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

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Chapter 3

Global genome repair proteins and their functions

3 Global genome repair proteins and their functions

In Figure 1 (see Appendix), a model is given for the GGR pathway of NER, its various stages and the factors that are involved in every stage. In this chapter, these factors are highlighted in the order in which they are thought to play their role, and their functions in NER are elaborated on in detail. In addition, attention is given to the interplay between factors – which plays an important role in the multistep process that NER is.

3.1 UV-DDB: a key factor assisting damage recognition

Several proteins and protein complexes have been proposed to play a role in the damage recognition step of GGR: the UV-damaged DNA binding factor (UV-DDB) (Chu and Chang, 1988; Hwang et al., 1999; Tang et al., 2000), XPA (Jones and Wood, 1993; Robins et al., 1991), the complex of XPA and RPA (Asahina et al., 1994; Li et al., 1995; Mu et al., 1996; Wakasugi and Sancar, 1999), XPC-hHR23B (Reardon et al., 1996; Sugasawa et al., 1998) and RPA (Burns et al., 1996; Reardon and Sancar, 2002). Although the XPC-hHR23B complex is now generally considered the damage recognition factor for most lesions, there is considerable evidence that UV-DDB plays an important role in assisting repair of several types of lesions. First and foremost, GGR of CPD is strictly dependent on UV-DDB (Fitch et al., 2003b; Hwang et al., 1999; Tang et al., 2000). Additionally, our own research as described in chapter 9 has pointed out that also the repair of low levels of 6-4 photoproducts by GGR is strongly enhanced by (though not dependent on) UV-DDB.

UV-DDB is a heterodimer of the DDB2 and DDB1 proteins (formerly called p48 and p127 or p125, respectively) and was originally purified as a factor that bound specifically to UV-irradiated DNA (Feldberg and Grossman, 1976). Later, this factor was isolated from cell extracts as the only factor to readily bind UV- or cisplatin-damaged DNA and was found to be missing from a subset of cells assigned to the xeroderma pigmentosum group E (Chu and Chang, 1988). It has only recently become clear that in fact all cells from XP-E are deficient in UV-DDB activity (see below). Indeed, UV-DDB displays a high affinity for UV-induced photolesions, especially 6-4PP – as a matter of fact, the highest of all putative damage recognition factors (Batty et al., 2000) – while in addition, the complex exhibits a moderate affinity for several other types of lesions (Batty et al., 2000; Chu and Chang, 1988; Feldberg, 1980; Feldberg and Grossman, 1976; Feldberg et al., 1982; Fujiwara et al., 1999; Payne and Chu, 1994; Wittschieben et al., 2005). Nevertheless, its role in NER has been controversial from the moment of its discovery, due to several complicating issues.

The first complication was the misclassification of several cells in XP group E. Based on cell fusion/complementation experiments using unscheduled DNA synthesis (UDS) as read-out, these assays were complicated by the high UDS levels (50-80% of normal cells) displayed by XP-E cells. Recent re-evaluations combined with sequencing of the *DDB2* gene instead classified cells in groups such as XP-F and XP-V (Itoh and Linn, 2001; Itoh et al., 2000; Ropic-Otrin et al., 2003). Given these problems, nowadays XP-E is assigned based on the absence of the UV-DDB activity and on mutations in *DDB2*, instead of cell complementation studies (as suggested by Cleaver et al., 1999). It should be noted that all XP-E causative mutations are found in the

DDB2 gene, with no mutations known in *DDB1*. The reason for this is likely that *DDB1* is an essential factor for development (Takata et al., 2004). Secondly, when UV-DDB has been included in *in vitro* NER reactions, contradictory findings have emerged, reporting that NER was either stimulated (Aboussekhra et al., 1995; Wakasugi et al., 2001), not effected (Araki et al., 2000; Rasic-Otrin et al., 1998) or even inhibited (Aboussekhra et al., 1995) [at high UV-DDB concentrations]; Kulaksiz et al., 2005) by UV-DDB. In any case, UV-DDB is not required for NER *in vitro*, leading to the suggestion that it might play a role in the repair of lesions within chromatin, rather than on naked templates (Rasic-Otrin et al., 1997). Unfortunately, its role in repair in the chromatin context is also a subject of dispute. Hara and co-workers measured excision of a single 6-4 photoproduct from a nucleosomal substrate and found rates to be comparable in extracts from HeLa cells, which contain functional UV-DDB, and from Chinese hamster ovary cells (Hara et al., 2000) expressing very low levels of UV-DDB (Hwang et al., 1998; Tang et al., 2000). The authors concluded that UV-DDB does not play a role in repair in chromatin at the nucleosomal level (Hara et al., 2000), but it cannot be excluded that the low level of UV-DDB present in these hamster cells was enough to stimulate repair, resulting in kinetics similar to HeLa cells. Alternatively, the procedure of making cell extracts from nuclei could abolish the effect UV-DDB has on NER in an intact nucleus, through the loss of some protein functions. It can also not be excluded that UV-DDB has an effect on higher levels of chromatin organisation than the nucleosome.

Finally, human XP-E and rodent cells, both of which are deficient in UV-DDB, show only a partial repair defect, i.e. only repair of CPD from non-transcribed DNA is absent. Although additionally in XP-E cells 6-4PP repair is mildly retarded compared to normal human cells at UV doses of 10 J/m² (Hwang et al., 1999) and 5 J/m² (chapter 9), at 30 J/m² repair in normal human and XP-E cells is indistinguishable (chapter 9). In contrast, 6-4PP repair in rodent cells is comparable to normal human cells; depending on the dose reaching completion within 4-8 hours following irradiation (Hwang et al., 1999; Tang et al., 2000 and chapter 9).

Taken together, these observations have led to the hypothesis that UV-DDB is not essential for damage recognition of all lesions but is necessary only for repair of those lesions that are otherwise poorly recognised by XPC-hHR23B, such as CPD (Hwang et al., 1999). In addition, our findings as described in chapter 9 suggest that UV-DDB may accelerate repair of lesions that are proper substrates for XPC-hHR23B, such as 6-4PP.

The role of CPD repair in cellular survival and mutagenesis

Interestingly, several types of normal rodent cells despite their lack of CPD repair show no reduced clonal survival following UV irradiation compared to normal human cells. This apparent discrepancy has been dubbed the 'rodent repairadox' (reviewed in Hanawalt, 2001). The CPD repair deficiency in rodent cells is partly thought to arise when rodent cells are grown in culture, as several observers have reported that repair of CPD declines when rodent cells are transferred from the skin to culture (Mullaart et al., 1988) and continues to decline following subsequent rounds of cell division (Ben-Ishai and Peleg, 1975; La Belle and Linn, 1984; Yagi, 1982). It appears that the underlying mechanism is the silencing by methylation of the *DDB2* gene (Hwang et al., 1998). At least in mouse cells, another factor contributing to the lower efficiency of CPD repair in rodent cells is the lack of transcriptional control over the *DDB2* gene by p53, leading to constitutively lower levels of UV-DDB and a lack of DNA damage-induced upregulation of *DDB2* protein levels (Tan and Chu, 2002; see also chapter 3.3).

Although interesting, these observations do not explain the high clonal survival of cultured rodent cells. The main difference between cultured human and rodent cells concerning their UV-survival instead appears to be their response to DNA damage checkpoints. Human cells exhibit a p53-dependent apoptotic response to UV damage (Smith and Fornace, 1997; Ziegler et al., 1994) as do mouse cells *in vivo* (Tron et al., 1998) whereas in cultured rodent cells this response is evidently absent.

