gene enabled us to identify it as the gene for XPF. Microinjection of *ERCC4* cDNA corrected the NER defect in XP-F cells and a mutation in this gene was identified in both alleles of a patient with XP-F [14]. Previously, it was found that ERCC1 is tightly associated with ERCC4, but was refractory to purification [16]. Purification of this complex was again aided by the use of tagged components (His-tagged ERCC1), and was shown to specifically cut DNA substrates containing single-strand loops at the single strand/double strand boundary [14]. The point of incision by the purified ERCC1–XPF (22 nucleotides 5′ of the lesion) is determined by RPA (W. L. de Laat, unpublished work). Moreover, the size of the excised patch (27–29 nucleotides) matches with the binding of a single molecule of RPA to DNA.

Gap filling

For the final steps of NER, components from other DNA-metabolizing processes are again utilized. The excision gap is filled by DNA polymerase δ and/or ε, assisted by proliferating cell nuclear antigen, RPA and replication factor C, and the newly synthesized DNA is ligated to the existing strand by DNA ligase I [8].

In practice, NER is more complicated than the above model suggests; pieces of the NER puzzle are still missing. The role of the XPE protein DNA-damage-binding protein [17] is not known. This protein (a heterodimer of 125 and 41 kDa) may assist in damage recognition by virtue of its capacity to specifically bind damaged DNA. However, in the *in vitro* NER assay, the DNA-damage-binding-protein activity has only an accessory function [8]. A newly recognized and poorly defined activity, initiation factor (IF7) [8], is necessary in the reconstituted *in vitro* NER reaction when highly purified activities are used. At present, the exact role, its composition and the gene(s) encoding IF7 are not known.

Transcription-coupled repair (TCR) versus global genome repair (GGE)

The elegant *in vitro* NER assay has permitted further insight into the mammalian repair mechanism. However, the relative contribution of chromatin dynamics, which are probably involved in the living cell, to the rate of NER cannot be assessed with substrates lacking a chromatin-like structure. In addition, an important limitation of the current *in vitro* assays is that the role of transcription in NER cannot be measured in these assays. This is of particular interest, since two subpathways of NER exist, a fast and efficient repair of lesions in the transcribed strand of RNAPII-driven genes (TCR) and the slower repair of untranscribed DNA (GGR) [18].

Damage recognition in the TCR pathway is probably performed by the elongating RNAPII complex itself. Since it is known that RNAPII stalls on UV-induced lesions [19], this paralysed RNAPII on DNA might serve as a substrate for the NER machinery. Both complementation groups of CS (CS-A and CS-B) exhibit deficient TCR but normal GGR. Recently, both genes have been cloned. *CSB* encodes a putative ATPase [20] belonging to the superfamily SWI/SNF-like helicases [21]. CSA belongs to a protein family containing WD repeats [22]. The role of CSA and CSB in repair, and possibly transcription (see below), is currently under investigation. Immuno-depletion, Western-blot screening and the yeast two-hybrid screening system, all used to study molecular interactions, have not revealed any clues so far. We have recently begun functional analysis of recombinant CSB (baculovirus-infected insect cells) and purification of His- and HA-tagged CSB. A possible function for CSA and CSB might be in recognizing stalled RNAPII and attracting excision proteins.

CSA, CSB and stalled RNAPII may play a role in inducing NER activity. However, initial damage recognition is by XPA, since, in XP-A cells, both NER subpathways are disrupted. Most of the XP complementation groups are deficient in both TCR and GGR, except XP-C which has a proficient TCR mechanism and is unable to remove pyrimidine dimers (the main UV-induced lesion) from untranscribed sequences [23]. Despite its specific role in GGR, the exact function of the XPC protein, which forms a heterodimer with the abundantly present HHR23B polypeptide [24], in NER is still obscure. However, the XPC–HHR23B complex is definitely required in NER *in vitro* [8,23]. The XPC protein on its own,

as well as when complexed iwth HHR23B, has a high affinity for single-stranded DNA. In addition to RPA, this complex may stabilize the partly unwound DNA structure after damage recognition and helicase action, or it may assist in the further assembly of the incision machinery by changing the structure of damaged DNA, competing with the favourable stalled RNAPII. Recombinant XPC (baculovirus system) is able to complement XP-C cell lysates in an *in vitro* assay; its function is stimulated when recombinant HHR23B is added [25].

