The XPC-hHR23B complex and nucleotide excision repair of DNA.

Dawn Patricia Batty.


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Abstract of thesis.

In human cells, nucleotide excision repair (NER) removes DNA damage caused by UV light and acts on a variety of other helix-distorting adducts. Xeroderma pigmentosum group C (XP-C) cells are deficient in NER and are complemented by a 125 kDa polypeptide (XPC) which is found complexed with a yeast Rad23 homologue, hHR23B. XPC (p125) and XPC-hHR23B complex were expressed in insect cells, hHR23B was expressed in E. coli and all were purified. Both XPC and XPC-hHR23B were active in complementing XPC-deficient cell extracts. In a reconstituted system using other purified NER factors, XPC alone possessed only weak repair activity and hHR23B gave a 10-fold stimulation.

An electrophoretic mobility-shift assay was set up in order to obtain the first quantitative measurements of the preference of XPC complex for damaged DNA. XPC-hHR23B had a 400-fold preference for binding UV damaged DNA over non damaged, a discrimination similar to that determined here in parallel for the UV damaged DNA binding factor, UV-DDB. Binding of XPC-hHR23B to UV damaged DNA showed a marked temperature-dependence. Association was detectable within seconds even at 0° C. Once bound, the damaged DNA-XPC-hHR23B complexes were stable and dissociation was slow. At least 50 % of the complexes were detectable 6 h after challenge with excess UV-damaged DNA at 30° C. XPC-hHR23B had a high affinity for (6-4) photoproducts as damage binding was little affected by enzymatic photoreactivation of the UV-irradiated probe. Mobility-shift competition assays showed that XPC-hHR23B had some affinity for DNA treated with cisplatin and alkylating agents. Both non and UV damaged single-stranded M13 were extremely efficient competitors of XPC-hHR23B binding. Synthetic single-stranded homopolymer DNA was a relatively poor competitor. This suggests that XPC-hHR23B interacts with structures formed within single-stranded or damaged DNA of mixed sequence. XPC-hHR23B may act as a major damage recognition factor in human NER.
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<table>
<thead>
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<th>Description</th>
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<tr>
<td>1,3-GTG</td>
<td>1,3-intrastrand d(GpTpG)-cisplatin crosslink</td>
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<td>1,2-GG</td>
<td>1,2-intrastrand d(GpG)-cisplatin crosslink</td>
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<tr>
<td>aa</td>
<td>amino acid</td>
</tr>
<tr>
<td>AEBSF</td>
<td>[4-(2-aminoethyl)benzenesulfonylfluoride, HCl]</td>
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<tr>
<td>AP</td>
<td>apurinic/apyrimidinic</td>
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<td>ATP</td>
<td>adenosine 5'-triphosphate</td>
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<tr>
<td>BER</td>
<td>base excision repair</td>
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<tr>
<td>bp(s)</td>
<td>base pair(s)</td>
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<td>BSA</td>
<td>bovine serum albumin</td>
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<td>cisplatin</td>
<td>cis-dichlorodiammineplatinum (II)</td>
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<tr>
<td>ds</td>
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<td>dithiothreitol</td>
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<td>EDTA</td>
<td>ethylenediaminetetraacetic acid</td>
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<td>ERCC</td>
<td>excision repair cross complementing</td>
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<td>ethidium bromide</td>
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<td>FPLC</td>
<td>fast protein liquid chromatography</td>
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<tr>
<td>FT</td>
<td>(column) flow through</td>
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<td>HEPES</td>
<td>N-2-hydroxyethylpiperazine-N-2-ethanesulphonic acid</td>
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<td>hHR23A/B</td>
<td>human homologue of Rad23 A/B</td>
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<td>High5</td>
<td><em>Trichoplusia</em> NI insect cell line</td>
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<td>IPTG</td>
<td>isopropyl β-D-thiogalactopyranoside</td>
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<tr>
<td>kDa</td>
<td>kilodalton</td>
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<td>$k_D$</td>
<td>equilibrium binding constant</td>
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<td>Luria-Bertani</td>
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<tr>
<td>MMS</td>
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<td>N-methyl-N-nitrosourea</td>
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<tr>
<td>NER</td>
<td>nucleotide excision repair</td>
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<tr>
<td>NP40</td>
<td>Nonidet-P40 (= Triton X-100)</td>
</tr>
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<td>nt(s)</td>
<td>nucleotide(s)</td>
</tr>
<tr>
<td>OD</td>
<td>optical density</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PBSA</td>
<td>phosphate buffered saline A</td>
</tr>
<tr>
<td>PCNA</td>
<td>proliferating cell nuclear antigen</td>
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<td>polymerase chain reaction</td>
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<td>packed cell volume</td>
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<td>PMSF</td>
<td>phenylmethylsulfonylfluoride</td>
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<tr>
<td>Pol</td>
<td>polymerase</td>
</tr>
<tr>
<td>RFC</td>
<td>replication factor C</td>
</tr>
<tr>
<td>RPA</td>
<td>replication protein A (single stranded binding protein)</td>
</tr>
<tr>
<td>rpm</td>
<td>revolutions per minute</td>
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<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
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<tr>
<td>Sf9</td>
<td><em>Spodoptera frugiperda</em> insect cell line</td>
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<tr>
<td>ss</td>
<td>single stranded</td>
</tr>
<tr>
<td>TBE</td>
<td>tris-borate EDTA buffer</td>
</tr>
<tr>
<td>TE</td>
<td>Tris-HCl EDTA buffer</td>
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<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
<tr>
<td>UV-DDB</td>
<td>UV-damaged DNA binding protein</td>
</tr>
<tr>
<td>wcE</td>
<td>whole cell extract</td>
</tr>
<tr>
<td>XP</td>
<td>xeroderma pigmentosum</td>
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Large amounts of rXPC have repair activity without rhHR23B

Binding of XPC-hHR23B to non and UV damaged DNA

Apparent $k_D$ for XPC-hHR23B binding to 2.5 kJ/m² UV damaged duplex DNA at 0°C

Apparent $k_D$ for XPC-hHR23B binding to 2.5 kJ/m² UV damaged duplex DNA at 37°C

Association of 50 ng XPC-hHR23B to 2.5 kJ/m² UV damaged duplex DNA at 0°C

Association of 50 ng XPC-hHR23B to 2.5 kJ/m² UV damaged duplex DNA at 30°C

Association of 50 ng XPC-hHR23B to 2.5 kJ/m² UV damaged duplex DNA at 30°C

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Association of 50 ng XPC-hHR23B to 2.5 kJ/m² UV damaged duplex DNA at 30°C

Association of 50 ng XPC-hHR23B to 2.5 kJ/m² UV damaged duplex DNA at 37°C

Association of 50 ng XPC-hHR23B to 2.5 kJ/m² UV damaged duplex DNA at 37°C

Association of 50 ng XPC-hHR23B to 2.5 kJ/m² UV damaged duplex DNA at 37°C

Association of 50 ng XPC-hHR23B to 2.5 kJ/m² UV damaged duplex DNA at 37°C

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Association of 50 ng XPC-hHR23B to 2.5 kJ/m² UV damaged duplex DNA at 37°C

 Association of 50 ng XPC-hHR23B to 2.5 kJ/m² UV damaged duplex DNA at 37°C

Comparison of damaged DNA binding activities of XPC-hHR23B and UV-DDB

Affinity of XPC-hHR23B for non damaged and UV damaged DNA

Affinity of XPC-hHR23B for non damaged and UV damaged DNA

Affinity of UV-DDB binding to 2.5 kJ/m² UV damaged duplex DNA at 30°C

Affinity of UV-DDB for non and UV damaged DNA

Independent binding of XPC-hHR23B and UV-DDB to UV damaged DNA

High affinity of XPC-hHR23B and UV-DDB for (6-4) photoproducts

Affinity of XPC-hHR23B and UV-DDB for (6-4) photoproducts

Affinity of XPC-hHR23B and UV-DDB for platinated DNA

Affinity of XPC-hHR23B for MMS and MNU damaged DNA

Affinity of UV-DDB for MMS and MNU damaged DNA

Affinity of XPC-hHR23B and UV-DDB for heat-denatured ss DNA and poly (dI*diC)

Affinity of XPC-hHR23B for non and UV damaged ss M13 DNA

Affinity of UV-DDB for non and UV damaged ss M13 DNA
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Chapter 1. Introduction.

Since the beginning of life on earth, evolution has been dependent on the ability of populations of organisms to adapt in response to an ever-changing environment. This ability to change is partially because DNA replication has an inherent error rate, and also because DNA is not chemically inert and can be damaged by a wide range of physical and chemical agents both inside cells and from the environment. This damage can result in the accumulation of mutations, which in some cases may increase an organism's chances of survival in a given environment but usually are detrimental to life. Accumulation of mutations in somatic cells plays an important role in the development of cancer and aging. Mutations in germ-line cells may even give rise to hereditary disease. To overcome some of these deleterious effects a variety of DNA repair systems evolved early in evolution.

1.1. DNA damage and repair mechanisms

There are numerous endogenous and environmental agents that can react with DNA and produce a wide variety of lesions. Major DNA lesions are presented together with their corresponding repair pathways in mammalian cells in Table 1. Essential cellular processes such as DNA replication and recombination are inherently error-prone, to some degree, and can lead to formation of mismatches in the DNA sequence. Natural metabolic processes in cells lead to the production of reactive metabolites (e.g. oxygen radicals) which can lead to base alterations and even strand breaks in the DNA double helix.
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<th>Type of DNA lesion</th>
<th>Repair pathway</th>
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<tr>
<td><strong>Endogenous</strong></td>
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<tr>
<td>DNA synthesis (incorporation of incorrect or damaged base or slippage)</td>
<td>mismatches</td>
<td>mismatch repair</td>
</tr>
<tr>
<td>DNA recombination</td>
<td>mismatches</td>
<td>mismatch repair</td>
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<tr>
<td>Reactive metabolites</td>
<td>base alterations (oxidation and alkylation), loss of bases and strand breaks</td>
<td>base excision repair, recombinalional repair, end-joining</td>
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<tr>
<td>(e.g. oxygen radicals)</td>
<td></td>
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<tr>
<td><strong>Environmental</strong></td>
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<tr>
<td>UV light</td>
<td>cyclobutane pyrimidine dimers, (6-4) photoproducts</td>
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<td>Ionizing radiation</td>
<td>single and double-strand breaks, loss of bases, base alterations</td>
<td>recombinalional repair, end joining base excision repair</td>
</tr>
<tr>
<td>Cross-linking agents</td>
<td>intra- and inter-strand crosslinks</td>
<td>recombinalional repair, nucleotide excision repair</td>
</tr>
<tr>
<td>(e.g. psoralen, cisplatin, mitomycin C)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alkylating agents</td>
<td>alkylated bases</td>
<td>DNA-alkyltransferases base excision repair nucleotide excision repair</td>
</tr>
<tr>
<td>Aromatic compounds</td>
<td>bulky adducts</td>
<td>nucleotide excision repair</td>
</tr>
</tbody>
</table>

Table 1. Major DNA lesions and repair pathways in mammalian cells (adapted from Friedberg *et al*, 1995).
1.1.1. UV damage

An important environmental genotoxic agent is the UV component of sunlight. The UV radiation spectrum has been sub-divided into three wavelength bands; UV-A (400 to 320 nm), UV-B (320 to 290 nm) and UV-C (290-100 nm). UV-C light causes the most damage to DNA as it contains wavelengths at or near the DNA maximum absorption wavelength of ~260 nm. Fortunately, UV-C is only weakly penetrating and most is filtered out by the ozone layer. However, this and UV-B light still have the ability to generate many lesions in DNA and these mainly occur at adjacent pyrimidine sites. Two types of photoproduct account for about 95% of these lesions, they are the cyclobutane pyrimidine dimer (CPD) and the (6-4) photoproduct (Friedberg et al., 1995).

CPDs are formed when two adjacent pyrimidines in the same DNA strand, absorb UV energy and form two covalent bonds at C5-C5 and C6-C6 positions. This produces a four-membered ring structure resulting from saturation of the C5=C6 double bonds in both pyrimidines (Figure 1.1). Cyclobutane pyrimidine dimers account for about 75% of all lesions produced by UV-B or UV-C light (Mitchell and Nairn, 1989). Theoretically, CPDs can exist in several isomeric forms, but by far the major product formed in double-stranded B-DNA is the cis-syn isomer (Friedberg et al., 1995). In denatured or single-stranded DNA the trans-syn isomer is found to some extent and ss DNA also allows formation of dimers between non-adjacent pyrimidines (Nguyen and Minton, 1988; Taylor and Brockie, 1988). Recent evidence suggests that cis-syn CPDs can be accommodated within the B-DNA double helix without much distortion however the trans-syn isomer causes more structural disturbance, especially at its 5' side (Friedberg et al., 1995).

The second most common type of UV lesion is the (6-4) photoproduct approximately one of these are formed for every three CPDs (Mitchell and Nairn, 1989). (6-4) photoproducts are formed most readily at sites where thymine is adjacent to cytosine (T-C) (Lippke et al., 1981) but at high UV doses
 (>5 kJ/m²) they also form to lesser extents at C-T, T-T and C-C sites. These lesions introduce major distortion in the DNA structure by formation of a stable bond between the two pyrimidines (5'-C⁶-C⁴ 3'). Figure 1.1 shows the structure of a thymine-cytosine (6-4) photoproduct.

Figure 1.1. UV-induced DNA lesions: cyclobutane pyrimidine dimers and (6-4) photoproducts.

1.1.2. DNA repair mechanisms – DNA damage reversal

The simplest way to remove certain sorts of DNA damage is through direct reversal in a single-enzyme reaction. Photolyases revert UV-induced dimers in
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a light-dependent reaction called photoreactivation (Sancar, 1990). Photolyases specific for cyclobutane pyrimidine dimers are widespread in both prokaryotes and eukaryotes including bacteria, fungi, fish, reptiles, amphibians and marsupials (Friedberg et al., 1995). However, they have not yet been found in placental mammals (Li et al., 1993; Yasui et al., 1994). Photolyases can bind to CPDs in the dark and are activated by absorbing a photon via intrinsic chromophores, when subjected to blue or near-UV light. The activated enzyme can then initiate the splitting of a dimer by electron transfer and thus restore the pyrimidines to their monomeric form. The enzyme is not inactivated by this process and can subsequently be released from DNA. Photolyases specific for (6-4) photoproducts have been found in some species including; Drosophila, Xenopus and plants (Todo et al., 1993). Recently, two genes with homology to photolyases have been identified in mammals but these have not yet been shown to have photoreactivation activity (van der Spek et al., 1996b; Yasui and McCready, 1998) and instead encode cryptochromes involved in regulation of the circadian clock (Griffin et al., 1999).

Direct damage reversal is also involved in the repair of alkyl damage in DNA and this process uses a class of enzymes called alkyltransferases. These proteins transfer the alkyl group from an alkylated base to an internal cysteine residue resulting in their own inactivation (Teo et al., 1984).

1.1.3. DNA repair mechanisms – double-strand break repair

The repair of double strand breaks (DSBs) in DNA is a more complex process and some of the mechanisms for this are outlined in figure 1.2. Strand breaks can occur during natural processes such as V(D)J-recombination and meiosis but also through exposure to environmental agents such as ionizing radiation and some chemicals. DSBs can be restored by end-joining of non-homologous ends using Ku80-Ku70 and other factors, but this is an error-prone pathway (Kanaar and Hoeijmakers, 1997). An alternative is to use homology-dependent
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Joining and ligation using: Ku80-Ku70, DNA-PK, ligase IV, XRCC4

5' to 3' degradation using: Rad50, Mre11, Xrs2

Homologous pairing, using: Rad51, 54, 55, 57, RPA

Branch migration and repair synthesis

Resolution of crossovers

Ligation

Rad52 recognition of DNA ends

DSB

DNA end-joining

5' to 3' degradation using: Rad50, Mre11, Xrs2

Figure 1.2. Model for double-strand break repair in yeast. In the case of homologous recombination, the homologous DNA molecule is indicated in grey. For the single-strand annealing pathway, grey areas represent repeats within the same DNA molecule. Filled arrows indicate new DNA synthesis.
recombination in the homologous recombination repair pathway or in single-strand annealing (SSA). These processes are initiated by a 5' to 3' exonuclease that generates long single-stranded 3' tails (White and Haber, 1990; Sugawara and Haber, 1992). In homologous recombination repair, one of these tails invades a homologous DNA duplex and the donor sequence can form a template for new DNA synthesis. The daughter strand can then anneal with another single-stranded tail on the other side of the DSB resulting in the formation of crossover points called Holliday junctions. Resolution of Holliday junctions restores the exact sequence into two resultant recombinant DNA molecules and so is an error-free pathway.

In the SSA pathway, the 5' to 3' degradation continues until a short region of homology is revealed in the same DNA molecule (e.g. within repeats) (Sugawara and Haber, 1992). Annealing of the homologous regions results in the formation of an intermediate containing non-homologous 3' ends which are removed using the Rad1-Rad10 complex (Fishman-Lobell and Haber, 1992; Yao et al., 1997) in yeast along with other factors. Following this new DNA synthesis and ligation can occur. This is an error-prone pathway and results in a deletion of DNA sequence, and also on occasion, a translocation.

1.1.4. DNA repair mechanisms - postreplication repair

If the DNA replication machinery encounters a damaged site, DNA synthesis can restart downstream of the lesion, leaving a gap. These single-stranded gaps opposite lesions in the newly replicated DNA strand can be filled in by postreplication repair, also known as daughter strand gap repair. This process involves homologous pairing and strand exchange with the undamaged sister DNA molecule (Friedberg et al., 1995). The functions of proteins involved in this process is, as yet, not well understood.
An alternative way to bypass a lesion is translesion synthesis, which is intrinsically less accurate. A polymerase involved in this process has recently been identified as defective in xeroderma pigmentosum group V (variant) cells (Masutani et al., 1999).

1.1.5. DNA repair mechanisms - mismatch repair

This process corrects base misincorporation errors after DNA synthesis and also acts on mispaired bases that can occur in recombination intermediates. A summary of the process in *E. coli* is presented in figure 1.3. Here, the mismatch is located and bound using the MutS homodimer. This intermediate is then bound by a MutH dimer which is subsequently activated by the binding of MutL, leading to single-strand incision either 5' or 3' to the damaged site (Grilley et al., 1990; Au et al., 1992). Repair is initiated only on the newly synthesized daughter strand as this is temporarily unmethylated. Subsequent excision initiates at the nick and proceeds towards the mismatch using the concerted action of MutS, MutL, the UvrD helicase and an exonuclease activity (Grilley et al., 1993). The reaction is completed by resynthesis over several hundred bases and ligation of the DNA backbone involving DNA polymerase III and possibly single-stranded binding protein (SSB). The DNA backbone is sealed by DNA ligase. A similar process occurs in human cells but the mechanism of discrimination of the template strand is as yet unknown. Mutations in the human genes encoding mismatch repair proteins are associated with hereditary non-polyposis colorectal cancer (HNPCC) (Fishel, 1994; Modrich, 1994).

1.1.6. DNA repair mechanisms - base excision repair

The process of base excision repair (BER) corrects minor base alterations that cause small structural distortions in the DNA duplex structure. The type of
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Figure 1.3. Model for mismatch repair in E. coli.
lesions acted on include those formed by endogenous oxidative events, ionizing radiation and simple alkylating agents. Examples of frequently induced and often highly mutagenic BER lesions are 8-oxoguanine, O6-methylguanine, uracil and thymine glycol (Beckman and Ames, 1997).

During BER in mammalian cells, the damaged base is released from the DNA backbone by a DNA glycosylase (Krokan et al., 1997). An AP endonuclease nicks 5' of the resultant apurinic/apyrimidinic (AP) site leaving a 5' deoxyribose residue. This is removed by DNA polymerase β, which has intrinsic AP lyase activity. Incorporation of the single correct nucleotide is also accomplished by DNA polymerase β (Mullen and Wilson, 1997) and this is followed by ligation, involving DNA ligase III and XRCC1 (Friedberg et al., 1995). A model for this “short patch” BER pathway is outlined in figure 1.4.

Occasionally, longer repair patches of 2-10 nucleotides are observed. This is likely to result from strand displacement in the 5' to 3' direction by DNA polymerase β or δ in the presence of PCNA. This generates a “flap” structure that is cleaved off by the structure-specific endonuclease, FEN-1 to allow ligation to occur. This is referred to as “long patch” base excision repair (Klungland and Lindahl, 1997). There appear to be no human BER-deficient syndromes, which may reflect the fact that disruptions of many of the genes involved lead to embryonic lethality in mouse models.

1.7. DNA repair mechanisms - nucleotide excision repair

Another excision repair process is nucleotide excision repair (NER). The major difference between NER and BER is that BER acts to remove a single, damaged residue after releasing the free base, while NER removes the damaged site as part of an oligonucleotide. NER will be discussed in more detail in section 1.2.
Figure 1.4. Model for short patch base excision repair in mammalian cells. A. A glycosylase catalyzes hydrolysis of the N-glycosyl bond to release the damaged base (represented here as an 8-oxo G residue) from DNA leaving an AP site. B. An AP endonuclease cleaves the DNA 5' to the AP site. C. AP lyase activity in Pol β removes deoxyribose (dRp) residue and incorporates the correct nucleotide. D. DNA ligase III (and XRCC1) seals the gap. E. Repaired duplex DNA.
as the XPC-hHR23B protein complex and its involvement in NER are the main subjects of this thesis.

### 1.1.8. An alternative excision repair pathway for UV lesions

In most organisms NER is thought to be the most important pathway for the removal of UV-induced DNA damage. In addition to NER, in some species, photoreactivation and BER are also involved in the repair of UV damage. For example, CPD-specific glycosylases have been identified in bacteriophage T4 and the bacterium *Micrococcus luteus* (Dodson et al., 1994). An alternative nucleotide excision repair pathway for UV lesions has recently been identified in *Schizosaccharomyces pombe* (and in the filamentous fungi *Neurospora crassa*) (Doetsch et al., 1995; Freyer et al., 1995; Yajima et al., 1995).

This process involves the use of a new type of nicking activity, called the *S. pombe* DNA endonuclease which makes an incision immediately 5' to the dimer (Bowman et al., 1994; Freyer et al., 1995). Following incision, either a 3' endonuclease or a 5' to 3' exonuclease (perhaps a FEN-1 homologue) may continue the repair reaction. Subsequent DNA synthesis and ligation may involve proteins used in other repair systems (Murray et al., 1994).

### 1.2. Nucleotide excision repair in *E. coli*

*E. coli* was the original model system for the study of nucleotide excision repair and I will later compare its mechanism to what is now known about the human NER system. The *E. coli* Uvr system consists of at least six structural genes (*uvrA, uvrB, uvrC, uvrD, polA* and *lig*). These genes have been characterized and
their products purified to homogeneity giving reagents that have been used in the biochemical reconstitution of partial and overall NER reactions.

As with NER studied in all organisms to date, the UvrABC system recognizes and eliminates a wide variety of DNA lesions including UV induced photoproducts (cyclobutane pyrimidine dimers and (6-4) photoproducts), AP (apurinic and apyrimidinic) sites (Lin and Sancar, 1989), bulky chemical adducts (alkylation damage, benzo(a)pyrene and AAF adducts) and damage formed by the action of crosslinking agents such as cis and trans platinum (Beck et al., 1985), psoralen and mitomycin C (Sancar and Rupp, 1983; Van Houten et al., 1986; Pu et al., 1989). In this section I will focus on the proteins involved in damage recognition in NER in both *E. coli* and human cells.

The overall process is as follows. A dimer of UvrA (Oh and Grossman, 1989; Mazur and Grossman, 1991) binds UvrB and loads onto the DNA (Orren and Sancar, 1989). The 5'-3' helicase activities of this complex allow limited ATP-dependent scanning for damaged bases. On encountering damage, UvrA2 dissociates to leave a highly stable preincision complex (Oh and Grossman, 1986) consisting of UvrB bound to DNA (Orren and Sancar, 1990; Visse et al., 1992). This is recognized and becomes bound by UvrC (Orren and Sancar, 1989). In the UvrBC complex, incisions are made 4-5 phosphodiester bonds 3' to the lesion and then at the 8th phosphodiester bond 5' to the lesion (Lin and Sancar, 1992). Binding or hydrolysis of ATP are important in most steps of the damage recognition and dual incision reaction (Husain et al., 1986; Oh and Grossman, 1989; Myles et al., 1991). UvrD then loads onto the free 3' end of the damaged oligonucleotide and displaces it using its intrinsic 3'-5' helicase activity. The gap is filled by DNA Pol I and sealed by *E.coli* DNA ligase. A possible model for this process is presented in Figure 1.5.
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Figure 1.5. Model for nucleotide excision repair in E. coli.
1.2.1. Properties of Uvr proteins involved in the damage recognition and incision reaction

Uvr

The 107 kDa UvrA protein is the only protein that by itself has a known catalytic activity, behaving as a weak ATPase/GTPase (Oh and Grossman, 1989). Several known motifs are present in the UvrA protein including 2 ATP binding sites, 2 C4 type Zn fingers, a helix-turn-helix motif and a polyglycine hinge region. Some of these motifs are involved in DNA binding and damage discrimination. Mutagenesis studies have shown that the polyhinge region in the C-terminal 44 amino acids of the protein is important for stabilization of UvrA binding at a damaged site (Claassen and Grossman, 1991). It contains a high number of glycine and proline residues - amino acids that allow a high degree of flexibility. It has been suggested that it is this region of UvrA that allows accommodation of the many types of damage eliminated by the UvrABC system - by a flexible "induced fit" mechanism. UvrA alone has a relatively low specificity for damaged DNA and appears to have a role in translocation and delivery of UvrB to a damaged site - after which it may dissociate from the complex (Orren and Sancar, 1990; Visse et al., 1992).

In contrast, the 76 kDa UvrB protein neither binds DNA by itself nor has any known catalytic activity, but when complexed with UvrA it acts as a DNA-dependent ATPase-helicase (5'-3'). This could provide the energy required to translocate along the DNA during the process of scanning for damage (Seeley and Grossman, 1989; Seeley and Grossman, 1990). The delivery of UvrB to a damaged site is dependent on its association with UvrA as it contains no intrinsic DNA binding activity (Yeung et al., 1986).

The 67.5 kDa UvrC protein binds to single-stranded DNA with high affinity, a property that has been exploited during its purification. When present at high concentrations, it shows a weak non-specific nuclease activity (Caron and Grossman, 1988). However, if added to the specific UvrB-DNA complex, the characteristic incisions occur at the 4-5th phosphodiester bond 3' and 8th
phosphodiester bond 5' to the lesion (Orren and Sancar, 1990; Thiagalingam and Grossman, 1991). Studies using mutant UvrB and UvrC proteins indicate that the UvrB subunit is important for the 3' incision whereas residues involved in the 5' incision reside in UvrC (Lin and Sancar, 1992). The association of UvrC with the UvrB-DNA complex activates both the 5' and 3' nuclease activities.

1.2.2. Incision intermediates and reaction mechanism

In the early 1990's, structural intermediates formed during repair by the UvrABC system were visualized directly using electron microscopy (Shi et al., 1992). Some kinking of the DNA was observed when bound by the UvrA2B complex but this was variable. However, when these complexes were treated with a high concentration of KCl (to remove UvrA) it was seen that the DNA in the UvrB-DNA complex is severely kinked – by approximately 130° (Shi et al., 1992). This result suggested that the propensity of DNA to unwind and bend at the site of a lesion is as important as the actual conformational change caused by the lesion itself.

Not all studies have supported the existence of unpaired DNA as an intermediate in UvrABC action (Gordienko and Rupp, 1997). However, there is now increasing evidence that an open intermediate does occur. Visse et al investigated the binding and incision of a specific cis Pt-GG adduct by UvrABC, using a combination of DNase I and MPE-Fe (II) hydroxyl radical footprinting (Visse et al., 1991). In agreement with earlier footprinting on specific psoralen and 6-O-methylguanine substrates, UvrAB binding resulted in enhanced DNase I cleavage sites 5' to the lesion. These enhanced cleavage sites were at different positions for the different types of damage studied, however the 5' incision remained constant – at the 8th phosphodiester bond 5' to the lesion. These incisions were found to be dependent on ATP hydrolysis which is required for the formation of a preincision complex. These preincision complexes can have slightly different structures depending on the type of damage encountered. The
MPE-Fe (II) footprinting revealed enhanced cleavage 5' and 3' to the lesion, indicating a relatively unwound or open structure. The area of enhanced cleavage occupies about 1 helical turn, which is similar to the unwinding induced on UvrAB binding and extends to the 5' and 3' incision sites.

These studies were extended using KMnO₄ footprinting (Visse et al., 1994). A slight increase in sensitivity was observed on preincision complex formation, but on addition of UvrC the sensitivity on the non-damaged strand close to the 3' incision site was increased dramatically, indicating this region may become single stranded after incision. This suggests the 3' end of the damaged oligonucleotide is no longer base paired as tension in the kinked DNA structure is released following the 3' incision. As UvrD has an affinity for single-stranded DNA this region could be available as an entry site for UvrD, which with its 3' to 5' helicase activity could then displace the damaged oligonucleotide.

