Nucleotide excision repair : complexes and complexities : a study of global genome repair in human cells

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Chapter 2

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2.1 Two modes of NER: GGR and TCR

In vivo NER consists of two subpathways. The most prominent pathway is the global genome NER (GGR) pathway which removes lesions from the entire genome. As highlighted by the existence of the inherited disorder xeroderma pigmentosum (discussed in chapter 2.2), this pathway plays an important role in preventing the accumulation of mutations in our genomic DNA that ultimately give rise to cancer. Our view of NER has come about by putting pieces from various sources together, resulting in the highly detailed understanding of NER we have today.

Starting with the observation that patients suffering from XP were defective in repair replication of DNA (Cleaver, 1968) progress has been made in leaps and bounds. After some initial confusion, eight complementation groups were established (discussed in chapter 2.2). Subsequently, the genes responsible were one by one cloned (Legerski and Peterson, 1992; Mudgett and MacInnes, 1990; Tanaka et al., 1990; Thompson et al., 1994; Weber et al., 1988; Weeda et al., 1990), and the proteins they encoded were characterised. Only the *XPE* and *XPV* genes remained elusive for a longer time (chapter 3.1; Masutani et al., 1999).

The next major step forward in the research of NER was the reconstitution of GGR *in vitro* using cell-free extracts (Masutani et al., 1993; Sugasawa et al., 1993; Wang et al., 1993; Wood et al., 1988) or purified proteins (Aboussekhra et al., 1995; Araujo et al., 2000; Bessho et al., 1997; Mu et al., 1995), combined with a variety of damaged DNA templates. These experiments have resulted in a detailed understanding of the role of each protein or protein complex in the reaction and have led to the insight that the NER reaction proceeds through various stages. However, several aspects of GGR have proven difficult to investigate *in vitro*: repair from chromatinised DNA (discussed in chapter 5) and the organisation of the NER complex, i.e. the interdependence of proteins recruited to the site of a lesion and their precise order of being incorporated into the NER complex (discussed in chapters 3, 6, 8 and 10).

Transcription-coupled repair: an outline

While GGR is dedicated to the removal of lesions from the entire genome, regardless of their location, on the other hand transcription-coupled NER (TCR) is a specialized pathway that removes lesions from the transcribed strand of active genes only (Mellon et al., 1987; Venema et al., 1992), and depends strictly on ongoing transcription. The relevance of TCR lies in the rapid removal of lesions that block transcribing RNA polymerase II (RNA pol II), ensuring that the vital process of transcription is obstructed for as short a period as possible.

To a large extent GGR and TCR use the same set of proteins. The major difference between them lies in the initial step, i.e. recognition of the DNA lesion. To this end GGR employs the specialised damage recognition proteins UV-DDB and XPC-hHR23B, whereas in TCR the stalling of RNA pol II on a lesion is thought to initiate repair. TCR additionally requires the involvement of the CSA and CSB proteins and XPA binding protein 2 (XAB2).

The CSB protein and its role in TCR are relatively well-studied compared to the other two TCR-specific proteins, but nonetheless the exact role of CSB in TCR remains unclear. The CSB protein is 168 kDa in size and contains seven conserved helicase domains (Troelstra et al., 1992). CSB is a member of the SWI2/SNF2 superfamily of ATPases and harbours a DNA-dependent

ATPase activity (Citterio et al., 1998; Guzder et al., 1996a) which drives a chromatin remodelling activity (Citterio et al., 2000; see also chapter 5.2). Inactivation of this ATPase activity does not fully abolish complementation by CSB of the recovery of RNA synthesis (RRS; explained in chapters 2.2 and 4.1) in CS-B cells (Citterio et al., 1998) indicating that other properties of CSB also play a role during TCR. One such role could be the DNA wrapping activity of CSB, as this activity only requires CSB to bind ATP, not to hydrolyse it (Beerens et al., 2005). Alternatively, CSB may function as a structural factor in the physical stabilisation of repair complexes.

Several interactions of CSB with other factors involved in TCR and transcription have been observed. Most observations suggest an early role after RNA polymerase stalling, for example in recruitment of subsequent (repair) factors. Firstly, CSB binds directly to elongating RNA pol II (Tantin et al., 1997), an interaction that is stabilised following the induction of DNA damage (van den Boom et al., 2004). CSB also interacts with CSA (Henning et al., 1995), but CSA itself does not interact directly with RNA pol II (Tantin et al., 1997). Indeed, translocation of CSA to the nuclear matrix following DNA damage is dependent on CSB protein (Kamiuchi et al., 2002), as does the interaction of CSA with hyperphosphorylated (elongating) RNA pol II (M.I. Fousteri, pers. comm.). Finally, the *S. cerevisiae* homolog of CSB, Rad26, is involved in transcription past damaged bases independent from NER or BER, reinforcing the idea that CSB is intimately involved in ongoing transcription. These data also suggest that CSB is present at TCR sites before CSA and recruits, or helps recruit, CSA to a TCR site.