The similar clinical phenotype of XP-E patients compared to other XP groups implies that in XP-E CPD are the primary cause for the introduction of mutations and the formation of cancer. Functional TCR removes CPD from the transcribed strand of genes, ensuring that XP-E cells can proliferate without major problems, but the price is paid in the form of a high damage load carried over into S-phase, leading to the increased introduction of mutations. In other XP groups too, CPD may be the main causative lesion for the introduction of mutations, given its three-fold higher induction compared to 6-4 photoproducts.

Finally, mouse keratinocytes *in vivo* repair CPD as poorly as cultured mouse cells do (Mitchell et al., 1990; Qin et al., 1995), raising the question why mice do not display an XP-E-like cancer phenotype. The answer might simply be that mice, and other rodents, do not only possess a hairy fur but also live a nocturnal life; they may therefore not be exposed to substantial doses of UV in the wild. Additionally, mice only live for a few years, so they would not need to prevent the formation of tumours so vigorously as longer living animals such as humans do.

3.2 XPC-hHR23B

Recent *in vitro* and *in vivo* experiments provide evidence that the heterodimer consisting of XPC and hHR23B is the initial damage sensor in GGR: the complex plays a crucial role in the subsequent formation of the NER complex (Sugasawa et al., 1998 and chapter 6), predominantly via its interactions with TFIIH (Sugasawa et al., 1998; Uchida et al., 2002; Yokoi et al., 2000) and by opening of the helix slightly (Evans et al., 1997b; Tapias et al., 2004). Although *in vivo* this heterodimer is bound and further stabilised by centrin 2 (Araki et al., 2001; Nishi et al., 2005; see also below), centrin 2 is dispensable in *in vitro* reactions.

In vitro, XPC-hHR23B has an affinity for a broad spectrum of DNA lesions, including the UV-induced photolesions 6-4PP and CPD and cisplatin- and NA-AAF adducts (Batty et al., 2000; Reardon et al., 1996; Sugawasa et al., 1998). Additionally, XPC-hHR23B binds to small bubbles devoid of a lesion (Sugasawa et al., 2001) and in fact, XPC-hHR23B displays quite a high affinity for undamaged DNA in general (Batty et al., 2000; Reardon et al., 1996). This characteristic is probably also responsible for the observation that *in vivo*, 90% of XPC-GFP in the nucleus is at any moment immobilised for short periods of time (a few seconds), most likely on DNA (D. Hoogstraten, manuscript submitted). Upon binding to DNA, XPC-hHR23B slightly opens the double helix (Sugasawa et al., 1998) and bends the DNA regardless of whether this is damaged or undamaged (Janicijevic et al., 2003). The resulting structure likely facilitates the recruitment of subsequent NER factors, most notably TFIIH (Sugasawa et al., 1998; Yokoi et al., 2000), XPA and RPA.

XPC and hHR23B perform distinct functions in the repair reaction. The 125 kDa XPC protein by itself can bind DNA and displays an affinity for damaged DNA. Furthermore *in vitro*, XPC can recruit TFIIH to damaged DNA (Yokoi et al., 2000) and function in NER (Reardon et

al., 1996; Sugasawa et al., 1996) without a requirement for hHR23B, while in mice *in vivo*, XPC can correct the (XP-C-like) repair defect in HR23-deficient cells (Ng et al., 2003). Thus, XPC is the subunit active in NER. In contrast, the 58 kDa hHR23B protein stimulates XPC-dependent repair in reconstituted NER *in vitro*, but does not allow NER in the absence of XPC (Araki et al., 2001; Reardon et al., 1996; Sugasawa et al., 1996), in line with the repair-deficiency observed in XP-C patients (who still express hHR23B). Instead of playing a direct role in repair, (h)HR23B protects XPC from proteasomal degradation (Araki et al., 2001; Ng et al., 2003), as does its *S. cerevisiae* homolog Rad23 with Rad4, the XPC homolog (Ortolan et al., 2004). XPC and hHR23B both reside in the nucleus (van der Spek et al., 1996) and almost all XPC is complexed with hHR23B (Masutani et al., 1994), but because of the abundance of hHR23B inversely only a fraction of hHR23B is complexed to XPC (Sugasawa et al., 1996; van der Spek et al., 1996). A small fraction of XPC is complexed with hHR23A, a close homolog of hHR23B (Araki et al., 2001); a mouse model showed that only in the absence of HR23B substantial amounts of XPC-HR23A are found (Ng et al., 2003). From the embryonic lethality of HR23A-HR23B double knockout mice (Ng et al., 2003), it can be inferred that these proteins serve essential functions outside NER that remain to be elucidated.

In mouse embryonic fibroblasts the first 6-8 hours post UV irradiation or post NA-AAF treatment, XPC is further stabilised in an HR23B-dependent manner resulting in a higher repair capacity (Ng et al., 2003) (the mechanism of this stabilisation is discussed in more detail in section 3.3). As mentioned above, constitutively high levels of XPC appear to be toxic to the cell (Ng et al., 2003; W. Vermeulen, pers. comm.), perhaps as a result of the affinity of XPC for normal dsDNA (Batty et al., 2000; Reardon et al., 1996) but more likely because of its high affinity for small bubble structures (Hey et al., 2002; Sugasawa et al., 2001). Such bubbles are routinely formed during transcription, so it is conceivable that XPC-HR23B will interfere with this process if present in too high concentrations. Therefore, it seems that under normal conditions (i.e. in the absence of DNA damage) XPC levels are kept low, and are post-translationally increased via stabilisation by HR23B only when necessary, i.e. when DNA damage is present.

XPC-hHR23B probes for helix destabilisation assisted by UV-DDB

The first step in the bipartite model for damage recognition in NER is probing for disturbed base stacking (see chapter 2.4). A DNA helix that is destabilised by a lesion is locally more flexible, making it easier for the DNA to bend and be bent. The NER factors with the highest affinity for damaged DNA, UV-DDB and XPC-hHR23B, both indeed bend damaged DNA upon binding (Fujiwara et al., 1999; Janicijevic et al., 2003). In addition, many DNA lesions themselves introduce a bend in DNA which will also facilitate binding of the damage-recognising proteins. For example, 6-4PP introduce a bend of $\sim 40^\circ$ in DNA, while also the base pairing is somewhat disturbed (Kim and Choi, 1995; Kim et al., 1995), and these lesions are good substrates for XPC-hHR23B and UV-DDB. In contrast, CPD only introduce an angle of $7-10^\circ$ and retain the hydrogen bonds between the A and T bases (Kim and Choi, 1995; Kim et al., 1995) and consequently these lesions are poorly recognised by the XPC complex, which requires the assistance of UV-DDB to initiate their repair. Interestingly, the introduction of two T:G mismatched base pairs in the CPD does not disturb the hydrogen bonds, but does considerably increase the angle formed in the DNA (Lee et al., 2004) coinciding with an increased binding affinity by XPC-hHR23B (Sugasawa et al., 2001).

In a series of DNA binding and repair experiments, Sugasawa and co-workers showed that

XPC-hHR23B is capable of binding to small bubble structures distorting the DNA helix, regardless of whether these contain a lesion (Sugasawa et al., 2001; Sugasawa et al., 2002). Given the absence of base pairing within a small bubble (for example, a cisplatin adduct is sensitive to KMnO_4 approximately 3 nt on either side of the lesion; Constantinou et al., 1999; Evans et al., 1997a; Tapias et al., 2004) it is likely that also these structures are easily bent upon binding of XPC-hHR23B. *In sum*, XPC-hHR23B appears to be responsible for sensing destabilisations in the DNA helix. The mechanism of action of UV-DDB is less well-characterised, but UV-DDB might detect destabilisations in a comparable manner. Although only essential for the repair of CPD, it seems likely that UV-DDB assists in the repair of several other types of lesions similarly to its stimulation of 6-4 photoproduct repair, i.e. at physiologically relevant low doses. Meanwhile, dual incision only occurs when a lesion is present within the bubble (or more general, the region of destabilised DNA) bound by XPC-hHR23B (Sugasawa et al., 2001). XPA or XPA in conjunction with RPA are likely to be responsible for this second, damage verification step of NER, possibly in cooperation with TFIIH (see chapters 3.4 and 3.5).