Zou et al. investigated the structural requirements and intermediates formed during repair using various bubble and Y-shaped substrates with and without a single benzo(a)pyrene diol epoxide (BPDE) adduct (Zou et al., 1997). A substrate containing a non-complementary 11 bp bubble region was bound efficiently by UvrA, but importantly this was only incised by UvrBC if it also contained an adduct. If the Y substrate contained a pre-existing 3' nick, UvrBC weakly made a 5' incision, but this was much more efficient in the presence of an adduct. A recent study (Zou and Van Houten, 1999) further investigated the structural intermediates formed by UvrABC, using a BPDE-adduct. An unpaired region of 3-6 bp was sufficient for the formation of the 3' incision by UvrBC but a more open structure of 10-12 bp was required for the 5' incision to occur. This implies that the 5' incision is triggered by the 3' incision itself or by the presence of an extended unpaired region. These studies substantiate the idea of a bipartite damage recognition step in NER in E. coli, where both a conformational change and modification of DNA bases are prerequisite for efficient discrimination between damaged and non damaged bases and their subsequent elimination.
1.2.3. NER in human cells

In recent years the overall mechanism of NER in humans has been elucidated. This has been done in parallel with extensive studies of NER in the yeast S. cerevisiae (Friedberg et al., 1995). NER in human cells is the main defense against the genotoxic effects of UV light but also acts on a wide variety of bulky, helix distorting lesions (Sancar, 1996; Wood, 1997), and to a lesser extent, more subtle modifications such as AP sites (Sancar, 1996). It has been shown that the rate of repair of these different lesions varies over several orders of magnitude and this may reflect the efficiency with which NER recognizes a particular adduct. It appears that the more a lesion distorts the normal DNA structure, the more efficiently it is recognized and this is reflected in its overall repair rate (Wood, 1999). For example, (6-4) photoproducts distort the DNA more than cyclobutane pyrimidine dimers (Kim et al., 1995) and are removed 5-10 times faster from the bulk of the genome (Mitchell and Nairn, 1989).

Humans that are deficient in this process suffer a rare, recessively inherited disorder, xeroderma pigmentosum (XP) (Cleaver and Kraemer, 1995). This is characterized by the early onset of severe photosensitivity, a high incidence of skin cancers and in some cases, associated neurological abnormalities. Cells derived from XP patients have been used in cell fusion studies and this resulted in the identification of seven complementation groups, XP-A through XP-G, each group reflecting a defect in a single protein required for the incision stage of NER. Table 2 shows a summary of human NER factors and their homologues in yeast.

In vitro reconstitution of the NER process has defined six core factors, some of which have multiple subunits, that are required for the repair of most damage in DNA (Aboussekhra et al., 1995; Guzder et al., 1995; Bessho et al., 1997). These proteins are XPA, the RPA heterotrimer, the XPC-hHR23B complex, the 6-9 subunit TFIIH complex and the two structure-specific endonucleases, XPG and the heterodimeric ERCC1-XPF.
<table>
<thead>
<tr>
<th>Human protein (complex)</th>
<th>S. cerevisiae homologue</th>
<th>Protein activity</th>
<th>Additional function?</th>
</tr>
</thead>
<tbody>
<tr>
<td>XPA</td>
<td>Rad14</td>
<td>DNA binding, damage recognition, binds to RPA, ERCC1 and TFIIH</td>
<td></td>
</tr>
<tr>
<td>RPA p70, p32, p14</td>
<td>RPA</td>
<td>ss DNA binding</td>
<td>DNA replication, recombination</td>
</tr>
<tr>
<td>XPC p125</td>
<td>Rad4</td>
<td>damage recognition</td>
<td></td>
</tr>
<tr>
<td>hHR23B p58, hHR23A</td>
<td>Rad23</td>
<td>ss DNA binding</td>
<td></td>
</tr>
<tr>
<td>UV-DDB p125, p48</td>
<td></td>
<td>damage recognition</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>ss DNA binding</td>
<td></td>
</tr>
<tr>
<td>TFIIH (core) XPB, XPD, p52, p62, p44, p34</td>
<td>Rad25, Rad3, Tfb2, Tfb1, Ssl1, Tfb4</td>
<td>3' to 5' helicase, 5' to 3' helicase</td>
<td>basal transcription</td>
</tr>
<tr>
<td>XPG</td>
<td>Rad2</td>
<td>3' structure-specific endonuclease</td>
<td></td>
</tr>
<tr>
<td>ERCC1</td>
<td>Rad10</td>
<td>5' structure-specific endonuclease</td>
<td>SSA recombination pathway</td>
</tr>
<tr>
<td>XPF</td>
<td>Rad1</td>
<td>3' structure-specific endonuclease</td>
<td></td>
</tr>
</tbody>
</table>

Table 2. Human NER factors and their homologues in S. cerevisiae.
Human NER involves the formation of an open, unwound intermediate, which spans a region of up to 24-32 bp around the lesion (see Figure 1.6). The formation of this open complex is ATP dependent and the earliest stages of opening absolutely require XPC-hHR23B and TFIIH - which contains two helicases of opposing polarities, XPB and XPD.

Full opening to give a structure that can be incised by XPG and ERCC1-XPF requires the presence of most of the core NER factors. XPA, RPA, XPC-hHR23B, TFIIH and XPG are involved, although the catalytic activity of XPG is not a requirement of this stage (Evans et al., 1997; Mu et al., 1997b; Wakasugi et al., 1997; Constantinou et al., 1999). Incision of the damaged strand on the 3' side of a lesion is made by XPG and usually occurs before 5' incision by ERCC1-XPF (O’Donovan et al., 1994; Mu et al., 1996). Dual incision results in the release of a damage-containing oligonucleotide 24-32 residues in length (Huang et al., 1992; Moggs et al., 1996). The gap is then filled by DNA polymerase δ/ε holoenzyme (Wood and Shivji, 1997) and sealed by a DNA ligase.

**1.2.4. Transcription-coupled repair**

Transcription-coupled repair a specialized mode of NER that removes DNA adducts faster from the transcribed strand of active genes than from the non-transcribed strand or the bulk of the genome (Bohr et al., 1985). It is believed that this process is initiated when RNA polymerase stalls at a lesion in the DNA (Hanawalt, 1994). This could serve as a damage recognition signal and somehow lead to the recruitment of other NER factors. A model for transcription-coupled repair in mammalian cells is shown in figure 1.7. Here, an actively transcribing RNA polymerase II holoenzyme stalls at a lesion in DNA causing further distortion and opening around the damaged site. The partially unwound site may be entered by TFIIH creating an open complex. RNA polymerase II retracts from the damaged site, possibly facilitated by the CSA and CSB factors. The TFIIH bound open complex then becomes bound by
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Damage recognition

5' [Image of damaged DNA] 3'

NER factors involved

XPA?, RPA?, XPC-hHR23B?

Open complex formation

5' [Image of open complex] 3'

XPC-hHR23B?, TFIH

Dual incisions

5' [Image of dual incisions] 3'

XPG, ERCC1-XPF

DNA repair synthesis

5' [Image of DNA repair synthesis] 3'

DNA Pol δ or ε, PCNA, RFC

Ligation of DNA backbone

5' [Image of ligation] 3'

DNA ligase

Figure 1.6. Overview of nucleotide excision repair (NER) in mammalian cells. ¶Represents a helix distorting lesion in DNA. NER factors involved in each stage are shown on the right.
XPG which makes an incision at the 3' side of the lesion. The transcription-coupled repair process can be completed by e.g. NER or BER depending on the type of lesion causing the transcription block. Humans deficient in transcription-coupled repair suffer a condition known as Cockaynes syndrome (CS) (Venema et al., 1990a; van Hoffen et al., 1993) which is associated with neurological and developmental problems. Classical CS is associated with mutations in the CSA or CSB genes (Troelstra et al., 1992; Henning et al., 1995). Some individuals suffer from a combined XP-CS syndrome and this is caused by mutations in the XPD, XPB or XPG genes (Vermeulen et al., 1993; Vermeulen et al., 1994a; Vermeulen et al., 1994b).

1.2.5. NER substrate characteristics

For a lesion to become a substrate for NER in human cells, it must cause both distortion of the DNA structure and a modification of the DNA chemistry. Naegeli et al termed this a 'bipartite' DNA damage recognition system (Hess et al., 1997b). For example, cyclobutane pyrimidine dimers are usually quite a weak NER substrate, but placement of non-complementary bases opposite the lesion increases the efficiency with which it is repaired - presumably by increasing the helical distortion (Mu et al., 1997a). Similarly, a 1,3 GTG cisplatin intrastrand crosslink is more distorting than the equivalent 1,2 GG crosslink (Bellon et al., 1991) and is repaired more efficiently. Placement of a non-complementary T residue opposite the 1,2 GG adduct increases the distortion and increases its repair efficiency up to the level of the 1,3 GTG cisplatin adduct (Moggs et al., 1997; Mu et al., 1997a). Distortion alone is not sufficient to create an NER substrate as DNA mismatches and small loops distort the DNA helix but are repaired very poorly, if at all (Hess et al., 1997a; Hess et al., 1997b; Moggs et al., 1997; Mu et al., 1997a). A modification of DNA chemistry in addition to mispairing is required to produce a good NER substrate.
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Figure 1.7. Model for transcription-coupled repair in mammalian cells. I. Helix-distorting lesion (▲) in DNA. II. Transcribing RNA polymerase II (pol II) stalls at the lesion causing further DNA distortion. III. TFIIH enters partially unwound site creating an open complex. Pol II retracts from damaged site possibly facilitated by CS factors. IV. TFIIH positions XPG which makes an incision 3' of the lesion. V. Different repair pathways can complete TCR depending on the type of lesion causing transcription block e.g. in the case of oxidative damage (left), BER may make the 5' incision. In the case of CPD's (right) XPA, RPA and ERCC1-XPF enter the damaged site and ERCC1-XPF makes the 5' incision. VI. DNA repair synthesis. VII. Re-initiation of RNA Pol II transcription.
1.2.6. **Putative damage recognition proteins in human NER: XPC-hHR23B**

Cells from patients with xeroderma pigmentosum are highly sensitive to the lethal and mutagenic effects of UV. Variations in the UV sensitivity found both within and between complementation groups generally reflect the level of residual repair. A notable exception to this is cells belonging to the complementation group C (XP-C), which are less sensitive than other XP groups to the lethal effects of UV (Tyrrell and Amaudruz, 1987), even though functional XPC protein is completely absent. Non-dividing XP-C cells exhibit a relatively high UV survival despite a residual repair capacity of only 10-15% of that seen in normal cells (Kantor and Hull, 1984). The XP-C complementing factor is the sole NER protein that is dispensable for transcription-coupled repair. The residual repair in XP-C cells is mostly confined to actively transcribed regions of the genome (Venema et al., 1990b; Venema et al., 1991) and this may explain the relatively high UV survival of this cell type.

The 106 kDa XPC protein copurifies with a tightly bound 43 kDa partner, hHR23B (Masutani et al., 1994) and the XPC-hHR23B complex has a high affinity for single stranded-DNA cellulose, a feature that has been exploited during its purification (Masutani et al., 1994; Shivji et al., 1994). XPC is a 940 residue protein and amino acids 114-311 and 503-940 show homology to the yeast NER factor Rad4 (Masutani et al., 1994). A region towards its C-terminus (776-800 aa) has been reported to contain the hHR23B interacting domain (Figure 1.8.) (Li et al., 1997). Its usual cellular partner, hHR23B is a 409 residue protein and is one of two human homologues of the yeast NER factor Rad23. Although XPC protein is quantitatively bound to hHR23B, hHR23B is much more abundant and the majority of this is found in free form *in vivo* (Sugasawa et al., 1996; van der Spek et al., 1996a).
Figure 1.8. Nucleotide and predicted amino acid sequence of XPC (pl25). Top numbers on the right are those of nucleotide residues and lower ones (in parentheses) are those of amino acids. Black boxed regions show homology to yeast Rad4 and the red boxed region indicates the hHR23B (and hHR23A) interacting domain of XPC.
This together with the presence of two forms of the human homologue of Rad23, suggest that these proteins may have multiple functions \textit{in vivo}.

Sugasawa \textit{et al} used a partially reconstituted NER system to look at the activity of XPC (p125) alone and together with hHR23B (Sugasawa \textit{et al.}, 1996). Here, a UV-irradiated plasmid was the substrate in repair synthesis assays and only weak repair was observed in the presence of XPC alone. With co-addition of hHR23B, repair was stimulated approximately 2-fold. Further work by the same group showed that hHR23A could stimulate XPC to a similar extent suggesting some level of functional redundancy of these proteins (Sugasawa \textit{et al.}, 1997). In support of this idea, XPC was shown to interact with both hHR23B and hHR23A \textit{in vivo}, using a yeast two-hybrid system (Li \textit{et al.}, 1997). In contrast to the above, Reardon \textit{et al} reported that XPC (p125) alone was sufficient in the repair of a T-T dimer or a substrate containing a cholesterol moiety in place of a base and inclusion of hHR23B had no effect (Reardon \textit{et al.}, 1996). In view of the uncertain and conflicting data on this subject, one of the goals of this thesis was to investigate the requirement for hHR23B in a fully reconstituted NER system using purified proteins.

hHR23B (and hHR23A) contains a ubiquitin-like (Ub-like) domain at its N-terminus, showing 30\% identity and about 59\% homology to ubiquitin (Masutani \textit{et al.}, 1994). In the yeast homologue, Rad23, this region is essential for its role in NER (Watkins \textit{et al.}, 1993). This protein also contains two ubiquitin-associated (UB-A) domains (amino acids 178-232 and 357-409) (Masutani \textit{et al.}, 1994) which in similar proteins are involved in ubiquitin metabolism (Figure 1.9.). Deletion studies identified the XPC-interacting domain to reside between residues 275 and 332 of hHR23B (Figure 1.10.) - which overlaps with the UB-A domain and a fragment consisting of this region is sufficient for the NER function of hHR23B \textit{in vitro} (Masutani \textit{et al.}, 1997). Therefore, the Ub-like sequence and the UB-A domains appear dispensable for the core NER reaction.
Chapter 1. Introduction.

Figure 1.9. Nucleotide and predicted amino acid sequence of hHR23B (p58). Top numbers on the right are those of nucleotide residues and lower ones (in parentheses) are those of amino acids. Boxed area I indicates the region of homology with ubiquitin, boxed areas II and III indicates the regions of homology with ubiquitin-associated domains (UBA’s).
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**Figure 1.10. Amino acid sequence alignment of hHR23B (p58) and hHR23A.** Numbers on the right are those of amino acids. Boxed/shaded areas indicate regions of identity and shaded areas indicate regions of similarity. Red boxed area indicates XPC interacting domain.
Unlike XPC-hHR23B, the homologous yeast Rad4-Rad23 complex is required for transcription-coupled repair as well as for repair of non-transcribed DNA. However, in contrast to mammalian cells the vast majority of the yeast genome is transcriptionally active (Holstege et al., 1998). There seems to be a fundamental, but not yet understood difference between the function of the yeast Rad4 and the mammalian XPC proteins. The yeast Rad7-Rad16 complex binds preferentially to UV damaged DNA (Guzder et al., 1997) and in yeast is only required for the repair of the genome as a whole (He et al., 1996; Wang et al., 1997). This has led to the suggestion that yeast Rad7-Rad16 is the “functional homologue” of mammalian XPC-hHR23B, but the biochemical basis for this apparent similarity in phenotype is unknown (Scott and Waters, 1997).

In transcription-coupled repair RNA polymerase stalls at a lesion in the DNA (Hanawalt, 1994) and signals the recruitment of other repair factors. In non-transcribed DNA, the XPC complex may carry out an analogous damage-detection role early in the NER process (Naegeli, 1995). Another clue to the role of XPC-hHR32B is that it along with TFIIH is required for the earliest detectable opening around a lesion - which could be diagnostic of the onset of NER (Evans et al., 1997). XPC complex has been found to co-purify with TFIIH through several chromatographic steps (Drapkin et al., 1994) and a similar interaction (dependent on Rad23) has been seen in yeast (Bardwell et al., 1994). These results suggest that TFIIH in combination with XPC complex may have a role in sensing the damaged strand as well as in opening during the NER process.

1.2.7. Putative damage recognition proteins in human NER: XPA

The XPA protein was the first human NER protein demonstrated to have a preference for binding to damaged DNA (Robins et al., 1991; Jones and Wood, 1993; Asahina et al., 1994). The yeast homologue of XPA, Rad14 has a similar preference (Guzder et al., 1993). The zinc finger containing region present in this protein is required for DNA binding (Tanaka et al., 1990; Kuraoka et al.,
1996; Buchko et al., 1998) and is essential for its function in NER. Recently, the structure of this domain was solved (Buchko et al., 1998; Ikegami et al., 1998). NMR spectra of XPA-DNA complexes suggest that DNA binds in a cleft lined with basic residues (Ikegami et al., 1998), which is large enough to accommodate either single or double-stranded DNA. DNA damaged by various agents including cisplatin and UV light is bound by XPA (Robins et al., 1991; Jones and Wood, 1993; Asahina et al., 1994; Wakasugi and Sancar, 1999). It appears that the affinity of XPA for a given lesion correlates with the extent of helical distortion associated with it. A damaged site may have some single-stranded character, and this was proposed as the main determinant for XPA recognition (Jones and Wood, 1993). A more recent analysis (Buschta-Hedayat et al., 1999) found that XPA binds with considerably higher efficiency to partially duplex DNA than to single-stranded substrates of the same length. Further, XPA appears to prefer binding to sites where base pairing is disrupted and may do so via hydrophobic interactions with aromatic base components that are abnormally exposed to the helical surface.

_In vitro_ studies have demonstrated that XPA interacts with a number of other NER proteins. The heterotrimeric single stranded binding protein RPA also shows some preferential binding to damaged DNA (Clugston et al., 1992; He et al., 1995; Burns et al., 1996; Wakasugi and Sancar, 1999) and its 70 kDa and 34 kDa subunits have been shown to interact with XPA (Li et al., 1995; Saijo et al., 1996). A co-complex of XPA and RPA has an increased affinity for damaged DNA (He et al., 1995; Wakasugi and Sancar, 1999) and the interaction between these two proteins is essential for NER (Li et al., 1995).

Association of XPA and RPA with the structure specific endonuclease ERCC1-XPF appears to increase the affinity for damaged sites (Nagai et al., 1995). XPA also contacts TFIIH (Park et al., 1995; Nocentini et al., 1997) and RPA interacts with both XPG and ERCC1-XPF nucleases (He et al., 1995; Bessho et al., 1997; de Laat et al., 1998). At any rate, XPA plays a critical DNA and protein binding role as it forms an integral part of the open preincision complex (PIC) and has a central role in NER. In its absence, cells have absolutely no capacity for NER.
and no incisions are made (Evans et al., 1997b). XPA and RPA may have a role in positioning the other repair factors relative to DNA and conferring strand specificity to the nucleases (de Laat et al., 1998).

1.2.8. **Putative damage recognition proteins in human NER: UV-DDB**

Human and monkey cells contain another binding activity that has a relatively high specificity for DNA damaged by UV (Feldberg and Grossman, 1976) and also recognizes DNA damaged by cisplatin (Chu and Chang, 1988), nitrogen mustard, denaturation and depurination (Payne and Chu, 1994). In fact it is the only damage-binding activity strong enough to be detected in crude extracts of mammalian cells. This UV-damaged DNA binding (UV-DDB) activity is absent in a subset of XP group E patients (Chu and Chang, 1988). XP-E cells show the mildest NER defect of all the XP groups, having about 50% of normal repair capacity.

UV-DDB is a protein complex of a 127 kDa subunit with variable amounts of a 48 kDa subunit (Hwang and Chu, 1993; Keeney et al., 1993). It is not yet completely clear whether the DNA binding activity resides in p127 or only in the complex. UV-DDB XP-E cells examined to date only contain mutations in the p48 gene (Nichols et al., 1996). Expression of p48 is damage inducible in a p53-dependent manner (Hwang et al., 1998).

A widely held view in the literature is that UV-DDB has only an accessory role in the repair of UV damaged DNA. In support of this, NER of a UV damaged DNA substrate was reconstituted in vitro, in the absence of UV-DDB (Aboussekhra et al., 1995; Bessho et al., 1997). Addition of the partially purified factor to the system stimulated repair only two-fold (Aboussekhra et al., 1995) and in another dual incision system had no effect (Kazantsev et al., 1996). However, microinjection of partially purified UV-DDB into XP-E cells lacking
the DNA binding activity restored NER activity (Keeney et al., 1994; Otrin et al., 1998), indicating a role for this factor *in vivo*, possibly only when damage is present in a chromatin context.

Recently, Hwang *et al.*, presented evidence that *in vivo* repair of cyclobutane pyrimidine dimers in the bulk of the genome is p48 dependent (Hwang *et al.*, 1999). Hamster cells show much less of this type of repair than do human cells and have no detectable p48 or UV-DDB expression (Hwang *et al.*, 1998). On transfection of hamster cells with human p48 cDNA, repair of cyclobutane pyrimidine dimers was increased to a level comparable with that seen in normal human cells. Therefore, *in vivo*, UV-DDB may somehow facilitate the identification of lesions, especially those that are poorly recognized by XPC-hHR23B, such as UV-induced cyclobutane pyrimidine dimers (Hwang *et al.*, 1999). However, this is not particularly consistent with the measured binding specificity of UV-DDB which has a greater affinity for (6-4) photoproducts in naked DNA than for cyclobutane pyrimidine dimers (Abramic *et al.*, 1991; Keeney *et al.*, 1993).

Intriguingly, the pl27 component of the UV-DDB protein has also been found in association with human and viral proteins thought to be involved in transcription activation, such as E2F1 (Hayes *et al.*, 1998) and the X protein of hepatitis B virus (Becker *et al.*, 1998). The significance of this is unknown.

### 1.3. Aims of this thesis

The overall aim of this study was to further our understanding of the role of the XPC-hHR23B complex in nucleotide excision repair. I wanted to determine if hHR23B is required for XPC (pl25) activity in repairing cisplatin damaged DNA in a fully reconstituted NER system using purified proteins. Further to this I wanted to investigate the DNA binding activity of the XPC-hHR23B complex and quantify any preference for binding to various types of damaged DNA.
Chapter 2. Materials and methods.

2.1. Polymerase chain reaction (PCR)

PCR was used to produce fragments of the XPC gene for expression in *E. coli* and subsequent antibody production. XPC fragment 1 was made using oligonucleotide 70481 as the forward and 74126 as the reverse primer to give an expected product of ~1 kbp (38.2 kDa protein). XPC fragment 2 used oligonucleotide 70481 as the forward and 74125 as the reverse primer to give an expected product of ~750 bp (27.6 kDa protein). XPC fragment 3 used oligonucleotide 70481 as the forward and 74124 as the reverse primer to give an expected product of ~430 bp (15.6 kDa protein). XPC fragment 4 used oligonucleotide 74123 as the forward primer and 70482 as the reverse primer to give an expected product of ~1.1 kbp (42.2 kDa protein). XPC fragment 5 used oligonucleotide 74123 as the forward and 74126 as the reverse primer to give an expected product of ~730 bp (28.1 kDa protein). Oligonucleotide details are as follows:

**Forward primers:** bold indicates the position of an incorporated *Sal* I site in forward primers.

70481.
TTTTGTCGACTCGAAACGCGCGGCCGGC primes at 120-138 nt in the XPC (p125) sequence.

74123.
TTTTGTCGACTGGGGATGACCTCAGGGAC primes at 398-416 nt in the XPC (p125) sequence.

**Reverse primers:** bold indicates the position of an incorporated *Not* I site in reverse primers. *Italics* indicate the position of an incorporated Stop codon.
TTCCGCGGCCGCTTAAGACACCCTGGAGGC primes at 1404-1386 nt in the XPC (p125) sequence.

TTTTGCGGCCGCTTATGGAAATGCCTGAGGTCATCCCC primes at 422-399 nt in the XPC (p125) sequence.

TTTTGCGGCCGCTTAGTGTGTGCCTCATGGACCCC primes at 729-711 nt in the XPC (p125) sequence.

TTTTGCGGCCGCTTAGACCAATTCCTCATCATCTCGAGC primes at 1010-987 nt in the XPC (p125) sequence.

Approximately 40 pmol pBluescript II KS (+) vector (Stratagene) containing XPC (p125) cDNA was used as template in PCR reactions with ~30 pmol each primer. 50 μl reactions also contained 2.5 U PFU polymerase (Stratagene) 1 x PFU buffer and 400 μM dATP/dTTP/dCTP/dGTP. A typical reaction cycle was as follows:

**Denaturation:** 94° C for 1 min  
**Annealing:** 55° C for 1 min  
**Extension:** 72° C for 3 min  
**Repeat:** 35 cycles  
**Soak:** 4° C

Approximately 4-5 μg each fragment was obtained from the PCR amplification. XPC fragments and pET28-C (Pharzigen) vector were then digested with 50 U Sal I (Boehringer Mannheim, 20 U/μl) and 50 U Not I (Boehringer Mannheim, 10 U/μl) in 1x Boehringer Mannheim buffer H for 4 h at 37 °C and DNA
purified from the reactions on Qiaquick PCR purification spin columns according to the supplier's instructions. DNA concentration and purity were determined by agarose gel electrophoresis (0.8 % gel, 1 x TBE).

2.2. Ligation

Routinely 1:1 ratios of gene fragments and vector were incubated at 16° C for ~4 h in 10 μl reactions containing 1x NEB T4 DNA ligase buffer and 400 U T4 DNA ligase (NEB, 400 U/μl). The 10 μl reaction mix was then used directly in transformations.

2.3. Preparation of competent E. coli cells

A single colony of TOP10 (Invitrogen) competent cells was inoculated into 50 ml of Luria-Bertani medium and grown overnight at 37° C. 4 ml of the resultant culture was used to inoculate 400 ml LB broth and grown at 37° C until an OD₅₉₀ of 0.375 was achieved (mid-log phase). Cells were collected by centrifugation for 10 min at 3000 rpm (~1600 x g), 4° C in a Sorvall GSA rotor and allowed to decelerate without the brake. Each cell pellet was then resuspended in 10 ml ice-cold CaCl₂ and cells collected by centrifugation for 5 min at 3000 rpm (~1100 x g), 4° C in a Sorvall SW34 rotor. Each cell pellet was then resuspended in 10 ml ice-cold CaCl₂ and incubated on ice for 30 min. Cells collected by centrifugation as previously. Finally cell pellets were resuspended in 2 ml ice-cold CaCl₂, incubated on ice for 1 h and then dispensed into pre-chilled tubes in 100 μl aliquots and stored immediately at -80° C.
2.4. **Transformation of DNA into *E. coli***

Routinely, 100 μl TOP10 competent *E. coli* cells were incubated on ice for 10 min with 100 ng closed circular plasmid DNA or 10 μl ligation mixes. Cells were then heat-shocked at 37° C (in a water bath) for 5 min and then returned to ice for 2 min. 1 ml of LB broth was added and cells allowed to recover by gentle shaking at 37° C for 1 h, prior to spreading onto LB plates containing the appropriate antibiotic selection. Transformants obtained after overnight incubation at 37° C. All resultant DNA plasmids were purified using Qiagen maxi-prep columns according to supplied instructions.

2.5. **Expression of XPC (p125) in *Pichia pastoris***

All transformation and expression methods were as stated in the *Pichia* expression kit manual (Invitrogen, # K1710-01).

2.6. **PCR screening of putative Pichia transformants***

Putative *Pichia pastoris* transformants were screened by PCR using a forward oligonucleotide which primes in the 5’ AOX1 region (67664) of the pPICZB/pPICZαA (Invitrogen) vectors and a reverse oligonucleotide which primes within the XPC gene sequence (67665). Oligonucleotide details were as follows:

67664.
CGACTGGTTCCAATTGACAAG primes from 855-875 nt in the 5’AOX1 region of pPICZB/ pPICZαA.
GGCTCTTCTTTGGGGGTTTCTC primes from 253-231 nt in the XPC gene sequence.

A typical screening reaction mix contained the following: ~30 pmol each oligonucleotide, 5 U *Thermus aquaticus* (Taq) polymerase (Boehringer Mannheim, 5 U/µl), 1x PCR buffer containing 15 mM MgCl₂ (supplied with Taq polymerase) and 400 µM dATP/dTTP/dCTP/dGTP. A sample of the putative transformant colony was inoculated into the reaction mix using a sterile Gilson tip.

A typical reaction cycle was as follows:

**Cell lysis:** 95° C for 15 min, 1 cycle  
**Denaturation:** 94° C for 1 min  
**Annealing:** 50° C for 1 min  
**Extension:** 72° C for 3 min  
**Repeat:** 35 cycles  
**Soak:** 4° C

### 2.7. Expression of XPC (p125) fragments for antibody production

5 N-terminal XPC fragments were designed. 3 of these constructs began at amino acid position 2 in the XPC sequence the remaining 2 started at amino acid number 95. XPC cDNA was used as template in PCR reactions to amplify the various fragments. In all cases the forward primer was designed to add a 5'Sal I site and the reverse primer added a stop codon and a 3' Not I site. These enabled insertion into pET28-C in such a way that the resultant proteins would be N-terminally 6 x His tagged.

The constructs were numbered 1-5 (1-3 having the same N-terminus) and the
predicted protein fragment sizes were 38.2, 27.6, 15.6, 42.2 and 28.1 kDa respectively. The constructs were transformed into *E.coli* strain BL21 DE3 and 50 ml test inductions carried out for 5 h at 30° C in LB broth containing 50 µg/ml kanamycin and 1 mM IPTG. On SDS PAGE analysis it was found one of the fragments was highly expressed and this was designated XPC fragment 5 and comprised amino acids 96 to 299 of the XPC sequence.

20 ml LB broth containing 50 µg/ml kanamycin was inoculated with the appropriate colony and grown at 37° C overnight. 5 ml of this was used to inoculate 200 ml LB media containing 50 µg/ml kanamycin and this was grown at 37° C until an optical density at 600 nm of 0.6 was achieved. Cells were collected by centrifugation and resuspended in 2 l fresh media and grown again to an optical density of 0.6. Cells were collected by centrifugation and resuspended in 2 l fresh media containing 50 µg/ml kanamycin and 1 mM IPTG. Induction was carried out for 5 h at 30° C and 1 ml samples were taken pre and post induction. Cells were collected by centrifugation and the pellet stored overnight at -80° C.