In addition, workers from the group of Cooper recently presented evidence that XPG when bound to a small DNA bubble (Dunand-Sauthier et al., 2005; Thorel et al., 2004) can interact with CSB, stimulating its ATPase activity (Sarker et al., 2005). These authors also showed that CSB together with XPG can interact with stalled RNA pol II ternary complexes (Sarker et al., 2005). Finally, the nuclease activity of XPG is inhibited in this situation but addition of TFIIH together with ATP relieves this inhibition (Sarker et al., 2005). Interestingly, a highly similar mechanism of NER endonuclease inhibition being relieved by TFIIH and ATP was observed before but in a simpler system using only a bubble DNA substrate, TFIIH, ATP and XPG or ERCC1-XPF (Winkler et al., 2001).

Much less is known about CSA. This 44 kDa protein lacks enzymatic activity; its main features are five WD repeats which may play a role in protein-protein interactions. CSA interacts with CSB and the p44 component of TFIIH (Henning et al., 1995). Moreover, CSA resides in a multi-subunit complex with the DDB1 protein (the large subunit of the UV-DDB damage-recognising heterodimer; see chapter 3.1), a ubiquitin-ligase activity in the form of cullin 4A and the COP9-signalosome (CSN) (Groisman et al., 2003). This complex associates constitutively with RNA pol IIa (the hypophosphorylated initiating form of RNA pol II) and after UV also interacts with RNA pol IIo (the hyperphosphorylated elongating form of RNA pol II). Also, after UV more CSN associates with the CSA complex, downregulating the ubiquitin-ligase activity (Groisman et al., 2003). However, the significance of these various associations and changing ubiquitin-ligase activity for TCR is as yet unknown.

XAB2 was only recently discovered as a protein implicated in TCR, transcription and pre-mRNA splicing (Nakatsu et al., 2000; Yonemasu et al., 2005). It consists mainly of 15 tetratricopeptide repeats. It interacts with XPA, CSA and CSB, and RNA pol II and its importance is underlined by the observation that mice lacking XAB2 suffer from pre-implantation lethality (Yonemasu et al., 2005). As yet, little more is known about XAB2.

2.2 Inherited disorders resulting from defective NER

The importance of the NER process at the organismal level is underlined by the existence of various rare autosomal recessive inherited disorders that are caused by mutations in proteins involved in NER (reviewed in Bootsma et al., 1998; Bootsma et al., 2001). The clinical appearance and a brief explanation of the most striking features of these diseases are given below.

Xeroderma pigmentosum (XP)
Patients suffering from XP display extreme sun-sensitivity, parchment skin (xeroderma) and freckles (pigmentosum) and a highly (>1000-fold) increased incidence of cancer in sun-exposed parts of the skin. Seven repair-deficient complementation groups can be distinguished (XP-A through XP-G), resulting from mutations in genes encoding NER proteins. Cells from all these complementation groups are compromised in GGR and with the exception of XP-C (Venema et al., 1991) and XP-E (Hwang et al., 1999) also in TCR. More severe cases of XP, mostly from complementation groups A and D also show mental retardation which probably arises from neuronal degradation following the accumulation of unrepaired damages in these cells. The observation that XP-C and XP-E are virtually free of neurological abnormalities suggests that removal by TCR of lesions from the transcribed strand of genes is critical for the development of neurological abnormalities and that functional TCR is sufficient to prevent these symptoms from occurring. When XP is diagnosed early, patients can be protected from sunlight which dramatically reduces their risk to develop skin tumours and accordingly increases their lifespan.

The eighth XP complementation group (XP variant or XP-V) remarkably is not deficient in NER, but bears a mutated DNA polymerase η, abrogating the possibility to replicate past CPD in an error-free manner (Masutani et al., 1999; see also chapter 1.2). Despite being NER-proficient, XP-V patients display a characteristic XP phenotype, including hypersensitivity of the skin to sunlight and a dramatic increase in sun-induced skin cancers.

Cockayne syndrome (CS)
CS is comprised of two complementation groups, CS-A and CS-B. At the cellular level CS is characterised by a lack of TCR but not GGR; moreover, CS cells do not display a recovery of RNA synthesis (RRS), i.e. they are unable to recover from the transcription inhibition that follows introduction of DNA damage, a phenomenon that has been related to the TCR defect (Mayne and Lehmann, 1982; Venema et al., 1990; see also chapter 4.1).