3.3 Post-transcriptional regulation of UV-DDB and XPC – regulating the GGR pathway via the damage-recognition step

Degradation of DDB2 following UV

The DDB2 subunit of UV-DDB tightly binds to UV-damaged chromatin, after which it is consumed by proteasome-mediated degradation (Chen et al., 2001; Fitch et al., 2003b; Raptic-Otrin et al., 2002). After this initial degradation, DDB2 levels are restored to their original levels within the next 24 hours, with an overshoot at 48 hours after UV, likely caused by the p53-dependent transcriptional activation of the *DDB2* gene (Hwang et al., 1999; Liu et al., 2000; Nichols et al., 2000). Recently, the molecular mechanism of DDB2 degradation has been clarified. Cullin 4A, an E3 ubiquitin-protein ligase, coimmunoprecipitates with UV-DDB (Shiyanov et al., 1999). This interaction results in the ubiquitination of DDB2, causing it to be degraded (Chen et al., 2001; Nag et al., 2001). Degradation of DDB2 in turn results in a decrease in UV-DDB damaged DNA binding activity (Chen et al., 2001). The hypothesis that follows from these observations, i.e. that cullin 4A is responsible for the observed degradation of UV-DDB following UV irradiation, has since been confirmed (Matsuda et al., 2005; Raptic-Otrin et al., 2002; Sugasawa et al., 2005).

Given the high affinity of UV-DDB for especially 6-4PP, it is conceivable that the ubiquitination and/or degradation of UV-DDB is necessary to allow its removal from this type of lesion before the subsequent action of other NER factors can take place. This view is supported by several observations: (i) degradation of UV-DDB is observed in NER-deficient cells as well – most relevantly including XP-C (Raptic-Otrin et al., 2002); (ii) the kinetics of 6-4PP repair and the degradation of DDB2 are highly similar, both occurring primarily in the first few hours after UV irradiation (Fitch et al., 2003b; Liu et al., 2000; Nichols et al., 2000; Raptic-Otrin et al., 2002); and (iii) ubiquitination of UV-DDB dramatically reduces its DNA binding activity (Sugasawa et al., 2005). It would be interesting to see whether mutations in DDB2 that prevent its ubiquitination give rise to a dominant negative effect on repair (of 6-4PP); the existence of a patient expressing a truncated DDB2 with a specific defect in 6-4PP repair but not CPD repair (Itoh et al., 1999) might be especially significant in this matter.

The known interactions between cullin 4A and UV-DDB were extended by Groisman and co-workers (Groisman et al., 2003). Two complexes of almost identical composition are present in the cell, each containing the DDB1 subunit of UV-DDB, the cullin 4A-based ubiquitin-ligase activity, the COP9-signalosome (CSN), which is a regulator of cullin-based ubiquitin-ligase activity, and either DDB2 or CSA but not both (Groisman et al., 2003). The DDB2-containing complex when engaged in GGR (after UV irradiation), loses its CSN and thus likely gains ubiquitin-ligase activity (Groisman et al., 2003). In contrast, the CSA-containing complex gained more CSN following UV irradiation, further downregulating this activity (Groisman et al., 2003). The implications of this latter phenomenon for TCR are not clear yet.

It is interesting to note that DDB2 is degraded to virtual absence during repair of a lesion for which it is not strictly required, i.e. 6-4 photoproducts, thus rendering it unavailable for repair of CPD, which do require DDB2. The most likely explanation seems that the observed depletion of DDB2 is an experimental artefact due to the single high dose of UV light used in laboratory experiments, a situation that would rarely if ever occur in nature. Rather, an all-day long continuous exposure to a low UV flux might lead to a balanced production and degradation of DDB2 – sufficient to cope with the rate of photolesion induction and resulting in a more or less equilibrated amount of DDB2. Given the periodicity and regularity of the induction of UV damage to the DNA it is also not surprising to find an upregulation of *DDB2* expression 48 hours following UV when, under laboratory conditions, ‘all damages have been removed’. In nature, it is highly likely that 48 hours following the introduction of UV damage, UV damage will again be introduced – the induction can thus be considered an adaptation process (also suggested in McKay et al., 2005).

Increased activity of XPC following UV

The finding that the UV-DDB-cullin 4A complex probably increases its activity when participating in repair is particularly interesting in the light of the second role that ubiquitination plays in NER: the monoubiquitination of XPC following the infliction of NER-types of DNA damage (Lommel et al., 2002; Ortolan et al., 2004; Sugasawa et al., 2005; Wang et al., 2005). In contrast to the ubiquitination of DDB2, ubiquitination of XPC does not result in its proteasomal degradation, because XPC is protected from degradation by its heterodimeric partner, HR23 (Araki et al., 2001; Ng et al., 2003; Ortolan et al., 2004). HR23B contains two ubiquitin-associated (Uba) domains that interact with ubiquitin. Thus, the sequence of events that occur following DNA damage is likely to be: XPC is monoubiquitinated, which leads to an enhanced interaction with HR23B; the resulting protection against degradation in turn increases overall levels of XPC, leading to the higher repair capacity of cells observed in the first hours after UV (Ng et al., 2003). Simultaneously, HR23 can prevent polyubiquitination of its partners thereby also precluding their proteasomal degradation (Ortolan et al., 2000).

Not only does ubiquitination of XPC prevent its degradation, it also increases the affinity of XPC for DNA in general, regardless of whether this is damaged or not (Sugasawa et al., 2005); moreover the ubiquitination is reversible, upon which the affinity of XPC for DNA decreases accordingly (Sugasawa et al., 2005). Remarkably, but in the light of the above maybe not surprisingly, ubiquitination of XPC is executed by the UV-DDB-cullin 4A complex (Sugasawa et al., 2005). Together, these findings result in a model for the early stage of GGR in which ubiquitination of the main damage recognition complexes plays an important role in their loading and unloading on the DNA. The model is depicted in Figure 2 (see Appendix). Adjusting the level of XPC post-transcriptionally allows the cell to rapidly regulate its GGR capacity, avoiding

the slower process of upregulation via transcription. Although XPC is actually transcriptionally upregulated following DNA damage (by p53), this upregulation occurs at a time when TCR is expected to have removed most lesions from transcribed DNA; see below). Similarly to DDB2 (see above), upregulation of XPC could prepare the cell for a 'second round' of DNA damage, e.g. exposure to UV light from the sun one day later.

Recently it was reported that SUMOylation of XPC following UV irradiation also can protect XPC from degradation (Wang et al., 2005). Although dependent on UV-DDB (like its ubiquitination), the co-dependence on XPA protein (Wang et al., 2005) could suggest that this modification occurs in a later stage of the NER reaction, when the complex is (nearly) completely formed.

Other XPC-regulating proteins

The levels of XPC are modulated by two more proteins. The small centromere protein centrin 2 (18kDa) assists in the stabilisation of XPC by hHR23B (Araki et al., 2001; Nishi et al., 2005). Recently it was found that centrin 2 stimulates NER both *in vitro* and *in vivo* via its interaction with XPC (Nishi et al., 2005). The established function of centrin 2 lies in centrosome duplication, suggesting a coupling between NER and replication, but evidence for such a coupling has yet to be reported.