### 2.8. Denaturing purification of XPC fragment 5 on Ni-NTA agarose

All subsequent steps except thawing, sonication and centrifugation were carried out at room temperature to prevent precipitation of denaturing agents. Cell pellet was resuspended in 20 ml buffer A (6 M guanidine hydrochloride, 0.1 M NaH$_2$PO$_4$, 10 mM Tris-HCl, pH 8.0) (5 ml per gramme pellet) and stirred at room temperature for 1 h. Insoluble material was removed by centrifugation in a Sorvall SS34 rotor at 13,000 rpm (20,000 x g) for 15 min at 4° C. A 15 ml 50 % slurry of NTA-Ni agarose beads (Qiagen) equilibrated in buffer A was added to the supernatant and stirred at room temperature for 1 h to allow binding. The slurry was loaded into a column and left for the beads to settle out for 1 h. The column was washed with 75 ml buffer A followed by 75 ml buffer B (8 M urea, 0.1 M NaH$_2$PO$_4$, 10 mM Tris-HCl, pH 8.0). The column was then washed with
75 ml buffer C (8 M urea, 0.1 M NaH₂PO₄, 10 mM Tris-HCl, pH 6.3) and finally with 75 ml PBSA (137 mM NaCl, 3.4 mM KCl, 10.1 mM Na₂HPO₄, 1.8 mM KH₂PO₄). The beads were removed from the column in 15 ml PBSA (50 % slurry) and used to immunize 3 rabbits (RWO27, 28 and 29) over a period of 84 days. On the 94th day the rabbits were sacrificed and bleedouts collected and tested.

2.9. SDS polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE gels containing 37.5:1 acrylamide:bisacrylamide (Severn Biotech) and 1 % SDS were prepared according to Sambrook et al, 1989 and run in a Mini-Protean II Cell system (BioRad). Proteins were generally separated on 10 %, 0.75 mm, SDS-polyacrylamide gels for 1-2 h at 100-200 V in 25 mM Tris-HCl, 192 mM glycine, 0.1 % SDS. Gels were Coomassie brilliant blue stained according to Sambrook et al, 1989 or silver nitrate stained with Silver stain plus (BioRad).

2.10. Western blotting

Proteins were separated on SDS-PAGE as in section 2.9 and then transferred overnight (~16 h) at 40 V onto Immobilon P (Millipore) polyvinylidene difluoride (PVDF) membrane in 25 mM Tris-HCl, 192 mM glycine, 20 % methanol as described previously (Sambrook et al., 1989). All incubations and washes were carried out at room temperature. The membrane was washed with PBSA containing 0.01 % Tween-20 and blocked with 5 % dried milk in PBSA containing 0.01 % Tween-20 for 1 h. The membrane was washed for 15 min with PBSA containing 0.01 % Tween-20 (3 changes of wash) and then a suitable dilution of antibody (1:2000 for RWO28 and 1:10,000 for anti-Rad23) added directly to the blocking buffer and incubated for 3 h. The membrane was then washed as previously described and incubated for 1 h with a 1:50,000
dilution of anti-rabbit secondary antibody in PBSA containing 0.01 % Tween-20. Detection was achieved using chemiluminescence (ECL™, Amersham Pharmacia Biotech).

2.11. Growing XPC and XPC-hHR23B baculovirus stocks

Two independent baculovirus stocks were kindly donated by Fumio Hanaoka, Osaka University. One contained XPC (p125) cDNA in the Invitrogen baculovirus expression vector, pVL1393. The other contained both XPC (p125) and hHR23B (p58) cDNA’s in a Clontech baculovirus expression vector, pAcW31. Both are polyhedrin promoter expression systems. Monolayers of ~2 x 10^7 Sf9 cells were routinely infected with 4 ml viral supernatant in 175 cm^2 flasks for 1h at room temperature on a rocking platform. Virus was then removed and the cells covered with 40 ml Grace’s medium (Imperial laboratories) containing 10 % (v/v) foetal bovine serum (Autogen Bioclear), 1 % (v/v) fungizone (Gibco) and 0.5 % gentamycin (Gibco). Cells were incubated at 27° C for 48/72 h and then collected by low-speed centrifugation. Virus containing supernatants were removed and used to infect more Sf9 cells until enough virus had been obtained for a large scale infection. To examine total cellular proteins the cell pellets were lyzed in 10 volumes (relative to packed cell volume, PCV) 2x sodium dodecyl sulphate (SDS) sample buffer (125 mM Tris-HCl, pH 6.8, 2 % (w/v) SDS, 10 % (v/v) glycerol, 4 % (v/v) 2-mercaptoethanol) and heated to 95° C for 5 min. Lysates were analyzed by SDS PAGE followed by Coomassie staining or Western blotting.

For large scale infections for protein purification, Sf9 cells were resuspended in 150 ml viral supernatant and incubated in spinner flasks at 27° C for 48 h. The infected cells were then collected by low-speed centrifugation and washed once in PBSA and frozen at -80° C until use.
Chapter 2. Materials and methods.

2.12. Preparation of extracts from infected Sf9 cells

The following is based on the procedure of Sugasawa et al with some modifications (Sugasawa et al., 1996). Infected cell pellets were resuspended in 8 x PCV of ice-cold NP lysis buffer (25 mM Tris-HCl, pH 8.0, 1 mM EDTA, 10 % (v/v) glycerol, 1 % (v/v) Nonidet P-40 (NP40), 1 mM DTT, 50 μM EGTA, 1 mM AEBSF, 0.2 μg aprotinin/ml, 2 μl leupeptin/pepsstatin A, chymostatin/ml PCV) and incubated on ice for 30 min. The insoluble fraction was pelleted by centrifugation at 4° C in a Sorvall SS34 rotor at 2500 rpm (800 x g) for 10 min. The supernatant fraction (S1) was removed and discarded and the pellet resuspended in 8 x PCV (10 ml) of ice-cold NP lysis buffer containing 300 mM NaCl and incubated on ice for 30 min. The insoluble fraction (P) was removed by centrifugation at 4° C in a Sorvall SS34 rotor at 10,000 rpm (12,000 x g) for 15 min and the resultant supernatant designated S2.

2.13. Purification of XPC-hHR23B from infected Sf9 cell extracts

Purification was based on the procedure of Sugasawa et al (Sugasawa et al., 1996). Supernatant S2 from XPC-hHR23B infected Sf9 cells was loaded onto an 8 ml phosphocellulose column equilibrated in buffer A (25 mM Tris-HCl, pH 7.5, 1 mM EDTA, 10 % (v/v) glycerol, 0.01 % (v/v) Triton X-100, 1 mM DTT, 1mM AEBSF) containing 0.3 M NaCl at a flow rate of 0.33 ml/min. The column was washed in the same buffer and bound material eluted using buffer A containing 1 M NaCl. The eluate was adjusted to 0.6 M NaCl by dilution with buffer A and loaded at a flow rate of 0.33 ml/min onto a 2 ml ss DNA cellulose column equilibrated in buffer A containing 0.3 M NaCl. The column was washed in the same buffer and bound material eluted using buffer A containing 1.5 M NaCl. Fractions were analyzed by SDS PAGE followed by Coomassie staining or Western blotting. XPC-hHR23B containing fractions were pooled and dialyzed into buffer containing 25 mM Tris-HCl, pH 7.5, 0.3 M NaCl, 0.5 mM EDTA, 2 mM DTT, 12.5 % (v/v) glycerol, 0.01 % Triton X-100 and 1 mM
AEBSF in the cold (4° C) for 6 h with two changes of buffer. Protein stored in 10 µl aliquots at -80° C until use.

2.14. Purification of XPC (p125) from infected Sf9 cell extracts

The XPC (p125) S2 fraction contained very little XPC protein and most was found in the insoluble fraction. The insoluble fraction (P) remaining after S2 was removed was resuspended in 8 x PCV (8 ml) NP lysis buffer containing 0.4 M NaCl and incubated on ice for 1 h. The fraction was clarified by centrifugation in a Sorvall SS34 rotor at 10,000 rpm (12,000 x g) at 4° C for 15 min. The supernatant (S3) was collected and a sample of this and the pellet (P2) analyzed by SDS PAGE and Coomassie staining. Supernatant S3 was adjusted to 0.6 M NaCl and loaded onto a 4 ml ss DNA cellulose column equilibrated in buffer A containing 0.6 M NaCl at a flow rate of 0.33 ml/min. The column was washed with the same buffer and bound material eluted with buffer A containing 1.5 M NaCl. Fractions were analyzed by SDS PAGE followed by Coomassie staining or Western blotting. XPC (p125) containing fractions were pooled and dialyzed into buffer containing 25 mM Tris-HCl, pH 7.5, 0.3 M NaCl, 0.5 mM EDTA, 2 mM DTT, 12.5 % (v/v) glycerol, 0.01 % Triton X-100 and 1 mM AEBSF in the cold for 6 h with two changes of buffer. Protein stored in 10 µl aliquots at -80° C until use.

2.15. Preparation of Manley whole cell extracts

Whole cell extracts from lymphoblastoid cells were made according to Manley and co-workers (Manley et al., 1983) with some modifications as described by Wood et al (Wood et al., 1995). All cell lines were cultured by Imperial Cancer Research Fund Central Cell Services department. Cell pellets, containing ~10⁹ cells, were resuspended in cold PBSA (4° C) and pelleted by centrifugation. The PCV was estimated and the pellet resuspended in 4 x PCV hypotonic lysis
buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 5 mM DTT, 2.5 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml pepstatin, 10 μg/ml chymostatin and 5 % aprotinin). Cells were incubated on ice for 20 min before breakage by 30 to 40 strokes using a Teflon homogenizer (Jencons Scientific).

Cells were examined by nigrosin dye exclusion and homogenization continued until ~90 % were broken. 4 x PCV sucrose-glycerol buffer (50 mM Tris-HCl, pH 8.0, 10 mM MgCl₂, 25 % sucrose, 50 % glycerol and 2 mM DTT) were very slowly added to the homogenate. 1 x PCV of cold (4° C) saturated ammonium sulphate solution (neutralized to pH 7.0 with 1 M NaOH) was slowly added with stirring at less than one revolution per second. The stirring was continued at 4° C for 45 min. The mixture was then poured into ultracentrifuge tubes and spun at 42,000 rpm in a Beckman SW55 ultracentrifuge rotor (170,000 x g) at 2° C for 3 h. The supernatant was removed and its volume measured.

Solid powdered ammonium sulphate (0.33 g/ml) was added slowly with slow mixing at 4° C. The solution was neutralized by adding 10 μl of 1 M NaOH/g ammonium sulphate and stirring continued for 30 min. The mixtures were then spun at 11,000 rpm in a Sorvall HB4 centrifuge rotor (20,000 x g) at 4° C for 60 min. The supernatant was discarded and the precipitated protein pellet was transferred into dialysis tubing using a 1 ml syringe (Plastipak). The tubes were placed in 2 l cold dialysis buffer (25 mM Hepes-KOH, pH 7.9, 100 mM KCl, 12 mM MgCl₂, 1 mM EDTA, 17 % glycerol and 2 mM DTT) and dialyzed for 12-14 h with mixing at 4° C. The extract removed from the tubing and spun in a microfuge for 10 min to remove insoluble proteins. The supernatant was frozen at -80° C in small aliquots (10-50 μl).

2.16. Expression of hHR23B in E. coli and its extraction

This was based on the procedure of Masutani et al (Masutani et al., 1997). E. coli strain BL21 (DE3) p11d-hHR23B was kindly donated by Fumio Hanaoka.
5 ml full-grown culture of BL21 (DE3) cells carrying the p11d-hHR23B construct was used to inoculate 1 l of LB media containing 50 μg/ml ampicillin and grown at 37°C to an optical density at 600 nm of 0.4. The cells were collected by centrifugation and resuspended in 1 l fresh LB media containing 50 μg/ml ampicillin and isopropylthiogalactoside (IPTG) added to 0.4 mg/ml. 1 ml samples were taken pre and post induction. The cells were induced at 37°C for 3 h, collected by centrifugation, washed once in PBSA and frozen overnight at -80°C.

The cell pellet was resuspended in 10 ml buffer L (25 mM Tris-HCl, pH 7.5, 1 mM EDTA, 25 mM NaCl, 1 mM DTT, 0.2 mM AEBSF and 10 μl aprotinin/ml) and sonicated for six 15 second bursts with 15 second intervals (on ice). The lysate was clarified by centrifugation in a Sorvall SS34 rotor at 19,000 rpm for 1 h at 4°C. NaCl was added to give a final concentration of 50 mM and glycerol (Fluka) added to 10 % (v/v). The lysate was frozen in a bath of dry ice and ethanol and stored at -80°C overnight.

The sample was thawed and loaded onto a 5 ml Q sepharose FPLC column equilibrated in buffer A (25 mM Tris-HCl, pH 7.5, 1 mM EDTA, 10 % glycerol (v/v), 1 mM DTT, 0.2 mM AEBSF, 10 μl aprotinin/ml and 50 mM NaCl) at a flow rate of 2 ml/min. The column was washed with 50 ml of the same buffer and bound material eluted with a linear gradient of 50 – 400 mM NaCl in buffer A. On SDS PAGE analysis it was seen that most hHR23B eluted between 100 and 150 mM NaCl. The hHR23B containing fractions were pooled and ammonium sulphate powder added to 35 % saturation (1.38 g) with stirring at 4°C. The precipitates were collected by centrifugation at 10,000 x g for 10 min at 4°C and the pellet stored overnight at -80°C.

The pellet was thawed, resuspended in 2 ml buffer B (25 mM Tris-HCl, pH 7.5, 1 mM EDTA, 10 % glycerol (v/v), 1 mM DTT, 0.2 mM AEBSF and 10 μl aprotinin/ml) and dialyzed overnight (~13 h) into buffer B containing 0.8 M ammonium sulphate at 4°C with one change of buffer. The insoluble material
was pelleted by centrifugation at 10,000 x g for 10 min at 4° C and the soluble fraction collected. This was loaded onto a 1 ml butyl sepharose column equilibrated in buffer B containing 0.8 M ammonium sulphate at a flow rate of 0.33 ml/min. The column was washed with the same buffer and eluted with a 15 ml linear gradient of 800–0 mM ammonium sulphate in buffer B. The majority of hHR23B eluted between 300 and 0 mM ammonium sulphate. hHR23B containing fractions were identified by SDS PAGE analysis, pooled and stored at 4° C overnight.

The hHR23B pool was loaded onto a 1 ml hydroxyapatite column equilibrated in buffer A containing 0.35 M KCl at a flow rate of 0.5 ml/min. The column was washed in the same buffer and eluted with a 15 ml linear gradient of 0–250 mM potassium phosphate in buffer A. On gel analysis most hHR23B was found to be in the column flow through (FT). The FT was dialyzed overnight (~13 h) into buffer A containing 50 mM NaCl with one change of buffer.

This was loaded onto a 1 ml FPLC Mono Q HR5/5 column equilibrated in buffer A containing 50 mM NaCl at a flow rate of 0.5 ml/min. The column was washed with the same buffer and eluted with a 20 ml linear gradient of 50–300 mM NaCl in buffer A. On SDS PAGE analysis hHR23B was found eluting between 150–200 mM NaCl, these fractions were pooled and frozen at -80° C until use.

2.17. Expression of His-tagged hHR23B in E. coli and its extraction

Expression and extraction based on the protocols of Masutani et al (Masutani et al., 1997). pET-23B-His construct was kindly donated by Fumio Hanaoka. 100 ng of the pET23B-His construct was used to transform 100 μl competent FB810 cells (BL21 (DE3) recA). Transformants were selected on LB plates containing 50 μg/ml kanamycin. A 4 ml overnight culture from a single colony was used to inoculate 4 x 50 ml LB broth containing 50 μg/ml kanamycin and
these were grown at 37° C to an optical density at 600 nm of 0.4 - 0.6. The cells were then resuspended in 4 x 500 ml fresh LB media containing 50 µg/ml kanamycin and induced with 0.4 mM IPTG for 2.5 h at 37° C. 1 ml samples were taken pre and post induction. Cells were then collected by centrifugation, washed once with ice-cold PBSA and frozen at -80° C overnight.

Cell pellets were each resuspended in 15 ml lysis buffer (20 mM sodium phosphate, pH 7.8, 0.3 M NaCl, pooled and protease inhibitors added (0.2 mM AEBSF, 0.2 µg leupeptin/ml, chymostatin, pepstatin A, aprotinin and 50 µM ethylene-glycol-bis (β-aminoethyl ether)-N,N,N′-tetraacetic acid, EGTA). The suspension was incubated on ice for 15 min in the presence of 1 mg lysozyme/ml, frozen in a bath of dry ice/ethanol and thawed at 4° C. After three cycles of freezing and thawing, the sample was centrifuged in a Sorvall SW38 rotor at 18,500 rpm for 30 min at 4° C. The cleared lysate was then purified as described below.

2.18. Purification of His-tagged hHR23B

The cleared lysate was diluted to 100 ml with lysis buffer and adjusted to 20 mM imidazole. All subsequent steps were carried out at 4° C. The lysate was loaded onto a 9 ml NTA-Ni agarose column (Qiagen) equilibrated in buffer C (20 mM sodium phosphate, pH 7.8 and protease inhibitors as above) containing 0.3 M NaCl and 20 mM imidazole at a flow rate of 0.4 ml/min. The column was washed with the same buffer and bound material eluted sequentially with buffer C containing 0.3 M NaCl and 60, 90 and 250 mM. The majority of His-tagged hHR23B eluted at 60 mM imidazole and was at a concentration of 150 µg/ml (~9 mg total protein). Glycerol was added to 10 % (v/v) to half of the preparation and frozen at -80° C.
2.19. Production of an antibody against hHR23B

The remaining 30 ml (~4.5 mg protein) was loaded onto a 4.5 ml NTA-Ni agarose (Qiagen) column equilibrated in buffer C (20 mM sodium phosphate, pH 7.8 and protease inhibitors as above) containing 0.3 M NaCl and 20 mM imidazole at a flow rate of 0.4 ml/min. The column was washed in the same buffer and then in 10 column volumes (45ml) PBSA. The matrix was resuspended in 9 ml PBSA to give a 50% slurry and this was used to immunize two rabbits (RWO30 and RWO31) 7 times over a period of 84 days. On the 94th day the animals were sacrificed, bleedouts collected and tested by Western blotting.

2.20. Affinity purification of RWO31 (hHR23B antiserum)

150 μg His-hHR23B was run on a 10% SDS PAGE gel and then transferred at 40 V to Immobilon-P membrane overnight. The membrane was stained for 5 min in Ponceau-S and the hHR23B band excised. The membrane was then washed in PBSA/0.25% Tween-20 and blocked in PBSA/0.25% Tween-20 containing 10% dried milk for 1 h. The membrane was then incubated with 500 μl RWO31 antiserum and 500 μl PBSA/0.5% Tween-20 overnight (~16 h) with rotation at 4°C. Unbound serum was removed and the membrane washed for 2 x 5 min with 30 ml PBSA/0.5% Tween-20 and then with 300 μl the same buffer. Antibody was eluted from the membrane strip using 100 μl of glycine buffer (5 mM glycine, 0.5% Tween-20, 500 mM NaCl, 0.01% BSA) of 3 different pH's (pH 3.0, 2.0 and 1.0). 3 washes of 100 μl were carried out at each pH and followed by neutralization with 100 μl 10x PBSA. The pH was checked to ensure it was close to pH 7.0 and each fraction tested by Western blotting.
2.21. Construction of closed circular duplex DNA containing a single 1,3-intrastrand d(GpTgG)-cisplatin crosslink

A 5-fold molar excess of 24-mer platinated oligonucleotide containing a single 1,3-intrastrand d(GpTgG)-cisplatin crosslink was annealed to the single-stranded form (+ strand) of bacteriophage M13mp18 DNA modified to contain a sequence complementary to the platinated oligonucleotide within the polycloning site. The 3' terminus of the oligonucleotide acts as a primer for complementary strand synthesis by T4 DNA polymerase. The newly synthesized DNA strand is covalently closed with T4 DNA ligase resulting in a circular DNA duplex containing the single cisplatin-DNA adduct at a specific site (Shivji et al., 1999).

**Annealing reaction**

25 μg single-stranded M13 DNA (500 ng/μl), 3.8 μl (380 ng) 5'-phosphorylated 24 mer oligonucleotide containing a single 1,3-intrastrand d(GpTgG)-cisplatin crosslink, 7.5 μl 10 x reaction buffer (identical to NEB buffer 2; New England Biolabs) and 13.7 μl water were incubated at 65° C for 5 min, 37° C for 30 min, 25° C for 20 min and finally 4° C for 20 min.

**Complementary strand synthesis and ligation**

75 μl annealed DNA, 12.5 μl 10 x reaction buffer, 2 μl 10mg/ml BSA, 4 μl 100 mM ATP, 12 μl 10 mM dNTP mixture (contains 10 mM each of dATP, dCTP, dGTP and dTTP), 15 μl T4 DNA polymerase (5 U/μl), 4 μl T4 DNA ligase (400 cohesive end units/μl) and 75.5 μl water were mixed on ice and incubated at 37° C for 3 h. Reaction products (1 μl of each 200 μl reaction) were analyzed on a 0.8 % agarose gel run in 1 x TBE buffer at 50 V overnight (~16 h).
Purification of closed circular duplex DNA containing a single 1,3-intrastrand d(GpTpG)-cisplatin crosslink

Closed circular duplex DNA was purified from nicked and linear forms by CsCl/EtBr density gradient centrifugation. For 8 x 2.0 ml gradients 15 g CsCl was dissolved in 9.5 ml water at 37° C for 1 h. 1.352 ml of this solution was aliquoted into 2.2 ml microfuge tubes. DNA samples were diluted to a final volume of 500 µl with TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) added to the tube containing the CsCl solution. The DNA/CsCl solutions were then transferred to 2 ml Quickseal polyallomer tubes (11 x 32 mm, Beckman #344625) using a wide bore needle and syringe. The tubes were covered with foil and 148 µl 10 mg/ml ethidium bromide (EtBr) solution added to each. The tubes were then heat-sealed and spun at 85,000 rpm in a TLA 100.2 (Beckman) ultracentrifuge rotor (270,000 x g) at 18° C for 24 h.

The centrifuge tubes were unloaded in the dark using a hand-held UV lamp (312 nm) to visualize the closed circular DNA. Using a wide bore needle and syringe ~500 µl DNA solution was removed from each tube. 500 µl water-saturated butanol was added, vortexed for 5 seconds and centrifuged for 1 min. The upper phase (pink due to EtBr) was removed and discarded and the extractions repeated until no EtBr remained in either phase. The resultant solution was diluted to 2.0 ml with TE buffer. The samples were then placed in a Centricon 100 or Centricon 30 ultrafiltration unit and centrifuged at 1000 x g for 30-60 min at 4° C to concentrate the DNA to approximately 50 µl. TE buffer was added to 2.0 ml and this step repeated 4 more times to remove the CsCl. The final DNA solutions were quantified by spectrophotometry and purity checked by agarose gel electrophoresis. Purified DNA was stored in aliquots at -80° C.
2.22. Digestion of closed circular duplex DNA containing a single 1,3-intrastrand d(GpTpG)-cisplatin crosslink with ApaL1

The 1,3-intrastrand d(GpTpG)-cisplatin crosslink is located within a unique ApaL1 restriction site. Resistance to cleavage by this enzyme is diagnostic for the presence of the cisplatin-DNA adduct. Before using a new preparation of the 1,3-intrastrand d(GpTpG)-cisplatin damaged DNA this method was used to verify that the lesion was present and at a specific site. 4 μl platinated DNA, 1 μl 10x NEB buffer 4, 1 μl 10 mg/ml BSA, 1 μl ApaL1 (10U/μl) and 3 μl water were incubated at 37° C for 3 h and analyzed by agarose gel electrophoresis on a 0.8 % agarose gel run at 50 V overnight (~16 h).

2.23. Analysis of NER DNA products by incision assay

Reaction mixtures were in a total volume of 8.5 μl in the fully defined reconstituted system or 10 μl for cell extract complementation. Reactions were carried out in a buffer containing 45 mM HEPES-KOH (pH 7.8), 70 mM KCl, 7 mM MgCl₂, 1 mM DTT, 0.3 mM EDTA, 12.5 % glycerol, 2.5 μg BSA, 0.025 % NP40 and 2 mM ATP. Complementation reactions were supplemented with 40 mM phosphocreatine (di-Tris salt) and 0.5 μg creatine phosphokinase (type I, Sigma). Complementation reactions contained 40 μg whole cell extract. Reconstituted reactions contained 50 ng RPA, 22.5 ng XPA, 10 ng XPC-hHR23B complex, 50 ng XPG, 20 ng ERCC1-XPF complex and 1.5 μl of HeLa TFIIH (Hep fr. IV, (Marinoni et al., 1997)). Any changes to these amounts are shown in Figure legends. Following pre-incubation for 10 min at 30° C (5 min for complementations of whole cell extracts), 50 ng platinated DNA substrate (Pt-GTG) was added and reactions incubated for 90 min (30 min for complementations of whole cell extracts) at 30° C.

Reactions were stopped by rapid freezing on dry ice. 6 ng of an oligonucleotide (4GP) complementary to the excised DNA fragment was added to each reaction.
mixture. This oligonucleotide contains 4 extra G residues at the 5' end (4GP) and was annealed to the excised products by heating at 95°C and then incubating at room temperature for 20 min. Oligonucleotide annealing and quantitation controls were also used. This consisted of annealing 5 ng oligonucleotide 4GP to 5 and 1 pg (0.55 and 0.11 fmol) of its complement respectively. The excision products and annealing controls were labeled with 0.1 U Sequenase v2.0 polymerase (USB) and 1 μCi [α-32P] dCTP (Amersham Pharmacia biotech, 3000 Ci/mmol), separated on a 14 % polyacrylamide denaturing gel, dried and visualized by autoradiography as described by Shivji et al (Shivji et al., 1999). For quantitation purposes, the dried gel was exposed to a phosphorimage screen for 2-3 days and analyzed using Image Quant software. The intensity of all the bands present in the oligonucleotide control lanes (C1 and C2) were counted to give an average conversion factor for each experiment between phosphorimage counts and fmol labeled oligonucleotide. In repair reactions, all reaction products between 24-32 nt were counted during quantification. This allowed NER activity to be presented in the units, fmol released oligonucleotide rather than relative phosphorimage counts.

2.24. Denaturing polyacrylamide gel electrophoresis

Denaturing 14 % polyacrylamide gels containing 19:1 acrylamide:bisacrylamide and 8.3 M urea (Sequagel™, National Diagnostics) were run in a Model S2 (GIBCO, BRL) sequencing gel electrophoresis apparatus at constant temperature (50°C).
2.25. Preparation of 136 bp UV irradiated probe for electrophoretic mobility-shift assay

The 136 bp DNA fragment was prepared from the vector pSE420 (Invitrogen) by sequential digestion with Nhe I and Eco RI restriction enzymes and separated from the vector fragment by electrophoresis on a 1.2 % agarose gel. The 136 bp fragment was excised from the gel and purified using Qiagen gel extraction spin columns. The concentration of the fragment was determined by spectrophotometry and purity checked by agarose gel electrophoresis.

~200 ng (~30 µl) of the Nhe I-Eco RI fragment was labeled on the Nhe I end using the Klenow fragment of E. coli polymerase I (New England Biolabs, NEB). 50 µl reactions contained: 1 µl Klenow polymerase (5 U/µl, NEB), 1x NEB Klenow buffer, 20 µM dATP/dTTP/dGTP and 65 µCi [³²P]dCTP (10 mCi/ml, Amersham Pharmacia biotech). Reaction was incubated for 15 min at room temperature and then 2 µl 2 mM mix of each dNTP (i.e. each to 80 µM) added and the reaction incubated at 30 ° C for 5 min, to ensure that the fragment was completely double-stranded. 2 µl 0.5 M EDTA (i.e. to 20 mM) was added to inhibit the Klenow reaction and the mix applied to 2 x G25 sephadex columns to remove unincorporated label. Samples of the eluate (1 µl) were placed on filter paper, scintillant added and counted in a liquid scintillation counter for 5 min to determine the labeling efficiency. The probe was then diluted 1:4 with TE buffer to give ~20,000 cpm/µl.

50 µl drops of the probe were then UV irradiated on ice at a dose rate of 10 J/m²/sec to give a total dose of 2.5 or 5 kJ/m². Routinely 0.5 µl of this probe (~1 ng) was used per reaction.
2.26. Production of bubble structure probes for electrophoretic mobility-shift assay

150 ng of the top strand (marked *) of each bubble substrate was 5' labeled with using 10 U T4 polynucleotide kinase (NEB) and 30 μCi γ-[ATP] (10 MBq/ml, Amersham Pharmacia biotech). 150 ng of radiolabeled oligonucleotide in 1 x T4 polynucleotide kinase buffer (NEB) in a 10 μl reaction at 37° C for 30 min. This was then mixed with a 4-fold molar excess of the indicated unlabelled partner. The DNA was heated to 95° C for 3 min, 68° C for 10 min, 37° C for 10 min and room temperature for 15 mins. The annealed substrate was purified on a non denaturing 10% polyacrylamide gel in 1 x TBE buffer run at 4° C. Labeled species were identified by autoradiography and excised. DNA was allowed to diffuse from the gel slice into TE buffer containing 50mM NaCl overnight at 4° C before filtering through an amicon Ultrafree-DA filter unit (Millipore). The probes were then diluted in the same buffer to an appropriate concentration and stored at -20° C.