In clinical appearance, the most striking difference between CS and XP is the absence of a cancer predisposition in the former, despite the presence of a hypersensitivity of the skin to sunlight in both. Several non-mutually exclusive explanations exist for the cancer-free phenotype of CS. Firstly, since GGR is functioning normally DNA damages can be removed from the entire genome, thus preventing the development of cancer through unrepaired lesions (as a comparison, XP-C is TCR-proficient but shows a typical high skin cancer incidence). Secondly, it is hypothesised that apoptosis efficiently gets rid of cells that could transform into cancer cells (Ljungman and Zhang, 1996). Finally, CS patients may die before they can develop cancer; the mean age of death is around 12 years. Furthermore CS patients are usually diagnosed at an early age after which they are protected from sunlight, further decreasing their chances of developing skin cancers. Although a cancer predisposition is absent CS patients do display a wide variety of clinical symptoms that are conversely absent in typical XP, ranging from dwarfism and cachexia

to mental retardation. These symptoms are thought to ultimately result from accumulating stalled RNA polymerases on DNA lesions in the transcribed strand. Normally removed during TCR, these interfere with transcription in non-dividing cells and replication in dividing cells, eventually leading to cell death.

In addition to the 'classical' CS, CS can be associated with XP resulting from mutations in the *XPB*, *XPD* or *XPG* genes. XP/CS patients display a clinical and cellular phenotype that combines features from XP and CS. The clinical symptoms encompass the skin cancer predisposition and hyperpigmentation of XP patients and the mental retardation of CS patients, whereas at the cellular level, cells display impaired GGR and TCR together with the inability to recover RNA synthesis following DNA damage (van Hoffen et al., 1999).

Trichothiodystrophy (TTD)

A third disorder associated with defective NER is the photosensitive form of TTD. Its main clinical features are sometimes summarised in the acronym PIBIDS, i.e. photosensitivity, ichtyosis, sulphur-deficient brittle hair and nails (the most typical feature and used as a diagnostic), impaired intelligence, decreased fertility and short stature. Like CS, TTD is not characterised by a cancer predisposition.

A small number of TTD cases are the result of mutations in the *XPB* and *TTDA* genes, but the vast majority of TTD-causative mutations are in the *XPD* gene. TTD mutations destabilise the TFIIH complex resulting in the cellular hallmark of TTD, reduced levels of TFIIH (Botta et al., 2002; Vermeulen et al., 2000). This, due to the dual role of TFIIH (see chapter 3.4), also compromises NER.

It has been hypothesised that the specific TTD features, which also include β-thalassaemia (Viprakasit et al., 2001), result from the gradual decrease of transcription in terminally differentiated cells while still high levels of transcription are required to produce e.g. cysteine-rich matrix proteins (in hair shafts) or β-globin (in erythrocytes) (de Boer et al., 1998). Whereas under normal circumstances, resynthesis of TFIIH can compensate for its instability, in these situations the level of TFIIH may become limiting.

The combined transcription/repair defect in TTD patients together with the absence of a cancer predisposition conflicts with the hypothesis that CS is cancer-free because GGR is functioning normally, since within TTD both GGR and TCR are affected. To explain this apparent discrepancy, it has been speculated that certain transcriptional responses necessary to transform a cell into a tumour cell are absent (Berneburg et al., 2000). Another explanation may be provided by the observation that NER in TTD cells is cell cycle dependent. As a result, dividing TTD cells display efficient repair of 6-4PP, coupled with TCR of CPD, which together might be sufficient to prevent TTD patients from displaying an increased skin cancer phenotype (Riou et al., 2004). Finally, TTD patients like CS patients may simply not live long enough to develop cancer.

Like CS, also TTD can occur in combination with XP (Broughton et al., 2001), resulting from mutations in the *XPD* gene. The occurrence of such combined disease phenotypes highlights the complex interactions that exist in the cell between GGR, TCR and transcription, and simultaneously stresses the fact that especially mutations in *XPD* can give rise to a wide variety of clinical outcomes.

2.3 Sequential incorporation of factors into the GGR complex

An important question regarding NER *in vivo* is its organisation. NER proteins have to constantly search the genome for damages and after their detection damages have to be removed to prevent the obstruction of transcription or replication. To accomplish this, NER can be organised in at least two principally different ways. Firstly, most or all proteins might form a pre-assembled holocomplex that moves through the nucleus as a single entity, the so-called 'repairosome'. Alternatively, single proteins and/or small subcomplexes might be attracted to the lesion one at a time in a sequential manner.