Finally, the unusual protein kinase C (PKC) ζ influences XPC and hHR23B at the post-translational level. Cells overexpressing PKC ζ show increased resistance to genotoxic agents that induce lesions repaired by NER, such as UVC and cisplatin, and display concurrent increased repair rates of the respective lesions (Louat et al., 2004). These effects appear to be a direct consequence of a 10- and 5-fold increase in levels of XPC and hHR23B protein, respectively, while their mRNA levels are unaffected (Louat et al., 2004). A putative role for PKC ζ in stabilising XPC fits well with similar findings concerning other members of the PKC family, which regulate various proteins involved in genomic stability such as DNA-PKcs (Bharti et al., 1998), hMSH2 and hMSH6 (Christmann et al., 2002; Humbert et al., 2002) and MGMT (Srivenuogal et al., 2000).

Regulation at the transcriptional level: a supervising role for p53

The tumour suppressor protein p53 is involved in NER through its regulation of DDB2 (Hwang et al., 1999) and XPC (Adimoolam and Ford, 2002) at the transcriptional level (reviewed in Ford, 2005). In p53-proficient cells, transcription of the *DDB2* gene is upregulated in a p53-dependent manner following DNA damage, resulting in an increased UV-DDB activity (Hwang et al., 1999). Also XPC is induced both at the protein and the mRNA level in a p53- and DNA damage-dependent manner (Adimoolam and Ford, 2002). Additionally, in p53-deficient cells the basal mRNA levels of the respective proteins are severely reduced compared to that in the p53-proficient counterparts (Adimoolam and Ford, 2002; Hwang et al., 1999). These results explain the earlier observation that efficient GGR of CPD requires the activation of p53 (Ford and Hanawalt, 1995; Ford and Hanawalt, 1997), since both XPC and UV-DDB are essential for repair of CPD via GGR, and could also provide a rationale for the reduced repair of, and sensitivity to, the bulky anti-benzo[a]pyrene-diol-epoxide adduct observed in p53^{-/-} cells (Wani et al., 2002; Wani et al., 2000). Finally, as mentioned in chapter 3.1, in mice the *DDB2* gene is not transcriptionally controlled by p53, partially explaining the lack of CPD repair in mouse (and possibly other rodent) cells (Tan and Chu, 2002).

3.4 TFIIH

TFIIH is a highly versatile protein, being involved in at least five (at last count) distinct cellular processes: RNA pol II transcription initiation (Flores et al., 1992; Gerard et al., 1991); RNA pol I transcription (Iben et al., 2002); global genomic NER (Drapkin et al., 1994; Schaeffer et al., 1993; van Vuuren et al., 1994; Wang et al., 1994); transcription-coupled repair (van Hoffen et al., 1999); and promoter-specific transactivation (Drane et al., 2004; Keriell et al., 2002; Liu et al., 2001). Furthermore, its CAK subcomplex contains a cyclin dependent kinase activity functioning in cell-cycle progression (Fisher and Morgan, 1994).

TFIIH is the largest factor in NER, both in number of subunits (ten) and in molecular weight (~450 kDa). Its subunits are XPB/p89, XPD/p80, p62, p52, p44, p34, the three subunits of the CAK subcomplex cdk7 (41 kDa), cyclin H (38 kDa) and MAT1 (32 kDa) and the 8 kDa small p8/TTDA, which was discovered only recently (Giglia-Mari et al., 2004; Ranish et al., 2004). There is some unclarity as to whether TFIIH is present in the cell as a complex harbouring all ten subunits ('holo-TFIIH') or whether several smaller subcomplexes exist beside holo-TFIIH.

Although holo-TFIIH can always be isolated from human cells, several early reports indicate the existence of other possible compositions for TFIIH. A 'core' has been found, consisting of XPB, XPD, p62, p52, p44 and p34, separate from the CAK complex (Rossignol et al., 1997); additionally XPD has been observed to complex with CAK, while the remaining factors constituted another complex (Drapkin et al., 1996; Reardon et al., 1996; Rossignol et al., 1997). In support of these findings it was found that (i) six-subunit core was functional in NER *in vitro* (Araujo et al., 2000; Mu et al., 1996) and (ii) several complexes, i.e. holo-TFIIH, the six-factor core and the smaller core (i.e., lacking XPD) were all functioning in transcription initiation *in vitro* (Tirode et al., 1999).

Other observations, on the other hand, indicate that in the nucleus holo-TFIIH is the predominant form, performing NER – and probably also all other functions of TFIIH. Firstly, as early as 1994 it was reported that microinjection of cells with an antibody against cdk7 led to an inhibition of NER (Roy et al., 1994). Secondly, pulldown of TFIIH with an antibody against cdk7 precipitated exclusively holo-TFIIH, with no TFIIH (measured by the presence of XPB and p62) remaining in the supernatant (Araujo et al., 2001). Thirdly, after local UV irradiation and immunofluorescent labelling, components of the CAK subunit localise at the site of local UV damage (Coin et al., 2006; M. Volker, unpublished observation). In short, there is an apparent contradiction between the supposed existence of several separate subcomplexes and reports suggesting that TFIIH is mainly present in holo-form in the cell. To add to the unclarity, in *S. cerevisiae* altogether different subcomplexes of TFIIH have been found, e.g. 'core' TFIIH comprises the homologs of XPD, p62, p52, p44 and MAT1, but not XPB and p34 (Chang and Kornberg, 2000). The lack of consistency between the various reports has led to the hypothesis that the observed variation in composition of TFIIH merely reflects differences in the various purification procedures and that in the cell holo-TFIIH is the predominant form of TFIIH (Araujo et al., 2001). If so, the question arises if and how CAK dissociates from core TFIIH to perform its function in the cell cycle; possibly, CAK is present in excess over core TFIIH.

Several enzymatic activities reside in TFIIH: XPB and XPD are DNA-dependent ATPases/DNA helicases (Roy et al., 1994; Schaeffer et al., 1994; Schaeffer et al., 1993; Sung et al., 1993; Weber et al., 1990), while cdk7 possesses kinase activity. The 3' to 5' helicase activity of XPB is essential in both NER and transcription initiation (Evans et al., 1997b; Guzder et al., 1994; Qiu et al., 1993; Sung et al., 1996); in contrast the 5' to 3' XPD helicase activity is necessary for NER

but dispensable for transcription initiation, where only the structural presence of XPD is required (Coin et al., 1999; Sung et al., 1988; Winkler et al., 2000). Still, functional XPD does enhance transcription when associated with the five-subunit 'small core' TFIIH (Tirode et al., 1999). In both NER and transcription TFIIH opens up a 10-12 nt stretch of helical DNA to form a single-strand bubble (Evans et al., 1997b; Holstege et al., 1996; Tapias et al., 2004). This structure might stabilise the binding between TFIIH and DNA, and in repair thus facilitate the further recruitment of NER factors to the NER complex via their interactions with TFIIH (Araujo et al., 2001; Evans et al., 1997b). The kinase activity residing in the CAK subcomplex targets different proteins depending on its associations. Association of CAK with core TFIIH confers a preference to phosphorylate the C-terminal domain of RNA pol II, stimulating promoter clearance and the start of transcription elongation (Rossignol et al., 1997; Tirode et al., 1999). On the other hand, CAK on its own phosphorylates cdk1, cdk2 and cdk4 (Nigg, 1996; Rossignol et al., 1997), promoting cell cycle progression (Fisher and Morgan, 1994). Finally, the yeast homolog of TFIIH was recently found to possess a ubiquitin-ligase activity residing in its Ssl1 subunit (the homolog of p44) (Takagi et al., 2005). This activity is involved in a transcriptional response and is not required for repair activity (Takagi et al., 2005). As yet its targets are unknown, but a likely possibility are transcriptional activators, for instance of DNA repair genes. It will be interesting to see whether the mammalian p44 subunit possesses a similar activity.