Oligonucleotide details as follows:

30 bp bubble

5' CCAGTGATCACATACGCTGTGCTAGGACACCCCCCCCCCCCCCCCCC
CCCCCCCCCCCCCCCCCAGCGCCACGTTGCCCACGTTGACCG 3' *

5' CGGTCAACGTGGGCAGACAACGTGGCGCTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT
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5’ CGGTCAACGTGGGCATTATCCCGGACAACGTGGCGCTGTTTTTTTTTTTTTTTGTGTCCTAGCACACATAGCTGGTATGTGATCACTGG 3’

5 bp bubble

5’ CCAGTGATCACATACCAGCTATGACCAGCTGTGCTAGGACACACCCCGAGAGCGCCACGTTTAGTCGGGATAAACATGCGCCACGTTGACCG 3’ *

5’ CGGTCAACGTGGGCACATTGTTATCCCGGACAACGTGGCGCTGTTTTTTTGTCCTAGACAGCTGTGCTAGGATATGTGATCACTGG 3’

3 bp bubble

5’ CCAGTGATCACATACCAGCTATGACCAGCTGTGCTAGGACACACCCCGAGAGCGCCACGTTTAGTCGGGATAAACATGCGCCACGTTGACCG 3’ *

5’ CGGTCAACGTGGGCACATTGTTATCCCGGACAACGTGGCGCTGTTTTTTTGTCCTAGACAGCTGTGCTAGGATATGTGATCACTGG 3’

2 bp bubble

5’ CCAGTGATCACATACCAGCTATGACCAGCTGTGCTAGGACACACCCCGAGAGCGCCACGTAGTCGGGATAAACATGCGCCACGTTGACCG 3’ *

5’ CGGTCAACGTGGGCACATTGTTATCCCGGACAACGTGGCGCTGTTTTTTTGTCCTAGACAGCTGTGCTAGGATATGTGATCACTGG 3’

1 bp bubble (C-C mismatch)

5’ CCAGTGATCACATACCAGCTATGACCAGCTGTGCTAGGACACACCCGAGAGCGCCACGTTAGTCGGGATAAACATGCGCCACGTTGACCG 3’ *
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5' CGGTCAACGTGGGCACATTGTATCCCGGCACGTGAAGATGTGGCGCTGGCTG
TGTCCTAGCATCGGATGGTACATGCTGTATGTGATCACTGG 3'

Double stranded 90 mer

5' CCAGTGATCACATACCAGCTATGACCATCGCTGTGCTTAGGACACCCCA
GCGCCACGTACGTGCGGATAAAATGTGGCCACGTGACC 3' *

5' CGGTCAACGTGGGCACATTGTATCCCGGCACGTGAAGATGTGGCGCTGGCTG
TGTCCTAGCATCGGATGGTACATGCTGTATGTGATCACTGG 3'

2.27. Electrophoretic mobility-shift assay

Routinely, 1 ng 136 bp 2.5 kJ/m² UV irradiated or bubble structure probe was
incubated with the appropriate protein (50/100 ng XPC-hHR23B or 0.5 ng
UV-DDB) for 1 h at 30° C. Reactions were carried out in bandshift assay buffer
(1 x, 40 mM Tris-HCl, pH 7.5, 5 mM MgCl₂, 0.1 mg/ml BSA, 1 mM DTT) in the
presence of 100 ng of the non-specific competitor DNA poly (dI•dC). 6x Gel
loading buffer (0.25 % bromophenol blue, 0.25 % xylene cyanol FF, 30 %
glycerol in water) was then added and the free and bound fractions separated
by non denaturing gel electrophoresis.

2.28. Non denaturing gel electrophoresis

Protein bound and free fractions from electrophoretic mobility shift assays were
separated on non denaturing 5 % polyacrylamide gels containing 37.5:1
acrylamide:bisacrylamide (Severn Biotech). I routinely used Protean II,
(BioRad) apparatus, and gels were run for ~2.5 h at 20 mA, cooled by circulating
water behind the gel plates. The gels were then dried at 80° C for 1 h and
2.29. Photoreactivation of UV-irradiated probe DNA

To remove cyclobutane pyrimidine dimers, 50 ng 2.5 kJ/m² UV-irradiated 136 bp probe DNA was treated with 200 ng *Anacystis nidulans* photolyase (kindly donated by A. P. Eker, Erasmus University, Rotterdam) in a 100 µl reaction containing 10 mM sodium phosphate buffer, pH 7.0, 0.1 M NaCl, 0.1 % BSA (w/v) and 5 mM DTT. Reaction mix was incubated at room temperature under 420 nm blue light (Philips TLDK 30W/03 lamp) for 30 min. To remove photolyase, 6 µl 10 % (w/v) sodium dodecyl sulphate (SDS) and 20 µg proteinase K (2 mg/ml, final concentration of 190 µg/ml) added and the reaction incubated at 37° C for 30 min. 120 µl phenol:chloroform:isoamyl (25:24:1) alcohol added, mixed and centrifuged to separate the two phases. 115 µl of the top phase removed, 115 µl chloroform added, mixed and centrifuged as previously. 110 µl of the top phase removed and mixed with 50 µl 7.5 M ammonium acetate, pH 7.5, 320 µl ice-cold ethanol and precipitated on dry-ice for 30 min. DNA recovered by centrifugation in a benchtop microfuge for 20 min at 13,000 rpm and washed with 70 % ethanol. DNA resuspended in 50 µl TE buffer and recovery checked by liquid scintillation counting.

2.30. T4 endonuclease V assay

Efficiency of photoreactivation treatment was tested using T4 endonuclease V (kindly donated by R. Stephen Lloyd, University of Texas, Galveston). T4 endonuclease V (0.15 mg/ml in 25 mM sodium phosphate buffer, pH 6.8, 1 mM EDTA, 100 mM NaCl) was diluted 10-fold in 25 mM sodium phosphate buffer, pH 7.0, 0.1 mg/ml BSA and 150 ng used to digest 4 ng photoreactivated probe DNA exposed to Biomax XR film (Sigma) overnight (~16 h). Reactions were quantitated using a phosphorimager and Image Quant software.
alongside the same amount of non damaged and 2.5 kJ/m² UV-irradiated, non photoreactivated probe. 10 µl reactions were in 25 mM sodium phosphate buffer, pH 7.0, 1 mM EDTA, 100 mM NaCl and were incubated at 37° C for 30 min. 1 µl 0.5 M EDTA added along with 6 µl formamide loading buffer (90 % deionised formamide (Fluka), 1 x TBE (90mM Tris-borate, pH 8.5, 2mM EDTA), 0.025 % xylene cyanol, 0.025 % bromophenol blue), samples heated at 95° C for 5 min and fragments separated on a denaturing 14 % polyacrylamide gel. The gel was then dried and exposed to Biomax XR film (Sigma) overnight.

2.31. Preparation of cisplatin damaged DNA for competition assays

pSE420 plasmid DNA (Invitrogen) was treated with cisplatin at a drug to nucleotide ratio of 1:100 to give approximately 50 lesions per plasmid (Hansson and Wood, 1989) (equivalent to the number of lesions in the UV-irradiated pSE420 competitor). A 50 µl reaction containing 10 µl 10 nM cisplatin and 6 µg pSE420, in platination buffer (3 mM NaCl, 0.5 mM Na₂HPO₄, 0.5 mM NaH₂PO₄) was allowed to react in the dark at 37° C for 14 h. Reactions were stopped by addition of NaCl to 0.5 M. DNA was precipitated in the presence of 2.5 M ammonium acetate, pH 7.5 with 2.5 volumes of ice-cold ethanol on dry-ice for 5 min, washed in 70 % ethanol and redissolved in TE buffer to a concentration of 100 ng/µl. Platinated DNA was stored at -80° C until required.

2.32. Preparation of MMS and MNU damaged DNA for competition assays

pSE420 plasmid DNA (Invitrogen) was treated with a concentration of methyl methanesulphate (MMS) and N-methyl-N-nitrosourea (MNU) to give approximately 50 lesions per plasmid. 10 µg pSE420 plasmid DNA was dissolved in 10 mM sodium phosphate buffer, pH 7.0 to give a concentration of 0.5 µg/ml. This was then mixed with 1 µl 50 mM MMS or MNU solution and
made up to 50 µl with the same sodium phosphate buffer and incubated at 37°C for 30 min. 100 µl 7.5 M ammonium acetate, pH 7.5 was added to each along with 350 µl ice-cold ethanol and incubated on dry-ice for 5 min to precipitate the DNA. DNA was pelleted by centrifugation in a bench top microfuge for 20 min at 13,000 rpm. DNA pellets were washed with 70% ethanol and each resuspended in 100 µl TE buffer to give 100 ng/µl stock solutions which were then stored at -80°C until required.

2.33. Preparation of radiolabeled DNA markers

0.5 µg Msp I digested pBR322 (1 mg/ml, NEB) was labeled using 2.5 U of the Klenow fragment of *E. coli* polymerase I (New England Biolabs, NEB, 5 U/µl), 15 µCi [³²P]dCTP (10 mCi/ml, Amersham Pharmacia biotech) and 1 x Klenow buffer (NEB) in a total volume of 25 µl. Reaction mix was incubated on ice for 30 min and then stopped by addition of 2 µl 0.5 M EDTA. Labeled markers were then diluted appropriately (usually 1:10) in TE buffer. Sizes of DNA markers after labeling is as follows: 623, 528, 405, 308, 243, 239, 218, 202, 191, 181, 161, 148, 124, 111, 91, 77, 68, 35, 27, 16 and 10 bp.
Chapter 3. Production and purification of XPC-hHR23B and antibodies.

3.1. Expression of XPC (p125) fragments and antibody production

As we had no detection method for XPC except functional assay, it was decided to produce various fragments of XPC (p125) to immunize rabbits for antibody production. On examination of XPC protein sequence it was found that the N-terminal half of the protein had the highest predicted level of antigenicity (data not shown) and so this region was used for antibody production.

Initial attempts to express the N-terminal half of XPC in E. coli were unsuccessful and so 5 smaller N-terminal XPC fragments were designed. 3 of these constructs began at amino acid position 2 in the XPC sequence and the remaining 2 started at amino acid number 95. XPC cDNA was used as template in PCR reactions to amplify the various fragments. These were inserted into pET28-C in such a way that the resultant proteins would be N-terminally 6x His tagged.

The constructs were numbered 1-5 (1-3 having the same N-terminus) and the predicted protein fragment sizes were 38.2, 27.6, 15.6, 42.2 and 28.1 kDa respectively. The constructs were transformed into E. coli strain BL21 DE3 and a test induction carried out for 5 h at 30° C using 1 mM IPTG. On SDS-PAGE analysis it was observed that one of the fragments had been expressed (Figure 3.1 A, fragment 5, lane +). This was designated XPC fragment 5 and comprises amino acids 96 to 299 of the XPC sequence.

A large scale induction (2 l) of this clone yielded approximately 12 mg of XPC fragment 5 which was purified under denaturing conditions on Ni-NTA agarose (Figure 3.1 B, lane 1). 3 rabbits (RWO27-29) were immunized with the protein bound beads. The RWO28 serum proved to be the most sensitive at
Figure 3.1. SDS-PAGE analysis of XPC fragment induction. The strains BL21 (DE3)/pET28-C-XPC fragment 1-5 were induced by IPTG, cells lysed and polypeptides separated by electrophoresis through a 10% SDS-PAGE gel. Proteins detected by Coomassie brilliant blue staining. A XPC fragment 1-5 and +, ~30/50 µg BL21 (DE3)/pET28-C-XPC fragment 1-5 cell lysate protein, pre/post induction, control and +, ~30/50 µg BL21 (DE3)/pET28-C vector (control) cell lysate protein, pre/post induction. * shows the position of induced XPC fragment 5. Gel shows BioRad Kaleidoscope markers (M1) on the left and prestained broad range markers (M2) on the right. B lane 1, ~1 µg Ni-NTA agarose-purified XPC fragment 5, Coomassie stained, lane 2, ~25 ng Ni-NTA agarose-purified XPC fragment 5, immunoblot using RW028 polyclonal antibody for detection. Numbers indicate position of BioRad Kaleidoscope markers.
recognizing both the antigen (Figure 3.1 B, lane 2) and XPC protein in whole cell extracts and was routinely used at a dilution of 1:2000. Figure 3.2 shows the presence of full length XPC protein in a normal lymphoblastoid cell extract (705 ORI) and XPA deficient (XP-A) cell extract (GM2345). No XPC protein is present in 2 XP-C lines (GM2246 and GM2249) and reduced levels in the XPC heterozygotic lines GM4237/4238. XPC deficient cell line GM2246 (xeroderma pigmentosum patient XP1BE) contains a 2 bp deletion and therefore lacks the AA dinucleotide at position 1492-1493 nt in at least one of its XPC alleles. This is expected to lead to premature termination of the protein by the introduction of a new stop codon 15 nt downstream. Northern blotting has shown the levels of XPC transcript in this cell line to be highly reduced and so the lack of detectable XPC protein is in agreement with this data. GM4237/4238 cell lines originate from the parents of patient XP1BE who was the source of line GM2246.

XPC deficient cell line GM2249 (xeroderma pigmentosum patient XP8BE) has been reported to contain at least two mutations. The first is the insertion of a GTG codon leading to an additional valine after Val697. The second is an A to C point mutation changing the penultimate residue in the polypeptide from Lys920 to Gln920. This is known to be one of the least UV sensitive XP-C lines and Northern blotting has detected near-normal levels of the XPC mRNA. Despite a complete XPC coding sequence no XPC protein was detectable in this cell line.

3.2. Characterization of an antibody against Rad23

hHR23B is a human protein which is homologous to *Saccharomyces cerevisiae* Rad23. At the start of this study we had no method for the detection of hHR23B. A polyclonal antibody raised against Rad23 was kindly donated by Simon Reed and Errol Friedberg and I found that this cross-reacts with a band of the expected size (~58 kDa) in human normal cell extracts (Figure 3.3, lanes 4
Figure 3.2. Specificity of RW028 polyclonal antibody. Approximately 50 µg of each whole cell extract (lane 1 705 ORI (normal), lane 2 GM2345 (XP-A), lane 3 GM4237, lane 4 GM4238, lane 5 GM2246 (XP-C) and lane 6 GM2249 (XP-C)) and lane 7 50 ng purified XPC-hHR23B complex (HeLa) were separated by electrophoresis through a 10% SDS-PAGE gel, transferred to Immobilon-P membrane and probed with RW028 antiserum (chemiluminescent detection). Numbers indicate positions of Kaleidoscope markers (BioRad).

Figure 3.3. Species cross-reactivity of Rad23 antiserum. Cell extracts were separated by electrophoresis through a 10% SDS-PAGE gel and then transferred to Immobilon-P membrane and probed with Rad23 antiserum (chemiluminescent detection). Lane 1 50 µg Xenopus high speed egg extract, lane 2 50 µg Droso phila embryonic extract, lane 3 50 µg CHO9 (Hamster) whole cell extract (wce), lane 4 50 µg HeLa wce, lane 5 50 µg 1BR3N wce. Numbers indicate positions of broad range pre-stained markers (BioRad).
and 5). A similarly sized cross reacting species was also observed in *Xenopus laevis* egg extract, CHO9 (hamster) cell extract and weakly in a *Drosophila melanogaster* embryonic extract (Figure 3.3, lanes 1, 2 and 3). The cross-reactivity of this antibody enabled us to use it as a tool to detect hHR23B during its subsequent expression and purification.

### 3.3. Attempts at expression of XPC (p125) in *Pichia pastoris*

It has not been possible to express full length XPC protein in *E.coli* and when XPC is expressed in insect cells the majority is insoluble (F. Hanaoka, personal communication). It is possible to purify XPC complex from HeLa cells but this method fails to yield much protein. One of the aims of this project was to determine whether XPC requires the presence of its co-purifying partner hHR23B for activity and so it was necessary to express the two components independently. For these reasons I attempted to express recombinant XPC (p125) in the methylotrophic yeast *Pichia pastoris*. This organism generates stable transformants by homologous recombination and these can be selected for using the drug Zeocin. Vectors were linearized in the 5' AOX1 region using Sac I to allow integration of the construct into the host 5' AOX1 region by gene insertion. Heterologous protein expression is driven by the alcohol oxidase promoter (PAOX1) which is induced during growth on methanol. To enable cloning and expression using the *Pichia* vectors (Figure 3.4 A) both the 5' and the 3' ends of the XPC (p125) gene required alteration. 2 stop codons upstream of the initiating methionine had to be removed to enable the 5' alpha factor secretion signal (present only in the pPICZαA vector) to be correctly fused onto the protein's N-terminus. The native downstream termination codon also needed to be removed to enable C-terminal 6x His and myc tagging of the protein. Both ends of the gene were altered using PCR which also added on the appropriate cloning sites (Kpn I and Not I). All the regions altered by PCR were sequenced on both strands to ensure that the required changes had been made and that no polymerase errors had been introduced.
Chapter 3. Proteins and antibodies.

Figure 3.4. Screening for transformants in *Pichia pastoris*. A. Two constructs were tested in the expression study: pPICZB. XPC (p125) is used for intracellular expression of His and myc tagged XPC. pPICZA. XPC (p125) is used for secreted expression of His and myc tagged XPC. **Relevant elements:** 5' AOX1; contains AOX1 promoter for methanol inducible expression and targets integration to the AOX1 locus. α-factor; *Saccharomyces cerevisiae* secretion signal. AOX1 TT; transcription terminator and poly A signal. Zeocin; *ble*, Zeocin resistance gene from *Streptalloteichus hindustanus*. B. Transformants were selected by Zeocin resistance and screened for the presence of the XPC cDNA by PCR using primers complementary to a 3' region of the XPC sequence. Reaction products separated on a 1% agarose gel and stained with ethidium bromide. **lanes 1 and 9,** pPICZB vector alone transformants, **lanes 2-5,** pPICZB + tagged XPC transformants, **lane 10,** pPICZB + untagged XPC transformant, **lane 11,** pPICZαA vector alone transformant, **lane 12,** pPICZαA + XPC transformant, **lane 6,** pPICZB plasmid DNA, **lane 7,** pPICZB + tagged XPC plasmid construct, **lane 8,** pPICZB + untagged XPC plasmid construct, **lane 13,** pPICZαA plasmid DNA, **lane 14,** pPICZαA + tagged XPC plasmid construct, **lane 15,** no DNA control. M = 100 bp ladder markers (Pharmacia).
Once the sequence had been verified the modified gene was subcloned into pPICZB (for intracellular expression) and pPICZαA (for secreted expression) at the Kpn I and Not I sites (Figure 3.4 A). A further construct was made using the original XPC cDNA to give an untagged version of XPC p125 in pPICZB. Sac I linearized constructs were transformed into Pichia strain GS115 by electroporation and transformants initially selected on Zeocin plates. Then further verification used a PCR assay with primers complementary to part of the XPC gene (Figure 3.4 B) and also using combinations of oligonucleotides complementary to the XPC gene and the 5' and 3' AOX1 regions of the construct (data not shown). Production of a 380 nt product after PCR showed the gene was present in the host in the correct orientation (Figure 3.4. B). Once verified a selection of transformants were used in small-scale expression studies. Induction time courses were carried out at 28-30°C over 5 days using methanol to a final concentration of 0.5%. SDS-PAGE and immunoblot analysis showed no detectable XPC expression (data not shown). On examination of the 5' sequence of the XPC gene, several consecutive unfavourable codons (as judged by a S. cerevisiae codon usage table) were identified. This could provide a possible explanation to the lack of XPC expression observed in Pichia. As we could obtain no XPC protein using this method we decided like others, to use a baculovirus expression system and attempt to overcome the insolubility problems that had been previously observed.

3.4. **Expression time course of XPC-hHR23B complex in Sf9 insect cells**

The baculovirus expression construct contained both XPC (p125) and hHR23B (p58) cDNA's in a Clontech baculovirus expression vector, pAcW31. Heterologous protein expression is driven from the polyhedrin promoter in this system. To determine the optimal time for harvesting the insect cells post infection a short expression time course was carried out.
Figure 3.5. Time course of expression of XPC and XPC-hHR23B in insect cells. A. 2.5 x 10^6 Sf9 insect cells were infected with 0.5 ml pAcW.XPC.hHR23B virus stock and cells harvested at the indicated times post infection. Cells were lysed and the soluble fraction (S2) collected by centrifugation. ~50 μg of each S2 supernatant and a sample of the insoluble fractions (P) was separated by electrophoresis through a 10% SDS-PAGE gel, transferred to Immobilon-P membrane and probed with RWO28 antiserum (chemiluminescent detection). B. 2.5 x 10^6 Sf9 insect cells were infected with 0.5 ml pVL.XPC virus stock and cells harvested at the indicated times post infection. Cells were lysed and the soluble fraction (S2) collected by centrifugation. ~50 μg of each S2 supernatant and a sample of the insoluble fractions (P) was separated by electrophoresis through a 10% SDS-PAGE gel, transferred to Immobilon-P membrane and probed with RWO28 antiserum (chemiluminescent detection). C. 2.5 x 10^6 Sf9 or High5 insect cells were infected with 0.5 ml pVL.XPC virus stock and cells harvested at the indicated times post infection. Uninfected control Cells were lysed in SDS sample buffer to give total cellular proteins (T). ~50 μg of each insect cell lysate or supernatant (S2) and a sample of the insoluble fractions (P) was separated by electrophoresis through a 10% SDS-PAGE gel, transferred to Immobilon-P membrane and probed with RWO28 antiserum (chemiluminescent detection). Numbers indicate positions of Kaleidoscope markers (BioRad).
Monolayers of Sf9 cells were infected with the pAcW.XPC-hHR23B virus and cells harvested 24 or 48 h post infection. Insect cells were first lysed in a NP40 containing lysis buffer without salt and then centrifuged to separate soluble (S1) and insoluble fractions (Sugasawa et al., 1996). The insoluble fraction was then extracted with the same NP40 containing buffer but this time containing 0.3 M salt. After centrifugation this gives the soluble supernatant fraction (S2) and an insoluble pellet (P). Samples of these fractions were separated by SDS-PAGE, transferred to Immobilon-P membrane and XPC protein detected using RW028 anti-XPC antibody (Figure 3.5 A). At 24 h post infection a small amount of XPC expression was detected in the S2 fraction and none in the insoluble sample. At 48 h post infection the total XPC expression was higher and about 40 % in the soluble S2 sample. There was also a high amount of XPC (about 60 % of the total) present in the insoluble fraction (P). The level of XPC protein in the supernatant (S2) fraction at 48 h was satisfactory and therefore when expression was scaled up Sf9 cells were harvested 48 h post infection.

3.5. Purification of XPC-hHR23B from infected Sf9 cells

Based on the procedure of Sugasawa et al (Sugasawa et al., 1996). 1 x 10⁹ SF9 cells were infected with the pAcW.XPC-hHR23B virus and harvested 48 h post infection where expression of XPC-hHR23B was both reasonably high and soluble. Insect cells were first lysed in a NP40 lysis buffer without salt and then centrifuged to separate soluble (S1) and insoluble fraction (P1). The insoluble fraction (P1) was then extracted with the same NP40 containing buffer but this time containing 0.3 M salt. After centrifugation this gives the soluble supernatant fraction (S2) and an insoluble pellet (P2).

The S2 fraction was purified on a phosphocellulose column. Samples of the S1 fraction, phosphocellulose load (L/S2), column flow through (FT), wash (W) and 1 M NaCl eluate (E) were separated by SDS-PAGE. Proteins were then transferred to Immobilon-P and XPC and hHR23B proteins detected
Figure 3.6. Purification of XPC-hHR23B from baculovirus infected Sf9 insect cells. 1 x 10^9 Sf9 insect cells were infected with 200 ml pAcW.XPC.hHR23B virus stock and cells harvested at 48 h post infection. Cells were lysed in NP lysis buffer containing no salt and the soluble fraction (SI) separated from the insoluble fraction (PI). PI was then extracted in NP lysis buffer containing 0.3 M NaCl to give the soluble fraction S2 (L). A. S2 (37.5 mg total protein) was purified on a phosphocellulose column. 20 µl of SI, the load (S2), the flow through (FT), 0.3 M wash (W) and the 1 M eluate (E) were separated on a 10 % SDS-PAGE gel and stained with Coomassie brilliant blue. B. The pooled elution from A (5.3 mg) was purified on a ss DNA cellulose column. 20 µl of SI, the load (S2), the flow through (FT), 0.3 M wash (W) and the 1 M eluate (E) were separated on a 10 % SDS-PAGE gel and stained with Coomassie brilliant blue. C. 20 µl of SI, the load (S2), the flow through (FT), 0.3 M wash (W) and the 1 M eluate (E) were separated on a 10 % SDS-PAGE gel, transferred to Immobilon-P membrane and probed with RWO28 (anti-XPC) and anti-Rad23 antisera (chemiluminescent detection). Numbers indicate positions of Kaleidoscope markers (BioRad).
Figure 3.7. Scheme for the purification of XPC-hHR23B from baculovirus infected Sf9 insect cells.
simultaneously using RWO28 (anti-XPC antibody) and an antibody to yeast Rad23 which cross-reacts well with the human homologue, hHR23B. XPC and hHR23B were detected in the S1, load (S2) and elution fractions (Figure 3.6 A). Some hHR23B was also detected in the flow through (FT) and wash (W) fractions.

The pooled eluate from the phosphocellulose column was then purified on a ss DNA cellulose column and eluted with 1.5 M NaCl. Samples of the load (L), flow through (FT), wash (W) and 1.5 M NaCl elution fractions were separated by SDS-PAGE and stained with Coomassie brilliant blue (Figure 3.6 B). The fractions at this stage were reasonably pure, except for a protein of approximately 33 kDa which can also be seen in the immunoblot shown in figure 3.6 A and therefore may be a breakdown product of the XPC protein. A duplicate gel was transferred to Immobilon-P and XPC and hHR23B proteins detected simultaneously using RWO28 (anti-XPC antibody) and an antibody to yeast Rad23 (Figure 3.6 C). Fractions 4 and 5 were pooled to give XPC-hHR23B pool 1 and fractions 3 and 6 were pooled to give XPC-hHR23B pool 2. XPC-hHR23B pools 1 and 2 were in 1.5 M NaCl, which is too high a salt concentration for use in in vitro repair assays and so were dialyzed for 6 h into buffer containing 0.3 M NaCl. Bradford assay (BioRad) showed that XPC-hHR23B pool 1 was at a concentration of 142 µg/ml (~67 µg total protein) and XPC-hHR23B pool 2 was at a concentration of 20 µg/ml (~68 µg total protein). A summary of the XPC-hHR23B purification scheme is shown in Figure 3.7.

3.6. **Expression time course of XPC (p125) in Sf9 and Hi5 insect cells**

The baculovirus expression construct contained XPC (p125) cDNA in the Invitrogen baculovirus expression vector, pVL1393 (Sugasawa et al., 1996). Heterologous protein expression is driven from the polyhedrin promoter in this system. To determine the optimal time for harvesting the insect cells post
infection a short expression time course was carried out. Monolayers of Sf9 cells were infected with the pVL.XPC virus and cells harvested 24 or 48 h post infection. Insect cells were lysed firstly in a NP40 containing lysis buffer without salt and then centrifuged to separate soluble (S1) and insoluble fractions. The insoluble fraction was then extracted with the same NP40 containing buffer but this time containing 0.3 M salt. After centrifugation this gives the soluble supernatant fraction (S2) and an insoluble pellet (P). Samples of these fractions were separated by SDS-PAGE, transferred to Immobilon-P membrane and XPC protein detected using RW028 anti-XPC antibody (Figure 3.5 B). At 24 h post infection some XPC expression was detected in the S2 fraction and none in the insoluble sample. At 48 h post infection the total XPC expression was higher but about 90 % of the XPC was present in the insoluble fraction (P). The level of XPC protein in the supernatant (S2) fraction at 48 h was very low and also low in the S2 fraction at 24 h and so it was decided to compare XPC expression in Sf9 cells to another insect cell type, Hi5.

Again a small expression time course was carried out and cells were harvested at either 48 or 72 h post infection and soluble S2 and insoluble P fractions collected. Total cell lysates from mock infected Sf9 or Hi5 cells were also prepared for comparison. XPC protein was detected using RW028 antibody as previously. The level of XPC protein in both Sf9 and Hi5 48 h S2 samples was similar as was the amount present in both insoluble (P) fractions. Again most of the XPC protein was insoluble. At 72 h the total XPC expression in both cell types was increased but the amount of soluble XPC (S2) was lower than that seen in the 48 h samples especially in the Hi5 cell type, where ~95 % of the XPC was insoluble (Figure 3.5 C). No XPC was detected in either Sf9 or Hi5 mock-infected insect cells.
3.7. Solubilization of XPC (p125) from infected insect cells and purification

As the XPC protein was mainly insoluble in both Sf9 and Hi5 cell types I decided to try a slightly different extraction procedure to attempt to gain more soluble protein. \(1 \times 10^9\) Sf9 cells were infected with the pVL.XPC virus and harvested 48 h post infection where expression of XPC was reasonably high but mostly insoluble. Insect cells were lysed firstly in a NP40 containing lysis buffer without salt and then centrifuged to separate soluble (S1) and insoluble fraction (P1). The insoluble fraction (P1) was then extracted with the same NP40 containing buffer but this time containing 0.3 M salt. After centrifugation this gives the soluble supernatant fraction (S2) and an insoluble pellet (P2). The P2 fraction was then extracted again with NP40 lysis buffer but containing 0.4 M NaCl. Centrifugation separated the soluble S3 and insoluble P3 fractions. Samples of all the fractions were separated by SDS-PAGE and stained with Coomassie brilliant blue (Figure 3.8 A).

The S1 fraction contained most of the non-specific insect cell proteins and P1 contained most if not all of the XPC. The S2 fraction also contained a lot of insect cell protein and some XPC but most was, as seen previously, present in the P2 fraction. Extraction of P2 with 0.4 M salt released more XPC protein into the soluble S3 fraction, about 50 % of the total XPC protein. Extraction of the P3 pellet with NP40 lysis buffer containing 1 M NaCl releases even more XPC protein into the soluble fraction (data not shown).