The first indications for the existence of a repair holocomplex originated from studies on NER in *S. cerevisiae*, in which part of the entire NER machinery could be isolated as being associated with TFIIH (Svejstrup et al., 1995) and, according to a later report, with Rad14, the *S. cerevisiae* homolog of XPA (Rodriguez et al., 1998). In addition, around the same time a multitude of other NER factor associations and isolation of partial or incomplete NER complexes was reported, including: XPC and TFIIH (Drapkin et al., 1994; Mu et al., 1995), TFIIH and XPG (Iyer et al., 1996; Mu et al., 1995), XPG and RPA (He et al., 1995), RPA and XPA (He et al., 1995; Li et al., 1995; Matsuda et al., 1995), and XPA and ERCC1-XPF (Li et al., 1995; Nagai et al., 1995), creating links between all the core NER factors. Taken together these observations led to the hypothesis that also in humans NER could be organised as a repairosome. Indeed, He and Ingles upon mild lysis of HeLa cells reported the isolation of a repair holocomplex (He and Ingles, 1997). The complex not only contained preincision proteins such as XPA, RPA, TFIIH, ERCC1 and XPG, but also RF-C, PCNA and even the DNA polymerases δ and ε (He and Ingles, 1997). Consistent with the presence of these latter proteins, the complex exhibited NER activity up to and including DNA resynthesis (He and Ingles, 1997).

The major advantage of organisation into a holocomplex is that a lesion can be processed immediately after it is detected, but a repair holocomplex also presents specific problems. In the nucleus damages have to be processed in dense heterochromatin, where access to the lesions might be restricted for a large holocomplex. Furthermore, the sheer size of such a massive complex (~1-2 MDa) might render it rather inefficient – in other words, its movement through the nucleus might be very slow – so that timely removal of lesions would be compromised. Rather, to ensure efficient repair the (damaged) DNA would have to be recruited to the repairosome which would reside at a fixed place within the nucleus, i.e. the organisation of repair should much resemble the organisation of replication and transcription. A final point of concern is the fact that many proteins have functions outside NER, such as TFIIH in transcription and RPA in DNA replication and homologous recombination. Permanent incorporation of these proteins into a repair holocomplex might distract them from functioning in other pathways.

A fundamentally different way of organisation is to incorporate every protein sequentially into the 'emerging' NER complex. Interestingly, the first indications for this manner of organisation were also obtained by studying interactions of *S. cerevisiae* NER proteins with TFIIH (Guzder et al., 1996b). Guzder et al. suggested that the conclusions of Svejstrup et al. were likely to be caused by two experimental artefacts. First, two proteins reported to copurify with His-tagged TFIIH bound by themselves to the nickel column used by Svejstrup and co-workers (Guzder et al., 1996b). Second, Svejstrup et al. had reported that several proteins eluted in the same fraction of a size-exclusion column (Svejstrup et al., 1995), but the column in question was very likely to possess too little resolving power to be able to see the small differences in size between the various

NER proteins. Using a different size-exclusion column, Guzder et al. found the NER proteins to elute in different fractions (Guzder et al., 1996b). The findings of He and Ingles (1997) may not stand up to close scrutiny either. Firstly, these authors used the same size-exclusion column as Svejstrup and co-workers (Svejstrup et al., 1995). Secondly, their XPA-affinity column might actually detect separate individual interactions between proteins that do not actually reside in a large complex, e.g. XPA and TFIIH, XPA and ERCC1. A further discussion of the possibility of experimental artefacts in the reports of repair holocomplexes can be found in chapter 8 and in Araujo and Wood (1999).

Most problems associated with a repair holocomplex are circumvented by sequential assembly: single proteins and small subcomplexes can access the DNA in tightly packed chromatin, and will diffuse significantly faster than a holocomplex, together ensuring rapid screening of the entire genome. Also, since most proteins only interact strongly at the site of a lesion, they can function freely in other pathways than NER when no damages are present. Recently much evidence has been put forward supporting the latter model (Araujo et al., 2001; Hoogstraten et al., 2002; Houtsmuller et al., 1999; Mone et al., 2004; Riedl et al., 2003) and chapters 6 and 8 and the idea of a repairosome in human cells has been virtually abandoned.

A related question can be asked about the disassembly of the repair complex. Is it dismantled at once – together with or immediately after the release of the oligonucleotide, or during/by the repair replication step – or sequentially, similar to its assembly? Little research has been done on this matter; most authors either assume that all preincision proteins leave at once some time shortly after incision or step over the subject altogether, leaving a blank space between incision and resynthesis.