NER and transcription deficiency syndromes

The efficient mechanism of TCR provides a quick resumption of the blocked essential transcription process, by removal of lesions from the transcribed strand only. Another functional link between NER and transcription is obvious from the dual role of TFIIH in both processes. The fact that the entire TFIIH complex probably acts as a functional unit in repair and transcription [26–28] suggests a similar role in the two distinct mechanisms. Analogous to NER, where TFIIH is proposed to be involved in the unwinding of the DNA double helix, a similar helix-opening function can be envisaged for loading and initiating RNAPII [29].

A specific correlation is apparent between TFIIH and the three NER-deficient complementation groups exhibiting the TTD features, XP-B, XP-D and TTD-A [26]. Within these groups a remarkable clinical heterogeneity is present: patients with classical XP symptoms (XP-D) or with combined XP and CS features or TTD (XP-B and XP-D) [26,30]. As noticed before, the CS and TTD features are difficult to explain by an NER defect; the link with TFIIH leads us to suggest that some of these features are due to a subtly crippled transcription. Therefore we propose to introduce the phenomenon of 'repair/transcription syndromes' for those NER groups that are associated with mutations in one of the TFIIH subunits (see Figure 2) [26]. Mutations in TFIIH may lead to only an affected NER function (classical XP) or inactive NER and concomitantly partly disrupt the efficient transcription function (XP/CS and TTD) (Figure 2). This model also provides an explanation for the non-photosensitive forms of CS and TTD, in which only the transcription function is slightly altered (Figure 2). We recently identified decreased transcription-initiation activity of a TFIIH complex that was purified from XP-B (XP11BE) cells [31]. This patient exhibited symptoms of both XP and severe CS. Besides NER and transcription function, the helicase activity of this mutated TFIIH was also reduced, whereas the stoichiometric composition was not changed. These data provide evidence for the proposed model of 'repair/transcription syndromes'.

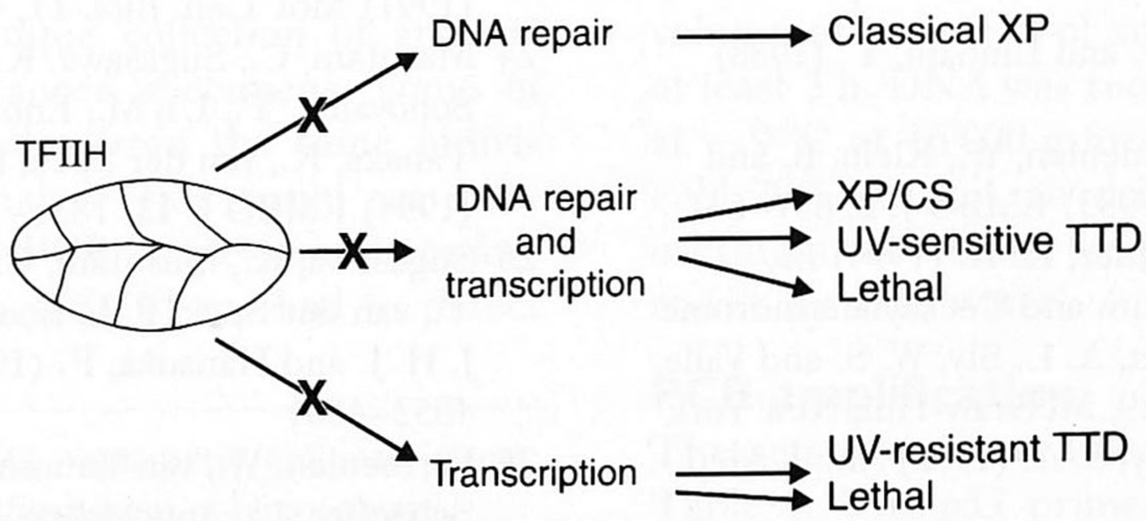
Difference between the CS and TTD transcription defect

An obvious question raised by this model is, how is the difference between CS and TTD (the additional hair and skin problems) rationalized? Mutations in TFIIH might not only affect the efficiency of TFIIH transcription activity, but also its stability. The salient TTD features,

Figure 2

Clinical consequences of mutations in TFIIH subunits

Mutations in TFIIH components affect excision repair or basal transcription, or both processes. This results in the indicated inherited diseases, which can be categorized as repair, repair/transcription and transcription syndromes.