After these extractions the XPC preparation S3 was relatively clean and so was loaded directly onto a ss DNA cellulose column. XPC was eluted with 1.5 M NaCl and pooled fractions 4-7 (XPC pool 1) and 8-11 (XPC pool 2) contained a single polypeptide of \(~125\) kDa corresponding to the XPC (p125) protein (Figure 3.8 B). XPC pools 1 and 2 were in 1.5 M NaCl, which is too high a salt concentration for use in \(in vitro\) repair assays and so were dialyzed for 6 h into
Figure 3.8. Purification of XPC (p125) from baculovirus infected Sf9 insect cells. A. $1 \times 10^9$ Sf9 insect cells were infected with 200 ml pVL.XPC virus stock and cells harvested at 48 h post infection. Cells were lysed in NP lysis buffer containing no salt and the soluble fraction (S1) separated from the insoluble fraction (P1) by centrifugation. P1 was then sequentially extracted in NP lysis buffer containing 0.3 and 0.4 M NaCl to give fractions S2 and P2 and S3 and P3 respectively. The indicated amounts of each fraction were separated by electrophoresis through a 10 % SDS-PAGE gel and stained with Coomassie brilliant blue. B. S3 (27 mg total protein) was loaded onto a 4 ml ss DNA cellulose column in buffer containing 0.6 M NaCl at a flow rate of 0.33 ml/min. The column was washed with buffer containing 0.6 M NaCl and eluted with 1.5 M NaCl. 5 µl of load (L) and flow through (FT) and 20 µl of each elution fraction was separated on 10 % SDS-PAGE gels and stained with Coomassie brilliant blue. Numbers indicate positions of Kaleidoscope markers (BioRad).
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1 x 10^9 infected Sf9 cells

Extract pellet in NP lysis buffer no salt → Discard supernatant (S1)

Extract pellet (P1) in NP lysis buffer 0.3 M salt → Discard supernatant (S2)

Extract pellet (P2) in NP lysis buffer 0.4 M salt → Discard pellet (P3)

SSDNA cellulose

0.6 M wash

1.5 M elution

Dialyze into 0.3 M salt

XPC

Figure 3.9. Scheme for the purification of XPC (p125) from baculovirus infected Sf9 insect cells.
buffer containing 0.3 M NaCl. Bradford protein assays (BioRad) were performed and XPC pool 1 was found to be at a concentration of 160 μg/ml (~460 μg total protein) and XPC pool 2 was at a concentration of 14.5 μg/ml (~54 μg total protein). A summary of the scheme used for the purification of XPC (p125) protein is shown in Figure 3.9.

3.8. Expression and purification of hHR23B

hHR23B protein expression was induced with 0.4 mg/ml IPTG at 37° C for 3 h (Figure 3.10 A and B) and then purified according to a scheme from Masutani et al (Figure 3.11) (Masutani, 1997). The bacterial lysate was first loaded onto a Q-sepharose (FPLC) column. The hHR23B containing fractions were identified by SDS-PAGE analysis (Figure 3.12 A), dialyzed into 800 mM ammonium sulphate, then loaded onto a Butyl-sepharose column. hHR23B containing fractions were identified by SDS-PAGE (Figure 3.12 B) and loaded directly onto a hydroxyapatite column. Unfortunately, SDS-PAGE analysis showed that most of the protein flowed through the hydroxyapatite column (Figure 3.12 C, lane FT) and so this step was repeated. Again hHR23B failed to bind so fractions from the first hydroxyapatite run were pooled, dialyzed into low salt and loaded onto a mono-Q FPLC column as the final purification step (Figure 3.12 D). The recombinant hHR23B protein cross-reacted with an antibody raised against yeast Rad23 (donated by E. Friedberg), both when purified and in crude cell extracts (Figure 3.10 B lanes 3, 4 and 5).

3.9. Expression of His-hHR23B and antibody production

The non-tagged purification scheme above did not yield very much hHR23B protein and the purification was quite laborious for an expressed protein. It was therefore decided to purify a 6 x His-tagged version of hHR23B. hHR23B protein expression was induced with 0.4 mM IPTG at 37° C for 2.5 h
Figure 3.10. SDS-PAGE and immunoblot analysis of *E.coli* expressed hHR23B. The strain BL21 (DE3) p11D-hHR23B was induced with IPTG, cells lysed and polypeptides separated by electrophoresis through a 10% SDS-PAGE gel. Proteins detected by staining with Coomassie brilliant blue (A) or immunoblotting (B). A. *lane 1* ~200 ng yeast Rad23 protein, *lane 2* ~40 μg BL21 (DE3) p11D-hHR23B cell lysate protein, pre induction, *lane 3* ~80 μg BL21 (DE3) p11D-hHR23B cell lysate protein, post induction, *lane 4* ~150 ng hHR23B protein purified by Q sepharose, butyl sepharose, hydroxyapatite and FPLC mono Q chromatography (5). Numbers indicate positions of Kaleidoscope size markers (BioRad). B. Proteins were transferred to Immobilon-P membrane and probed with Rad23 antiserum (chemiluminescent detection). *lane 1* ~10 ng purified recombinant Rad23 protein, *lane 2* ~8 μg BL21 (DE3) p11D-hHR23B cell lysate protein, pre-induction, *lane 3* ~16 μg BL21 (DE3) p11D-hHR23B cell lysate protein, post induction, *lane 4* ~50 ng partially purified hHR23B, *lane 5* ~20 ng purified hHR23B. Numbers indicate positions of broad range prestained size markers (BioRad).
Figure 3.11. Scheme for the purification of hHR23B (p58) from induced *E. coli* cells.
Figure 3.12. Purification of recombinant hHR23B. Clarified E.coli cell lysate from 1 l induced culture was purified over 4 successive columns. Proteins separated by electrophoresis through 10 % SDS-PAGE gels and stained using Coomassie brilliant blue. Numbers indicate the positions of either broad range prestained or Kaleidoscope markers (BioRad). A. Purification on a 5 ml Q-sepharose column, lane R23, ~200 ng recombinant Rad23 protein (donated by E. Friedberg), lane L, sample of load, lane FT, sample of flow through and fractions from the 50-400 mM NaCl elution gradient. B. Purification on a 1 ml Butyl-sepharose column, fractions from part of the 800-0 mM NH4(SO4)2 elution gradient. C. Purification on a 1 ml Hydroxyapatite column, lane R23, ~200 ng recombinant Rad23 protein (donated by E. Friedberg), lane FT, sample of flow through and fractions from part of the 0-250 mM PO4 elution gradient. D. Purification on a 1 ml FPLC Mono-Q column, fractions from part of the 50-300 mM NaCl elution gradient.
Chapter 3. Proteins and antibodies.

(Figure 3.13 A) and then purified based on a protocol from Masutani et al (Figure 3.14) (Masutani et al., 1997). The crude *E. coli* lysate was purified on a NTA-Ni agarose column and step eluted with buffer containing increasing concentrations of imidazole (Figure 3.13 B). Virtually all of the His-hHR23B was eluted in the 60 mM imidazole fraction and ran as a single band at a similar position to the yeast homologue Rad23 (donated by Errol Friedberg).

About 4.5 mg protein of the protein was rebound to a NTA-Ni agarose column and after washing the matrix was resuspended in 9 ml PBSA to give a 50 % slurry and this was used to immunize two rabbits (RWO30 and RWO31) 7 times over a period of 84 days. On the 94th day the animals were sacrificed, bleedouts collected and tested. Rabbit RWO31 gave the best signal for hHR23B in Western blots, giving only a single band in whole cell extracts. However, when tested against purified XPC protein, RWO31 was found to cross-react with it non-specifically (Figure 3.15 A). The antibody was therefore affinity purified against His-hHR23B antigen, immobilized on Immobilon-P membrane and eluted with glycine buffers of pH 3, 2 and 1. Samples of each eluate were tested by Western blotting against 320 ng purified XPC and 380 ng His-hHR23B. pH 3 eluates were found to contain most of the RWO31 antibody, but these fractions also cross-reacted with purified XPC. pH 2 eluates contained RWO31 antibody specific only for hHR23B and pH 1 washes contained no activity (Figure 3.15 B).

3.10. SDS-PAGE and immunoblot analysis of purified XPC, XPC-hHR23B and His-hHR23B

As part of my project was to determine if XPC (p125) required its co-purifying partner hHR23B for activity we had to be sure that the XPC preparation contained no contaminating hHR23B. 800 ng XPC, 710 ng XPC-hHR23B and 380 ng His-hHR23B were separated by SDS-PAGE and their purity checked by silver staining (Figure 3.16 A). XPC ran as a single polypeptide at the expected
Figure 3.13. SDS-PAGE and immunoblot analysis of E.coli expressed His-hHR23B. The strain FB810-His.hHR23B was induced with IPTG, cells lysed and polypeptides separated by electrophoresis through a 10% SDS-PAGE gel. Proteins detected by staining with Coomassie brilliant blue. A. lane 1 ~40 μg FB810-His.hHR23B cell lysate protein, pre induction, lane 2 ~50 μg FB810-His.hHR23B cell lysate protein, post induction. B. Clarified cell lysate was loaded onto 9 ml Ni-agarose column at a flow rate of 0.4 ml/min, washed with buffer containing 20 mM imidazole and step eluted with 60, 90, 250, 300 and 400 mM imidazole. 2 μl of the load (L), flow through (FT) and 20 μl of each fraction were separated on a 10% SDS-PAGE gel alongside ~200 ng yeast Rad23 (R23) and stained with Coomassie brilliant blue. Numbers indicate positions of broad range prestained size markers (BioRad).
Induced BL21 (DE3) cells

Extract pellet with lysozyme no salt

Freeze-thaw 3x

Adjust to 20 mM imidazole

20 mM imidazole wash

Ni-Agarose

60/90/250 mM imidazole elutions

His-hHR23B Ni-Agarose Raise Antibody

Affinity purify

Figure 3.14. Scheme for the purification of His-hHR23B from induced E. coli cells.
size of ~125 kDa and contaminating proteins were almost undetectable. XPC-hHR23B contained detectable amounts of the XPC (p125) and hHR23B (p58) polypeptides but was less pure containing a major contaminant of ~33 kDa. This may be a breakdown product of the XPC protein as it sometimes is detected in Western blots using the anti-XPC antibody RWO28 (Figures 3.5 A and B and 3.6 A). His-hHR23B ran at a similar position to the hHR23B in the XPC-hHR23B preparation and contaminants were barely detectable.

To further check the purity of these preparations all samples were analyzed by Western blotting. In figure 3.16 B, 320 ng XPC, 280 ng XPC-hHR23B and 304 ng His-hHR23b were separated by SDS-PAGE alongside 50 µg of a whole cell extract deficient in XPC (XP-C) protein (GM2498) and 50 µg of a normal lymphoblastoid whole cell extract (GM0892A) which contains XPC. The proteins were transferred to Immobilon-P membrane and XPC (p125) detected using the RWO28 anti-XPC antibody. As expected XPC (p125) was present in all samples tested except for the XP-C whole cell extract and the His-hHR23B preparation. The XPC deficient extract GM2498 is derived from xeroderma pigmentosum patient number XP9BE which is the identical twin brother of patient XP8BE the donor of cell line GM2249. They therefore share identical XPC mutations (for details see section 3.1).

Similarly, in figure 3.16 C, 320 ng XPC, 280 ng XPC-hHR23B and 304 ng His-hHR23b were separated by SDS-PAGE alongside 50 µg of a whole cell extract deficient in XPC (XP-C) protein (GM2246), 50 µg of a normal lymphoblastoid whole cell extract (GM0892A) and 375 ng yeast Rad23 protein. The proteins were transferred to Immobilon-P membrane and hHR23B (p58) detected using the affinity purified RWO31 (anti-hHR23B) antibody. As expected hHR23B was detected in all samples except the XPC preparation. Unlike the anti-Rad23 antibody used earlier in this study (Figure 3.3 and 3.10 B), RWO31 did not cross-react with the yeast homologue, Rad23, despite the high degree of homology between these two proteins (~34%).
Figure 3.15. Affinity purification of RW031 anti-hHR23B antibody.  
A. Cross-reactivity of crude RW031 antibody. lane 1 320 ng XPC, lane 2 280 ng XPC-hHR23B and lane 3 304 ng His-hHR23B lane 4 50 µg XP-C (GM2246) whole cell extract (wce) and lane 5 50 µg normal (GM0892A) wce were separated by electrophoresis through a 10 % SDS-PAGE gel. Proteins were transferred to Immobilon-P membrane and hHR23B protein detected by probing with RW031 antiserum. B. lane 1 320 ng XPC and lane 2 380 ng His-hHR23B were separated by electrophoresis through 10 % SDS-PAGE gels. Proteins were transferred to Immobilon-P membrane and hHR23B protein detected by probing with samples of pH 3, 2 and 1 elutions of RW031 affinity purified antiserum. Numbers indicate positions of prestained kaleidoscope size markers (BioRad).
Figure 3.16. SDS-PAGE and immunoblot analysis of purified recombinant XPC, XPC-hHR23B and His-hHR23B.  

**A.** lane 1 800 ng XPC, lane 2 710 ng XPC-hHR23B and lane 3 380 ng His-hHR23B were separated by electrophoresis through a 10% SDS-PAGE gel and detected by silver staining (BioRad). 

**B.** lane 1 320 ng XPC, lane 2 280 ng XPC-hHR23B, lane 3 304 ng His-hHR23B, lane 4 50 μg XP-C (GM2498) whole cell extract (wce) and lane 5 50 μg normal (GM0892A) wce were separated by electrophoresis through a 10% SDS-PAGE gel. Proteins were transferred to Immobilon-P membrane and XPC protein detected by probing with RW028 antiserum. 

**C.** lane 1 320 ng XPC, lane 2 280 ng XPC-hHR23B, lane 3 304 ng His-hHR23B, lane 4 50 μg XP-C (GM2246) whole cell extract (wce), lane 5 50 μg normal (GM0892A) wce and lane 6 375 ng Rad23 were separated by electrophoresis through a 10% SDS-PAGE gel. Proteins were transferred to Immobilon-P membrane and hHR23B protein detected by probing with affinity purified RW031 antiserum. Numbers indicate positions of high range prestained size markers (BioRad).
Chapter 4. NER activity of purified recombinant XPC-hHR23B.

4.1. Activity of recombinant XPC-hHR23B complex and XPC in complementation of repair deficient XP-C extracts

XPC-hHR23B complex and XPC (p125 alone) were produced in insect cells infected with recombinant baculovirus carrying the corresponding genes as in sections 3.4-3.7. The activity of these proteins was initially measured by testing their ability to complement an XPC deficient whole cell extract (GM2634) in an in vitro complementation incision assay. This assay uses a DNA substrate containing a single 1,3, intrastrand d(GpTpG) cisplatin-crosslink and dual incision products were detected by direct end-labeling as described previously (Shivji et al., 1999).

50 µg protein of the XPC-deficient extract GM2634 showed no repair activity (Figure 4.1. A) therefore dual incision of the platinum adduct is XPC-dependent. 50 µg HeLa S3 whole cell extract is included for comparison. Recombinant XPC-hHR23B was active in this assay (Figure 4.1. A). 20 ng XPC-hHR23B gave a high level of complementation of the XP-C deficient extract. 40 ng XPC-hHR23B also showed complementation activity but at a level about 30 % less than when the reaction was carried out using 20 ng. This suggested that high amounts of XPC-hHR23B can actually inhibit the dual incision reaction. 100 ng of XPC-hHR23B in buffer containing 1.5 M NaCl (direct eluate from the ss DNA cellulose column) inhibited dual incision to the background level indicating that the reaction is also salt-sensitive. The dual incision activity of 25 µg GM2634 was also successfully complemented using 25 µg AG08802 XP-G deficient extract, as a positive control.
Chapter 4. NER activity of XPC-hHR23B.

Figure 4.1. Activity of recombinant XPC-hHR23B complex and XPC in the complementation of an XP-C deficient extract. Reactions each contained 50 ng DNA substrate containing a single cisplatin lesion and were incubated at 30° C for 30 min. Oligonucleotides excised during repair were detected by annealing to 5 ng of an oligonucleotide complementary to this region which contained a 5'-GGGG overhang. Excised oligonucleotides carrying the platinum adduct were labeled by a Sequenase reaction using [32P]dCTP, separated on a denaturing 14 % polyacrylamide gel and visualized by autoradiography. A. Reactions contained 50 µg XP-C deficient whole cell extract (wce) GM2634 (except lane 1, 50 µg HeLa wce and lane 6, 25 µg GM2634 wce complemented with 25 µg XP-G deficient (AG08802) extract and were complemented with the indicated amount of recombinant XPC-hHR23B complex (in 0.3 M NaCl). * indicates XPC-hHR23B in 1.5 M NaCl direct from the ssDNA cellulose column elution. B. Reactions contained 50 µg XP-C deficient whole cell extract (wce) GM2634 (except lane 1, 50 µg HeLa wce) and were complemented with the indicated amount of recombinant XPC in 0.3 M salt.
XPC (p125) alone was also active in complementation of the same XPC-deficient extract (Figure 4.1. B) indicating that recombinant XPC can successfully associate with the native hHR23B present in this extract to give a repair proficient complex. 16 ng XPC gave a high level of complementation but higher amounts again appear to be inhibitory to the reaction. It appears the inhibition is mediated by the XPC (p125) subunit of the XPC-hHR23B complex.

### 4.2. Repair activity of recombinant XPC-hHR23B complex in a fully reconstituted in vitro incision assay

I then went on to test the activity of recombinant XPC-hHR23B in a fully reconstituted incision assay using the same DNA substrate. Reactions contained XPA, RPA, TFIH, XPG, ERCC1-XPB and between 30 and 180 fmol recombinant XPC-hHR23B. A reaction supplemented with 35 fmol XPC-hHR23B purified from HeLa cells is included for comparison. Dual incision was dependent on the presence of XPC-hHR23B complex (Figure 4.2. A, lanes 2-7). The repair reaction was maximal supplemented with around 50-100 fmol of the recombinant complex and again higher amounts of XPC-hHR23B (above 150 fmol) gave a reduction in dual incision activity. The activity of recombinant XPC-hHR23B in this assay appeared to be similar to that of the native complex purified from HeLa cells. 35 fmol HeLa XPC-hHR23B gave a similar repair signal to the 30 and 50 fmol recombinant complex lanes (Figure 4.2. A, lanes 2-4). The quantitation presented in Figure 4.2. B reveals that the maximal release of cisplatin containing oligonucleotide was about 1 fmol. The reactions each contain approximately 11 fmol of lesion therefore about 10 % of the substrates are repaired during the 90 min incubation at 30 °C.
Figure 4.2. Activity of recombinant XPC-hHR23B in the reconstituted repair system.

A. Reactions contained a DNA substrate containing a single cisplatin lesion, XPA, RPA, TFIIH, ERCC1-XPF, XPG and the indicated amounts of baculovirus expressed XPC-hHR23B complex or 35 fmol XPC-hHR23B complex purified from HeLa cells. Reactions were incubated at 30°C for 90 min and oligonucleotides excised during repair were detected by annealing to 5 ng of an oligonucleotide complementary to this region which contained a 5'-GGGG overhang. Excised oligonucleotides carrying the platinum adduct were labeled by a Sequenase reaction using [32P]dCTP, separated on a denaturing 14% polyacrylamide gel and visualized by autoradiography. Cl and C2 are quantitation controls derived from annealing 5 ng of the 5'-GGGG oligonucleotide to 5 and 1 pg its complement respectively and labeling as above.

B. Quantitation of the data in part A.
4.3. Stimulation of XPC activity by hHR23B in a fully reconstituted in vitro incision assay

XPC protein in cells is found tightly bound to a human homologue of the yeast Rad23 protein, usually hHR23B but also less frequently to hHR23A. To test whether XPC (p125) needs the hHR23B partner for dual incision activity, the two proteins were expressed and purified separately. XPC from recombinant baculovirus infected Sf9 insect cells and hHR23B (-/+ His tag) from E. coli. The XPC preparation was free of hHR23B, as determined by immunoblotting with an anti-hHR23B antibody (Figure 3.16. C). As a further check against possible contamination with insect Rad23 homologue we used an antibody against yeast Rad23 (S. Reed and E. Friedberg, unpublished) that cross-reacts with human hHR23B and Rad23 homologues in other species (Figure 3.3.). No cross-reacting material was found in the XPC preparation (data not shown).

Reactions contained a fixed amount of XPC protein (150 fmol) and were carried out in the absence of hHR23B or in the presence of increasing amounts of this protein (1-92 fmol). XPC without hHR23B had little repair activity in this assay, giving dual incision products marginally above the background level seen in complete XPC dropout reaction (Figure 4.3. A, lanes 1 and 3). Addition of hHR23B greatly stimulated the reactions (by at least 10-fold) with repair activity reaching 80 % of its maximum after adding 50 fmol of hHR23B, a similar amount to the amount of preformed XPC-hHR23B complex that gave maximum repair activity in figure 4.2. A. Addition of higher amounts of hHR23B did not significantly increase the repair activity of XPC (data not shown) which remained at a similar level to the XPC-hHR23B purified from HeLa cells (Figure 4.3. A, lane 1). In the absence of XPC, addition of hHR23B gave no effect showing that the stimulation is mediated through an interaction of hHR23B with XPC.
Chapter 4. NER activity of XPC-hHR23B.

Figure 4.3. Stimulation of rXPC activity by rhHR23B in the reconstituted repair system.  
A. Reactions contained a DNA substrate containing a single cisplatin lesion, XPA, RPA, TFIIH, ERCC1-XPF, XPG and -/+ 151 fmol baculovirus expressed XPC and the indicated amounts of recombinant hHR23B (E. coli). For comparison 35 fmol XPC-hHR23B complex purified from Hela cells was included. Reactions were incubated at 30°C for 90 min and oligonucleotides excised during repair were detected by annealing to 5 ng of an oligonucleotide complementary to this region which contained a 5' -GGGG overhang. Excised oligonucleotides carrying the platinum adduct were labeled by a Sequenase reaction using [32P]dCTP, separated on a denaturing 14 % polyacrylamide gel and visualized by autoradiography. C1 and C2 are quantitation controls derived from annealing 5 ng of the 5' -GGGG oligonucleotide to 5 and 1 pg of its complement respectively and labeling as above. B. Quantitation of the data in part A.
Figure 4.4. Stimulation of rXPC activity by a fragment of hHR23B in the reconstituted repair system. A. Reactions contained a DNA substrate containing a single cisplatin lesion, XPA, RPA, TFIIH, ERCC1-XPF, XPG and -/+ 15 ng baculovirus expressed XPC and lane 2 no hHR23B, lane 3 38 ng recombinant hHR23B (E.coli) or lane 4 44 ng hHR23B fragment (amino acids 277-332). 20 ng baculovirus expressed rXPC-hHR23B complex is included for comparison (lane 1). Reactions were incubated at 30° C for 90 min and oligonucleotides excised during repair were detected by annealing to 5 ng of an oligonucleotide complementary to this region which contained a 5' -GGGG overhang. Excised oligonucleotides carrying the platinum adduct were labeled by a Sequenase reaction using [32P]dCTP, separated on a denaturing 14 % polyacrylamide gel and visualized by autoradiography.
XPC activity in this reconstituted system could be stimulated by just a fragment of hHR23B. This was kindly donated by F. Hanaoka and consists of amino acids 277-332 containing only the XPC-interactive domain of this protein. In figure 4.4. 15 ng XPC alone had no repair activity but on addition of either 38 ng hHR23B or 44 ng hHR23B 277-332, dual incision products are observed. This is consistent with the results observed by Masutani et al. who defined the XPC interactive domain of hHR23B and produced the hHR23B 277-332 fragment (Masutani et al., 1997). 20 ng recombinant XPC-hHR23B preformed complex was included for comparison and gave higher repair activity than either XPC plus hHR23B or XPC plus hHR23B 277-332.

XPC activity can also be stimulated by the other human homologue of yeast Rad23, hHR23A with which it shows extensive sequence homology (57 % identity, 76 % similarity). The XPC interactive domain is conserved in hHR23A and hHR23B but is also highly retained in the yeast Rad23 protein and thus may also mediate the interaction of Rad23 with Rad4. Therefore I decided to test whether yeast Rad23 could stimulate XPC activity in the same way as its human homologues, hHR23B and hHR23A. 22 fmol XPC was tested alone or in combination with 104 fmol hHR23B or 186/279 fmol yeast Rad23 (kindly donated by E. Friedberg). Again XPC alone showed little repair activity (Figure 4.5. A and B) and was stimulated at least 4-fold by the addition of hHR23B. There is an indication that Rad23 could stimulate XPC as addition of 186 fmol yeast Rad23 weakly increased the activity of XPC (about 2-fold) but inclusion of 279 fmol Rad23 showed no effect. Further titrations of Rad23 protein are necessary for any meaningful comparison of its activity with hHR23B. However, from the result presented here, it seems that despite the relatively high homology between Rad23 and hHR23B (~34 % identity overall) and highly conserved XPC/Rad4 interactive domain the stimulation achieved using this protein was much less than has been observed with hHR23B (Figure 4.3. A and B).

To investigate the residual activity of XPC in the absence of hHR23B, large amounts of XPC (0.25-2 pmol) were used in reconstituted repair reactions and
Figure 4.5. Stimulation of rXPC activity by rhHR23B and rRad23 in the reconstituted repair system. A. Reactions contained a DNA substrate containing a single cisplatin lesion, XPA, RPA, TFIH, ERCC1-XPF, XPG, 22 fmol baculovirus expressed XPC and lane 1 no hHR23B, lane 2 104 fmol hHR23B and the indicated amounts of recombinant yeast rRad23 (E.coli). Reactions were incubated at 30°C for 90 min and oligonucleotides excised during repair were detected by annealing to 5 ng of an oligonucleotide complementary to this region which contained a 5’-GGGG overhang. Excised oligonucleotides carrying the platinum adduct were labeled by a Sequenase reaction using [32P]dCTP, separated on a denaturing 14% polyacrylamide gel and visualized by autoradiography. C1 and C2 are quantitation controls derived from annealing 5 ng of the 5’-GGGG oligonucleotide to 5 and 1 pg of its complement respectively and labeling as above. B. Quantitation of the data in part A.
Figure 4.6. Large amounts of rXPC have repair activity without rhHR23B. A. Reconstituted repair reactions contained a DNA substrate containing a single cisplatin lesion, XPA, RPA, TFIH, ERCC1-XPF, XPG and the indicated amounts of baculovirus expressed XPC-/+ 2.2 pmol recombinant hHR23B (E.coli). Reactions were incubated at 30°C for 90 min and oligonucleotides excised during repair were detected by annealing to 5 ng of an oligonucleotide complementary to this region which contained a 5' -GGGG overhang. Excised oligonucleotides carrying the platinum adduct were labeled by a Sequenase reaction using [³²P]dCTP, separated on a denaturing 14 % polyacrylamide gel and visualized by autoradiography. C1 and C2 are quantitation controls derived from annealing 5 ng of the 5' -GGGG oligonucleotide to 5 and 1 pg of its complement respectively and labeling as above. B. Quantitation of the data in part A.
compared with ∆X activity in the presence of a fixed excess amount of hHR23B (2.2 pmol). About 2 pmol XPC gave clear repair activity in the absence of hHR23B, but the reaction efficiency of the maximum signal obtained with XPC in the presence of hHR23B (Figure 4.6. A and B). This amount of XPC is about 40-fold molar excess over the amount of XPC-hHR23B complex required for the same level of repair activity (Figure 4.2). 2 pmol XPC in the presence of 2.2 pmol hHR23B gave lower repair activity than the maximum, showing again that large amounts of the XPC-hHR23B complex can actually inhibit the repair reaction.

4.4. Discussion

In this chapter I wanted to address two main questions:

- Are recombinant XPC-hHR23B and XPC (p125) active in repair and if so what is the optimum amount to use?

- How much stimulation is affected by hHR23B on XPC in a reconstituted system?

Here, I found that both the XPC-hHR23B complex and XPC (p125) alone are active in the complementation of XPC-deficient cell extracts and in a fully reconstituted system. Both used a defined substrate containing a single platinum adduct. The amount XPC-hHR23B had to be carefully titrated as large amounts (in excess of about 100 fmol in reconstitutions or above ~30 ng in complementations) appear inhibitory to NER. XPC activity in fully reconstituted NER was stimulated up to 10-fold by the addition of hHR23B protein. Very little repair activity was observed without hHR23B unless a huge molar excess (40-fold) of XPC was used. Activity of XPC (p125) may also be stimulated by addition of a fragment of hHR23B (amino acids 277-332) and possibly by the yeast homologue, Rad23.
The involvement of hHR23A and hHR23B in NER in human cells has been the subject of recent study, with varying results, as has the study of the yeast homologue, Rad23. Two previous studies investigated whether the hHR23B partner was needed for XPC activity.

Sugasawa et al, looked at the repair of UV irradiated SV40 minichromosomes and naked plasmid DNA treated with either UV or N-acetoxy-2-acetylfluorene (AAF) in a repair synthesis assay using cell extracts depleted of hHR23B (Sugasawa et al., 1996). In all situations a ~2-fold stimulation of repair was observed on addition of XPC alone but further ~2-fold enhancement of the repair signal was observed upon co-addition of hHR23B. Further work by the same group showed that this stimulation could also be achieved by the second human homologue of yeast Rad23, hHR23A (Sugasawa et al., 1997) and by a small fragment of hHR23B (amino acids 277-332) which is highly conserved in both hHR23A and Rad23 (Masutani et al., 1997).