Additionally, most subunits of TFIIH perform specific non-enzymatic functions. The C-terminal domain of XPB stimulates incision by ERCC1-XPF and deletion of this domain, whilst not interfering with the structure or helicase activity of XPB nevertheless results in abrogated 5' NER incision (Coin et al., 2004; Evans et al., 1997b). Curiously, a direct interaction between ERCC1-XPF and the C-terminus of XPB has yet to be reported. XPD is the subunit that anchors CAK to TFIIH (Coin et al., 1999; Drapkin et al., 1996; Rossignol et al., 1997). The p62 subunit contains a PH domain that interacts with XPG (Gervais et al., 2004). Deletion of this domain resulted in an abolished *in vitro* NER activity of TFIIH without apparently affecting its transcriptional activity or its structure (Gervais et al., 2004). If *in vivo* mutations in or the deletion of this domain would have an analogous effect, it raises the intriguing question why there is no XP complementation group with mutated p62. The p52 subunit stimulates the helicase activity of XPB through a direct interaction (Jawhari et al., 2002); in a similar manner, XPD helicase activity is stimulated by the p44 subunit (Coin et al., 1998). Finally p8/TTDA functions as a stabilising factor (Coin et al., 2006; Giglia-Mari et al., 2004; Ranish et al., 2004). p8/TTDA furthermore stimulates the helicase activity of XPB (Coin et al., 2006) although apparently p8/TTDA and XPB do not interact directly (Coin et al., 2006). p8/TTDA does interact with XPD and with p52 (Coin et al., 2006); perhaps p8/TTDA thus stimulates XPB indirectly.

Possible roles for TFIIH in detecting chemical alterations and in regulating dual incision

Apart from helix unwinding TFIIH might perform other functions in NER. Rad3 is the *S. cerevisiae* homolog of XPD and is able to bind to a 3'-single-strand extension of dsDNA and from there can unwind duplex DNA in the 5' to 3' direction (Sung et al., 1987). This helicase activity can be blocked by damages in the strand to which Rad3 binds, while damages in the other strand pose no obstacle for unwinding (Naegeli et al., 1992; Naegeli et al., 1993). This strand-specific block may thus serve as both a damage sensor and as a strand discrimination signal (to determine in which strand of a disturbed DNA duplex the damage resides), which led to the hypothesis that this mechanism could contribute to damage recognition by NER (Naegeli et al., 1992; Naegeli et al.,

1993). The structure formed after the blocking of the helicase stabilises the binding between TFIIH and the DNA (Naegeli et al., 1992; Naegeli et al., 1993), and thus facilitates the further recruitment of NER factors to the NER complex via their interactions with TFIIH. When the helicases are not blocked by a lesion, the complex may disassemble (Wood, 1999), precluding gratuitous repair.

A recent finding by the group of Egly adds another layer of complexity to the damage recognition step of NER. Coin et al. reported that phosphorylation of XPB at lysine 751 leads to a specific inhibition of XPB activity in NER but not in transcription (Coin et al., 2004). This phosphorylation specifically inhibits the 5'-incision by ERCC1-XPF by preventing its recruitment to the NER complex, without affecting either the helicase or the transcriptional activity of XPB. Although these findings provide evidence suggestive of another regulatory mechanism in NER, further research is required to resolve some important questions that remain, including: is the phosphorylation status indeed actively changed *in vivo* after the induction of DNA damage, as their findings suggest; does the phosphorylation occur in XP(-C) mutant cells; and what proportion of total XPB is phosphorylated and what is the time course of phosphorylation and dephosphorylation. It will be interesting to observe whether cells expressing a mutated XPB incapable of phosphorylating lysine 751 show a distinct phenotype besides impaired NER.

3.5 XPA

As explained above, initially it was suggested that the 30 kDa XPA protein was directly involved in the first step of NER: damage recognition. The reason for this was twofold; firstly, XP-C cells only show compromised GGR whereas in XP-A cells also TCR is affected. Secondly, early biochemical characterisations showed that purified XPA has an increased affinity for damaged over undamaged DNA (Jones and Wood, 1993; Robins et al., 1991). These observations led to models in which XPA was thought to recognise all lesions after which XPC would be responsible for initiating the GGR pathway, for instance by stabilising an open intermediate complex that in TCR would be stabilised by RNA pol II (Mu et al., 1996; Reardon et al., 1996; van der Spek et al., 1996).

Shortly hereafter, it was found that XPA interacts with RPA (He et al., 1995; Lee et al., 1995; Li et al., 1995; Matsuda et al., 1995) and the resulting complex displayed a higher affinity for damaged DNA than XPA alone (Li et al., 1995). The XPA-RPA complex thus seemed a likely candidate for global damage recognition. Wakasugi and Sancar performed DNA binding and repair assays on an oligonucleotide harbouring a single 6-4 photoproduct. They found that damage removal was faster when XPA and RPA were the first factors to bind to DNA compared to when XPC was the first protein presented to DNA, and they concluded that RPA and XPA are the initial damage sensing factors of human NER (Wakasugi and Sancar, 1999).

On the other hand, evidence has accumulated supporting the view that XPC-hHR23B is the damage recognition factor for GGR. Using NA-AAF-treated adducted plasmids in an *in vitro* damage recognition-competition assay Sugasawa and co-workers showed that XPC-hHR23B is likely the damage detector that initiates NER *in vitro* (Sugasawa et al., 1998), while we have shown that *in vivo*, recruitment of XPC to UV lesions is independent of functional XPA whereas vice versa XPA nor RPA in the absence of XPC localise to UV lesions (chapters 6 and 8). The difference in the *in vitro* findings has been attributed partly to the shorter probe fragments used by Sancar's laboratory, but mainly to the lack of competitor DNA in their experiments (Batty et al., 2000). Further *in vitro* experiments by the group of Egly have corroborated the findings

by Sugasawa et al. and additionally have indicated that XPA is not incorporated into the NER complex until after the recruitment of TFIIH (Riedl et al., 2003). Although from our experiments described in chapter 6 it is technically not possible to exclude that *in vivo* XPA is actually recruited to the complex before TFIIH, Coin and co-workers recently observed that in TTD-A cells, which have a defect in helix opening during NER, TFIIH is recruited to sites of local UV but XPA is not (Coin et al., 2006), corroborating the *in vitro* data and further strengthening the notion that XPC and TFIIH are the first two factors to be involved in NER. Damage recognition in TCR, meanwhile, has become thought of as being triggered by stalling of the transcribing RNA polymerase at a lesion (originally proposed by Mellon and Hanawalt, 1989; Mellon et al., 1987), obviating the need for a dedicated damage-recognising protein in TCR.

Several reports by the group of Naegeli have indicated that XPA specifically binds to bent DNA duplexes, a feature that is incompatible with a function in direct lesion recognition but is characteristic of architectural proteins (Missura et al., 2001; Camenisch et al., 2006). As mentioned above, such bending of DNA is introduced upon binding of the damage-recognition proteins UV-DDB and XPC-hHR23B. During this binding XPA does not directly interact with (or ‘recognise’) the lesion, instead performing an ‘indirect readout’, in agreement with the wide substrate specificity of NER.

Damage verification by XPA: regulation of the endonucleases in cooperation with TFIIH?