From a conceptual point of view it is conceivable that one or more proteins are released from the complex before repair is completed. The major advantage of this 'early release' would be that proteins quickly become available to initiate other repair events after having executed their function in the current one, leading to more effective repair. The only consistently reported early release is that of XPC-hHR23B leaving the NER complex upon the recruitment of XPG (Riedl et al., 2003; Tapias et al., 2004; Wakasugi and Sancar, 1998). In addition, Riedl et al. find that XPA and TFIIH leave the complex upon the arrival of ERCC1-XPF (Riedl et al., 2003). Measurements of the mobility of XPC-GFP in a live nucleus (Hoogstraten, submitted) suggest that XPC is attached to the NER complex significantly shorter, for approximately 2 minutes, than other factors such as ERCC1-XPF (Houtsmuller et al., 1999), TFIIH (Hoogstraten et al., 2002) and XPA (chapter 8) which are involved in NER for ~4-5 minutes. These latter findings on the one hand provide support for the notion that XPC leaves the complex before other NER proteins (although not necessarily directly following entry of XPG into the complex), but are ambiguous towards an early exit for TFIIH and XPA.

Kinetic modelling of the NER reaction confirms that an early release of XPC-hHR23B can give rise to enhanced repair rates or alternatively, lower the required concentration of XPC-hHR23B in the nucleus (Politi et al., 2005). This latter effect might be more important since XPC-hHR23B at higher concentrations is toxic to the cell (Ng et al., 2003; W. Vermeulen, pers. comm.). In this context it is also relevant to note that the nuclear concentration of XPC-hHR23B is lower than that of other investigated NER proteins (Araujo et al., 2001).

On the other hand, not all proteins necessarily dissociate immediately after dual incision: Riedl and co-workers found that no functional XPG was released following dual incision (Riedl et al., 2003). Although consistent with a possible role of XPG in the recruitment of late NER

factors, most notably PCNA (Gary et al., 1997), the fact that Riedl et al. did not measure release of XPG directly via Western blotting but indirectly via activity (incision) assays leaves the possibility open that XPG is released, but in an inactive form. Measurements of the mobility of XPG following UV irradiation indicate that XPG is bound to the complex for ~4 min, i.e. comparable to the binding time of other NER factors (except XPC) (D. Zotter, manuscript in preparation), suggesting that XPG is released simultaneously with the other NER factors (with the possible exception of XPC) following dual incision. In chapter 10, we address the possible release of factors from the NER complex utilising an *in vivo* competition assay consisting of a dual UV irradiation combined with the use of DNA synthesis inhibitors and NER-deficient cells. Our findings are consistent with a model in which all preincision NER proteins are released following dual incision, except RPA, which remains bound to assist in the DNA resynthesis stage. XPC may be released directly after incision, while the other preincision repair proteins stay associated with the lesion site and assist in the recruitment of the postincision factors (RF-C, PCNA, DNA polymerases and DNA ligase I).

2.4 NER substrate specificity: the bipartite recognition model

A striking feature of the GGR pathway of NER is its wide substrate specificity. GGR is capable of processing a large variety of lesions, ranging from the large chemical adducts formed by benzo[a]pyrene, via the small intrastrand crosslinks formed by cisplatin, to the purely structural (non-adduct) change of UV-induced photolesions. In contrast, processes such as BER or DSBR deal with a narrow set of lesions and repair by these processes is initiated by lesion-specific proteins. Although human MMR utilises one factor, hMSH2-hMSH6, to detect all possible single base pair mismatches, these mismatches (present in only a limited number of possibilities) all display a similar structure. The general structure of insertion/deletion loops, which are processed by the other MMR-initiating factor hMSH2-hMSH3, is also quite constant.

The prevailing model to explain the mechanism of damage recognition in NER is the 'bipartite' model put forward by workers from the lab of Naegeli, who found that a small mismatched bubble and a non-disturbing chemical modification to the DNA were only processed by NER when they were combined (Hess et al., 1997). The bipartite model thus suggests that NER probes the DNA for two types of changes to the normally stacked double helix. Firstly, NER processes (i.e. 'recognises') lesions that disturb this base stacking, usually giving rise to small bubble structure (Gunz et al., 1996; Hess et al., 1997). Secondly, NER will only proceed to the incision stage if concurrently with the helix distortion the chemical composition of the DNA is changed (Hess et al., 1997). Which proteins in the GGR pathway operate in which stage of this bipartite recognition is discussed in the next chapter.