In contrast, Reardon et al looked in a reconstituted system at the repair of both a cyclobutane pyrimidine (T<>T) dimer and a substrate containing a cholesterol moiety attached to the DNA backbone in place of a base (Reardon et al., 1996). In this system XPC alone was sufficient for repair activity and no effect was seen on addition of hHR23B. The level of repair of the cholesterol moiety appeared higher with 2.5 ng XPC alone than with 5 ng XPC or 7.5 ng XPC-hHR23B which is very different from the data presented here. These results however seem fairly variable as in another panel of the same figure overall repair of the same adduct with similar amounts of protein (2 ng XPC with excess hHR23B) gave very much lower dual incision products (at least 10-fold). The activity of XPC alone was comparable to that of XPC in the presence of hHR23B and not higher as shown earlier in the same figure. The cyclobutane pyrimidine dimer was repaired very inefficiently with either XPC alone or with XPC-hHR23B, to a much lower level than had been seen with the cholesterol moiety in some experiments. It is possible that some other inhibitory factor is present in one of the protein preparations, keeping the overall repair activity low.
There is also the possibility of contamination of some of those workers' protein preparations with hHR23B, as TFIIH, XPG and XPF-ERCC1 were purified from HeLa cells and not recombinant. Contaminating hHR23B could mask any stimulatory effect observed on addition of this protein as even very small sub-equimolar amounts of hHR23B can stimulate XPC activity (Figure 4.3. and data not shown). Small amounts of these preparations were immunoblotted with an antibody against hHR23B but it is plausible that hHR23B could be present but below the limits of detection. In yeast, the homologue of hHR23B, Rad23 is known to interact with TFIIH and so the TFIIH preparation could contain low levels of the hHR23B protein. The reconstituted system used in my studies was much more defined and all proteins except TFIIH were in recombinant form and so much less likely to contain contaminating hHR23B activity. The results presented in this chapter are more in agreement with the work of Sugasawa et al than with Reardon et al. However, they show a much larger effect of hHR23B than has been observed previously. One reason for this may be that the non specific background in the dual incision assay used here is less than the background in the repair synthesis assays. Possible roles for hHR23B in repair are discussed in chapter 8.
Chapter 5. Binding of XPC-hHR23B to UV damaged DNA.

5.1. Binding of XPC-hHR23B to non damaged and UV damaged DNA

XPC-hHR23B is a candidate damage recognition factor in NER for several reasons. Firstly, this is the only core NER factor not required for transcription-coupled repair – a specialized mode of NER specific for repairing the transcribed strands of active genes (Venema et al., 1990). Transcription-coupled repair is probably initiated when RNA polymerase II becomes stalled at a damaged site in DNA. This stalled polymerase could act as a DNA damage signal in itself therefore obviating the need for a further NER damage recognition factor. Secondly, XPC-hHR23B binds strongly to single-stranded DNA cellulose indicating it has some DNA binding activity (Masutani et al., 1994; Shivji et al., 1994).

I wanted to quantify the affinity of XPC-hHR23B for UV damaged and non damaged DNA and explore the association and dissociation kinetics of the process. In order to do this an electrophoretic mobility-shift assay was established, to allow quantification of DNA binding. I used a 136 bp restriction fragment, terminally labeled with $^{32}$P and filled to give blunt ends. Blunt ends were chosen to avoid any possible complications arising from binding of XPC-hHR3B to single-stranded tails. The labeled fragment, designated “probe” was then either left undamaged or UV-irradiated to a final dose of 1.25 or 2.5 kJ/m$^2$ at a dose rate of 10 J/m$^2$/sec. In the absence of competitor DNA we observed only retention of the labeled DNA in the wells of the gel, presumably in large DNA-protein aggregates (Figure 6.9. A). With inclusion of 10 to 100 ng of competitor poly (dI•dC) in reaction mixtures containing 1 ng labeled probe, a
Figure 5.1. Binding of XPC-hHR23B to non damaged and UV damaged DNA. A. A 136 bp duplex (1 ng) with no damage or UV damaged at a dose of 1.25 kJ/m² or 2.5 kJ/m² was incubated at 30°C for 1 h with the indicated amount of XPC-hHR23B. Free and bound fractions were separated on a non denaturing 5% polyacrylamide gel. M = DNA size markers. B. Quantitative analysis of the data in part A.
defined band shift could be observed. Reactions were incubated at 30° C for 1 h prior to loading. XPC-hHR23B band shifts were strongly observed on 1.25 or 2.5 kJ/m² UV damaged DNA but barely detectable on the non-damaged probe (Figure 5.1. A and B). Increasing amounts of the probe were shifted with higher UV dose and also with higher amounts of protein. At the high UV doses and high XPC-hHR23B concentrations, slower migrating shifted species were formed, possibly due to XPC-hHR23B binding to multiple sites of damage in one probe molecule. For subsequent experiments a UV dose of 2.5 kJ/m² was chosen, which yields an estimated 3 cyclobutane pyrimidine dimers and 1 (6-4) photoproduct per 136 bp probe.

5.2. *Apparent equilibrium binding constants* (*k_D*) *for XPC-hHR23B binding to UV damaged DNA*

As all the assays were performed in the presence of 100 ng poly (dI•dC) competitor DNA an accurate equilibrium binding constant (*k_D*) could not be determined. However for comparative purposes an apparent *k_D* was estimated for XPC-hHR23B binding to the 2.5 kJ/m² UV damaged probe at different temperatures. Increasing amounts of XPC-hHR23B were incubated with 1 ng probe at either 0, 30 or 37° C and the band shifted forms quantified with a phosphorimager to determine the point at which 50 % saturation was achieved. At 0° C (Figure 5.2. A and B) approximately 14 % of the probe was shifted at the maximum point and 50 % saturation was achieved at about 110 nM XPC-hHR23B (~155 ng). The equilibrium binding showed some temperature dependence and at 30° C (Figure 5.3. A and B) approximately 36 % of the probe was shifted at the maximum point and 50 % saturation was achieved at about 95 nM XPC-hHR23B (~140 ng). At 37° C, again a slightly higher proportion of the probe was shifted with approximately 38 % shifted at the maximum point. Here, 50 % saturation was achieved with about 60 nM XPC-hHR23B (~80 ng). It appears that at higher temperatures, XPC-hHR23B-DNA complexes reach equilibrium at lower XPC-hHR23B concentrations and the maximum level of
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Figure 5.2. Apparent $k_D$ for XPC-hHR23B binding to 2.5 kJ/m$^2$ UV damaged duplex DNA at 0° C. A. The indicated amounts of XPC-hHR23B were incubated for 1 h at 0° C with 1 ng 136 bp 2.5 kJ/m$^2$ UV damaged probe. Free and bound fractions were separated on a non denaturing 5% polyacrylamide gel. M = DNA size markers. B. Quantitative analysis of the data in part A.
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Figure 5.3. Apparent kD for XPC-hHR23B binding to 2.5 kJ/m² UV damaged duplex DNA at 30°C. A. The indicated amounts of XPC-hHR23B were incubated for 1 h at 30°C with 1 ng 136 bp 2.5 kJ/m² UV damaged probe. Free and bound fractions were separated on a non-denaturing 5% polyacrylamide gel. M = DNA size markers. B. Quantitative analysis of the data in part A.
Figure 5.4. Apparent $k_D$ for XPC-hHR23B binding to 2.5 kJ/m$^2$ UV damaged duplex DNA at 37° C. A. The indicated amounts of XPC-hHR23B were incubated for 1 h at 37° C with 1 ng 136 bp 2.5 kJ/m$^2$ UV damaged probe. Free and bound fractions were separated on a non denaturing 5% polyacrylamide gel. B. Quantitative analysis of the data in part A.
binding is higher at 30 or 37° C than at 0° C. In subsequent experiments 50 or 100 ng XPC-hHR23B was used and the incubation temperature was generally 30° C as this gives a good level of band shifted material.

5.3. Association rate of XPC-hHR23B to UV damaged DNA

To measure the association rate of XPC-hHR23B to the UV damaged probe the protein complex was incubated with 1 ng probe at 30° C for times between 15 seconds and 15 minutes (Figure 5.5. A and B). The amount of band shifted material was quantified as previously and it was observed that at this temperature 50% saturation had already been achieved by the earliest time point showing that the association rate of XPC-hHR23B to UV damaged DNA at 30° C is very rapid. In attempt to get a more accurate measurement of association rate a similar experiment was carried out at the lower temperature of 0° C (Figure 5.6. A and B). At this temperature association rate was slow enough to be accurately measured but 50% saturation was still achieved within 10 min.

5.4. Dissociation rate of XPC-hHR23B from UV damaged DNA

To determine the dissociation rate of XPC-hHR23B from UV damaged DNA protein-DNA complexes were allowed to form with 1 ng probe and 50 ng XPC-hHR23B (in the presence of 100 ng poly (dI•dC) competitor) at 30° C for 1 h. The reactions were then challenged by adding in 200 ng excess non damaged or 2.5 kJ/m² UV damaged plasmid DNA and incubated at 30, 37 or 42° C for 3 or 6 h (Figure 5.7. A and B-D).

At 30° C only about 10% of the XPC-hHR23B-DNA complexes had dissociated using excess undamaged plasmid DNA at the 3 h point and at 6 h this level had
Figure 5.5. Association of 50 ng XPC-hHR23B to 2.5 kJ/m² UV damaged duplex DNA at 30°C. A. 1 ng 136 bp 2.5 kJ/m² UV damaged probe was incubated with 50 ng XPC-hHR23B for the indicated time at 30°C, lane 1 no protein. Free and bound fractions were separated on a non-denaturing 5% polyacrylamide gel. M = DNA size markers. B. Quantitative analysis of the data in part A.
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Figure 5.6. Association of 50 ng XPC-hHR23B to 2.5 kJ/m² UV damaged duplex DNA at 0°C. A. 1 ng 136 bp 2.5 kJ/m² UV damaged probe was incubated with 50 ng XPC-hHR23B for the indicated time at 0°C. lane 1 no protein. Free and bound fractions were separated on a non denaturing 5% polyacrylamide gel. M = DNA size markers. B. Quantitative analysis of the data in part A.
dropped no further. The apparent increase in the amount of DNA bound at the 6 h point is probably not significant and just demonstrates the variability seen in this experiment. Variability is observed as XPC-hHR23B binding to UV damage is very stable and is difficult to dissociate without an increase in temperature. When using UV damaged plasmid DNA, at the 3 h point the amount of XPC-hHR23B-DNA complexes had dropped to about 50 % of the original amount, but again at the 6 h point the level of complexes had dropped no further. At 37 °C with non damaged competitor 75 % of the XPC-hHR23B-DNA complexes remained at the 3 h point, dropping to about 55 % at 6 h. With UV damaged competitor, after 3 h only about 15 % of the original protein-DNA complexes were observed and this level remained the same at 6 h post challenge. At 42 °C with non damaged competitor about 65 % of the original XPC-hHR23B-DNA complexes remained at the 3 h point and by 6 h post challenge less than 10 % of the complexes remained. With UV damaged competitor, at the 3 h point more than 90 % of the original protein-DNA complexes had already dissociated and the level of bound material remained the same at 6 h. From this experiment alone it can be seen that XPC-hHR23B distinguishes between non and UV damaged DNA and that once bound to UV damaged sites the complexes formed are stable and relatively difficult to dissociate despite addition of a large excess of UV damaged competitor DNA.

To attempt to find a more accurate measurement of dissociation rate of XPC-hHR23B-DNA complexes a further, more detailed dissociation time course was carried out over a 4 h period at 37° C. Again, XPC-hHR23B was allowed to reach equilibrium with 1 ng UV damaged probe and then was challenged with 200 ng non or UV damaged plasmid DNA. 50 % of the XPC-hHR23B-DNA complexes were dissociated by about 90 min when challenged with UV damaged competitor but when challenged with excess non damaged DNA about 55 % of the complexes remained at the 4 h point. This again emphasizes the stability of XPC-hHR23B’s interaction with UV damaged DNA and is in agreement with the previous dissociation experiment. The true rate of
Figure 5.7. Dissociation of 50 ng XPC-hHR23B from 2.5 kJ/m² UV damaged duplex DNA at 30, 37 and 42°C. A. 1 ng 136 bp 2.5 kJ/m² UV damaged probe was incubated with 50 ng XPC-hHR23B for 1 h at 30°C, lane 1 no protein, lane 2 1 h association. Dissociation was attempted by the addition of excess (200 ng) non damaged or 2.5 kJ/m² UV damaged closed circular plasmid DNA and incubation for the indicated time at 30, 37 or 42°C. Free and bound fractions were separated on a non denaturing 5% polyacrylamide gel. M = DNA size markers. B, C, D. Quantitative analysis of the 30, 37 and 42°C data in part A respectively.
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Figure 5.8. Dissociation of 50 ng XPC-hHR23B from 2.5 kJ/m$^2$ UV damaged duplex DNA at 37° C.  

A. 1 ng 136 bp 2.5 kJ/m$^2$ UV damaged probe was incubated with 50 ng XPC-hHR23B for 1 h at 30° C, lane 1 no protein, lane 2 1 h association. Dissociation was attempted by the addition of excess (200 ng) non damaged or 2.5 kJ/m$^2$ UV damaged closed circular plasmid DNA and incubation for the indicated time at 37° C. Free and bound fractions were separated on a non denaturing 5 % polyacrylamide gel. M = DNA size markers. 

B. Quantitative analysis of the data in part A.
dissociation is probably close to that seen with excess UV damaged DNA as this should act as a sink for XPC-hHR23B's UV binding activity. With excess non-damaged DNA in the reaction what we observe is likely to be a result of continual dissociation from the competitor and reassociation with the probe.

5.5. Binding of XPC (p125) and hHR23B (p58) to non and UV damaged DNA

A question of interest is in which component of the XPC-hHR23B complex does the DNA binding activity reside. Electrophoretic mobility-shift assays were used to test the binding of XPC-hHR23B complex and XPC (p125) and hHR23B individually and in combination (Figure 5.9. A). The indicated proteins were preincubated at 30° C for 15 min to allow protein interactions to occur and then 1 ng of labeled DNA probe UV irradiated to a total dose of 0, 2.5, 5 or 10 kJ/m² added and incubation continued for a further hour. UV dose-dependent binding was observed as expected for XPC-hHR23B complex. There was also damage-dependent binding with XPC (p125) alone. However, the intensity of bound complexes observed with XPC (p125) alone and in combination with hHR23B was about 10-fold lower than for the pre-formed XPC-hHR23B complex. No binding was observed for hHR23B alone. As the high DNA binding activity of the complex could not be reconstituted from the individual components, it is difficult to be certain that XPC (p125) alone really does have a lower affinity for UV damaged DNA than when complexed to hHR23B.

Figure 5.9. B. shows an attempt to supershift XPC-hHR23B-DNA complexes using an antibody against yeast Rad23 which also strongly recognizes the human homologue hHR23B. As negative controls, lanes using XPC alone were included and also unrelated antibody (against XPF) was used to attempt to show specificity of any supershifted complexes. No supershifts were observed in any case but other unexpected observations were made. XPC-hHR23B in
Figure 5.9. Binding of XPC-hHR23B complex, hHR23B and XPC to non damaged and UV damaged DNA. 

A. Specified proteins (lane 1 no protein) were preincubated for 15 min at 30°C then 1 ng 136 bp duplex with no damage or UV damaged at a dose of 2.5, 5 or 10 kJ/m² was added and incubation continued for 1 h. Free and bound fractions were separated on a non denaturing 5% polyacrylamide gel. 

B. A 136 bp duplex (1 ng) with no damage or UV damaged at a dose of 5 kJ/m² was incubated at 30°C for 30 min with the indicated amount of XPC-hHR23B (lane 1 no protein) or XPC then 1 μl anti-Rad23 (2) or anti-XPF (3) serum added where shown and incubation continued for a further 30 min. Free and bound fractions were separated on a non denaturing 5% polyacrylamide gel. M = DNA size markers.
combination with either of the two antisera gave a weak shift with non damaged probe whereas XPC-hHR23B alone did not. The intensity of XPC-hHR23B-UV DNA complexes also increased with addition of either antibody. Similarly, XPC (p125) alone did not bind the non damaged probe but in combination with either antibody a shift was observed. A weak shift was observed for XPC (p125) incubated with UV damaged probe which was also increased in the presence of serum. In contrast to XPC-hHR23B complex, binding of the XPC (p125) component to either non or UV damaged probe was quite similar in the presence of either antibody. It appears that there may be a factor present in antisera which stabilizes both XPC and XPC-hHR23B allowing them to interact more readily with DNA – whether damaged or not. This effect appears more pronounced with XPC alone as both non and UV-irradiated probe were bound to equal extents and the binding to non damaged DNA in the presence of sera was actually about 3-fold higher than the binding of XPC-hHR23B to the same non damaged probe.

5.6. Discussion

In this chapter I have looked quantitatively at the binding of XPC-hHR23B to UV damaged DNA using electrophoretic mobility-shift assays. I found XPC-hHR23B binds specifically to a 136 bp UV damaged DNA fragment. Under the conditions used here (100 ng poly (dI•dC) competitor), very little band shifted material was observed using a non damaged DNA probe. Damage binding was both UV dose and protein concentration dependent. When either variable was increased a higher proportion of XPC-hHR23B-DNA complexes was observed and new, slower migrating species were seen. These probably represent two or more XPC-hHR23B complexes binding independently to two or more damaged sites in one probe molecule. At 30° C, equilibrium binding reaches 50 % saturation at an XPC-hHR23B concentration of about 95 nM. Equilibrium binding showed modest temperature dependence. Higher temperatures lead to the 50 % saturation point being achieved at lower protein
concentrations and an increase in the maximum level of bound material. Association and dissociation rates from UV damage were also temperature dependent. Association was too rapid to be accurately measured at 30° C and so instead was measured on ice. Even at this temperature the 50 % saturation point was achieved within 10 minutes of addition of XPC-hHR23B to the reaction. XPC-hHR23B-damaged DNA complexes are stable and difficult to dissociate, even in the presence of a large excess of non or UV damaged competitor DNA. If the temperature was raised to 37 ° C, dissociation rate could be measured and after addition of excess UV-irradiated competitor DNA, 50 % was dissociated after about 2 h. With non damaged competitor DNA at this temperature, 50 % dissociation still wasn’t achieved 4 h, post challenge. In this situation there is the complication that because of weak binding to non damaged DNA, reassociation with the damaged probe can occur. A probable explanation for these results is that association with both UV and non damaged DNA is rapid but dissociation rate of XPC-hHR23B from UV damaged DNA is much slower than from non damaged DNA.

The first time DNA binding activity of XPC-hHR23B was directly observed was in 1996 by Reardon et al using electrophoretic mobility-shift assays (Reardon et al., 1996). They reported that both XPC-hHR23B and XPC alone had a high affinity for a 60 bp duplex substrate and that this affinity was only slightly increased (~1.5 times higher) on UV-irradiation of the probe. These experiments were carried out with no competing DNA in the reaction mixtures and most of the probes were not completely double-stranded but contained a small 4-5 bp overhang at either end. They also showed binding of XPC-hHR23B to single-stranded oligonucleotides and their “double-stranded” probes also contained contaminating single-stranded oligonucleotide. In 1999 the same group also showed electrophoretic mobility-shifts with XPA, RPA and XPC on both a single (6-4) photoproduct and on non damaged DNA (Wakasugi and Sancar, 1999). Again, no competing DNA was included in their reaction mixtures. They also found no damage specific footprint around a single (6-4) photoproduct for XPC-hHR23B, XPA or RPA or any combinations of these factors.
In 1998 Sugasawa et al looked at the DNA binding activity of XPC-hHR23B by a type of immunoprecipitation pull down assay (Sugasawa et al., 1998). Here, the complex was incubated with a mixture of two differently sized plasmids (200 ng of each) and bound complexes immunoprecipitated using an antibody to XPC protein. If neither plasmid was damaged both were present to equal extents in the XPC-bound fraction, but if one was damaged with UV or AAF only the damaged DNA was present. This showed the selectivity of XPC-hHR23B for damaged over non damaged DNA and the importance of titrating out the non specific DNA binding activity of the complex by inclusion of non damaged competitor. If one of the plasmids was damaged with cisplatin about 80 % of the DNA present in the bound fraction was cisplatin damaged but this fraction also contained some non damaged DNA. This suggested that cisplatin damaged DNA is not as efficiently bound by XPC-hHR23B as UV or AAF, although it was not commented on in the manuscript. The pull-down method is qualitative and didn’t result in measurements of the relative affinity of XPC complex for damaged DNA. In the same paper Sugasawa et al also directly examined the binding of XPC-hHR23B to a single (6-4) photoproduct by DNase I footprinting. They found a region covering approximately 30 bp was protected on both the damaged and non damaged strands. No protection was observed when a control fragment containing no damage was used. This is the only stable footprint ever to be observed for any NER protein.

In 1998, two papers were published regarding the damage-specific binding of the yeast NER complex Rad4-Rad23 – the presumed homologue of XPC-hHR23B. Guzder et al used DNA mobility-shift assays to demonstrate UV-specific binding of Rad4-Rad23 and that this binding appeared specific for (6-4) photoproducts as it was not diminished on photoreactivation of the UV-irradiated probe (Guzder et al., 1998). No binding was observed on non damaged DNA and again 100 ng of non damaged double-stranded DNA was included in their reaction mixtures. Jansen et al observed UV and AAF-specific binding by Rad4-Rad23 on electrophoretic mobility-shift assay and very low binding of this complex to non damaged DNA (Jansen et al., 1998). Their reactions contained 10-100 ng poly (dI•dC) as non damaged competing DNA.
Although the reports of Sugasawa, Guzder and Jansen et al included no quantitative data or measurements of association and dissociation the results presented here are in qualitative agreement with their general conclusion that XPC-hHR23B and Rad4-Rad23 show preferential binding to UV damaged DNA. It seems the lack of preferential binding to damaged DNA observed by the Sancar group could be adequately explained by the consistent lack of inclusion of competing non damaged DNA in their reaction mixtures. Thus the specific binding to DNA damage is masked by the general ability of the XPC-hHR23B to interact and bind to DNA, whether damaged or not. It is also possible that even weakly-bound protein-DNA complexes could be stabilized when loaded on a polyacrylamide gel and result in a mobility-shift. The presence of contaminating single-stranded DNA in many of the substrate preparations and the fact that their “double-stranded” substrates are in fact not completely double-stranded also complicates the interpretation of the results of Reardon et al and Wakasugi et al. They themselves showed XPC-hHR23B had a similarly high preference for single-stranded DNA. The binding observed with non damaged “double-stranded” preparations is probably a mixture of XPC-hHR23B interacting with double and single-stranded species. There is a consistent reduction in the amount of free faster migrating single-stranded DNA as well as a reduction in free double-stranded probe with increasing XPC-hHR23B concentration. This is concurrent with an increase in the proportion of protein bound species – which appear to migrate at similar gel positions independent of the whether the probe is single or double-stranded.

Under our assay conditions, no stable DNA binding was found for XPA and/or RPA on non or UV damaged substrates (data not shown) and only weak binding for the XPC (p125) alone. No DNA binding was observed for hHR23B alone but as the strong, specific damage binding activity of the complex could not be reconstituted from its individual components these results are difficult to interpret. It is possible that XPC and hHR23B need the presence of other NER factors to bring the two proteins together or put them in the correct conformation to allow complex formation. A candidate for this could be TFIIH as yeast studies have shown an interaction between this and Rad23, the
homologue of hHR23B. The XPC-hHR23B complex has also been shown to co-purify with TFIIF through several columns (Drapkin et al., 1994; van der Spek et al., 1996) and to interact with TFIIF in immunoprecipitation studies (S. Araujo, unpublished results). In the presence of either Rad23 or XPF crude antiserum the binding of XPC (p125) to both non and UV damaged DNA was much increased. The intensity of XPC-hHR23B-DNA complexes showed a slight increase however damage specificity was retained. This indicates there may be a factor present in serum that can interact with and stabilize the conformation of XPC (and to a lesser extent XPC-hHR23B) allowing it to bind DNA more strongly. This result is not just due to the presence of non specific carrier proteins in serum as inclusion of 5 µg BSA in reaction mixtures does not affect DNA binding efficiency. It is possible that XPC (p125) alone is relatively unstable and any chaperone proteins present in serum could recognize this partially unfolded polypeptide and fold it into a more favorable conformation for DNA binding. Any such factor could be isolated by fractionation of serum and tested with XPC (p125) directly. However, at present it is difficult to determine whether XPC (p125) alone really does have less affinity and specificity for UV damaged DNA than when complexed with hHR23B.
Chapter 6. Comparison of damaged DNA binding activities of XPC-hHR23B and UV-DDB.

6.1. Affinity of XPC-hHR23B for non damaged and UV damaged DNA - by competition assay

The experiments in the previous chapter showed that XPC-hHR23B has an affinity for UV damaged DNA. To obtain a direct measurement of the preference of XPC-hHR23B for UV damaged DNA a competition assay was devised. Reaction mixtures were set up to contain an excess of either non damaged plasmid DNA or plasmid DNA irradiated with 2.5 kJ/m² UV light to give the same lesion density as in the band-shifted probe. Examples of competition with non damaged and UV damaged DNA are shown in figure 6.1. A and B. Based on the amounts of competitor needed to reduce binding to the labeled probe to 50 %, the preference for UV damaged DNA over non damaged is about 400-fold (Figure 6.1. C). The standard binding reactions contained 100 ng poly (dI•dC) but this should not interfere with the measurement of relative affinity. However, this is a minimal estimate for the binding preference for the following reason. The probe is 136 bp and contains an average of 3 cyclobutane pyrimidine dimers and 1 (6-4) photoproduct, so depending on the size of the binding site and to which lesions XPC-hHR23B is actually binding, the preference could actually be up to about 100 times higher.

6.2. Binding of UV-DDB to non and UV damaged DNA

Ultraviolet damaged DNA binding factor (UV-DDB) is a known mammalian damage binding protein that is defective in a subset of XP-E patients. UV-DDB is the only mammalian NER factor whose damage binding activity is
Figure 6.1. Affinity of XPC-hHR23B for non damaged and UV damaged DNA. A. A 136 bp DNA duplex (1 ng) UV damaged at a dose of 2.5 kJ/m² was incubated at 30°C for 1 h with 100 ng XPC-hHR23B (lane 1 no protein) in the presence of the indicated amounts of competing non damaged closed circular plasmid DNA (lane 2 no competitor). B. A 136 bp DNA duplex (1 ng) UV damaged at a dose of 2.5 kJ/m² was incubated at 30°C for 1 h with 100 ng XPC-hHR23B (lane 1 no protein) in the presence of the indicated amounts of competing 2.5 kJ/m² UV damaged closed circular plasmid DNA (lane 2 no competitor). Free and bound fractions were separated on a non denaturing 5% polyacrylamide gel. C. Quantitative analysis of the data in parts A and B.
Figure 6.2. Apparent $k_p$ for UV-DDB binding to 2.5 kJ/m² UV damaged duplex DNA at 30°C. A. The indicated amounts of UV-DDB were incubated for 1 h at 30°C with 1 ng 136 bp non or 2.5 kJ/m² UV damaged probe. Free and bound fractions were separated on a non denaturing 5% polyacrylamide gel. M = DNA size markers. B. Quantitative analysis of the data in part A.
strong enough to be detected in crude cell extracts and we thought it would be of interest to compare the DNA binding activities of this to XPC-hHR23B. The UV-DDB protein complex was purified in the laboratory by a visiting worker, Vesna Rapic Otrin, who kindly donated some of the preparation (Takao et al., 1993; Otrin et al., 1998).

Binding of UV-DDB to UV and non damaged DNA was tested in the same electrophoretic mobility-shift assay as described previously. As all the assays were performed in the presence of 100 ng poly (dI•dC) competitor DNA an accurate equilibrium binding constant \( k_D \) could not be determined. However, to allow comparison with XPC-hHR23B, an apparent \( k_D \) was estimated for UV-DDB binding to 2.5 kJ/m² UV damaged DNA at 30°C. Between 0.05 and 10 ng UV-DDB were incubated with 1 ng UV irradiated probe DNA at 30°C for 1 h. 10 ng UV-DDB was also incubated with 1 ng non irradiated probe to examine binding to non damaged DNA.

Very little binding was observed with the non damaged probe despite the high amount of UV-DDB showing that this protein’s activity is specific for UV damaged DNA. The band-shifted material did not run as a single species even at low UV-DDB concentrations (Figure 6.2, A and B). Between 0.05 and 1 ng UV-DDB most of the shifted material is seen at position B1 as three closely migrating species. These may be due to one molecule of UV-DDB binding the probe in three different conformations. Band shifted material was also present in position B2 even with some of these low amounts of UV-DDB. At 2-10 ng UV-DDB shifted material is observed at positions B1, B2 and B3 but the amount of binding at the B1 position appears to be saturated at about 2-3 ng UV-DDB. The proportion of shifted material at positions B2 and B3 increased between 2 and 10 ng UV-DDB. Material shifted to positions B2 and B3 probably represents independent binding of two and three molecules of UV-DDB to one molecule probe, respectively. 50% saturation was achieved at about 0.4 nM (≈0.5 ng) whereas with XPC-hHR23B at this temperature it was at about 95 nM (≈140 ng).
Figure 6.3. Affinity of UV-DDB for non damaged and UV damaged DNA. A. A 136 bp DNA duplex (1 ng) UV damaged at a dose of 2.5 kJ/m² was incubated at 30° C for 1 h with 0.5 ng UV-DDB in the presence of the indicated amounts of competing non damaged or 2.5 kJ/m² UV damaged closed circular plasmid DNA. Free and bound fractions were separated on a non denaturing 5% polyacrylamide gel. M = DNA size markers. B. Quantitative analysis of the data in part A.
It appears that UV-DDB has about 200-fold higher affinity for UV damaged DNA than XPC-hHR23B. Experiments were also carried out to measure the damage discrimination ability of UV-DDB an example of which is shown in figure 6.3. A. At the 50 % binding level, UV damaged DNA was a 300-fold better competitor than was non damaged DNA (Figure 6.3. B). It appears that although UV-DDB shows a 200-fold greater affinity for UV damaged DNA than XPC-hHR23B, its ability to discriminate between damaged and non damaged sites is similar.