As mentioned above, XPC-hHR23B has a high affinity even for undamaged DNA, and is frequently immobilised for short periods of time in the nucleus, most likely to DNA (D. Hoogstraten, submitted). XPC-hHR23B in turn interacts quite strongly with TFIIH (Araujo et al., 2001; Drapkin et al., 1994; Yokoi et al., 2000), so ‘proto-NER complexes’ consisting of XPC-hHR23B and TFIIH bound to DNA probably form regularly. It is important that these erroneous proto-NER complexes do not reach the incision stage, to prevent numerous strand breaks from being introduced into the genome. XPA is likely to play an important role in the second part of the bipartite damage recognition by NER, i.e. detection of the chemical modification of the DNA – often referred to as ‘damage verification’ (Missura et al., 2001; reviewed in Thoma and Vasquez, 2003). XPA might execute this damage verification in several different ways.

Firstly, binding of XPA to a lesion site could (further) stabilise a bona fide NER complex. If no lesion is present the complex is not stabilised by XPA and disassembles. As shown in chapter 10, the partial NER complex formed in XP-A cells following UV irradiation appears to be stable, seemingly arguing against this mechanism. In this case however, there are lesions present in the partial complexes which is likely to increase their stability (see chapters 3.2 and 3.4).

In addition to further stabilising an NER complex when a lesion is present, several observations indicate that XPA might perform a crucial role in directly and indirectly regulating the endonuclease activities, providing the second line of defence against aberrant cutting. First, the presence of XPA is required to allow recruitment of ERCC1-XPF (Li et al., 1995 and chapter 6). Also the incorporation of XPG into the NER complex has been reported to depend on XPA *in vitro* (Riedl et al., 2003), although *in vivo* this is not observed (chapter 6). Second, XPA might stimulate or even permit incision by XPG and ERCC1-XPF. This stimulation (or permission) could be an indirect effect that depends on the function of the TFIIH helicases. The mechanism can be inferred from two observations. Firstly, the presence of XPA specifically stimulates ATP hydrolysis activity of the TFIIH helicase subunits in the presence of a DNA lesion (Winkler et al., 2001). Addition of RPA further increases this stimulation (Winkler et al., 2001). Secondly,

this ATP hydrolysis is required to stimulate dual incision (Mu et al., 1996; Svoboda et al., 1993). Thus, XPA could be essential for endonuclease activity in the presence of a lesion. Inversely, if XPA by virtue of its affinity for damaged DNA is not able to detect a lesion within the complex, it will not associate with the NER complex, preventing the incision step.

Supporting a mechanism in which XPA stimulates the endonucleases either directly or indirectly is the observation that in XPA-deficient cells XPG is incorporated into the complex (chapter 6) but does not incise (Evans et al., 1997a). In contrast, in XPF-deficient cells both XPA and XPG are incorporated into the complex (chapter 6). In this situation, some authors have found that XPG is capable of making an incision (Evans et al., 1997a); others found that the presence of ERCC1-XPF is required before XPG can incise (Tapias et al., 2004). Despite this discrepancy, in both cases XPA was absolutely required for incision by XPG. Also, the presence of p8/TTDA which stimulates XPB helicase activity and promotes open complex formation (Coin et al., 2006) is required for XPA localisation to sites of UV damage; concurrently p8/TTDA not only promotes 5' incision by ERCC1 but also 3' uncoupled incision by XPG (Coin et al., 2006). The latter could be a direct effect of the stimulated open complex formation, an indirect effect of XPA recruitment to the complex, or (maybe most likely) a combination of both.

Taking all data together, XPA is not a principle damage recognition factor but instead a damage verification (or demarcation) protein in a later stage of NER, functioning to ascertain that the DNA covered by the NER complex indeed harbours a lesion (Missura et al., 2001; Sugasawa et al., 2001). It is noteworthy that the proposed roles for TFIIH and XPA in this stage of NER are very similar in nature, overlap to a large extent, and could likely occur in concert or even in synergy. Additionally, RPA is also likely to assist in this stage of NER (see below).

3.6 RPA

The RPA protein is a heterotrimer consisting of a large (70 kDa), middle (34 kDa) and small (17 kDa) subunit that displays an extraordinarily high affinity for single-strand DNA. Many DNA metabolic processes that involve ssDNA intermediates, including replication, recombination and various repair pathways, utilise RPA. Covering the ssDNA, RPA protects it from attacks by endonucleases and prevents the formation of unwanted secondary structures.

RPA is able to bind to ssDNA in two modes. In the one mode, it binds to a stretch of 8-10 nucleotides; in the other, far more stable (~100 fold) mode it binds 30 nucleotides (Blackwell and Borowiec, 1994; Kim et al., 1994; Kim et al., 1992). The stretches of ssDNA formed by the helicase action of TFIIH are of essentially the correct length for RPA to bind in 8-10 nt mode. After binding to this bubble, RPA might change to the 30 nt conformation, thereby creating stretches of ssDNA similar in length to the oligomer released after dual incision. Apart from playing a role in ssDNA protection, RPA is absolutely required for dual incision (Coverley et al., 1991). Interestingly, RPA is important for the proper positioning and stimulation of the NER endonucleases, XPG and ERCC1-XPF (de Laat et al., 1998b; He et al., 1996; Matsunaga et al., 1996). RPA binds with a specific polarity to the undamaged strand during NER. From this position it coordinates the binding of and incision by XPG and ERCC1-XPF. At its 3' end, RPA interacts with and stimulates activity of ERCC1-XPF in the opposing strand, resulting in the incision 5' to the lesion. Conversely, at its 5' end it allows the binding of XPG which can then incise 3' to the lesion (de Laat et al., 1998b).

RPA is also an essential component in the DNA resynthesis step of NER (see chapter 3.10). Thus, it is the only protein that is involved in both the pre- and post-incision stages of NER. The possibility that RPA remains localised to the undamaged DNA strand after excision of the damaged oligo, and possible consequences of its 'dual role' for the formation of the preincision NER complex are discussed in chapter 10.

RPA complements XPA in damage verification

Although it has been suggested that RPA (either alone or in combination with XPA) is implicated in the damage recognition step by virtue of a preference for cisplatin- (Clugston et al., 1992) and UV-damaged DNA (Burns et al., 1996) over undamaged DNA, or by direct interaction with a psoralen lesion in the absence of other repair factors (Reardon and Sancar, 2002), there is no support for these claims within the context of what else is known about the NER reaction. Our experiments in fact show that RPA does not translocate to sites of UV damage in XP-C cells (chapter 8) providing evidence against RPA as the initial damage recognizing factor. The binding of RPA to damaged DNA may actually reflect its role in damage verification, where it cooperates with XPA. While XPA detects a kink in the DNA (see above), RPA specifically binds to the unwound single-strand DNA at the site of the lesion (Missura et al., 2001). If either the kink or the single-strand are absent from the DNA, XPA or RPA respectively will not bind to the NER complex, in turn abrogating incision by the endonucleases.

Recently it has become doubtful whether XPA and RPA actually coexist in a complex in the nucleus as was originally thought (He et al., 1995; Lee et al., 1995; Li et al., 1995; Matsuda et al., 1995). *In vivo* diffusion measurements of XPA-GFP provided no evidence for an interaction of XPA with (the much larger) RPA, and in XP-A cells RPA readily relocates to sites of subnuclear UV irradiation (chapter 8). These results suggest that both proteins normally only interact with each other in the context of an NER complex.