brittle hair and ichthyosis, suggest specific problems in (terminally) differentiated keratinocytes (producing hair and cornified envelope). A decreased instability might become overt in these highly specialized cells, since most of the genome is transcriptionally silent (no *de novo* synthesized TFIIH) and focused on high level production of structural hair and skin proteins. Recently, evidence for this stability concept was provided by cellular transcription studies of an unusual patient with TTD. This particular patient (TTD1RO) exhibited a reversible and sudden worsening of hair and skin problems immediately after an episode of fever [32], suggesting the presence of a temperature-sensitive mutation in XPD (TTD1RO belongs to XP group D). Transcription in the cells of this patient, as measured by the incorporation of tritiated uridine, was sensitive to higher temperatures when compared with (isogenic) TTD1RO cells that were corrected with *XPD* cDNA.

Different NER-deficient mice, mimicking mutations in patients with XP, CS and TTD, have been generated in our laboratory, via gene targeting in pluripotent mouse embryonal stem cells. These mouse models will hopefully validate the proposed concept in the near future and shed light on the complex genotype–phenotype relationship in these syndromes. Moreover, these mice may serve as a valuable tool for carcinogenic studies in relationship to specific DNA-repair defects.

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Detection of *p53* mutations in oral cancer samples using a sensitive PCR-based method

H. Steingrimsdottir*, J. Penhallow*, F. Farzaneh†, N. Johnson* and M. Tavassoli*
*RCS Dental Science/Department of Oral Medicine and Pathology and †Department of Molecular Medicine, King's College School of Medicine and Dentistry, Caldecot Road, London SE5 9RW, U.K.

Introduction

Mutations in the conserved regions of the *p53* tumour suppressor gene are commonly detected in a variety of human cancers [1–3]. However, these are not ubiquitous and determination of the precise *p53* mutations in primary tumour samples is important for elucidation of the basic mechanisms involved and for prognostic assessment of the likely course of disease. Furthermore this may help in optimizing disease management/therapy. Mutations in the *p53* gene can occur in any of the ten conserved exons 2–11 [4], but more than 95% affect exons 4–9. Because of the absence of common hot spots in the *p53* gene, it has become necessary to amplify each exon separately and to analyse them invidually [5]. A further complication is the small amount and poor quality of DNA usually obtained from archival material. Aberrations in the *p53* gene have frequently been found in organ squamous cell carcinoma (SCC) [6–8]. However, characterization of *p53* mutations in large panels of oral SCC has in the past been hampered by the very small amounts of tissue available from most of these tumours. We have a large (>200) and unique collection of archival oral cancer and precancer specimens, some of which are serial samples from the same individual. In order to analyse this panel we have designed a two-round PCR single-strand conformation polymorphism (SSCP) method to detect mutations in exons 2–9 of the *p53* tumour suppressor gene. Modifications have also been made to the silver-staining method which have improved the feasibility and sensitivity of this detection method for SSCP analysis. Using this protocol we have so far detected mutations in exons 2–9 of the *p53* gene in 68% malignant and 29% premalignant samples of a panel of 32 archival oral tumour samples.

Abbreviations used: SCC, squamous cell carcinoma; SSCP, single-strand conformation polymorphism.

Materials and methods

DNA extraction

DNA was extracted from 4 μm-thick paraffin sections. Briefly, the cut sections were dewaxed with three changes of 500 μl of xylene each time followed by centrifugation at 16 000 ***g*** for 3 min. Xylene was removed with three changes of ethanol, each followed by centrifugation at 16 000 ***g*** for 3 min. The resulting pellets were air-dried and resuspended in 1 × lysis buffer (100 mM NaCl, 10 mM Tris/HCl, pH 8.0, 25 mM EDTA and 0.5% SDS). The tissues were digested with proteinase K (0.1 mg/ml) at 50 °C for 2–3 days, adding fresh enzyme each day. DNA was precipitated by the addition of an equal volume of propan-2-ol and stored at −20 °C for at least 3 h. DNA was recovered by centrifugation at −9 °C at 16 000 ***g*** for 30 min, washed in ice-cold 70% ethanol, air-dried and finally dissolved overnight at room temperature in 100 μl of sterile distilled water.

PCR amplification

The sets of primers used for PCR are shown in Table 1. The *p53* primers were designed using