6.3. Independent binding of XPC-hHR23B and UV-DDB to UV damaged DNA

A question of interest is whether XPC-hHR23B and UV-DDB proteins would readily interact to form a co-complex on DNA. The mobility-shift assay was used to test this and was carried out under two conditions, with or without 100 ng non damaged competitor DNA in the reaction mixtures. The assay used amounts of each protein that shifted approximately equal amounts of probe: 100 ng XPC-hHR23B and 0.5 ng UV-DDB. The results for the two conditions were essentially the same (Figure 6.4. A and B). A mixture of the two proteins gave two independent band shifts of the same size seen with the individual proteins. The sum of the intensities of the UV-DDB-DNA complexes and the XPC-hHR23B-DNA complexes in a mixture of both proteins together was about the same as the sum of the intensities when both factors were added separately. No new bands were observed of higher mobility that would indicate a co-complex of the two proteins on DNA.
Figure 6.4. Independent binding of XPC-hHR23B and UV-DDB to UV damaged DNA. A. A 136 bp duplex (1 ng) UV damaged at a dose of 2.5 kJ/m² was incubated at 30°C for 1 h with the indicated amount of XPC-hHR23B and/or UV-DDB with or without 100 ng non damaged competitor DNA. Free and bound fractions were separated on a non denaturing 5% polyacrylamide gel. M = DNA size markers. B, C. Quantitative analysis of the data in part A in the absence or presence of competing non damaged DNA respectively.
6.4. High affinity of XPC-hHR23B and UV-DDB for (6-4) photoproducts

Enzymatic photoreactivation can be used to specifically and efficiently remove cyclobutane pyrimidine dimers from UV irradiated DNA. The principal photoproducts formed by UV-C irradiation of DNA at the doses used here are cyclobutane pyrimidine dimers (CPD's) and (6-4) photoproducts, in a ratio of 3:1. *Anacystis nidulans* photolyase was added to an aliquot of the 2.5 kJ/m² UV irradiated probe and illuminated with 420 nm photoreactivating blue light. T4 endonuclease V is an enzyme that specifically cuts close to cyclobutane pyrimidine dimers and was used to analyze the efficiency of the photoreactivation treatment (Figure 6.5. C). Nicks were formed in the 2.5 kJ/m² UV irradiated probe as expected but no nicking was observed in this after photoreactivation treatment, indicating that all the CPD's had been removed. Some weak nicking was apparent in the non damaged DNA probe and so this may contain a few UV lesions just through being exposed for short periods of time to visible light or from fragment purification using a UV light box.

Electrophoretic mobility-shift assays showed that removal of CPD's gave a small reduction (~20 %) in the binding of XPC-hHR23B, but no discernible reduction in the binding of UV-DDB, which in fact appeared to increase (Figure 6.5. A and B). The results indicate that XPC-hHR23B has on the order of a 10-fold stronger binding affinity for (6-4) photoproducts than for cyclobutane pyrimidine dimers, as 80 % of the binding is attributable to only 25 % of the total photoproducts.

It has been established that UV-DDB binds to both cyclobutane pyrimidine dimers and to (6-4) photoproducts, with about 10-fold higher affinity for the latter. However, because the affinity of UV-DDB for CPD's is only 2-fold above that for non damaged DNA, the binding to such lesions cannot be detected in the presence of excess competitor DNA. This probably explains why we observe no effect of photoreactivation on UV-DDB binding under the conditions.
Figure 6.5. High affinity of XPC-hHR23B and UV-DDB for (6-4) photoproducts. A. A 136 bp duplex DNA (1 ng) UV damaged at a dose of 2.5 kJ/m² with or without photoreactivation was incubated for 1 h at 30°C with 100 ng of XPC-hHR23B or 0.5 ng UV-DDB. Free and bound fractions were separated on a non denaturing 5% polyacrylamide gel. M = DNA size markers. B. Quantitative analysis of the data in part A. C. To check efficiency of photoreactivation treatment the 136 bp duplex DNA (4 ng) non damaged or UV damaged at a dose of 2.5 kJ/m² with or without photoreactivation was incubated for 30 min at 37°C with the indicated amount of T4 endonuclease V. Reactions were stopped by addition of EDTA and fragments were separated on a denaturing 14% polyacrylamide gel. Arrows indicate positions of major T4 endonuclease V nicks. M = DNA size markers.
used here and is consistent with previous observations where competitor DNA has been included in reactions.

6.5. Affinity of XPC-hHR23B and UV-DDB for platinum damaged DNA - by competition assay

XPC-hHR23B is required in vitro for the repair of a substrate containing a single specific platinum adduct (Figure 4.1. A and B and Figure 4.2. A) but no direct measurement of binding to cisplatin damaged DNA has been made. To obtain a measurement of affinity of XPC-hHR23B and UV-DDB for platinum lesions, reactions were set up to contain increasing excess amounts of plasmid DNA treated with the chemotherapeutic drug cisplatin. This is a drug used with varying success in the treatment of human cancers. Although about 90% of testicular cancers can be cured by cisplatin chemotherapy, a significant problem is the development of resistant tumours (Andrews and Howell, 1990; Timmer-Bosscha et al., 1992). The basis for the therapeutic effectiveness of this drug is not yet well understood but its cytotoxic action against tumour cells is thought to be mediated through formation of cisplatin-DNA adducts which may inhibit DNA replication and transcription (Treiber et al., 1994; Mello et al., 1995; Zamble and Lippard, 1995). The effectiveness of this treatment must also reflect to some extent, the efficiency with which cisplatin-DNA adducts are recognized and repaired by the NER system. XP cells show increased sensitivity to killing by the drug cisplatin (Fraval et al., 1978) indicating the importance of understanding the involvement of the NER process in repairing these lesions and thus, possibly decreasing the efficiency of chemotherapy treatments.

DNA was treated with an amount of cisplatin expected to give the same lesion density to that present in the band-shifted probe (Hansson and Wood, 1989). The main lesion formed should be the 1,2-GG platinum intrastrand crosslink which is relatively poorly repaired by NER but the DNA will also contain 1,3-
Figure 6.6. Affinity of XPC-hHR23B and UV-DDB for platinated DNA. A. A 136 bp DNA duplex (1 ng) UV damaged at a dose of 2.5 kJ/m² was incubated at 30°C for 1 h with 100 ng XPC-hHR23B or 0.5 ng UV-DDB (lane 1 no protein) in the presence of the indicated amounts of competing platinated closed circular plasmid DNA (lane 2 and 3 no competitor). Free and bound fractions were separated on a non denaturing 5% polyacrylamide gel. M = DNA size markers. B, C. Quantitative analysis of the data in part A for XPC-hHR23B and UV-DDB respectively. Competition by non damaged (n.d.) closed circular plasmid DNA shown in red for comparison.
GTG platinum intrastrand crosslinks – the substrate used in the repair assays presented in chapter 4 (Calsou et al., 1992; Moggs et al., 1997). An example of competition with platinated DNA is shown in figure 6.6. A. Based on the amounts of competitor needed to reduce binding to the labeled probe to 50 %, the preference of XPC-hHR23B for platinum damaged DNA over non damaged is about 2-fold (Figure 6.6. B). UV-DDB shows a similar level of preference (Figure 6.6. C).

6.6. Affinity of XPC-hHR23B and UV-DDB for alkyl damaged DNA – by competition assay

It is known that NER generally removes bulky, helix-distorting lesions but in vitro it can also act on less bulky alterations including AP sites, O6-methylguanine and mispaired bases. MMS and MNU are simple alkylating agents that cause a variety of lesions in DNA that are usually repaired by the base excision repair pathway or by direct damage reversal (Lindahl and Wood, 1999). The main lesion formed with both of these agents is N7-methylguanine but this appears to be quite well tolerated in cells. They also cause other more deleterious lesions to be induced such as N3-methyladenine which blocks DNA replication and O6-methylguanine which is the main mutagenic lesion. O6-methylguanine can still base pair with cytosine but can also mispair with thymine, causing transitional mutations. In cells this lesion is generally removed from DNA by direct reversal using a specific methyltransferase which transfers the methyl group to one of its own cysteine residues but it can also be repaired by NER (Lindahl and Wood, 1999).

It is of interest to examine the affinity of XPC-hHR23B and UV-DDB for this type of lesion as these are two of the putative damage recognition proteins in the NER pathway. It was shown by Payne and Chu that UV-DDB recognizes some methylated DNA products including C5-methylcytosine and to a lesser
Chapter 6. DNA binding activities of XPC-hHR23B and UV-DDB.

Figure 6.7. Affinity of XPC-hHR23B for MMS and MNU damaged DNA. A. A 136 bp DNA duplex (1 ng) UV damaged at a dose of 2.5 kJ/m^2 was incubated at 30^o C for 1 h with 100 ng XPC-hHR23B (lane 1 no protein) in the presence of the indicated amounts of competing MMS or MNU damaged closed circular plasmid DNA (lane 2 no competitor). Free and bound fractions were separated on a non denaturing 5% polyacrylamide gel. M = DNA size markers. B, C Quantitative analysis of the data in part A for MMS and MNU damaged DNA respectively. Competition by non damaged (n.d.) closed circular plasmid DNA shown in red for comparison.

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A

MMS damaged MNU damaged
pSE420 (ng) pSE420 (ng)

M 1 2

25 50 100 200 25 50 100 20

M = DNA size markers.

UV-DDB bound

Free

UV damaged DNA (2.5 kJ/m^2)

B

Quantitative analysis of the data in part A for MMS and MNU damaged DNA respectively.

C

Competition by non damaged (n.d.) closed circular plasmid DNA shown in red for comparison.

Figure 6.8. Affinity of UV-DDB for MMS and MNU damaged DNA. A. A 136 bp DNA duplex (1 ng) UV damaged at a dose of 2.5 kJ/m^2 was incubated at 30° C for 1 h with 0.5 ng UV-DDB (lane 1 no protein) in the presence of the indicated amounts of competing MMS or MNU damaged closed circular plasmid DNA (lane 2 no competitor). Free and bound fractions were separated on a non-denaturing 5 % polyacrylamide gel. M = DNA size markers. B, C. Quantitative analysis of the data in part A for MMS and MNU damaged DNA respectively. Competition by non damaged (n.d.) closed circular plasmid DNA shown in red for comparison.
extent N6-methyladenine (Payne and Chu, 1994). Binding of UV-DDB (or XPC-hHR23B) to O6-methylguanine or other lesions induced by treatment with MMS or MNU has not been directly studied.

To obtain a measurement of the affinity of XPC-hHR23B and UV-DDB for alkyl damaged DNA, reaction mixtures were set up to contain an excess of either MMS or MNU damaged plasmid DNA which had been treated to give a similar lesion density to the band shifted probe. An example of competition for XPC-hHR23B binding to UV damage using MMS and MNU damaged DNA is shown in figure 6.7. A. Based on the amounts of competitor needed to reduce binding to the labeled probe to 50%, the affinity of XPC-hHR23B for MMS and MNU damaged DNA over non damaged is at least 6-fold (Figure 6.7. B and C).

Similar experiments were carried out to investigate the affinity of UV-DDB for this type of lesion and an example of this is shown in figure 6.8. A. The affinity of UV-DDB for MMS damaged DNA over non damaged appears to be about 3-fold and 4-fold for MNU damage. The spectrum of DNA damage produced by these two chemicals is essentially similar but MNU gives a higher proportion of O6-methylguanine than MMS and this is the lesion that may occasionally be repaired by NER. It appears that alkyl damage is a better competitor than platinum damaged DNA for both proteins and XPC-hHR23B may have a slightly higher affinity for this type of damage than UV-DDB.

6.7. Affinity of XPC-hHR23B and UV-DDB for heat-denatured ss DNA, ss M13 DNA and poly (dI•dC) - by competition assay

XPC-hHR23B has long since been assumed to be a single-stranded DNA binding protein due its ability to bind strongly to single-stranded DNA cellulose, a matrix commonly used for its purification (Masutani et al., 1994; Shivji et al., 1994). Direct binding to single-stranded oligonucleotide probes has
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Figure 6.9. Affinity of XPC-hHR23B and UV-DDB for heat denatured ss DNA and poly (dl.dC). A 136 bp DNA duplex (1 ng) UV damaged at a dose of 2.5 kJ/m² was incubated at 30°C for 1 h with 100 ng XPC-hHR23B (A) or 0.5 ng UV-DDB (B) in the presence of the indicated amounts of competing heat-denatured ss DNA or poly (dl.dC). Lanes 1 and 2 no protein and no competitor respectively. Free and bound fractions were separated on a non-denaturing 5% polyacrylamide gel. M = DNA size markers.
been shown by Reardon et al but no quantitative measurements were taken (Reardon et al., 1996). Therefore, a comparison of the affinity of XPC-hHR23B for single versus double stranded DNA has not so far been possible. UV-DDB has been reported to bind to non damaged single-stranded M13 DNA by Patterson and Chu, in similar competition mobility-shift assays but with 700-fold less affinity than its binding to (6-4) photoproducts. It also appeared to have a slightly higher affinity for UV-irradiated ss M13 DNA (Patterson and Chu, 1989).

Poly (dI•dC) has often been used as non specific competitor DNA in electrophoretic mobility-shift assays. It is of interest to measure the affinity of XPC-hHR23B and UV-DDB for this synthetic copolymer.

Results of a mobility-shift competition assay using heat denatured plasmid DNA and poly (dI•dC) with XPC-hHR23B are shown in figure 6.9. A and in figure 6.9. B for UV-DDB. 50 ng heat denatured ss DNA was sufficient to virtually abolish the binding of XPC-hHR23B to UV damage. With UV-DDB, this amount of ss DNA still allows a UV-dependent band shift to be observed although the shift itself is within a smear of complexes. UV-DDB binding to the UV damaged probe is almost undetectable with 200-300 ng heat denatured ss DNA.

If no poly (dI•dC) is included in the band shift reaction using XPC-hHR23B, there is no defined band shift, just a smear, and most of the probe-XPC-hHR23B complexes are present as large aggregates in the wells of the gel (Figure 6.9. A). Inclusion of 10 ng poly (dI•dC) allows some band-shifted material to be observed but 100 ng (the amount used in all other included band shift assays) was found to allow virtually all the protein-DNA complexes to enter the gel and so was used routinely. XPC-hHR23B must bind to poly (dI•dC) to a similar level as seen with non damaged plasmid DNA as competition is only seen with between 300 and 500 ng poly (dI•dC). UV-DDB-DNA complexes can be seen strongly with 50 or 100 ng poly (dI•dC) and appear less sensitive to the absence
of this competitor as shifts at a similar level are observed even if no poly (dl•dC) is included (data not shown). However, inclusion of 200-500 ng poly (dl•dC) strongly inhibited the binding, indicating the importance of titrating in the amount of non damaged competitor included in band shift assays for each protein tested. It appears UV-DDB may bind more strongly to this synthetic copolymer than to non damaged plasmid DNA of random sequence.

To more accurately determine the affinity of XPC-hHR23B and UV-DDB for single-stranded DNA, ss M13 was used as there should no problems with re-annealing of this substrate (in contrast to heat-denatured duplex DNA) and it is also possible to UV-irradiate. Figure 6.10. A, shows an example of non and UV damaged ss M13 competition with XPC-hHR23B. XPC-hHR23B shows a high affinity for non damaged ss M13 DNA as only ~3 ng is required to inhibit binding by 50 % (Figure 6.10. B). It appears XPC-hHR23B has a ~70-fold higher affinity for non damaged single-stranded M13 DNA than non damaged double-stranded plasmid DNA. Inclusion of between 0.25 and 0.5 ng UV damaged ss M13 inhibits XPC-hHR23B-probe complexes by 50 %. This shows that XPC-hHR23B has an affinity for UV damage in single-stranded M13 at least equivalent to that which it has for UV damage in double-stranded DNA, i.e. at least 400-fold higher than its affinity for non damaged ds plasmid DNA.

Figure 6.11. A, shows an example of a similar experiment with UV-DDB. UV-DDB also shows a high affinity for single-stranded DNA as ~12 ng is sufficient to inhibit binding by 50 % (Figure 6.11. B). Therefore, UV-DDB has an affinity for non damaged single-stranded M13 DNA ~25-fold higher than its affinity for non damaged double-stranded plasmid DNA. Again, UV-irradiated ss M13 is an even better competitor of UV-DDB binding and only 3 ng is required to reduce this by 50 %. UV-DDB has an affinity for UV damage in single-stranded M13 DNA about 3-fold less than its affinity for UV damage in double-stranded DNA, but about 100-fold higher than for non damaged ds plasmid DNA.
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Figure 6.10. Affinity of XPC-hHR23B for non damaged and UV damaged ss M13 DNA.

A. A 136 bp DNA duplex (1 ng) UV damaged at a dose of 2.5 kJ/m² was incubated at 30°C for 1 h with 100 ng XPC-hHR23B (lane 1 no protein) in the presence of the indicated amounts of competing non damaged or 2.5 kJ/m² UV damaged ss M13 DNA (lane 2 no competitor). Free and bound fractions were separated on a non-denaturing 5% polyacrylamide gel. M = DNA size markers. B. Quantitative analysis of the data in part A.
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Figure 6.11. Affinity of UV-DDB for non damaged and UV damaged ss M13 DNA. A. A 136 bp DNA duplex (1 ng) UV damaged at a dose of 2.5 kJ/m² was incubated at 30°C for 1 h with 0.5 ng UV-DDB (lane 1 no protein) in the presence of the indicated amounts of competing non damaged or 2.5 kJ/m² UV damaged ss M13 DNA (lane 2 no competitor). Free and bound fractions were separated on a non denaturing 5% polyacrylamide gel. M = DNA size markers. B. Quantitative analysis of the data in part A.
6.8. Discussion

The damage binding activities of XPC-hHR23B and UV-DDB have not been directly compared in the same assay and so any assumptions made about their relative affinities for lesions in DNA have so far been through indirect evidence. I wanted to address 3 main questions in this chapter:

- What is the level of discrimination between non damaged and UV damaged DNA for XPC-hHR23B?

- What are the respective affinities of XPC-hHR23B and UV-DDB for UV damaged DNA and are they binding to the same type of UV lesion?

- What are their relative affinities for other types of DNA damage repaired by the NER process?

**Preferential binding to UV damaged DNA and the (6-4) photoproduct**

Using mobility-shift assays, I found that UV-DDB has an affinity for UV damaged DNA about 200-fold higher than XPC-hHR23B as 50 % saturation of binding occurs at ~0.4 and 95 nM respectively. This high affinity may explain why UV-DDB is the only UV damaged DNA binding protein strong enough to be detected in crude cell extracts (Hirschfeld et al., 1990). The affinity of UV-DDB for UV damage presented here is much less than the 10,000-fold preference estimated by Hwang and Chu (Hwang and Chu, 1993). They used a 148 bp probe UV irradiated to a total of 5 kJ/m², twice the dose I used here. This would give a 2-fold higher yield of (6-4) photoproducts and other UV lesions and therefore the affinity of UV-DDB for UV damage appears higher. In addition, it appears that their estimates of relative affinity for various damage types were qualitative rather than quantitative like the results presented in this
It is also possible that different UV-DDB preparations have varying specific activities, because for example, they contain different proportions of the p48 subunit. This has been shown to be important in activating the damaged DNA binding activity of the p125 subunit in the UV-DDB heterodimer (Hwang et al., 1998).

Enzymatic photoreactivation had very little effect on the binding of either protein to a UV damaged probe and so both appear to have a much higher affinity for (6-4) photoproducts than cyclobutane pyrimidine dimers. This is in accordance with previous observations on UV-DDB binding by Feldberg and Grossman (Feldberg and Grossman, 1976), Abramic et al., (Abramic et al., 1991), Treiber et al. (Treiber et al., 1992) and Payne and Chu (Payne and Chu, 1994) which all showed either a much lower affinity for T-T cyclobutane pyrimidine dimers or no affinity at all. Reardon et al showed binding of UV-DDB to (6-4) photoproducts and trans-syn dimers but a low affinity for cis-syn T-T dimers (Reardon et al., 1993). In general, it seems that the affinity of UV-DDB for (6-4) photoproducts is higher than the affinity for cis-syn dimers. Binding to these and other types of pyrimidine dimers can only be detected when that is the only lesion type present, the protein is highly purified and no non-damaged competitor DNA is included in reaction mixtures.

XPC-hHR23B had been shown by Sugasawa et al. to bind specifically to a (6-4) photoproduct and to give a stable footprint (Sugasawa et al., 1998). They also showed that XPC-hHR23B binds preferentially to UV, AAF and to a slightly lesser extent, platinum damaged DNA in co-immunoprecipitation assays. Guzder et al showed UV specific binding for the yeast homologue Rad4-Rad23, which was also virtually unaffected by photoreactivation of the UV damaged probe (Guzder et al., 1998). My results with XPC-hHR23B are consistent with the yeast study showing a much higher affinity of XPC-hHR23B for (6-4) photoproducts than cyclobutane pyrimidine dimers.

Although both XPC-hHR23B and UV-DDB are preferentially binding the same lesion type, when mixed together, independent band shifts still occur and no
new slower migrating species are formed. From this we can say there was no
evidence for formation of an XPC-hHR23B-UV-DDB-DNA co-complex on UV
damaged DNA. Despite having a 200-fold higher affinity for UV damaged
DNA, UV-DDB appears to have a similar level of discrimination between non
and UV damaged DNA as XPC-hHR23B. XPC-hHR23B has a 400-fold
preference for UV damaged DNA over non damaged and UV-DDB has a
300-fold preference. These binding preferences are minimal estimates and
could be even higher depending on the actual size of the binding sites of these
proteins and where damaged sites are positioned on the probe molecule.

**Preferential binding to platinum damaged DNA**

The affinity of XPC-hHR23B and UV-DDB for platinum lesions appears to be
quite low being only about 2-fold over their affinity for non damaged DNA.
UV-DDB had first been shown to bind to platinum lesions directly by Chu and
Chang but no measurements of affinity were taken (Chu and Chang, 1988). The
affinity of UV-DDB for both cis and trans platinum adducts has however, been
tested previously by Payne and Chu (Payne and Chu, 1994). They found
UV-DDB had a relatively high affinity for cisplatin damage – about 40-fold less
than its affinity for (6-4) photoproducts. Trans-platinum adducts were bound
much less – with about 1000-fold less affinity than a (6-4) photoproduct.

Here I found UV-DDB had an affinity for cisplatin about 150-fold less than its
affinity for UV damage in general. Most if not all of the binding to UV
damaged DNA in my assays was due to interaction with (6-4) photoproducts
and so the affinity estimated for UV-DDB binding to platinum damaged DNA is
a little less than had been previously observed. XPC-hHR23B also appeared to
have a low affinity for this type of damage despite being required for the repair
of a platinum lesion *in vitro*. A possible explanation for this is that detection of
platinum lesions is through a number of factors acting in combination (eg. XPC,
XPA, RPA and TFIIH). It is also possible that the DNA was not damaged by
incubation with cisplatin to the extent we expected and therefore contained less lesion density (especially the 1,3-GTG platinum intrastrand crosslink – which is well repaired by NER) and a higher proportion of non damaged DNA than we planned. This could explain the lower apparent affinity of UV-DDB for this type of DNA damage and the lower than expected affinity of XPC-hHR23B.

In addition to the work presented here I spent a considerable time attempting to use a probe containing a single, defined platinum lesion in electrophoretic mobility shift assays. The probe was a blunt ended 323 bp restriction fragment and contained a centrally located 1,3-GTG intrastrand platinum crosslink. I tried various salt, BSA, glycerol, CaCl₂, MgCl₂ and poly (dI·dC) concentrations and different combinations of XPC, hHR23B, XPA, RPA and TFIIH but under no conditions tested observed defined, reproducible band shifts. However, there was some evidence of protein-DNA complex formation as in some conditions large labeled aggregates were present in the wells of the gel. It is possible that the probe used in these studies was too large and the poly (dI·dC) concentration too low to observe efficient binding.

I also attempted DNase I footprinting with combinations of XPC, hHR23B, XPA, RPA and TFIIH on the same substrate. Again various buffer conditions were tried and extensive DNase I titrations, but no specific protection was observed. However, at high XPC-hHR23B concentrations there was overall protection of the whole substrate, indicating it was indeed bound by this factor, but non specifically in the conditions tested. A similar observation was made by Wakasugi and Sancar, who tried DNase I footprinting XPC-hHR23B on a substrate containing a single (6-4) photoproduct but included no competing poly (dI·dC) in their reaction mixes (Wakasugi and Sancar, 1999). Again, it is plausible that with further titration of buffer conditions and protein:probe ratio in combination with a higher concentration of poly (dI·dC), specific binding could be observed. In general it appears cisplatin is a relatively poor substrate for XPC-hHR23B.
Chapter 6. DNA binding activities of XPC-hHR23B and UV-DDB.

**Preferential binding to alkyl-damaged DNA**

XPC-hHR23B was found to have at least a 6-fold higher preference for binding to alkyl damaged DNA than non damaged DNA and a 3-4 fold preference was found for UV-DDB. Binding of XPC-hHR23B to this type of DNA damage has not been studied previously, but NER can act weakly on O\(^6\)-methylguanine lesions – which are produced by MNU and to a lesser extent MMS and so some affinity for this type of damage is to be expected. Payne and Chu showed that UV-DDB binds N\(^6\)-methyladenine and C\(^5\)-methylcytosine residues with an affinity 400 and 1000-fold less than for (6-4) photoproducts respectively (Payne and Chu, 1994). Interaction of UV-DDB with O\(^6\)-methylguanine or other lesions induced by MMS or MNU treatment hadn’t been tested. In my assay, UV-DDB had a 3-4-fold higher affinity for MMS/MNU damaged DNA than non damaged which is in the order of 100 to 150-fold less than its apparent affinity for UV damaged DNA. It appears XPC-hHR23B and UV-DDB have similar levels of affinity for alkyl damaged DNA. It must be stressed that the results presented on preferential binding of XPC-hHR23B and UV-DDB to both alkyl and platinum damaged DNA are as yet only preliminary. Further work is required under conditions where lesion yield in the competitor DNA is accurately measured and also using competitor DNA with single defined lesions to give more direct evidence for these interactions.

**Low affinity for poly (dI•dC) synthetic copolymer**

The affinity of XPC-hHR23B for poly (dI•dC) is basically the same as for non damaged double-stranded plasmid DNA. Inclusion of between 10-100 ng poly (dI•dC) in reaction mixtures was necessary to allow specific damage binding to occur. Without poly (dI•dC) present, all of the probe-protein complexes aggregated in the wells of the gel. UV-DDB appears less sensitive to the absence of poly (dI•dC), probably due to its higher affinity for UV damaged DNA.
DNA. Binding of UV-DDB to poly (dI•dC) is in the same range or may actually be slightly higher than its binding to non damaged plasmid DNA.

**High affinity for heat-denatured and single-stranded M13 DNA**

XPC-hHR23B appears to bind strongly to heat denatured (single-stranded) DNA. Experiments using M13 DNA allowed quantitation of the affinity of XPC-hHR23B and UV-DDB for both non and UV damaged single-stranded M13 DNA. It appears that both proteins recognize and bind to ss M13 and bind it with higher affinity when it contains UV damage. XPC-hHR23B shows a 70-fold higher affinity for non damaged M13 than non damaged ds DNA, whereas UV-DDB shows only a 25-fold higher affinity for ss M13 over ds non damaged DNA. UV damage in M13 ss DNA is at least as well recognized by XPC-hHR23B as it is in ds DNA. However, UV-DDB has an affinity for UV damage in ss M13 DNA 3-fold less than in ds DNA. The results presented here are in qualitative agreement with the work of Reardon et al who showed binding of XPC-hHR23B to a single-stranded oligonucleotide in a mobility-shift assay (Reardon et al., 1996). Previous work by Payne and Chu showed UV-DDB bound to ss DNA and slightly better to UV damaged ss DNA (Payne and Chu, 1994). The work presented here is in agreement with this data but adds further quantitation of the affinity of UV-DDB (and XPC-hHR23B) for damage in single and double-stranded DNA.

The NER process acts on a wide variety of lesions and so it is likely that the damage recognition components recognize a common feature of these. The presence of a lesion in duplex DNA is known to cause some local distortion and possibly the generation of a partially opened single-stranded region. Generally, the more helix-distorting a lesion is, the more efficiently it is repaired by NER and so a current hypothesis is that it is this distorted duplex structure that is the main determinant for recognition. The fact that both XPC-hHR23B and UV-DDB appear to bind UV damage in both single and double-stranded DNA.
introduces a puzzle as to what exactly is being recognized. It is possible that these factors bind directly to lesions but as they both have affinity for several different types of DNA damage this seems unlikely. XPC-hHR23B and UV-DDB bind strongly to UV damaged single-stranded M13 DNA and so a distorted DNA duplex appears not to be the main determinant for recognition. However, M13 DNA is probably not completely single-stranded as it is DNA of mixed sequence and so is very likely to contain some level of secondary structure. It may be that single-stranded M13 can actually form structures in these regions of secondary structure similar to those formed in damaged duplex DNA. It is also possible that these factors interact with the partial single-strandedness present at a damaged site in duplex DNA and therefore bind avidly to both non and UV damaged single-stranded DNA.

The single-stranded binding activity of XPC-hHR23B and to a lesser extent UV-DDB may give us an important clue to how a damaged site is recognized. The higher level of binding to non and UV damaged ss M13 DNA by XPC-hHR23B is also the main difference observed in the DNA binding activities of these two factors. For these reasons further work was carried out to attempt to characterize XPC-hHR23B’s affinity for different types of single-stranded DNA and this is presented and discussed in chapter 7.
Chapter 7. Single-stranded DNA binding activity of XPC-hHR23B.

7.1. Comparison of the affinity of XPC-hHR23B for different types of single-stranded DNA – by competition assay

In the previous chapter I showed XPC-hHR23B had a high affinity for both non and UV damaged ss M13 DNA. To investigate the structural requirements for binding to these substrates I compared the affinity of XPC-hHR23B for single-stranded M13 and synthetic single-stranded homopolymer DNA by a mobility-shift competition assay. Homopolymers such as poly (dT), poly (dC) and poly (dA) are completely single-stranded and no secondary structure can arise from Watson-Crick base pairing.