Taking above data together, it appears that XPA and RPA cooperate in the damage verification step of NER. Both play distinct, complementary and essential roles in the recruitment, positioning and activation of the endonucleases XPG and ERCC1-XPF, providing a double-check mechanism that will not allow dual incision to take place unless it is clear that a damage is present in the initial complex formed by XPC-hHR23B and TFIIH. As speculated above, TFIIH could provide further assistance in this step via its kinetic proofreading mechanism (chapter 3.4).

3.7 XPG

The 133 kDa XPG protein is a structure-specific endonuclease of the FEN-1 family (Liever 1997). It cuts specifically at or near the junction between the 3' end of single-stranded DNA and the 5' end of double-stranded DNA. In NER, XPG incises the damage-containing strand at a distance of 2-8 nt 3' of the lesion (Matsunaga et al., 1995; O'Donovan et al., 1994a; O'Donovan et al., 1994b).

Interactions between XPG, several subunits of TFIIH, and CSB have been observed (Araujo et al., 2001; Gervais et al., 2004; Iyer et al., 1996; Mu et al., 1995), although some investigators did not detect interactions between CSB and XPG (van Gool et al., 1997). XPG, TFIIH, CSB and RNA pol I were later found to reside in a complex that promotes RNA pol I synthesis (Bradsher et al., 2002), providing a rationale for the interactions described in previous reports.

Alternatively or additionally, the interactions between XPG, TFIIH and CSB in undamaged cells may reflect interactions that are important in the formation of a protein complex active in TCR (chapter 2.1), much like the interaction between XPC and TFIIH in undamaged cells (Araujo et al., 2000) reflects their concerted action in early steps of GGR (Yokoi et al., 2000).

Apart from its endonuclease activity, XPG contributes structurally to the formation of the NER complex (Constantinou et al., 1999; Evans et al., 1997a), and the structural presence of XPG – not its endonuclease activity – is necessary to permit ERCC1-XPF to cleave (Constantinou et al., 1999; Mu et al., 1997; Wakasugi et al., 1997). Recently, the crucial structure for this stimulatory activity was mapped to reside in the so-called spacer region of XPG, a >600 aa domain between its conserved nuclease domains that bears no clear homology to known proteins (Dunand-Sauthier et al., 2005; Thorel et al., 2004). Deletions in this domain abolish stable interaction with TFIIH and hence abrogate both the proper functioning of XPG in NER and its stimulation of ERCC1-XPF incision (Dunand-Sauthier et al., 2005; Thorel et al., 2004). In addition, the spacer domain is of critical importance to the preference of XPG to cleave bubble structures (Dunand-Sauthier et al., 2005; Thorel et al., 2004). XPG also contains a PCNA-binding domain, which could function to attract PCNA to the repair complex (Gary et al., 1997; Warbrick, 1998). However, even though XPG readily relocates to damage sites in XP mutant cells from groups XP-A and XP-F (chapter 6), PCNA does not (Green and Almouzni, 2003; Katsumi et al., 2001; Miura and Sasaki, 1996), indicating that this domain alone is not sufficient to recruit PCNA.

XPG participates in several other pathways besides NER. At least one of the functions of XPG outside NER is essential, as knockout XPG mice are not viable (Harada et al., 1999), whereas XP-G patients expressing truncated XPG can live (albeit with a much shortened lifespan). Indeed, no XP-G patients exist that are null for XPG. In XP-G patients, the small fragment of XPG expressed must be just enough to perform the essential function. It has been reported that the *S. cerevisiae* homolog of XPG, Rad2, is required for efficient RNA pol II transcription (Lee et al., 2002), but whether this is the critical role that causes the XPG-null lethality is not clear. XPG also plays a role in BER, where it activates the thymine glycol glycosylase NTH1 (Bessho, 1999; Klungland et al., 1999).

3.8 Order of binding of RPA, XPG and XPA

After the initial complex of XPC-hHR23B and TFIIH is formed, XPG, XPA and RPA are recruited to the NER complex (Riedl et al., 2003 and chapters 6 and 8). The order of their binding is unclear, and several reports present evidence for various different orders of binding of these respective proteins.

XPG first?

Interactions between TFIIH and XPG have been observed repeatedly (Araujo et al., 2001; Iyer et al., 1996; Mu et al., 1995). Of all NER factors XPG in fact displays the strongest interaction with TFIIH (Araujo et al., 2001) and XPG relocates to local UV-induced damages in the absence of functional XPA and XPF (chapter 6). The suggestion from these data, i.e. that XPG is recruited to the NER complex predominantly by its interactions with TFIIH is corroborated by recent findings. As mentioned above, deletions in the spacer domain of XPG cause weakened interactions with TFIIH and a consequently reduced incorporation into the NER complex

(Dunand-Sauthier et al., 2005; Thorel et al., 2004). These observations provide support for the idea that XPG is the first factor to be recruited following TFIIH, before both XPA and RPA (de Laat et al., 1999). The obvious lack of a genetic RPA mutant has so far prevented *in vivo* experiments into NER complex formation in the absence of RPA, leaving the possibility open that the incorporation of RPA into the complex precedes, and maybe even is necessary for, that of XPG. The advent of siRNA knockdown techniques has now made it possible to create temporary mutants in otherwise essential genes; thus, assessing NER complex formation after knockdown of RPA should be able to provide insights into this matter.

RPA first?

How RPA is attracted to the NER complex is unclear. RPA interacts only weakly with TFIIH (Araujo et al., 2001) and has not been reported to interact with XPC, ruling out a clear-cut recruitment via protein-protein interactions in the early stages of complex formation. RPA does interact with XPG (He et al., 1995) but this observation merely creates a chicken-and-egg type of question, i.e. both proteins may assist in recruiting the other, once the first of them is in the complex – but which is the first? Also the findings by Tapias and co-workers from the group of Egly are not conclusive. When added to ternary complexes of damaged DNA, XPC and TFIIH only the simultaneous addition of RPA, XPA and XPG led to further opening of the helix (Tapias et al., 2004); the possibility that RPA binds first before being activated by the arrival of XPA and/or XPG is however not ruled out by these observations.

In severe XP-B/CS mutant cells, the XPB subunit of TFIIH is presumed to be inactive in NER helix unwinding (Hwang et al., 1996), resulting in incomplete opening of the helix. Relocalisation of RPA to locally induced UV damage in these cells is nevertheless efficient (M. Volker, unpublished observation) suggesting that full opening around the lesion is not required to allow incorporation of RPA into the NER complex. As mentioned above, RPA can position and stimulate incision by XPG when RPA is pre-bound to a DNA substrate (de Laat et al., 1998b), suggesting that RPA is required to be present in the NER complex before XPG arrives. It has recently been observed that incomplete helix opening in TTD-A cells precludes XPA from being incorporated into the NER complex *in vivo* (Coin et al., 2006); it will thus be interesting to observe the behaviour of RPA following local UV irradiation in TTD-A cells.

Taken together, most data seem to indicate that the small stretches of ssDNA created by XPC-hHR23B and (even not fully functional) TFIIH and/or its weak protein-protein interactions with TFIIH are sufficient for RPA to join the NER complex. This is supported by the observation that following local UV irradiation, RPA efficiently relocalises to the subnuclear UV-damaged regions even in the absence of XPA or XPG (chapter 8). Given these observations and given the role RPA has in governing the endonucleases, it is conceivable that RPA is the first protein following TFIIH to be recruited to the NER complex.

XPA first?