One goal of this series of experiments was to explore the binding of XPC-hHR32B to defined secondary structures, specifically small “bubbles” within a duplex. For this reason, the electrophoretic mobility-shift assay was modified slightly to use a duplex formed by annealing two complementary 90-mer oligonucleotides, rather than the 136 bp restriction fragment used in previous chapters.

Results of a mobility-shift competition assay using ss M13, poly (dT) and poly (dC) as competitors is shown in figure 7.1. A. A P32-labeled 90 bp duplex formed by the annealing of two complementary oligonucleotides was UV-irradiated to a total dose of 3 kJ/m². This dose should give approximately 3 cyclobutane pyrimidine dimers and 1 (6-4) photoproduct per probe molecule. To compete for XPC-hHR23B binding to this probe, reactions included 30 or 300 ng ss M13, poly (dT) or poly (dC). Inclusion of 30 ng ss M13 reduced binding to the probe by about 40 % and 300 ng by approximately 75 %, showing
Figure 7.1. Affinity of XPC-hHR23B for ss M13, ss poly (dT) and ss poly (dC). A. A 90 bp DNA duplex (1 ng) UV damaged at a dose of 3 kJ/m² was incubated at 30°C for 1 h with 50 ng XPC-hHR23B (lane 1 no protein) in the presence of the indicated amounts of competing ss M13 DNA, ss poly (dT) or ss poly (dC) (lane 2 no competitor). Free and bound fractions were separated on a non-denaturing 5% polyacrylamide gel. M = DNA size markers. B. Quantitative analysis of the data in part A.
Figure 7.2. Affinity of XPC-hHR23B for ss M13, ss poly (dT), ss poly (dC) and poly (dA).

A. A 90 bp DNA duplex (1 ng) UV damaged at a dose of 3 kJ/m² was incubated at 30° C for 1 h with 50 ng XPC-hHR23B in the presence of the indicated amounts of competing ss M13 DNA (−/+ UV), ss poly (dT), ss poly (dC) (−/+ UV) or ss poly dA. Free and bound fractions were separated on a non denaturing 5% polyacrylamide gel. M = DNA size markers.

B, C, D, E. Quantitative analysis of the data in part A for ss M13 DNA (−/+ UV), ss poly (dT), ss poly (dC) (−/+ UV) and ss poly (dA) respectively.
ss M13 is a good competitor in this assay (Figure 7.1. B). 30 ng poly (dT) reduced binding by about 20 % and with 300 ng binding was still 75 % of that seen without competitor. 30 ng poly (dC) reduced binding to the probe by about 40 % but inclusion of 300 ng still allowed 40 % of the original labeled XPC-hHR23B-DNA complexes to be observed. It appears XPC-hHR23B requires some level of secondary DNA structure to allow single-stranded binding to occur with high efficiency.

To compare XPC-hHR23B’s affinity for UV lesions in ss M13 and homopolymer DNA a similar experiment was carried out including both UV-irradiated (2.5 kJ/m²) ss M13 and poly (dC) (Figure 7.2. A). Consistent with the previous data, inclusion of either 5 or 20 ng non damaged ss M13 reduced XPC-hHR23B binding by 40 and 60 % respectively. As seen previously (Figure 6.10. A and B) UV-irradiated (2.5 kJ/m²) ss M13 is a very good competitor with only 2 ng required to inhibit binding by 90 % and with 10 ng, XPC-hHR23B binding is almost undetectable. Poly (dT) or poly (dA) are both poor competitors and inclusion of 300 ng of each still allowed 70 and 95 % binding respectively. Again, XPC-hHR23B appears to have a slightly higher affinity for poly (dC) than the other homopolymers and 300 ng of this reduced binding to about 40 %. UV-irradiation of poly (dC) slightly increased XPC-hHR23B’s affinity for this probe but only by a further 5-10 %.

This data suggests that XPC-hHR23B recognizes and binds to specific secondary DNA structures and does not interact directly with lesions as these are present in UV-irradiated poly (dC) and this is approximately 1000-fold less efficient at inhibiting binding than UV-irradiated ss M13.

Single-stranded binding activity of XPC-hHR23B was examined directly by mobility-shift assay in figure 7.3. Here, a 90 nt single-stranded oligonucleotide of mixed sequence either non-damaged or UV-irradiated at a dose of 3 kJ/m² was used as probe. Shifted material consisting of 3 individual closely positioned bands was strongly observed with non damaged probe. These may
Figure 7.3. Affinity of XPC-hHR23B for ss DNA. A 90 nt single-stranded oligonucleotide (1 ng) either non damaged or UV damaged at a dose of 3 kJ/m² was incubated at 30°C for 1 h with 50 ng of XPC-hHR23B. Free and bound fractions were separated on a non denaturing 5% polyacrylamide gel. * marks the position of new bound species observed on UV-irradiation of the probe.
Figure 7.4. Structures of the 90 bp DNA substrates used in this study. 90 bp oligonucleotides were annealed and purified by non denaturing gel electrophoresis. Size of the unpaired "bubble" region of each substrate is indicated on the right. Asterix (*) indicates 5'-terminal labeling with $^{32}$p.
consist of XPC-hHR23B binding the single-stranded probe in 3 different conformations. After UV-irradiation the same shifted bands are seen and at a similar intensity but 2 additional bands (marked *) are now observed. These could represent XPC-hHR23B binding in two further conformations at specific UV lesions, which were not available prior to UV-irradiation.

### 7.2. Affinity of XPC-hHR23B for DNA bubble structures

As it appears XPC-hHR23B binds to structures within double and single-stranded DNA I wanted to investigate whether this complex would interact with specific DNA bubble structures even in the absence of lesions. DNA "bubble" structures (Figure 7.4.) were formed by annealing labeled, 90 nt oligonucleotides containing internal regions of non-complementarity (C's on one strand and T's on the opposite). This results in the formation of 90 bp DNA duplexes containing centrally located, unpaired bubble regions of 5, 3, 2 and 1 (C-C mismatch) bp. A completely double-stranded 90 bp probe was also made for control purposes. These were then used as probes in a mobility-shift assay with XPC-hHR23B (Figure 7.5.). XPC-hHR23B was found to bind all substrates very weakly and independently of the size of the unpaired bubble region. Despite a high affinity for ss M13 and single-stranded oligonucleotides, XPC-hHR23B has an affinity for single-stranded DNA with flanking duplex regions equivalent roughly equivalent to its affinity for non damaged duplex DNA.

It was possible that the single-stranded regions in these probes were too small to allow XPC-hHR23B to interact and so further 90 bp bubble probes were prepared containing unpaired regions of 30 and 15 bp. These were again tested by mobility-shift assay for interaction with XPC-hHR23B and still only very weak binding occurred (Figure 7.6. A). Note that the mobility of the 90-mers decrease with increasing bubble size, compare migration of free probe 0 bp bubble versus 5 bp bubble (Figure 7.5.) and free probe migration in figure 7.6. A.
Figure 7.5. Low affinity of XPC-hHR23B for DNA bubble structures. A 90 bp duplex (1 ng) containing unpaired regions of 5, 3, 2, 1 or 0 bp was incubated at 30°C for 1 h with 50 ng XPC-hHR23B. Free and bound fractions were separated on a non-denaturing 5% polyacrylamide gel. M = DNA size markers.
Figure 7.6. Affinity of XPC-hHR23B for DNA bubble structures. A. A 90 bp duplex (1 ng) containing unpaired regions of 30, 15 or 5 bp either non damaged or UV damaged at a dose of 3 kJ/m² was incubated at 30°C for 1 h with 50 ng of XPC-hHR23B. Free and bound fractions were separated on a non denaturing 5% polyacrylamide gel. M = DNA size markers. 
B. Quantitative analysis of the data in part A.
To check that XPC-hHR23B would bind these substrates in the presence of damage, the 30, 15 and 5 bp bubble probes were UV-irradiated to a total dose of 3 kJ/m² and again used as probes in mobility-shift assays (Figure 7.6.A). UV-irradiation increased the affinity of XPC-hHR23B for each probe about 4-fold (Figure 7.6.B) indicating that XPC-hHR23B binds to secondary structure in DNA but only that specifically formed around a damaged site or in single-stranded DNA of mixed sequence.

Figure 7.7. A and B demonstrates that the affinity of XPC-hHR23B for the smaller bubble structures (2, 1 and 0 bp) is also increased after UV-irradiation of these probes. The UV-dependence is unexpectedly small here and there are less sequential pyrimidines than in the larger bubble substrates and thus less photoproducts are formed on UV-irradiation. The 30 and 15 bp bubbles have a large number of pyrimidines (C's on one strand and T's on the other) in the unpaired region and thus are likely to contain several lesions after UV treatment.

7.3. Discussion

XPC-hHR23B was found to have a high affinity for ss M13 DNA and also for a single stranded oligonucleotide in both cases, with and without UV damage. The complex has a low affinity for single-stranded homopolymer even when UV-irradiated, thus suggesting that the secondary structure in ss DNA of mixed sequence is an important prerequisite for binding. XPC-hHR23B interacts only weakly with DNA duplexes containing internal single-stranded bubble structures and binding to these is increased after UV-irradiation. The bubbles were formed by having unpaired regions consisting of strings of cytosine on one strand and thymine on the opposite surrounded by duplex regions. These single-stranded bubbles may structurally be similar to synthetic homopolymers and contain little secondary structure hence XPC-hHR23B has only a weak affinity for these probes. The 1 bp bubble was actually a C-C mismatch rather than a C opposite a T. This was chosen because genetic experiments in S. pombe
Figure 7.7. Affinity of XPC-hHR23B for DNA bubble structures. A. A 90 bp duplex (1 ng) containing unpaired regions of 5, 2, 1 or 0 bp either non damaged or UV damaged at a dose of 3 kJ/m² was incubated at 30°C for 1 h with 50 ng of XPC-hHR23B. Free and bound fractions were separated on a non denaturing 5% polyacrylamide gel. M = DNA size markers. B. Quantitative analysis of the data in part A.
carried out by Fleck et al suggested that in the absence of msh-dependent mismatch repair, NER can act on this particular lesion (Fleck et al., 1999). As this is an NER substrate under some circumstances we wanted to determine if XPC-hHR23B would recognize and bind it. Neither the 1 or the 2 bp bubbles were bound more efficiently than the duplex 90-mer and so the question remains, exactly what kind of distortion is recognized by XPC-hHR23B? It is possible that these bubble structures are rather flexible and perhaps a distinct conformation is required for XPC-hHR23B recognition.

It is possible that XPC-hHR23B has two independent DNA binding activities, one for mixed sequence single-stranded DNA (eg. M13) and the other for structural distortion present at the site of a lesion. Another explanation may be there the slight opening and single-stranded nature at the site of a lesion in DNA is somehow mimicked in the secondary structure present in single-stranded DNA of mixed sequence. This can be recognized and bound by the XPC-hHR23B complex.

Binding of XPC-hHR23B to a single-stranded DNA has been assumed for several years as this protein interacts strongly with ss DNA cellulose, a matrix commonly used for its purification (section 3.5 and 3.7). In my purification procedure, XPC and XPC-hHR23B are eluted from this column using 1.5 M salt, showing the strength of this interaction. Binding of XPC-hHR23B to a single-stranded oligonucleotide was first reported by Reardon et al, using mobility-shift assays (Reardon et al., 1996). Binding of this complex to other types of single-stranded DNA structures has not been investigated previously.
Chapter 8. Discussion and concluding remarks.

8.1. Possible roles of hHR23 proteins

In chapter 4 of this thesis I presented data showing that hHR23B stimulated the activity of XPC (p125) about 10-fold in a reconstituted repair reaction. In cells hHR23B is much in excess of XPC and most is found in free form. A maximum of 20% of the hHR23B was estimated to be in complex with XPC (van der Spek et al., 1996) leading to the speculation that it may have other cellular functions. Recently, several groups have published data connecting Rad23 homologues to cell cycle progression and ubiquitination pathways. The C-terminal UB-A domain of hHR23A has been reported to be involved in the interaction of this factor with HIV-1 Vpr protein (Withers-Ward et al., 1997; Dieckmann et al., 1998). HIV-1 Vpr has a number of functions in the viral life cycle and expression of this protein leads to cell cycle arrest of lymphocytes in G2 phase. This hHR23A-Vpr interaction was reported to be important in cell cycle arrest induced by Vpr and overexpression of hHR23A (or the C-terminal Ub-A domain) leads to partial alleviation of this arrest (Withers-Ward et al., 1997).

Earlier this year, hHR23A was identified as a substrate for E6-associated protein-mediated ubiquitination and a low level of ubiquitinated forms of hHR23A could be detected in mammalian cell extracts (Kumar et al., 1999). A small fraction of hHR23A protein was found to be degraded in a cell cycle-dependent manner at the G1/S transition. This may reflect the small fraction of hHR23A involved in NER and it is possible that hHR23A degradation at the G1/S boundary serves as part of a signal indicating the completion of repair such that cells can exit G1 and enter S phase.

The UB-like domain of hHR23B has recently been shown to interact with the multi-ubiquitin chain binding domain of S5a (Hiyama et al., 1999)—one of the regulatory subunits of the 26 S proteosome. In vitro, hHR23B was also reported
to inhibit lysozyme degradation in reticulocyte extracts, without its own degradation. This showed the hHR23B protein was stable and is in agreement with yeast studies showing the high stability of Rad23 (Watkins et al., 1993). The Ub-like domain in Rad23 does not mediate its degradation in yeast (Watkins et al., 1993) and it seems similar with the human homologues. hHR23B may regulate the proteolysis of ubiquitinated proteins by the 26 S proteosome via its interaction with S5a (Hiyama et al., 1999). In summary it appears that Rad23 homologues may form a link between DNA repair, the ubiquitin pathways and possibly also cell cycle progression.

We don’t yet understand the mechanism of stimulation of XPC by Rad23 homologues and as this can be achieved using only a fragment of hHR23B it is possible that this component has little direct role in repair. Conceivably this fragment may stabilize XPC protein and put it in the right conformation for repair. In yeast it has been reported that Rad23 promotes formation of a complex between TFIIH and Rad14 (the homologue of human XPA), such that there is co-immunoprecipitation of TFIIH and Rad4 (the homologue of human XPC) (Guzder et al., 1995). By inference, it appears that Rad23 may act as a molecular matchmaker between the damage recognition components of NER. The fragment of hHR23B used in chapter 4 is highly conserved in yeast Rad23. It is possible that this region may not only contain the XPC interacting domain of XPC but also the TFIIH interacting domain and therefore the stimulation observed could be due to increased complex formation between XPC and TFIIH leading to more efficient repair.

8.2. XPC-hHR23B as a damage recognition factor in NER

In chapters 5 and 6 I showed that the XPC-hHR23B complex preferentially bound to UV damaged DNA and to some extent, to DNA damaged by alkylating agents and cisplatin. Sugasawa et al, recently provided evidence that XPC-hHR23B could be involved in the earliest damage sensing step of NER and
indeed could initiate the whole process (Sugasawa et al., 1998). They carried out kinetic experiments in which two differently sized plasmids were separately preincubated, one with XPC complex and the other with the remaining NER factors. On combining the two reaction mixtures, it was found that the plasmid preincubated with XPC was preferentially repaired - indicating that XPC-hHR23B is the earliest factor involved in NER. Furthermore, DNase I footprinting showed that the XPC complex binds directly to a (6-4) photoproduct and changes the conformation around the lesion (Sugasawa et al., 1998). The protein-DNA contacts spanned a region approximately 30 nt on both strands around the lesion and is the only stable footprint observed for any human NER protein around a damaged site. Studies of the yeast homologue, Rad4-Rad23 have also shown preferential binding to damaged DNA (Guzder et al., 1998; Jansen et al., 1998). Thus, XPC-hHR23B may initiate NER by sensing and binding tightly to distortions, locally modifying the DNA duplex so that the remaining repair machinery can enter at the site of damage.

For some time, XPA was the only known DNA damage-dependent binding factor in mammalian NER. As described above, however, the XPC-hHR23B complex was recently reported to have a stronger damage-binding capacity and probably to act prior to XPA (Sugasawa et al., 1998). Another study using similar methods challenges this view, concluding that the XPC complex does not show preferential binding to damage, does not act before XPA in the repair mechanism and does not show a stable footprint on a (6-4) photoproduct (Wakasugi and Sancar, 1999). The lack of inclusion of non specific competitor DNA in their reactions may explain the differing results of this group (see chapter 5 for further discussion).

I conclude that XPC-hHR23B complex is a factor with a high affinity for both non and UV damaged DNA but when present in a mixture of the two, shows a high level of discrimination and binds preferentially to DNA containing damaged sites. A probable reason for this is as follows: it is reasonable to expect that the high association rate of XPC-hHR23B to UV damage is matched by a similar high level of association to non damaged DNA but this cannot be
observed under our conditions due to the presence of excess non damaged competing poly (dI•dC) in the reactions. Without poly (dI•dC) the protein-DNA complexes were not apparent as discrete bands but as large aggregates in the wells of the gel (Figure 6.9. A). The dissociation rate of XPC-hHR23B from UV damaged DNA is very slow (Figure 5.7.). This provides the main level of specificity because the dissociation rate from non damaged DNA must be more rapid otherwise we would not be able to determine association rate to damaged DNA under our conditions. This is because all our reactions contain 100-fold excess of poly (dI•dC) in relation to the probe and the affinity of XPC-hHR23B for this synthetic copolymer appears the same as for non damaged plasmid DNA (Figure 6.9.A).

8.3. **Substrate specificity of XPC-hHR23B**

DNA lesions in the template strand of active genes transcribed by RNA polymerase II are repaired faster than those in non-transcribed strands or inactive regions of the genome (Bohr et al., 1985). This phenomenon is referred to as transcription-coupled repair and requires active transcription, NER factors and in addition the CSA and CSB proteins (Troelstra et al., 1992; Hanawalt, 1994) (Figure 1.7.). XPC-hHR23B is the only core NER factor not required for transcription-coupled repair. Mu and Sancar showed that a substrate containing a thymine-thymine pyrimidine dimer within a 10 nt unpaired bubble did not require XPC-hHR23B for its repair (Mu and Sancar, 1997). This may be analogous to the structure formed when a transcribing RNA polymerase encounters and stalls at a lesion in DNA and thus may mimic transcription-coupled repair, although CSA and CSB proteins are not required for damage excision. The bubble probes used in my study (chapter 7) may also mimic a transcription bubble, explaining the low affinity of XPC-hHR23B for this structure, as *in vivo* this would become bound by RNA polymerase II with no requirement for XPC-hHR23B. Similarly, a substrate containing a particular type of cholesterol moiety in place of a nucleoside does not require
XPC-hHR23B for its repair (Mu et al., 1996). It is possible that the cholesterol substitution causes significant unwinding in its immediate vicinity, generating a similar transcription bubble-like structure.

Generation of an open complex in DNA is an essential intermediate in the NER pathway as this is the substrate for the two site-specific NER endonucleases, XPG and ERCC1-XPF (Evans et al., 1997a). These cut at the junction between single and double-stranded DNA. XPC-deficient cell extracts are severely deficient in generating an open complex around a lesion in vitro along with cell extracts deficient in components of the TFIH helicase complex (Evans et al., 1997b), again showing a link of XPC-hHR23B to the very earliest stages of NER.

A possible mechanism consistent with these observations is that XPC-hHR23B recognizes and binds to the distortion present around a damaged site. This binding causes further slight opening which is stabilized by the presence of XPC-hHR23B due to the complex having single-stranded binding activity. This open intermediate may then be bound by other NER factors and further opened by the helicase activities of the TFIH complex to give the structure required for action of the NER endonucleases. This open intermediate probably has some kind of bubble structure and by this stage in the reaction the requirement for XPC-hHR23B may be over. When an RNA polymerase stalls at a damaged site in transcription-coupled repair an open, bubble intermediate is already present, thus obviating the need for XPC-hHR23B.

**8.4. Model for damage recognition in non-transcribed DNA**

As discussed in sections 1.2-1.2.2, the overall process of damaged strand recognition in *E. coli* is energy dependent, although UvrA can preferentially bind damage without ATP. It is expected that in human cells damage recognition would also have a requirement for ATP. None of the putative human damage recognition proteins (XPA, XPC-hHR23B and UV-DDB) require
energy for DNA binding. All have some single-stranded binding activity and may mainly associate with DNA through hydrophobic interactions with the unpaired bases. It is not clear whether the individual binding specificities of XPA-RPA, XPC-hHR23B and UV-DDB for damaged DNA are sufficient to account for the efficiency with which lesions are eliminated from the genome, and damage recognition could be a concerted reaction with many components working together.

The recognition-incision stage of NER in human cells as a whole requires ATP (Calsou and Salles, 1994; Evans et al., 1997a). The only two ATPases known in this system are XPB and XPD and these form helicase subunits with opposing polarity in the TFIIH complex. This may suggest a role for TFIIH in damage recognition or specifically in identification of the strand containing a lesion. Evidence for this idea is found in the yeast NER process. The yeast homologue of XPD, Rad3, is also an ATP-dependent helicase. Translocation of Rad3 along DNA is blocked at a damaged site - on the strand on which it is moving (Naegeli et al., 1992; Naegeli et al., 1993; Sung et al., 1994). Such stalling of helicase translocation could be important in identification of the damaged strand as well as in opening the region around the lesion.

In the model presented in Figure 8.1, a DNA adduct causes some intrinsic local distortion which in non-transcribed DNA (the bulk of the genome) is recognized by XPC-hHR23B. Binding of XPC complex to the lesion causes partial opening of the DNA structure. This opening allows entry of TFIIH (which has an affinity for the XPC complex) to the damaged site (Evans et al., 1997b; Mu et al., 1997). This may occur in concert with entry of XPA and RPA into the preincision complex. In an ATP-dependent step, the DNA strands could then be translocated through the repair machinery by the 5' to 3' helicase activity of XPD and the 3' to 5' helicase activity of XPB. The DNA becomes stalled when the helicases encounter the lesion. This serves to identify the damaged strand and results in the formation of a fully opened preincision complex. Incisions are then made sequentially on the damaged strand. XPG
Figure 8.1. Model for nucleotide excision repair in non-transcribed DNA in human cells.
makes an incision 2 to 9 phosphodiester bonds 3' to the lesion and then ERCC1-XPF cuts 16 to 25 phosphodiester bonds on the 5' side. The distance between the incisions is constant, with a modal size of 27 nt independent of the type of lesion repaired (Moggs et al., 1996), indicating that this is dictated by the size and shape of the repair complex. The 24-32 nt oligonucleotide containing the damage is then released - and there is evidence to suggest that this is protein bound (Mu et al., 1996) - possibly by one of the damage recognition proteins, for example XPC-hHR23B. The gap in the duplex is filled by a PCNA-dependent DNA polymerase and sealed by a DNA ligase to regenerate the intact DNA structure.

8.5. **Comparison of the E. coli and human NER systems**

The parallels in mechanism between the prokaryotic and eukaryotic systems are remarkable, considering that the mammalian NER reaction requires 15-18 polypeptides for damage recognition and incision, while the *E. coli* system requires only three. The similarities include:

(1) A distortion-recognition factor that is energy-independent (UvrA), though the search for the damaged strand is ATP-dependent and requires two different factors (UvrA₂B). This is reminiscent of the energy-independent damage-binding factors XPC and XPA, which in turn bind to the ATP-dependent TFIIH in mammalian cells.

(2) A strand-separating helicase (UvrB in prokaryotes and TFIIH in eukaryotes) to create an open preincision complex.

(3) 3' incision is normally the first, rate-limiting step in both mechanisms.

(4) UvrBC is a structure-specific endonuclease in *E. coli* as are ERCC1-XPF and XPG in mammalian cells.
(5) Unpaired structures eliminate the need for UvrA, but a lesion is still necessary for incision. This is similar to cases where a lesion found in an unpaired structure no longer requires XPC-hHR23B for its repair.

There are, however, differences between the two systems. It appears that repair in eukaryotes has evolved in a different way. Many of the factors are dual function and appear to have been recruited from other aspects of DNA metabolism. For example, TFIIH also functions as a general transcription factor, RPA has a function in DNA replication and ERCC1-XPF may also be involved in recombinational repair. Nevertheless, the end result is a remarkable unification of the overall strategy.

8.6. Future directions and concluding remarks

In this thesis I found that XPC has a requirement for the presence of hHR23B for full activity and that the complex as a whole preferentially binds to UV damaged DNA. The binding preference of this complex for UV damage was quantified as being ~400-fold, similar for that determined in parallel for UV-DDB. Preliminary work showed some preference of XPC-hHR23B for alkyl and platinum damage. Further work using DNA probes containing specific single lesions could give more definitive quantification of the binding preferences for all these types of DNA damage. Likewise, actual determination of the number of damaged sites in randomly damaged DNA competitor molecules would give greater confidence in the observed binding preferences.

XPC-hHR23B was found to bind strongly to ss M13 DNA and a ss oligonucleotide of mixed sequence. However, this complex interacted poorly with DNA bubble structures and ss homopolymer DNAs showing the importance of some level of secondary structure for this interaction. Further investigation of the binding requirements of XPC-hHR23B will add to our understanding of what exactly XPC-hHR23B recognizes. DNA lesions cause
some level of distortion and single-strandedness in DNA which could be a feature required for an XPC-hHR23B interaction. The lack of binding of this complex to DNA bubble structures was discussed in detail in chapter 7. One possibility is that this type of structure is too flexible in the bubble region and that a more rigid, defined structure is required for binding. Generation of substrates containing small 1 or 2 bp loops could give a structure more reminiscent of a photoproduct as these should be fully base paired, except at the site of the loop. This type of substrate may also contain intrinsic bending which could also be required for recognition by XPC-hHR23B.

The more general question of why is hHR23B required for XPC activity still requires further work. Further studies using the two components individually in DNA binding assays may shed light on this matter. It has been suggested that the XPC-hHR23B interaction is mediated by hydrophobic interactions (Masutani et al., 1997), therefore the role of hHR23B may be to stabilize XPC and help put it into the correct conformation for binding to damaged DNA. In the future, crystallization of XPC and XPC-hHR23B-damaged DNA complexes may be possible, giving direct information on the residues involved in DNA binding and possibly also on the DNA conformation in the complex.

XPC-hHR23B and UV-DDB were found to have similar binding preferences for UV and other types of DNA damage. However the intrinsic affinity of UV-DDB for UV damaged DNA (as distinct from damage discrimination ability) was about 200-fold higher than determined for XPC-hHR23B. This gives some explanation as to why UV-DDB is the only damaged DNA binding factor detectable in crude cell extracts. The presence of human DNA binding proteins with overlapping specificities raises the question of why we need two such similar activities and whether these proteins can substitute at all for each other at the cellular level. It is likely that these factors have as yet undetermined differences in binding requirements and cellular roles. This is the first time the two proteins have been directly compared and further direct comparison should help elucidate the differences between the two. My work has shown XPC-hHR23B to have a higher affinity for ss M13 DNA both with and without
UV damage than UV-DDB. This was the only significant difference observed between the two complexes binding specificities. Further studies with different types of ss DNA and DNA bubble structures as presented in chapter 7 for XPC-hHR23B, could be carried out with UV-DDB to further investigate this apparent difference in specificity. A sub-section of XP-E cells are deficient in UV-DDB activity and show mild UV sensitivity – although less than that observed with XPC-deficient cell lines. This shows that these proteins must have slightly different cellular roles. Similar to XP-C cells, those from XP-E patients have been reported to be defective in the removal of CPDs from non-transcribed DNA (Hwang et al., 1999).

UV-DDB is not required for reconstitution of NER in vitro (Aboussekhra et al., 1995) but microinjection of the protein into UV-DDB deficient XP-E cells corrects their DNA repair defect showing the importance of this protein in vivo (Keeney et al., 1994). It may be that this factor is important for the detection of DNA lesions in a chromatin context. The use of mini-chromosome substrates containing specific lesions may be helpful for in vitro analysis of binding of UV-DDB and XPC-hHR23B to more physiological DNA structures. The putative yeast homologue of XPC complex is Rad4-Rad23 and in contrast to XPC-hHR23B this factor is required for both transcription-coupled NER and repair of non-transcribed regions. At present there is no known yeast homologue of UV-DDB and it is possible that the activity of the Rad4-Rad23 complex (possibly in combination with the Rad7-Rad16 complex) functions in all aspects of damage recognition in yeast. In higher eukaryotes a number of damage recognition factors appear to have evolved with overlapping substrate specificities, and may have as yet unknown specialized repair activities.

The mechanism of DNA damage recognition is key to understanding how different types of lesions are detected and repair is initiated in cells. The identification of a human core NER protein with strong damage binding activity enables us to further dissect this important issue and future research should shed light on the questions that currently remain unresolved.
Bibliography


Gordienko, I. and Rupp, W.D.: UvrAB activity at a damaged DNA site - is unpaired DNA present. EMBO J 16 (1997) 880-888.


Lin, J.J. and Sancar, A.: A new mechanism for repairing oxidative damage to DNA - (A)BC excinuclease removes AP sites and thymine glycols from DNA. Biochemistry 28 (1989) 7979-7984.


Scott, A.D. and Waters, R.: The Saccharomyces cerevisiae rad7 and rad16 genes are required for inducible excision of endonuclease III sensitive sites, yet are not needed for the repair of these lesions following a single UV dose. Mutat Res DNA Repair 383 (1997) 39-48.


van der Spek, P.J., Kobayashi, K., Bootsma, D., Takao, M., Eker, A.P. and Yasui, A.: Cloning, tissue expression, and mapping of a human photolyase homologue with similarity to plant blue light receptors. Genomics 37 (1996b) 177-82.