Based on *in vitro* experiments in which (i) XPA was necessary to allow binding of both RPA and XPG to the complex, and (ii) incorporation of RPA stimulated binding of XPG, Riedl and co-workers suggested the order XPA → RPA → XPG (Riedl et al., 2003). However, as mentioned above and shown in chapters 6 and 8, local UV irradiation experiments using XP cells show recruitment of XPG and RPA to the NER complex to be independent of XPA, indicating that XPA incorporation into the complex is at least no prerequisite for the recruitment of RPA and

XPG and might not even precede one or both proteins. Conversely, XPG is dispensable for the incorporation of XPA into the NER complex (chapter 6), although it is possible that a truncated fragment of XPG present in the XP-G/CS cells used in these experiments is responsible for the observed recruitment of XPA.

No fixed order of binding of XPG, RPA and XPA?

Maybe the most satisfactory explanation for the inconsistent findings in the order of binding of XPG, RPA and XPA to the NER complex is that these factors bind in no specific order, and the idea of a fixed and strictly sequential order of binding in this phase of complex formation is incorrect. Instead, binding of any one of these factors to the complex could facilitate binding of the others by enforcing certain conformational changes (Riedl et al., 2003; Tapias et al., 2004) as well as through protein-protein interactions. The abovementioned findings from the group of Naegeli also indicate that XPA and RPA fulfil complementary roles in damage verification, without a specific requirement for either XPA or RPA to be present in the complex before the other protein (Missura et al., 2001).

In a recent publication, Politi and co-workers show that in a simple model 'disordered' binding of two factors at the second or third step of NER results in somewhat faster repair than strict sequential assembly (Politi et al., 2005). Although the authors do not describe models in which more than two factors bind in such way, it is conceivable that a similar disordered binding of three factors during one step would result in a similarly enhanced repair rate and thus present an advantage to the cell.

3.9 ERCC1-XPF

The second NER endonuclease consists of ERCC1 (33 kDa) and XPF (103 kDa), which mutually stabilise each other to form a heterodimer. ERCC1-XPF has a structural specificity complementary to that of its counterpart XPG, making a nick in ssDNA-to-dsDNA transitions at the 5' end of the ssDNA near the dsDNA (de Laat et al., 1998a; Sijbers et al., 1996). In contrast to XPG which cuts close to the lesion at the 3' side, ERCC1-XPF cuts further away (15-24 nt) at the 5' side of the lesion (Matsunaga et al., 1995; Sijbers et al., 1996). Although the XPF subunit contains the active nuclease domain (Enzlin and Scharer, 2002), the presence of ERCC1 is indispensable for nuclease activity. Additionally, the middle domain of ERCC1 interacts with XPA (Bessho et al., 1997; Li et al., 1995) and it was recently proposed that ERCC1 is also responsible for the interactions with DNA (Tripsianes et al., 2005).

Incorporation of ERCC1-XPF into the NER complex, allowing it to perform incision, depends on functional XPA (Li et al., 1995 and chapter 6) and the physical presence but not the nuclease activity of XPG (Constantinou et al., 1999; Mu et al., 1997; Wakasugi et al., 1997). While according to some reports, XPG in contrast is still capable of making an incision in the absence of ERCC1-XPF, giving rise to an uncoupled 3' incision (Evans et al., 1997b; Mu et al., 1996), a requirement for nuclease-active ERCC1-XPF in order for XPG to be able to incise has also been reported (Tapias et al., 2004). It has been shown that in the case of dual incision the cut by XPG is made prior to the cut by ERCC1-XPF (Evans et al., 1997b; Mu et al., 1996). All *in vitro* and *in vivo* evidence indicates that ERCC1-XPF is the final factor to be included in the NER complex before dual incisions are made.

Although uncommon, XP-F patients do exist; in contrast until recently no patients were known carrying mutations in ERCC1. When the first ERCC1 patient was reported he showed a dramatic phenotype lacking growth and development and died at the age of 13 months (N.G.J. Jaspers, manuscript in preparation). These observations correlate well with the phenotype of ERCC1^{-/-} mice, which show a more severe phenotype (including neonatal lethality) than other NER-deficient mice (Friedberg and Meira, 2006; McWhir et al., 1993; Weeda et al., 1997) indicating that ERCC1 and XPF have one or more important functions outside NER.

ERCC1-XPF is known to be involved in several other repair pathways apart from NER, but the details of these roles remains to be elucidated. Relatively well-characterised is the involvement of ERCC1-XPF in the repair of interstrand crosslinks (ICL), for which it is essential (Busch et al., 1997; Kuraoka et al., 2000; Niedernhofer et al., 2004). Additionally, ERCC1-XPF is implicated in homologous recombination. In *S. cerevisiae*, mutant *rad10* or *rad1* (the homologs of ERCC1 and XPF, respectively) interferes with mitotic recombination (Aguilera and Klein, 1989; Klein, 1988; Prado and Aguilera, 1995; Schiestl and Prakash, 1988; Schiestl and Prakash, 1990; Zehfus et al., 1990). Since the effect of *rad10-rad1* double mutants is not more severe than that of single mutants it is very likely that also in this process ERCC1-XPF acts as a dimer, i.e. a structure-specific endonuclease. Also in mammalian cells, ERCC1 mutations result in a defect in the resolution of intermediates of homologous recombination (Sargent et al., 2000; Sargent et al., 1997). Furthermore, ERCC1-XPF is required for targeted gene replacement in mouse embryonic stem cells (Niedernhofer et al., 2001), and it is involved in telomere maintenance, removing the 3' overhang from uncapped telomeres (Zhu et al., 2003).

The combination of an inability to deal with intermediates in ICL repair and in HR during mitosis and defective telomere maintenance is likely to explain why ERCC1 and XPF null mice and patients display a much more severe phenotype than 'pure NER' knockouts or XP patients.

3.10 DNA resynthesis and ligation

After dual incision has taken place and the damaged oligo is removed, DNA resynthesis can commence, followed by ligation. In *in vitro* repair reactions the proteins involved in DNA replication during S-phase are capable of adequately carrying out these steps (Aboussekhra et al., 1995; Araujo et al., 2000; Shivji et al., 1995); therefore, it is generally assumed that they occur in a way and order similar to DNA replication.

As an early step PCNA is recruited, presumably shortly after or together with RF-C, because RF-C is the protein that loads PCNA onto DNA. This ATP-dependent loading occurs specifically at ds- to ssDNA transitions with a 3'-OH end, a structure characteristically formed after 5' incision by ERCC1-XPF and oligomer removal. In XP cells that cannot execute dual incision, PCNA does not associate with chromatin – i.e., does not participate in NER – after UV (Aboussekhra and Wood, 1995; Miura et al., 1992; Miura et al., 1996), an observation that is consistent with the idea that primed DNA resulting from dual incision is essential for PCNA to bind to DNA. The homotrimeric ring-shaped PCNA encircles DNA and acts as a clamp that slides along DNA. PCNA interacts with the replicative DNA polymerases δ and ϵ (reviewed in Maga and Hubscher, 2003) and enhances their processivity during replicative DNA synthesis (Maga and Hubscher, 1995; Podust and Hubscher, 1993; Prelich et al., 1987) by keeping them tethered to the DNA.

In *in vitro* NER systems, each of the DNA polymerases δ and ϵ can resynthesise the excised strand (Aboussekhra et al., 1995; Araujo et al., 2000; Shivji et al., 1995). Little research has been done as yet to determine whether there is discrimination between the two polymerases and if so, what the basis is for such discrimination. Both these polymerases have a high fidelity, making NER DNA resynthesis essentially error-free. Finally, *in vitro* experiments have shown that the remaining nick can be sealed by DNA ligase I (Aboussekhra et al., 1995; Araujo et al., 2000), which is also involved in ligation of the lagging-strand Okazaki fragments during normal cellular DNA replication. In chapter 10, *in vivo* research is presented addressing the involvement of DNA pol δ and pol ϵ and DNA ligase I in NER.